MAINSTREAM ATTACHED GROWTH PARTIAL NITRITATION AND ANAMMOX: DESIGN AND OPTIMIZATION

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Abstract

There is a significant need to remove ammonia from municipal wastewater to meet increasingly stringent regulations set by Canada, US, and Europe. Although existing conventional biological wastewater treatment technologies are shown to achieve effective ammonia treatment, they are substantially limited by increased operational intensity and cost. Due to these limitations, other cost-effective biological treatment technologies, such as partial nitritation/anammox (PN/A), have become a more attractive solution for nitrogen removal at wastewater resource recovery facilities (WRRF). A moving bed biofilm reactor system (MBBR) operating under a novel design strategy using elevated total ammonia nitrogen (TAN) loading rate has shown promise to achieve robust partial nitritation and the oxidation of TAN with limited oxidation of nitrite without the need for intense operational measures. However, the novel and promising design strategy using elevated TAN loading rate was applied at higher influent TAN concentrations that are typically greater than concentrations in mainstream municipal wastewater. Therefore, the objective of this dissertation is to investigate and optimize the design and performance of a promising elevated loaded partial nitritation MBBR technology for mainstream, municipal wastewater treatment followed by downstream anammox to complete the design of a robust, stable, energy-efficient, and low operational cost total nitrogen removal PN/A system for mainstream wastewaters.

The first specific objective of the dissertation is to isolate the optimal design parameter of a mainstream elevated loaded partial nitritation MBBR system. The results identifies optimal distinct elevated surface area loading rate (SALR), hydraulic retention

time (HRT), and airflow rate that achieve stable partial nitritation performance (i.e., optimum total ammonia nitrogen (TAN) removal kinetics and percent NO_x as nitrite) in a mainstream elevated loaded partial nitritation MBBR system. The study shows that TAN SALR, HRT, and airflow rate significantly affect TAN surface area removal rates (SARR) and percent NO_x as nitrite and, as such, identifies the optimal design parameters (TAN SALR, HRT and airflow rate) of a mainstream elevated loaded partial nitritation MBBR system. A TAN SALR of 5 g TAN/m^2·d, HRT of 2h and airflow rate of 1.5 L/min are identified to provide stable partial nitritation performance with a TAN SARR of 2.3 ± 0.3 g TAN/m^2·d and a percent of NO_x as nitrite of 84.8 ± 1.2% in the mainstream elevated loaded partial nitritation MBBR system.

The second specific objective further identifies a new design configuration and the mechanism of nitrite oxidation suppression of the mainstream elevated loaded partial nitritation MBBR technology. The results identifies a unique design strategy using an zelevated TAN SALR of 5 g TAN/m^2·d to achieve cost-effective, stable, and elevated rates of partial nitritation in an MBBR system under mainstream conditions. The elevated loaded partial nitritation MBBR system achieves a TAN SARR of 2.01 ± 0.1 g TAN/m^2·d and NO_2^--N: NH_4^+-N stoichiometric ratio of 1.15:1, which is appropriate for downstream anammox treatment. The elevated TAN SALR design strategy promotes nitrite-oxidizing bacteria (NOB) activity suppression rather than a reduction in NOB population as the reason for the suppression of nitrite oxidation in the mainstream elevated loaded partial nitritation MBBR system. NOB activity is limited at an elevated TAN SALR, likely due to thick biofilm embedding the NOB population and competition for dissolved oxygen (DO) with ammonia-oxidizing bacteria for TAN oxidation to nitrite within the biofilm structure, which ultimately limits the uptake of DO by NOB in the system.

The third specific objective of this research characterizes the effects of distinct mixing and aeration strategies on the performance of the mainstream elevated loaded partial nitritation MBBR technology. This is addressed through a study investigating and comparing the kinetics, biofilm characteristics, and embedded biomass of three distinct mixing and aeration strategies employed to operate the mainstream elevated loaded partial nitritation MBBR system. The study compares the conventional mixing and aeration condition, continuous aeration with mechanical paddle & aeration, and recirculation pump & aeration utilized to optimize the partial nitritation MBBR system to achieve low DO effluent concentrations for optimal downstream anammox treatment. The results show that maintaining mixing and aeration in the elevated loaded partial nitritation MBBR system with recirculation pump & reduced aeration achieves lower effluent DO concentration and stable partial nitritation with appropriate NO_2^--N:NH_4^+-N stoichiometry ratio of 1.09:1 for subsequent anammox treatment compared to operation with continuous aeration or mechanical paddle & aeration.

The fourth specific objective of this research investigates the promising elevated loaded PN/A configured system for nitrogen removal under mainstream conditions. This is achieved through the operation of the elevated loaded partial nitritation MBBR system following the anammox unit as a combined two-stage system for nitrogen removal at mainstream municipal concentration. The elevated loaded partial nitritation MBBR system provides optimal NH_4^+-N:NO_2^--N stoichiometric effluent ratio of 1:1.17, resulting in the successful operation of a downstream anammox unit with a total nitrogen removal rate at 0.22 ± 0.2 g $N/m^2/d$ and total nitrogen removal efficiency at 74.1 ± 0.7%. The average NO_2^--N to NH_4^+-N molar removal ratio is 1.05 ± 0.1 from the anammox unit. Also, the anammox bacteria (AnAOB) gene copies are at $3.28 \pm 0.7 \times 10^8$, a value significantly higher than the AOB and NOB gene copies at $9.17 \pm 1.1 \times 10^4$ and 6.23 ± 1.0, respectively. This confirms that anammox activity is established and nitrogen removal is primarily through the anammox process. The results and overall system performance demonstrate that the combined two-stage mainstream elevated loaded partial nitritation/anammox MBBR system has shown promise and offers great insights for further advancement of the anammox process at mainstream municipal wastewaters.

Finally, the economic evaluation and cost comparative analyses conducted show that compared to the conventional biological nitrification/denitrification process for nitrogen removal, the two-stage elevated loaded PN/A system offers a 57.6% savings on energy cost, 100% savings on chemical cost, and 68.7% savings on the cost of sludge disposal. Therefore, the two-stage elevated loaded PN/A system, in addition to high nitrogen removal efficiency, reduced footprint, and ease of operation, is also economically favorable and reduces the overall operational cost of wastewater treatment system by 61.6%, thus saving up to an average of 3.7 million CAD every year.

Preface

This dissertation is an original work performed by Juliet Ikem under the supervision of Dr. Robert Delatolla. The dissertation contains four manuscripts that are prepared for publication in peer-reviewed journals. The version of these manuscripts are presented in Chapters 3 to 6 of this dissertation. For each manuscript, the title, reference, authorship, and author contributions are provided below.

Manuscript #1 – Thesis Chapter 3:

A version of the manuscript presented in this chapter has been submitted to the journal of Bioprocess and Biosystems Engineering in 2023. Ikem, J., Schopf, A., Chen, H., & Delatolla, R. "*Optimized design of a stable, long term and robust attached growth mainstream partial nitritation system*."

Juliet Ikem contributed to the experimental design, performed experimental operations, performed data collection and analysis, interpretation of results, and wrote the manuscript.

Alexander Schopf contributed to the experimental design and interpretation of the results.

Huiyu Chen contributed to the experimental design, performed experimental operations, and performed data collection and analysis.

Robert Delatolla (supervisor) conceptualized the research ideas, guided the research activities and data interpretation, and planned and revised the manuscript.

Manuscript #2 – Thesis Chapter 4:

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Juliet Ikem contributed to the experimental design, performed experimental operations, performed data collection and analysis, interpretation of results, and wrote the manuscript.

Huiyu Chen contributed to the experimental design, performed experimental operations, and performed data collection and analysis.

Robert Delatolla (supervisor) conceptualized the research ideas, guided the research activities and data interpretation, and planned and revised the manuscript.

Manuscript #3 – Thesis Chapter 5:

A version of the manuscript presented in this chapter will be submitted in Fall 2023:

Ikem, J., Chen, H., & Delatolla, R. "*Assessment of mixing and aeration strategies on elevated loaded moving bed biofilm reactor for mainstream partial nitritation*".

Juliet Ikem contributed to the experimental design, performed experimental operations,

performed data collection and analysis, interpretation of results, and wrote the manuscript.

Huiyu Chen contributed to the experimental design, performed experimental operations, and performed data collection and analysis.

Robert Delatolla (supervisor) conceptualized the research ideas, guided the research activities and data interpretation, and planned and revised the manuscript.

Manuscript #4 – Thesis Chapter 6:

A version of the manuscript presented in this chapter will be submitted in Fall 2023:

Ikem, J., Chen, H., & Delatolla, R. ”*Elevated loaded combined two-stage partial nitritation/anammox for mainstream municipal wastewater treatment*”.

Juliet Ikem contributed to the experimental design, performed experimental operations, performed data collection and analysis, interpretation of results, and wrote the manuscript.

Huiyu Chen contributed to the experimental design, performed experimental operations, and performed data collection and analysis.

Robert Delatolla (supervisor) conceptualized the research ideas, guided the research activities and data interpretation, and planned and revised the manuscript.

I am aware of the University of Ottawa Academic Regulations; I certify that I have obtained written permission from each co-author to include the above materials in my dissertation. The above material describes work completed during my full-time registration as a graduate student at the University of Ottawa.

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List of Abbreviations and Acronyms

Anammox – Anaerobic Ammonia Oxidation

AnAOB- Anammox Bacteria

AOB – Ammonia Oxidizing Bacteria

BOD – Biological Oxygen Demand

cBOD – Carbonaceous Biological Oxygen Demand

CLSM – Confocal Laser Scanning Electron Microscope

C/N - COD/Nitrogen

COD – Chemical Oxygen Demand

Cq- Quantification Cycle

ddPCR- Droplet Digital Polymerase Chain Reaction

DNA – Deoxyribonucleic Acid

DO – Dissolved Oxygen

EPS –Extracellular Polymeric Substance

FA - Free Ammonia

FNA – Free Nitrous Acid

HRT – Hydraulic Retention Time

IFAS – Integrated Fixed Film Activated Sludge

K_O -Oxygen Half-Saturation Coefficient

LC50 – Lethal Concentration for 50% Mortality

MBBR – Moving Bed Biofilm Reactor

MTBL- Mass Transfer Boundary Layer

N/DN-Nitrification and Denitrification

NOB – Nitrite Oxidizing Bacteria

PCR – Polymerase Chain Reaction

PN – Partial Nitritation

PN/A – Partial Nitritation and Anammox

RNA - Ribonucleic Acid

SALR – Surface Area Loading Rate

SARR – Surface Area Removal Rate

SCOD – Soluble Chemical Oxygen Demand

TAN – Total Ammonia Nitrogen

TSS – Total Suspended Solids

VPSEM – Variable Pressure Scanning Electron Microscope

VSS – Volatile Suspended Solids

WRRF – Wastewater Resource Recovery Facility

WSER-Wastewater System Effluent Regulations

CHAPTER 1 - INTRODUCTION

1.1 Background

Chronic toxicity of ammonia as a single compound or in combination with other substances can cause long-term harmful effects on aquatic organisms. Exposure of fish to chronic ammonia levels causes reduced reproductive capacity, gill damage, hyperplasia, and a significant reduction in the growth rates of young fish (Park et al., 2018). Thus, to prevent the chronic toxicity of ammonia to aquatic lives and environments, discharge guidelines for ammonia release in wastewater effluent have been established around the world.

To minimize the adverse impact of the released ammonia on the aquatic environments, the EU, the US, and Canada have set standards to regulate ammonia discharge to receiving water bodies (EEC, 1991; Oleszkiewicz, 2015; US EPA, 2013). In Canada, the federal government has implemented the Wastewater Systems Effluent Regulations (WSER) under the Canadian Fisheries Act. This regulation defines municipal wastewater discharge limits for carbonaceous biochemical oxygen demand (cBOD) and total suspended solids (TSS) as an average of 25 mg/L, total residual chlorine (TRC) as an average of 0.02 mg/L and maximum unionized ammonia (NH_3) concentrations expressed as nitrogen at 15^{o}C ± 1^{o}C as 1.25 mg/L. The regulations further define that municipal wastewater effluent must pass an acute lethality test (LC50), with less than 50% mortality rate of rainbow trout being reported after 96 hours of exposure to 100% wastewater effluent (Environment Canada, 2010). The total ammonia nitrogen (TAN) concentration between 15-20 mg TAN/L has been reported to fail the LC50 test (Di Giulio and Hinton, 2008). Also, under the WSER, the LC50 test allows for additional testing with increased sampling frequency for ammonia toxicity. Therefore, the addition of this test and the influent TAN concentrations conventionally between 15-20 mg TAN/L for combined sewer systems and between 30-40 mg TAN/L in separated sewer systems across Canada. There is a need for more cost-effective, energy-efficient, and sustainable treatment options for TAN removal in water resource recovery facilities (WRRFs).

Conventionally to mitigate the toxic effects of TAN release in waters and comply with discharge limits, conventional biological nitrification/denitrification (N/DN) process have been applied at WRRFs. However, the intensive energy consumption, external carbon source, and excess sludge production associated with conventional biological N/DN process have challenged its economic viability and sustainability as a current and future means of nitrogen removal. Considering these challenges, advanced process and technologies have been developed in the last decades focused on improving cost-effectiveness, performance, and resource recovery to meet sustainable goals of zero to net-positive energy facilities. One example of this advanced process and technology in municipal wastewaters include partial nitritation (PN)-anaerobic ammonia oxidation (anammox) (PN/A) (Kartal et al., 2010; Vlaeminck et al., 2012; Wang et al., 2021; Zhang et al., 2020).

The PN/A process has resulted in the progression towards energy neutrality and net-positive energy. PN/A involves two consecutive reactions, where TAN is aerobically partially oxidized to nitrite by ammonia-oxidizing bacteria (AOB); subsequently, the anammox bacteria (AnAOB) utilize nitrite as an electron acceptor to convert the remaining TAN to nitrogen gas. Compared to the conventional biological N/DN process for nitrogen removal, the PN/A process consumes 60% less oxygen and 100% less organic carbon source and produces approximately 80% less sludge (Jetten et al., 1997; Mulder et al., 1995; Wett et al., 2013). Therefore, some studies have calculated a 30-40% reduction in overall nitrogen removal costs with the use of the PN/A process relative to the conventional biological N/DN process (Hoekstra et al., 2019; Joss et al., 2009). Over the past decade, PN/A has been successfully implemented to treat sludge digester centrate, as a sidestream treatment system, within municipal WRRFs. Centrate is characterized by approximate TAN concentrations of 500-1000 mg TAN/L and temperatures of 25-30^{o}C. Municipal sidestream PN/A treatment is well established, with over 200 full-scale installations in the world (Lackner et al., 2014; Malovanyy et al., 2015b; Van der Star et al., 2007; Wett, 2006). Despite the demonstrated capacity of PN/A process for sidestream treatment, the feasibility of PN/A for the treatment of mainstream municipal wastewaters remains a challenge, with no full-scale installations to date (Bunse et al., 2020; Li et al., 2018). Mainstream municipal wastewater characteristics such as high C/N ratios of 7-12 g COD/g-N, low TAN concentrations of 20-40 mg TAN/L, seasonal temperature variations of 10-25^{o}C and low-temperature values of < 10^{o}C in many countries limits AOB and AnAOB growth rates and also increase the challenge of achieving effective nitrite-oxidizing bacteria (NOB) suppression (Cao et al., 2017a; Laureni et al., 2016; Vlaeminck et al., 2012). The activity of NOB is detrimental in PN/A systems, as NOB can compete with AOB for available dissolved oxygen (DO) and AnAOB for available nitrite (Malovanyy et al., 2015a; Van Tendeloo et al., 2021). Therefore, effective suppression of NOB populations or activity is important for the successful implementation of PN/A at mainstream municipal wastewaters (Al-Omari et al., 2015; Xu et al., 2015).

Biofilm-based treatment systems show the potential to address and manage the challenges of mainstream PN/A process. A biofilm-based technology such as the moving bed biofilm reactor (MBBR) is generally advantageous in that they offer long biomass retention times needed to retain slow-growing AOB and AnAOB populations. In addition, biofilm structures result in substrate gradients that can suppress NOB populations or NOB activity (Brockmann and Morgenroth, 2008; Gilbert et al., 2014b; Lotti et al., 2015a; Pérez et al., 2014; Piculell et al., 2016a). The possibility of effective suppression of NOB in PN/A systems has been investigated using one-stage or two-stage MBBR configurations. A one-stage configured system performs the PN/A process in a single reactor, while a two-stage configured system separates the PN process from the anammox process, such that each process occurs in its own reactor and the entire treatment systems hence occur in two reactors that are usually operated in series. Studies exist mostly for one-stage PN/A configuration (Gilbert et al., 2014b; Gu et al., 2018a; Gustavsson et al., 2020; Iannacone et al., 2021; Laureni et al., 2016), with recent research focusing on two-stage configuration due to challenges associated with process instability and insufficient NOB suppression in one-stage configured systems (Gu et al., 2018b). The two-stage configuration has been reported to offer the opportunity to optimize the PN and anammox process independently and ultimately achieve higher nitrogen removal rates as compared to the one-stage configured system (Kowalski et al., 2019a). However, optimization of PN in a single reactor has been shown to require complex and often multiple operational strategies resulting in a high operational intensive system (Delgado Vela et al., 2015).

Several operational strategies have been utilized to suppress NOB populations or activity in a PN/A MBBR systems under mainstream conditions. Through bioaugmentation of AOB biomass from sidestream reactor to mainstream, AOB populations or activity often becomes significantly dominant than NOB in the system, resulting in NOB populations or activity suppression (Thomson et al., 2016). Alternating feed between mainstream and sidestream in an MBBR system, where the sudden switch to high substrate concentration and temperature inhibits NOB populations or activity (Piculell et al., 2016a). Another common operational strategy that has been employed in an MBBR system is the implementation of constant dissolved oxygen (DO)/TAN ratio of 0.17 in the reactor bulk solution to establish oxygen limiting conditions for NOB populations or activity suppression (Bian et al., 2017). Recently, the application of ammonia-based aeration control through the manipulation of effluent ammonia concentration and aeration rate have also been utilized to establish oxygen limiting condition in an MBBR system that allows NOB populations or activity to be selectively suppressed (Schraa et al., 2020). Additionally, AOB has a higher oxygen affinity than NOB; as such, low DO concentration (e.g. 0.15-0.22 mg O_2/L) has been employed in an MBBR system to selectively inhibit NOB populations or activity (Chen et al., 2018; Laureni et al., 2016). On the other hand, studies have explored alternating aerobic and anoxic conditions through intermittent aeration resulting in transient anoxic conditions inhibiting NOB growth, as NOB populations are shown to have a longer lag time than AOB while transiting from anoxic to aerobic phase (Malovanyy et al., 2015a; Trojanowicz et al., 2021).

Notwithstanding the potential of NOB suppression using the above-listed control strategies, these strategies are all operationally intensive and have demonstrated difficulty in achieving long-term suppression of MBBR NOB populations or activity, overall process stability, and stable effluent quality. For example, the bioaugmentation and alternating feed strategy from sidestream to mainstream have been shown to cause effluent TAN concentrations to increase, resulting in the need for an additional polishing step to improve effluent quality in an MBBR system (Lemaire et al., 2014). Moreover, repeated exposure to sidestream effluent could result in a microbiome shift to a more resilient community that could inhibit nitrite accumulation rate (Cabrol et al., 2016; Piculell et al., 2016b). DO/TAN ratio control has been demonstrated not to provide long-term NOB populations or activity suppression in an MBBR system (Bartrolí et al., 2010) Ammonia-based aeration control is a complex process and utilizes sophisticated sensors that require continual monitoring to control DO and ammonia concentrations in the system and as such operational intensity is high. In addition, operating the MBBR system under DO limitations could inherently limit AOB growth, thus, affecting the overall process rate (Chen et al., 2018; Laureni et al., 2015; Pérez et al., 2014). Similarly, intermittent aeration has been shown ineffective in sustaining long-term NOB populations or activity suppression and can potentially contribute to increasing N_2O emission, a potent greenhouse gas (Schraa et al., 2020; Trojanowicz et al., 2016). Thus, there is a need to explore other strategies that are low operationally intensive and able to achieve stable, robust, and long-term NOB populations or activity suppression under mainstream conditions.

Recently, Schopf et al., (2019) have demonstrated the feasibility of using a design strategy as opposed to an operational control strategy to achieve stable and robust PN in an MBBR system. The use of a design strategy resulted in a no operational control measures being required to achieve stable PN. The authors employed elevated TAN loading rates as a design strategy to achieve PN in an MBBR system fed with TAN concentrations of 125 mg

TAN/L to replicate commercial and industrial wastewaters, which are higher influent TAN concentrations than traditional municipal mainstream concentrations. This strategy has the potential to provide the necessary design for a high-rate, small footprint, and low operational system for mainstream PN. However, there is no current literature on using elevated TAN loading rates as a PN operational control strategy under mainstream conditions. Therefore, there is a theoretical and practical knowledge gap on the application and optimization of this promising elevated loaded PN MBBR technology to mainstream wastewater treatment and the possibility of combining this system with a downstream anammox as an enhanced total nitrogen removal system from mainstream municipal wastewaters.

1.2 Research objectives

The overall objective of the research is to investigate and optimize the design and performance of a promising elevated loaded PN MBBR technology for mainstream, municipal wastewater treatment followed by downstream anammox to complete the design of a robust, stable, energy-efficient, and low operational cost total nitrogen removal PN/A system for mainstream wastewaters. This research focuses on isolating critical design parameters and control strategies to optimize the design of a PN MBBR system and integrate it within an PN/A MBBR system to guide future piloting and full-scale installation.

The study will achieve the following specific objectives:

I. Characterize the effects of surface area loading rate (SALR), hydraulic retention time (HRT), and airflow rate on TAN removal kinetics and nitrite accumulation as percent NO_x and isolate the optimal design parameter of the mainstream elevated loaded PN MBBR system.

II. Identify new design configuration and the mechanism of nitrite oxidation suppression of the mainstream elevated loaded PN MBBR technology through the application of microbial and molecular analyses.

III. Characterize the effects of mixing and aeration strategies on biofilm characteristics, embedded biomass, and overall system performance of the mainstream elevated loaded PN MBBR technology to achieve low DO effluent concentrations for optimal PN/A integration and design.

IV. Evaluate the performance of the promising elevated loaded PN/A configured system for nitrogen removal under mainstream conditions.

1.3 Thesis organization

The dissertation is organized in the form of a manuscript-based thesis composed of seven chapters.

Chapter 1 describes the background information of this study, including overall and specific research objectives, in addition to a description of the thesis organization.

Chapter 2 presents a literature review on fundamental knowledge regarding biological nitrogen removal from municipal wastewater via conventional nitrification and denitrification and focuses on alternative cost-effective nitrogen pathways such as PN and anammox using attached growth technologies.

Chapter 3 (manuscript #1) is a research article titled "*Optimized design of a stable, long term and robust attached growth mainstream partial nitritation system".* Please note that the co-author list and their contributions to this research article and all research articles included in this thesis are provided in the preface section of this thesis. The article is prepared and has been submitted for publication in *Bioprocess and Biosystems Engineering* in 2023. This study optimizes the design of the promising elevated loading rate PN MBBR system to remove TAN from mainstream municipal wastewater. Specifically, the research isolates the optimal TAN surface area loading rates (SALR), hydraulic retention time (HRT), and airflow rate of a mainstream elevated loaded PN MBBR system.

Chapter 4 (manuscript #2) is a published research article titled "*Design strategy and mechanism of nitrite oxidation suppression of elevated loading rate partial nitritation system".* This article has been published in *Frontiers Microbiology* in 2023. The study provides a new design configuration to achieve an appropriate effluent stoichiometric ratio from a partial nitritation MBBR system for downstream anammox treatment. In addition, this study identifies the mechanism of nitrite oxidation suppression of mainstream elevated loaded PN MBBR systems. In particular, the study characterizes the performance of a two-reactor in series designed mainstream elevated loaded PN MBBR system, determines the effects of elevated TAN loading rate on biofilm thickness, biofilm mass, biofilm density, and embedded cells, and how these characteristics influence the performance of the mainstream PN MBBR system; and quantitate the AOB and NOB population counts within the attached growth, biofilm community.

Chapter 5 (manuscript #3) is a research article titled "*Assessment of mixing and aeration strategies on elevated loaded moving bed biofilm reactor for mainstream partial nitritation*". This article is in preparation for submission for publication. This research investigates the kinetics, biofilm characteristics and embedded biomass of three distinct mixing and aeration conditions, continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration, employed to operate the elevated loaded PN MBBR systems under mainstream conditions. Specifically, the study compares, the conventional mixing and aeration condition, continuous aeration with mechanical paddle & aeration, and recirculation pump & aeration, utilized to optimize the elevated loaded PN MBBR system to achieve low DO effluent concentrations for optimal downstream anammox treatment.

Chapter 6 (manuscript #4) is a research article titled "*Elevated loaded combined two-stage partial nitritation/anammox for mainstream municipal wastewater treatment*". This article is in preparation for submission for publication. In this research, an elevated loaded PN/A MBBR system was operated its performance was evaluated as a combined two-stage configured system for nitrogen removal under mainstream conditions.

Chapter 7 summarizes the main conclusions of the research and includes a cost benefit analysis of PN/A for a Canadian city, a discussion of the contributions of the work, practical implications, and a recommendation of future studies.

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CHAPTER 2 - LITERATURE REVIEW

2.1 Ammonia in wastewater

Nitrogen enters the wastewater treatment in the form of ammonia, which is formed through hydrolysis and mineralization of organic nitrogen such as urea, a major constituent of urine within the sewer system (Ye et al., 2018). In water, ammonia can exist in two forms, the unionized or free phase ammonia (NH_3) associated with chronic toxicity and ionized ammonium (NH_4^+), the less toxic compound. The sum of unionized ammonia and ionized ammonium is referred to as total ammonia nitrogen (TAN). The speciation of unionized ammonia and ionized ammonium in a given aqueous solution (wastewater) is usually dependent on the pH and temperature of the water. The fraction of unionized ammonia can be calculated with equations 2.1 and 2.2, where pKa is the acid dissociation, and T is the temperature (^oC) of the solution (Metcalf and Eddy, 2014).

$$\mathrm{pKa} = 0.09 + \frac{2730}{273 + \mathrm{T}} \qquad 2.1$$

$$\%\mathrm{NH_3} = \frac{1}{1 + 10^{\mathrm{pka-pH}}} \times 100\% \qquad 2.2$$

Previous studies have shown that ammonia is considered one of the most concerning deleterious substances in the aquatic environment because of its chronic toxicity in surface water (Park et al., 2018). Ammonia differs from most other toxic compounds in water in that it is produced endogenously by fish, which usually rely on a diffusion gradient for excretion. However, elevated concentrations of ammonia in water may reduce or inhibit ammonia excretion leading to a buildup of ammonia in the plasma of the fish that can cause proliferation of gill tissues, increase ventilation rates, and ultimately lead to death (Brinkman et al., 2009; Park et al., 2018). As a result of these harmful impacts of ammonia, several jurisdictions in US, EU and Canada have instituted discharge limits for unionized ammonia or TAN to protect humans, the environment, and aquatic lives (Oleszkiewicz, 2015).

2.2 Nitrification and denitrification

2.2.1 Nitrification

Nitrification is a two-step biological process by which TAN is sequentially oxidized to nitrite and nitrate. As shown in equation 2.3, nitritation is the first step where ammonia is oxidized to nitrite. The nitritation proceeds through two consecutive intermediate oxidation reactions; TAN is first oxidized to hydroxylamine catalyzed by the enzyme ammonia monooxygenase (AMO). The substrates for AMO are ammonia, oxygen and two electrons (equation 2.4). In the second reaction, hydroxylamine is further oxidized to nitrite catalyzed by the hydroxylamine oxidoreductase (HAO), using oxygen from water and additional molecular oxygen as a terminal electron acceptor (equation 2.5). The distinct microbial group that catalyzes the nitritation process is the ammonia-oxidizing bacteria (AOB). AOB encompasses five different genera affiliated to the β- and γ- a subclass of the phylum *Proteobacteria* (Ferrera and Sanchez, 2016; Ge et al., 2015). The dominant AOB in the conventional municipal wastewaters treatment facilities belongs to the genus *Nitrosomonas* (Young et al., 2016).

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \qquad 2.3$$

$$NH_3 + O_2 + 2\,e^- \xrightarrow{AMO} NH_2OH + H_2O \qquad 2.4$$

$$NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5H^+ \ + 4e^- \qquad 2.5$$

The second step in nitrification is known as nitratation. Nitratation is the oxidation of nitrite to nitrate catalyzed by enzyme nitrite oxidoreductase (NOR). An additional oxygen atom of nitrate is derived from water, and two electrons are released for energy production during this process (equation 2.6). The distinct microbial group that catalyzes the nitratation process is the nitrite-oxidizing bacteria (NOB). The NOB belongs to seven genera in four bacterial phyla, α-, β- and γ- a subclass of *Proteobacteria, Nitrospinae, Nitrospirae*, and *Chloroflexi.* The genus *Nitrospira* is the dominant NOB in conventional municipal wastewaters treatment facilities (Mehrani et al., 2020).

$$NO_2 + H_2O \xrightarrow{NOR} NO_3^- + 2H^+ \ + 2e^- \qquad 2.6$$

The combination of nitritation and nitratation (nitrification) is represented in equation 2.7.

$$NH_4^+ + 1.86O_2 + 1.98HCO_3^- \rightarrow \ 0.98NO_3^- + 0.019C_5H_70_2N + 0.9411H_2O + 1.88H_2CO_3 \qquad 2.7$$

For each gram of NH_4^+-N oxidized to NO_3^--N, 4.25 g of O_2 is utilized, 0.16 g of biomass ($C_5H_7O_2N$) is formed, and 7.17 g of alkalinity as $CaCO_3$ is consumed (Metcalf and Eddy, 2014). A schematic diagram of the nitrogen cycle including the other key nitrogen assimilation and dissimilation pathways, such as anaerobic ammonia oxidation (ANAMMOX) and denitrification, is shown in Figure 2.

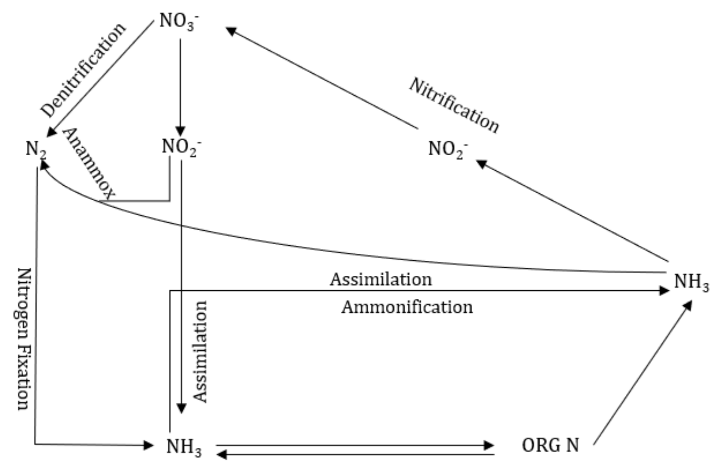

Figure 2.1 Nitrogen cycle.

2.2.2 Heterotrophic denitrification

Denitrification is the reduction of nitrate to nitrogen gas. It is a process that occurs in four sequentially reductive steps. In the first step, nitrate is reduced to nitrite in a process known as denitratation (equation 2.8). Then, nitrite is further reduced to dinitrogen gas (denitritation), with nitric oxide and nitrous oxide as second and third intermediates, respectively (equations 2.9, 2.10 and 2.11). The denitrification process is sequentially catalyzed by enzymes nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). It is also generally considered that the nitrogen reduction rate decreases as the oxidation state increases; as such, during denitrification to nitrogen gas, no accumulation of intermediates occurs (Pan et al., 2012). The capability for heterotrophic denitrification is widespread among bacteria, archaea, and eukaryotes. However, in an engineered system such as wastewater treatment, the denitrification process is mediated by bacteria belonging to the *Proteobacteria* and *Bacteroidetes* phyla (Lu et al., 2014).

$$2NO_3^- + 4H^+ + 4e^- \rightarrow 2NO_2^- + 2H_2O \qquad 2.8$$

$$2NO_2^- + 4H^+ + 2e^- \rightarrow 2NO + 2H_2O \qquad 2.9$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \qquad 2.10$$

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \qquad 2.11$$

2.2.3 Limitations of conventional biological nitrification and denitrification process

The combination of nitrification and denitrification process (Figure 2.2) has been widely adopted to remove excessive nitrogen compounds in the form of organic nitrogen and TAN from municipal wastewater. While nitrification requires intensive aeration, denitrification, on the other hand, requires an amply supply of organic carbon sources for effective TAN removal. These requirements can potentially increase the energy for aeration, pumping and sludge processing by as much as 30-50% (Farazaki and Gikas, 2019; Qiu et al., 2021; Rahimi et al., 2020). Furthermore, the nitrification/denitrification process have also been identified as significant contributors to N_2O emissions. This is a significant concern as N_2O has been considered the most dominant ozone-depleting agent (Perez-Garcia et al., 2017). Considering these limitations, there is extensive research focusing on improving the removal efficiency, cost, energy requirement, and sustainability of the biological TAN removal system in municipal wastewater by exploiting other alternative TAN removal pathways such as partial nitritation and anammox and nitritation-denitritation process.

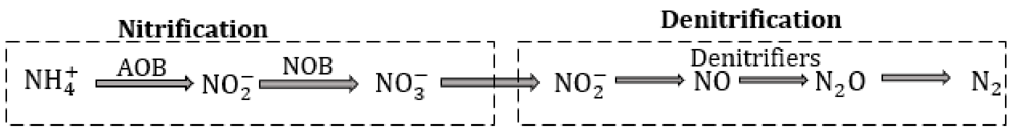

Figure 2.2 Nitrification and denitrification process.

2.3 Partial nitritation and anammox

2.3.1 Partial nitritation

The partial nitritation (PN) process is defined as the oxidation of approximately 55% of the influent TAN to nitrite by the AOB (equation 2.12). In a PN system, the focus is on halting further nitrite oxidation to nitrate through effective NOB populations or activity suppression (Piculell et al., 2016a). Therefore, several operational control strategies such as temperature, free ammonia & free nitrous acid, dissolved oxygen (DO) control, DO/TAN ratio control, residual TAN concentration, hydraulic retention time (HRT), and TAN loading rate (Bunse et al., 2020; Gilbert et al., 2014b; Kowalski et al., 2019a, 2019b; Ma et al., 2015; Trojanowicz et al., 2016) have been developed to either suppress NOB populations or the NOB activity.

$$NH_4^+ + 0.83O_2 \rightarrow 0.45NH_4^+ + 0.55NO_2^- + 0.55H_2O + 1.1H^+ \qquad 2.12$$

Temperature control

The operation of PN systems within critical optimal temperature for the AOB population can provide an ideal condition for NOB out-selection (Liu et al., 2020). The AOB has a faster growth rate under elevated temperatures ranging from 20°C to 25°C. On the other hand, NOBs can oxidize nitrite faster at temperatures as low as 12°C (Hwang and Oleszkiewicz, 2007). Although recent studies have reported the ability to suppress NOB activity at temperatures between 11°C and 20°C (Lotti et al., 2015b; Ma et al., 2015), maintaining other control parameters such as DO/TAN ratio, pH, residual TAN concentration, and TAN loading rate with temperature control are more effective strategies in achieving efficient NOB populations or activity suppression (Akaboci et al., 2018; Isanta et al., 2015; Kowalski et al., 2019b; Laureni et al., 2016).

Free ammonia & Free nitrous acid

Free ammonia (FA) and free nitrous acid (FNA) can be controlled through the manipulation of TAN concentration, temperature or pH to selectively suppress NOB populations or activity (Paredes et al., 2007; Soliman and Eldyasti, 2018). It has been reported that 7.5-8.5 is the optimal pH range for the inhibition of NOB populations or activity (He et al., 2012). At high pH values > 7.2, FA increases, while FNA increases at low pH of < 4.5 (Fumasoli et al., 2017). Although FA and FNA can inhibit AOB and NOB, nitrite oxidizers are more sensitive to FA and FNA concentration in the system. Previous studies have reported concentration values of 0.1-5.0 mg-N/L (Bae et al., 2001; Liu et al., 2020) and 0.026-0.220 mg-N/L (Zhou et al., 2011) of FA and FNA respectively as inhibitory thresholds to NOB without significantly affecting the AOB populations or activity.

Dissolved oxygen control (continuous and intermittent aeration)

Dissolved Oxygen (DO) is a common substrate for both AOB and NOB. However, AOB has a higher affinity for oxygen than NOB. This is because the oxygen half-saturation coefficient (K_O) of AOB is reported to be between 0.2-0.5 mg O_2/L while the $K_{O,\ NOB}$ for NOB is between 0.7-2.0 mg O_2/L (Picioreanu et al., 1997; Schramm et al., 1999). Therefore, low DO concentration or oxygen-limited conditions can provide AOB with a competitive advantage over NOB. In this regard, low DO concentration under 0.5 mg O_2/L has been employed for effective NOB populations or activity suppression (Yang et al., 2017). Although, other studies such as Gilbert et al. (2014) and Regmi et al. (2014) could not achieve NOB populations or activity suppression at similar low DO concentrations. This was attributed to the proliferation of the *Nitrospira* like NOB with a high affinity for oxygen that can adapt to low DO concentrations. As a result, long-term NOB suppression was identified as a significant challenge (Van Tendeloo et al., 2021). Moreover, in recent studies, the $K_{O,AOB}$ of AOB (0.41 mg O_2/L) have been reported higher than NOB (0.05 mg O_2/L) (Malovanyy et al., 2015a); as such, high DO concentration was utilized to encourage AOB dominance over NOB (Chen et al., 2019), with emphasis on suppression of *Nitrospira* like NOB (Regmi et al., 2014).

On the other hand, rather than controlling DO concentration through continuous aeration, it is also believed that intermittent aeration under low (< 0.05 mg O_2/L) or high (> 1.5 mg O_2/L) DO concentration can be applied to suppress NOB populations or activity (Bunse et al., 2020; Gilbert et al., 2014a; Gustavsson et al., 2020; Miao et al., 2016). This is possible as NOB are shown to have a longer lag time than AOB while transitioning from anoxic to aerobic, due to the possible inhibition by intermediate products such as hydroxylamine phases (Ge et al., 2015; Ma et al., 2016). Therefore, with intermittent aeration, the systems are usually set to have a more extended non-aerated period to facilitate NOB suppression (Bunse et al., 2020). Although, other studies have suggested longer aerated times than non-aerated times to promote AOB dominance over NOB in the system (Yang et al., 2015). While these contradictory findings could be attributed to varying experimental conditions and reactor configurations, DO control strategies still require optimization to further consolidate functional organisms and enhance process stability (Li et al., 2018).

DO/TAN ratio & residual TAN concentration

Maintaining an appropriate DO/TAN ratio has been described as a more effective control strategy to suppress NOB than controlling the DO concentration in the bulk solution (Cao et al., 2017a). Typical in DO/TAN ratio control strategy, two different closed loops are implemented; one is to monitor the TAN concentration in the bulk liquid, and the second is to control DO concentration in the bulk liquid and maintain both concentrations (DO/TAN) within a constant ratio ideally less than 1. The aim is to establish a strong oxygen limiting condition that allows NOB populations or activity to be selectively suppressed (Pérez et al., 2014). However, this is mainly applicable for biofilm-based systems due to the diffusion gradients intrinsic to the biofilm that leads to the spatial organization of AOB in the outer layer of the biofilm and NOB below the AOB. Therefore, NOB is usually more susceptible to oxygen limiting conditions (Cao et al., 2017a; Liu and Yang, 2017).

Furthermore, besides manipulating the DO/TAN ratio, it is also believed that maintaining appropriate concentrations of TAN concentration in the bulk solution (residual TAN concentration) encourages NOB out-selection. For example, it has been reported that at residual TAN concentrations between 2-5 mg TAN/L in the bulk solution, AOB was more competitive for DO due to their increased population, which caused preferentially use of DO for TAN oxidation to nitrite as such, limiting DO concentration available to maintain NOB populations or activity (Gao et al., 2014; Hu et al., 2013; Regmi et al., 2014; Yeshi et al., 2016). However, relying on retaining high residual TAN concentration for NOB repression could worsen the effluent quality (Pérez et al., 2014) and indirectly increase operational cost, especially where additional polishing treatment is required.

Hydraulic retention time

In municipal wastewater treatment, due to low TAN concentration, short HRT will be preferred in practice to achieve elevated loading rates in the system to ensure high substrate availability providing optimal conditions for selective NOB populations or activity inhibition (Li et al., 2018). Moreover, short HRT is primarily important as at high HRT (e.g, > 6h) the loading rate decreases in the system. And at this low loading rate, the system is operating under TAN limited condition, resulting in an increased oxygen transfer efficiency, prompting NOB populations or activity (Liang et al., 2016). As such, it is suggested that HRT are kept short and optimal to achieve stable PN performance (Chen et al., 2019; De Clippeleir et al., 2011). Although studies have shown possible NOB activity suppression at longer HRT (6-12h); these disparities can be attributed to different operational conditions and reactor configurations used in each study. However, short HRTs are usually recommended from a practical standpoint, especially where additional energy savings are necessary (De Clippeleir et al., 2011).

TAN loading rate

Total ammonia nitrogen (TAN) loading rates are typically controlled using design parameters such as influent TAN concentration and HRT. Although in an attached biofilm-based system, change in TAN loading rate can be achieved through variation of carrier fill fraction (Schopf et al., 2019). Studies have demonstrated that TAN loading rate could affect effluent composition generated in a PN reactor. For instance, operating at an elevated TAN loading rate suppresses NOB activity due to the preferential uptake of oxygen by the AOB population, resulting in high nitrite accumulation in the effluent. Therefore, an elevated TAN loading rate has been proposed as a more practical strategy to control the performance of

PN systems (Choi et al., 2018; Rodriguez-Sanchez et al., 2014). Besides considering the complexity in regulating other operational control parameters such as DO on a large scale, the TAN loading rate design strategy could offer a more effective and long-term NOB populations or activity suppression with no additional operational control requirement (Daalkhaijav and Nemati, 2014; Schopf et al., 2021).

2.3.2 Anaerobic ammonia oxidation

Anaerobic ammonia oxidation (anammox) is the anaerobic autotrophic oxidation of TAN to with nitrite as an electron acceptor producing nitrogen and nitrate as key end products under anoxic conditions (Mulder et al., 1995). Ever since the discovery and isolation of anammox bacteria (AnAOB) in wastewater treatment facilities (Mulder et al., 1995); today, anammox-based processes are widely explored as an alternative for TAN removal in municipal wastewaters (Gustavsson et al., 2020; Hoekstra et al., 2019; Laureni et al., 2016). The group of bacteria responsible for anammox are found within the five genera of order *Brocadiales* (Kartal et al., 2007); and they are proceeded by the name *Candidatus*, which is used when species are well characterized using molecular tools but not studied in pure culture (Strous et al., 1999). C*andidatus Kuenenia* and *Candidatus Brocadia* are the dominant AnAOB found in the conventional municipal wastewaters treatment facilities (Cui et al., 2019). The AnAOB is characterized by a slow growth rate with a doubling time of 4-15 days, strict growth requirements, low biomass yield, and long start-up time (Strous et al., 2006, 1999). As such, seeding from a previous anammox system is important to reduce the start-up time of the new anammox system.

$$NH_4^+ + 1.32NO_2^- + 0.06HCO_3^- + 0.13H^+ \quad 2.13$$

$$\rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

Importantly, anammox systems are usually operated under anoxic conditions and like AOB, AnAOB are chemolithotrophs; as such do not require organic carbon; instead, they use an inorganic carbon source for cell synthesis. In this regard, anammox-based processes are more sustainable, cost-effective and energy-efficient options for TAN removal at mainstream municipal wastewaters, as they can significantly reduce aeration and organic carbon demand (Li et al., 2018).

2.4 Mainstream partial nitritation and anammox process in municipal wastewaters

The combination of PN and anammox (PN/A) (equations 2.12 and 2.13) is the most common anammox-based process for TAN removal from municipal wastewaters (Figure 2.3). Currently, research focus has been towards possible implementation of PN/A process for treatment of mainstream municipal wastewaters (Agrawal et al., 2018; Bunse et al., 2020; Chen et al., 2018; Gustavsson et al., 2020; Laureni et al., 2016; Lotti et al., 2015b; Qiu et al., 2021; Schraa et al., 2020; Trojanowicz et al., 2016).

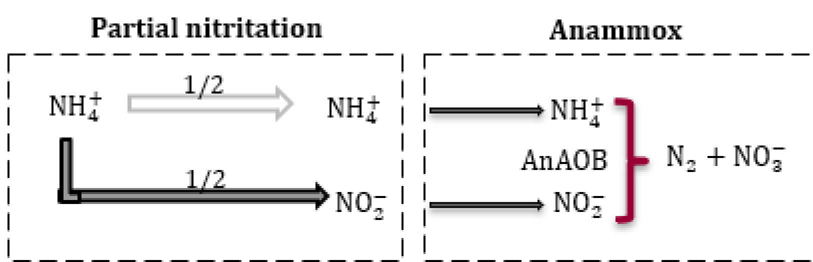

Figure 2.3 Schematic of partial nitritation and anammox process.

The direct application of PN/A process in mainstream municipal wastewaters is gaining interest because of the strategic significance of energy conversion. The PN/A process offer clear advantages over the conventional nitrification and denitrification process in that they, i) consume 60% less oxygen and 50% less alkalinity as approximately 55% of the influent TAN in the feed is oxidized to nitrite; ii) eliminate the need for organic carbon since AnAOB are autotrophic bacteria, and no organic carbon is needed for cell growth; and iii) produces 80% less sludge than the conventional nitrification-denitrification process (Winkler and Straka, 2019). In addition, there is also the ability to separate nitrogen removal from carbon removal in PN/A process and maximize energy recovery through enhanced carbon pre-treatment in an anaerobic digester for biogas generation (Cao et al., 2017a; Qiu et al., 2021). Therefore, the integration of mainstream PN/A process bears the potential to bring WRRFs close to energy neutrality (Laureni et al., 2016; Trojanowicz et al., 2021).

2.5 Biofilms

A biofilm is a complex structure and assemblage of microbial cells bonded with self-produced extra polymeric substances (EPS) adherent to inert or living surfaces (Costerton, 1999). Through intracellular interaction together with the properties of the biofilms are different from the free-living bacterial cells (Donlan, 2002; Flemming et al., 2016; Konopka, 2009; Suarez et al., 2019).

The mass transfer and diffusion rule govern the rate at which substrates, oxygen, and nutrients enter the biofilm. In a biofilm, the mass transfer boundary layer (MTBL), also called the diffusion layer, separates it from the bulk liquid. The decreased flow in the MTBL does not allow substrates' free flow to continue, requiring the substrates to diffuse slowly through the MTBL. The transfer of substrates from the bulk liquid to the biofilm can be enhanced through aeration or mechanical mixing (Metcalf & Eddy, 2014; Rittmann and McCarty, 2001; WEF, 2011).

The substrate concentration at the biofilm surface decreases with biofilm depth as the substrate diffuses into the biofilm layers and gets degraded. Also, the depth of layer diffused (mass transport inside the biofilm) by the substrate is dependent on biofilm porosity, biofilm thickness, molecular diffusivity of the substrate, and biofilm structure (Rusten et al., 2006; Taherzadeh et al., 2012). As such, biofilms are termed to be diffusion-limited. In a biofilm, the diffusion limitations form steep gradients, which result in a structured micro-environment. Examples are found in biofilm used for nitrification or PN/A process, here stratification of microbial communities typically occurs with AOB situated close to the oxygenated bulk liquid, NOB in the next layer after the AOB, and if anaerobic conditions are established AnAOB could establish in deeper layers of the biofilm (Suarez et al., 2019).

2.6 Biofilm Technology

2.6.1 The moving bed biofilm reactor (MBBR)

Over two decades, the MBBR has been established as a robust, flexible, and compact technology for wastewater treatment. The MBBR uses designed plastics carriers such as the AnoxK™5 carriers (AnoxKaldnes, Lund, Sweden) for biofilm attachment which are held in suspension throughout the reactor by aeration, water recirculation or mechanical mixing. The carriers are mixed in the reactor to promote transfer of substrates from the bulk solution to the biofilm. The process designs with MBBRs are based on the concept that wastewater treatment can be achieved using single or multiple reactors in series. And each reactor within the treatment scheme has its specific treatment function and the ability to promote the development of highly active specialized biomass that results in high removal efficiencies and increased process stability (WEF, 2011). The MBBR can be used in varieties of continuous flow-through systems for BOD or TAN removal in wastewater treatment facilities. The versatility of MBBR allows the system to be operated at varying loading rates by manipulating design parameters such as HRT or influent substrate concentrations. This provides the opportunity to design high-rate systems that are compact and capable of meeting treatment standards in a short HRT (Melin et al., 2004).

2.7 Mainstream PN/A MBBR systems

Mainstream PN/A using the MBBR technology have been studied under two major configurations, one-stage and two-stage configurations (Chen et al., 2018; Gu et al., 2018b; Gustavsson et al., 2020; Laureni et al., 2016; Schraa et al., 2020; Trojanowicz et al., 2021). While the one-stage MBBR configurations are mostly studied due to the lower cost of infrastructure than two-stage configured systems, the latter offers the opportunity to optimize the PN and anammox process independently and maintain a balanced ratio between functional bacteria groups (AOB and AnAOB) to achieve higher volumetric nitrogen removal rates. Compared to the one-stage configuration in which PN process known as a rate-limiting step can become oxygen rate-limited when combined with anammox process that requires low DO concentrations of < 0.2 mg O_2/L (Szatkowska et al., 2007); the operation of MBBR as a two-stage PN/A system can achieve higher nitrogen removal rate with 0.06 kg-N/m^3/d being the highest reported for real wastewater and 0.09 kg-N/m^3/d being the highest reported for synthetic wastewater (Gu et al., 2018b; Kowalski et al., 2019a).

2.8 Challenges in mainstream municipal wastewater

Though several studies are ongoing to optimize and develop the application of PN/A process for mainstream wastewater treatment, several challenges that must be addressed, includes, i) high C/N (7-12 g COD/g-N) ratio of mainstream wastewaters that may cause the proliferation of heterotrophic bacteria that could outcompete AOB and AnAOB populations in PN/A system (Cao et al., 2017b; Gustavsson et al., 2020); ii) slow growth rate of autotrophs especially under ammonia limiting condition that could prolong reactor start-up and also affect process stability; iii) long-term NOB populations or activity suppression since NOB can compete with AOB for available DO and AnAOB or denitrifiers for nitrite in PN/A system, respectively (Li et al., 2018; Van Tendeloo et al., 2021).

The first and second challenges can be addressed by adding a carbon removal process to control the effluent C/N ratio before the PN/A process and employing biofilm-based systems that offer long biomass retention time. However, the key to achieving high TAN removal rates, process stability, and successful implementation of PN/A process for the treatment of mainstream municipal wastewaters relies on maintaining long-term effective suppression of NOB populations or activity (Cao et al., 2017a).

Although studies have reported the possibility of achieving NOB populations or activity suppression by employing varying operational control strategies under mainstream conditions (Chen et al., 2018; Gustavsson et al., 2020; Laureni et al., 2016; Schraa et al., 2020; Trojanowicz et al., 2016); there is yet a consensus on which is most efficient. This is because most employed operational control strategies are conditional with different limitations (Table 2.1) and could fail if any or all operational conditions are not met (Liu et al., 2020; Xu et al., 2015).

Table 2.1 Overview of operational strategies employed for NOB suppression in mainstream MBBR systems

Operational control strategies	**Operating conditions**	**Nitrate production (%)**	**Limitations**	**References**
Continuous aeration with DO setpoint at 1.2-3.0 mg O_2/L	HRT: 3.8h, and Temp: 11-23^{o}C,	17 -44%	i) Maintaining continuous aeration with increased organic load could decrease nitritation rates and cause further nitrate to build up in the system.	(Gustavsson et al., 2020)
Intermittent aeration, DO ≤ 0.5 mg O_2/L	HRT: 2.6h, Temp:10-20^{o}C, and ratio of aerated to non-aerated periods in a cycle: ½-$^{1/3}$; total time of a cycle: 30-82 minutes.	40%	i) Intermittent aeration did not support effective NOB suppression and promoted higher N_20 production. ii) There could be increased energy and operational cost over long-term operations.	(Gustavsson et al., 2020)
DO/TAN ratio and pH control	HRT: 6-12 h, Temp: 10^{o}C, pH: 8.6 (dosing 0.125M aqueous solution of sodium hydroxide), and aeration rate: 0.1L/min	14%	i) There could be increased energy and operational cost over long-term operations.	(Kowalski et al., 2019b)
Residual TAN concentration	HRT:18h, continuous aeration with DO set at 0.5 mg O_2/L, Temp: 25^{o}C and pH: 6.6	NA	i) Needs to be combined with other control strategies. ii) An extra polishing treatment to meet stringent effluent quality.	(Malovanyy et al., 2015b)
Alternating feed from sidestream to mainstream with limited biofilm thickness	Alternating feed from sidestream to mainstream with limited biofilm thickness	NA	i) An additional polishing treatment step is required to meet stringent effluent quality. ii) Repeated exposure to sidestream effluent could result in a microbiome shift to a more resilient community that could inhibit the nitrite accumulation rate.	(Piculell et al., 2016a)

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CHAPTER 3 - OPTIMIZED DESIGN OF A STABLE, LONG-TERM AND ROBUST ATTACHED GROWTH MAINSTREAM PARTIAL NITRITATION SYSTEM

3.1 Context

Chapter 3 presents the research article titled *Optimized design of a stable, long term and robust attached growth mainstream partial nitritation system.* The article has been submitted for publication to the journal of Bioprocess and Biosystems Engineering in 2023. The study identifies the optimal distinct elevated surface area loading rates (SALR), hydraulic retention times (HRTs), and airflow rates that achieve stable PN performance in a mainstream elevated loaded PN MBBR system.

3.2 Abstract

A sustainable and cost-effective control system to achieve stable mainstream partial nitritation (PN) is essential to transition the anammox process to mainstream municipal wastewater treatment. This study identifies the optimal distinct elevated surface area loading rate (SALR), hydraulic retention time (HRTs), and airflow rate that achieve stable PN performance (i.e., optimum total ammonia nitrogen (TAN) removal kinetics and percent NO_x as nitrite) in a mainstream elevated loaded PN MBBR system. The study shows that TAN SALR, HRT, and airflow rate significantly affect TAN surface area removal rates (SARR) and percent NO_x as nitrite and, as such, identifies the following optimal design parameters of a mainstream elevated loaded PN MBBR system: TAN SALR of 5 g TAN/$m^2 \cdot$d, HRT of 2h and airflow rate of 1.5 L/min. This design resulted in stable and robust PN performance exhibiting a TAN SARR of 2.3 ± 0.3 g TAN/$m^2 \cdot$d and a percent NO_x as nitrite of 84.8 ± 1.2%.

3.3 Introduction

Conventional nitrification and denitrification systems are well-established and widely implemented for biological nitrogen removal in water resource recovery facilities (WRRFs) within numerous countries (Winkler and Straka, 2019). The nitrification process includes the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB) and the subsequent conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). In the biologically mediated denitrification process, nitrate is reduced to nitrogen gas by heterotrophic denitrifiers using an organic carbon source (Modin et al., 2011). Organic carbon is often externally sourced and supplied to wastewater treatment trains that are required to meet stringent effluent total nitrogen discharge regulations. Although conventional nitrification/denitrification process are widely employed in treatment facilities for nitrogen removal, they are subject to significant drawbacks such as; intensive aeration demands, organic carbon requirements, and increased sludge cell yields when compared to other cost-effective alternative nitrogen removal pathways (Farazaki and Gikas, 2019; Li et al., 2018; Ma et al., 2020). In addition, both nitrification/denitrification are associated with N_2O emission, a potent greenhouse gas (Farazaki and Gikas, 2019; Qiu et al., 2021; Rahimi et al., 2020). These practical limitations have created a need to shift toward less energy-intensive, sustainable, and cost-effective technologies to advance and upgrade WRRFs.

Partial nitritation (PN) and anaerobic ammonia oxidation (anammox), collectively referred to as PN/A, is an energy-efficient and sustainable alternative to conventional nitrification/denitrification process (Jetten et al., 1997; Mulder et al., 1995; Wett et al., 2013). The PN/A process enables nitrogen removal to be achieved with 60% less aeration, no carbon source addition, and with sludge production reduced by 80% compared to the combined nitrification/denitrification process (Agrawal et al., 2018; Dosta et al., 2015; Wang et al., 2021). PN/A is a two-step process in which the PN process results in approximately half of the influent ammonia being oxidized to nitrite by AOB and, subsequently, the anaerobic ammonia-oxidizing bacteria (AnAOB) oxidizing the remaining ammonia to nitrogen gas in the presence of nitrite as an electron acceptor (Mulder et al., 1995). To date, the PN/A process have been successfully employed to treat elevated ammonia concentrations originating from industrial wastewaters and anaerobic digesters in sidestream municipal wastewater treatment systems (Daigger et al., 2011; Malovanyy et al., 2015b; Wett, 2007). The number of worldwide installations of PN/A industrial wastewater systems are greater than 50 facilities, and sidestream PN/A treatment systems in Europe, Asia, and North America currently exceed 200 full-scale facilities (Cao et al., 2017; Lackner et al., 2014; Li et al., 2018).

The direct implementation of the PN/A process to mainstream municipal wastewater treatment is of great interest to further progress efforts of WRRFs to achieve net zero energy or positive energy production within wastewater facilities while minimizing the impact of discharged wastewaters to receiving waters (Laureni et al., 2016). To date, studies have established the possibility of mainstream PN/A process through experimental evidence (Chen et al., 2019b, 2019a; Gu et al., 2018; Laureni et al., 2016; Lotti et al., 2015). However, it remains difficult to accumulate nitrite and maintain the ideal NH_4^+ :NO_2^- metabolic ratio in mainstream wastewaters due to the challenge of sustaining the suppression of NOB populations or their activity (Cao et al., 2017; Malovanyy et al., 2015a). NOB are detrimental to the PN/A process, as NOB activity readily oxidizes nitrite and hence prevents the AnAOB from performing the subsequent anammox process; thus stymying the entire PN/A process (Malovanyy et al., 2015a; Pérez et al., 2014; Piculell et al., 2016b; Van Tendeloo et al., 2021). In regards to achieving effective NOB population or activity suppression, the moving bed biofilm reactor (MBBR), a biofilm-based technology, has been studied to achieve stable PN for subsequent anammox operations across a range of operational control strategies (Gustavsson et al., 2020; Kowalski et al., 2019a, 2019b; Piculell et al., 2016a). MBBR systems have been considered advantageous for PN as the biofilm structure may enable the AOB biomass to be preferentially retained within the system biofilms due to the availability of both ammonia and dissolved oxygen (DO) in the bulk liquid phase while outcompeting the NOB population for the shared substrate of DO within the biofilm structure. Hence, the NOB population is susceptible within PN MBBR systems to DO-limiting conditions that can suppress their population size or activity (Pérez et al., 2014).

Several studies on mainstream PN MBBR systems rely on employing various operational control strategies to establish oxygen-limiting conditions to selectively inhibit NOB populations or NOB activity to achieve stable PN (Laureni et al., 2019). Kowalski et al., (2019b) demonstrated the potential for a stable PN process with NOB activity suppression using a combination of DO/total ammonia nitrogen (TAN) ratio control and NOB inhibition using free ammonia (FA). Other studies have explored intermittent system aeration under low DO concentrations and continuous system aeration conditions under low DO setpoints between 0.15-0.22 mg O_2/L to selectively inhibit NOB populations or activity (Chen et al., 2018; Laureni et al., 2016). In contrast, rather than maintaining low DO concentrations in the bulk solution, NOB suppression has also been reported at high DO concentrations greater than 4 mg O_2/L within systems that exhibit thin biofilms and alter the feed stream between mainstream wastewaters at 15°C and synthetic reject water at 30°C. The synthetic reject waters elevated FA concentrations caused the inhibition of NOB activity in these systems (Piculell et al., 2016a). In addition, a recent study has employed ammonia-based aeration control to selectively suppress NOB populations or activity (Schraa et al., 2020). Thus, it is evident that various operational control strategies in an MBBR system can be used to achieve PN. However, these control strategies are all operationally intensive and do not demonstrate long-term significant NOB population and/or activity suppression, which has directly limited the application of mainstream PN/A MBBR systems (Gustavsson et al., 2020; Van Tendeloo et al., 2021).

Recently, Schopf et al. (Schopf et al., 2019) have demonstrated the feasibility of using a passive and zero operational intensity design strategy to achieve stable, long term and robust PN in an MBBR system. In particular, Schopf et al. (2019) employed elevated TAN loading rates as a design strategy to achieve PN in an MBBR system fed with TAN concentrations of 125 mg TAN/L, which are higher than conventional mainstream municipal concentrations. This design strategy (elevated TAN loading rate) has the potential to provide the necessary design for a high-rate, small footprint, and low operational intensity system for stable, long term and robust mainstream PN. However, no studies in the current literature have evaluated the potential of elevated TAN loading rate as a PN design and control strategy under mainstream conditions. Therefore, this study aims to optimize the design of the elevated loading rate PN MBBR system to remove TAN from mainstream municipal wastewater. In particular, the study determines the effects of distinct TAN surface area loading rates (SALRs), hydraulic retention times (HRTs), and airflow rates on TAN removal kinetics and nitrite accumulation as percent NO_x and isolates the optimal design of a mainstream elevated loaded PN MBBR system.

3.4 Materials and methods

3.4.1 Experimental setup

Three parallel 2 L MBBR reactors, PN_1, PN_2 and PN_3, with identical dimensions, volumes, and fill fractions of 9.5% were operated in this study (Figure S3.1). The reactors were filled with high-density polyethylene AnoxK™5 carriers (AnoxKaldnes, Lund, Sweden) with a protected biofilm surface area of 800 m^2/m^3. Synthetic wastewater was fed to the reactors with a peristaltic pump, and the reactors were continuously aerated from the base using an air pump. The air was dispersed by an air diffuser stone connected to a regulator to allow adequate control and provide continuous uniform mixing and DO to the reactors. All reactors were operated at ambient temperature, and no external pH or temperature control was applied.

3.4.2 Reactor inoculation and startup

The AnoxK™5 carriers were seeded carriers harvested from a single bench scale PN MBBR system operated at elevated TAN loading rates. Prior to use in the single bench scale PN MBBR study, the carriers were seeded from a biological oxygen demand (BOD) removal municipal integrated film-activated sludge (IFAS) wastewater treatment system located in Hawkesbury, Ontario, Canada. Harvested carriers from the use in the single bench scale PN MBBR study were distributed into three identical reactors, PN_1, PN_2, and PN_3, all designed to run in parallel under the same operational conditions: influent TAN of 41.1 ± 1.2 mg TAN/L, TAN SALR of 7 g TAN/$m^2 \cdot$d, HRT of 2h, DO concentration of 6.5 ± 0.2 mg O_2/L, pH of 7.5 ± 0.1, temperature of 19.8 ± 0.2°C. The initial phase of the study was performed until each of the reactors, PN_1, PN_2, and PN_3, demonstrated TAN surface area removal rate (SARR) of greater than 2.9 g TAN/m^2·d and percent of total oxidized TAN as nitrite (NO_x as nitrite) of greater than 80%, which indicated that stable PN was achieved. These conditions in the initial phase were maintained for a minimum of six weeks, during which time the three reactors were tested to validate steady-state operation, with steady-state operation defined as ± 10% fluctuation in TAN removal rate and percent NO_x as nitrite.

3.4.3 Reactor operation

In the preliminary experiment, the reactor PN_1 was operated at various target TAN SALRs of 4.0, 5.0, 6.0, 6.5, and 7.0 g TAN/m^2·d, HRTs of 1.0, 1.4, 1.6, 2.0, 2.2, and 2.5h and airflow rates of 1.0, 1.5, 2.0, 3.5 and 4.0 L/min. PN_1 was operated for at least two weeks at each TAN SALR, HRT, and airflow rate value in addition to demonstrating steady operation at each design parameter investigated in this study (Figure S3.2, S3.3). The preliminary operation of PN_1 was used to establish TAN SALR, HRT, and airflow rate values that showed distinct effects on the performance of the elevated loaded PN MBBR system. In addition, the operation of PN_1 was also used to identify the potential critical HRT (2h) and airflow rate (1.5 L/min) value, which was used in the subsequent experiment to isolate the optimum TAN SALR of the mainstream elevated loaded PN MBBR system.

To determine the optimal TAN SALR of the mainstream elevated loaded PN MBBR system, the target influent TAN concentrations applied to reactor PN_1 were 25, 30, 40, and 45 mg TAN/L corresponding to target TAN SALRs of 4.0, 5.0, 6.0, and 7.0 g TAN/m^2·d. Based on the results from the preliminary operation of PN_1, the HRT and airflow rate were maintained at 2h and 1.5 L/min, respectively (Table 3.1). During the isolation of the optimal TAN SALR, the PN_1 reactor was operated for seven weeks. A minimum of three triplicated data points were obtained to quantify the kinetics at each of the applied TAN SALRs of 4, 5, 6, and 7 g TAN/m^2·d. The TAN SALR of 5 g TAN/m^2·d was identified as the optimal value and was used to isolate further critical optimum design parameters, HRT and airflow rate investigated in this study.

Table 3.1 Operating conditions at distinct design parameters, TAN SALR, HRT, and airflow rate (average ± 95% confidence interval)

Reactor	Design Parameters	Measured Influent TAN concentration (mg TAN/L)	Measured SALR (g TAN/m^2·d)	HRT (h)	Airflow rate (L/min)	DO (mg O_2/L)	Temperature (°C)	pH
PN_1	TAN SALR	25.4 ± 0.9	4.0 ± 0.1	-	-	-	-	-
		31.0 ± 1.4	4.9 ± 0.2	-	-	-	-	-
		38.1 ± 2.1	6.0 ± 0.1	2.0 ± 0.3	1.5 ± 0.1	6.9 ± 0.1	20.2 ± 0.2	7.7 ± 0.2
		44.6 ± 1.3	7.0 ± 0.2	-	-	-	-	-
PN_2	HRT	25.4 ± 0.1	5.2 ± 0.4	1.6 ± 0.3	-	-	-	-
		30.6 ± 0.3	5.0 ± 0.2	2.0 ± 0.3	-	-	-	-
		35.8 ± 0.5	5.2 ± 0.3	2.2 ± 0.2	1.5 ± 0.1	7.0 ± 0.1	19.5 ± 0.2	7.8 ± 0.3
		40.9 ± 0.2	5.1 ± 0.4	2.5 ± 1.2	-	-	-	-
PN_3	Airflow rate	31.5 ± 0.2	-	-	1.0 ± 0.2	6.7 ± 0.1	-	-
		-	-	-	1.5 ± 1.2	6.8 ± 0.3	-	-
		-	5.2 ± 1.2	2.0 ± 0.2	2.0 ± 0.2	6.8 ± 0.2	19.8 ± 0.1	7.8 ± 0.2
		-	-	-	4.0 ± 0.6	6.9 ± 0.3	-	-

To ascertain the optimal HRT that results in optimum performance of the mainstream elevated loaded PN MBBR system. The reactor, PN_2, was operated at varying distinct HRTs of 1.6, 2.0, 2.2, and 2.5h, with applied target influent TAN concentrations at 25, 30, 35, and 40 mg TAN/L, corresponding to the identified optimal target TAN SALR of 5 g TAN/m^2·d and airflow rate maintained at 1.5 L/min (Table 3.1). The PN_2 was operated for a period of seven weeks during isolation of the optimal HRT. Triplicated samples were collected across three days and three data points. The identified optimal design HRT of 2h was subsequently applied to isolate the optimum airflow rate.

To confirm the optimal airflow rate that demonstrates optimum PN performance, reactor PN_3 was operated at varying distinct airflow rates of 1.0, 1.5, 2.0, and 4.0 L/min. The reactor was operated at a constant optimal TAN SALR of 5 g TAN/m^2·d and HRT of 2h (Table 3.1). The PN_3 was run for a duration of seven weeks during the isolation of the optimal airflow rate. Once steady-state was reached, three triplicate data points across three days were obtained.

Each design (combination of TAN SALR, HRT, and airflow rate) for a total of twenty-two weeks at each condition was repeated a minimum of three times to enable the study to progress through all the various conditions and verify the repeatability of the generated results. The optimal design of TAN SALR of 5 g TAN/m^2·d, HRT of 2h, and an airflow rate of 1.5 L/min were further operated for an additional 60 days until steady-state was reached, and samples were collected in triplicate and analyzed.

3.4.4 Wastewater feed

The synthetic wastewater was prepared based on the recipe by Delatolla et al., (2009) and modified by Schopf et al., (2019). Synthetic wastewater simulating postcarbon removal municipal wastewater treatment was used in this study. The specific composition of synthetic wastewater at a TAN SALR of 4 g TAN/m^2·d is as follows (per L of synthetic wastewater): 0.12 g $(NH_4^+)_2SO_4$ (corresponding in a concentration of approximately 25 mg NH_4^+-N/L), 0.325 g $NaHCO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, and 0.003 g $FeSO_4 \cdot 7H_2O$. Trace nutrients (per L of synthetic wastewater): $MnCl_2 \cdot 4H_2O$: 0.10 mg, $Na_2MoO_4 \cdot 2H_2O$: 0.03 mg, $CuSO_4 \cdot 5H_2O$: 0.10 mg, $CoCl_2 \cdot 6H_2O$: 0.001 mg, $ZnSO_4 \cdot 7H_2O$: 0.03 mg. The carbon source composition (per L of synthetic wastewater): glucose 4.86 mg, sodium acetate 2.59 mg, and peptone 4.86 mg resulting in a sCOD concentration of 25 mg sCOD/L mimicking postcarbon effluent (Schopf et al., 2019). At TAN SALRs of 5, 6, and 7 g TAN/m^2·d, the wastewater composition for all ingredients was adjusted and augmented proportionally.

3.4.5 Analytical methods

Wastewater influent and effluent samples of each reactor were collected at a minimum of three times per week. Standard methods were used to quantify TAN (Nessler-4500C-NH_3), nitrite (4500B- NO_2^-), and nitrate (4500A- NO_3^-), using a DR 5000 spectrophotometer (HACH, Loveland, CO, USA). The sCOD, total suspended solids (TSS), and volatile suspended solids (VSS) were measured using standards methods (APHA, 1998); HACH 8000, 2540 D-TSS, and 2540 E-VSS, respectively. The DO and temperature were measured using a HACH Flexi HQ30d DO probe meter (HACH, Loveland, CO, USA), and the airflow rate was controlled and measured using a Dwyer VFA -24 Visi-Float acrylic airflow meter (DWYER, Michigan City, IN, USA). The pH was measured using a SympHony VWR pH probe (VWR, Canada, Ontario).

3.4.6 Statistical analyses

The student *t*-test was used to validate statistical significance, with a *p*-value of less than 0.05 considered significant. The correlation between investigated the design parameters was determined using a Spearman's rank correlation test with a *p*-value less than 0.05 indicating significance. Error bars in the figures indicate 95% confidence intervals.

3.5 Results and discussion

3.5.1 Optimal TAN SALR

The findings from the preliminary study informed the operation of the reactor PN_1 at a consistent HRT of 2.0 ± 0.3h, airflow rate of 1.5 ± 0.1 L/min, DO concentration of 6.9 ± 0.1 mg O_2/L, pH of 7.7 ± 0.1 and temperature of 20.2 ± 0.2°C; to identify the TAN SALR 4.0, 5.0, 6.0 and 7.0 g TAN/m^2·d that achieve optimal PN performance in the mainstream elevated loaded PN MBBR system (Figure 3.1). The evaluated TAN SALR 4, 5, and 6 g TAN/m^2·d applied in this study and their respective TAN SARR demonstrate a first-order reaction with respect to the bulk liquid TAN concentration (linear relation between TAN SALR values of 4 and 5 g TAN/m^2·d and TAN SARR of 1.5 ± 0.1 and 2.2 ± 0.1 g TAN/m^2·d) that transitions to a mixed-order reaction (transition between first-order and zero-order reaction at an TAN SALR of 5 g TAN/m^2·d and TAN SARR 2.2 ± 0.1 g TAN/m^2·d) to a zero-order reaction (linear slope of approximately zero-order, between TAN SALR of 5 to 7 g TAN/m^2·d and TAN SARR 2.2 ± 0.1 to 2.4 ± 0.2 g TAN/m^2·d) (Figure 3.1A).

The TAN SARR, 1.5 ± 0.1 g TAN/m^2·d of the system loaded at TAN SALR of 4 g TAN/m^2·d is statistically significantly lower than the TAN SARR of 2.2 ± 0.1 g TAN/m^2·d measured at a TAN SALR of 5 g TAN/m^2·d. This lower TAN SARR at TAN SALR of 4 g TAN/m^2·d, indicates that the kinetics were likely operated at first order with respect to the bulk liquid TAN concentration, as the system is operating at TAN substrate concentration mass transfer rate limited condition. Meanwhile, the TAN SARRs values of 2.2 ± 0.1, 2.2 ± 0.1, and 2.4 ± 0.2 g TAN/m^2·d measured at TAN SALRs of 5, 6, and 7 g TAN/m^2·d, respectively, show no statistically significant difference. The lack of distinction between the kinetics at these varying TAN SALRs of 5, 6, and 7 g TAN/m^2·d indicates that the reactions are zero-order, as the reaction rate no longer increases in relation to TAN SALR values. Hence, the systems transition from TAN substrate concentration mass transfer rate limited while exhibiting first-order reaction relation to a DO concentration mass transfer rate limited zero order reaction as TAN SARR becomes independent of TAN SALR and TAN concentrations (Ehrich et al., 1995).

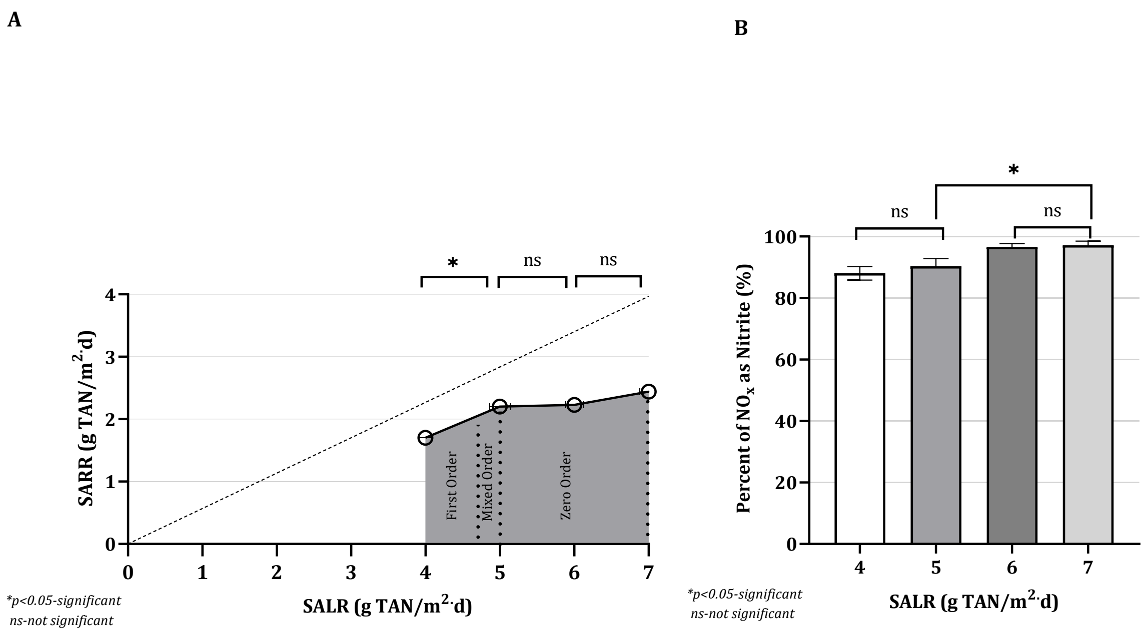


Figure 3.1 PN MBBR performance: **(A)** TAN SARR across TAN SALR, with 56.7% removal (equal to metabolic NH_4^+/NO_2^- ratio) represented with a diagonal dashed line (average ± 95% confidence interval) and kinetics indicated within the dark gray shaded area with vertical dotted lines; **(B)** Percent NO_x as nitrite across TAN SALR (average ± 95% confidence interval).

The dashed line in Figure 3.1a indicates 56.7% TAN oxidation, which approximates the ideal TAN oxidation efficiency required for anammox process based on the theoretical $NH_4^+:NO_2^-$ stoichiometric ratio of 1:1.32 (Strous et al., 1997). An increase in TAN oxidation efficiency from 37.5 ± 0.9 to 45.7 ± 0.6% is shown between the TAN SALRs of 4 and 5 g TAN/$m^{2}\cdot$d. While the TAN oxidation efficiency did not statistically differ between TAN SALRs of 6 and 7 g TAN/$m^{2}\cdot$d; between the TAN SALRs of 5 and 7 g TAN/$m^{2}\cdot$d, the TAN oxidation efficiency decreases from 45.7 ± 0.6 to 33.7 ± 0.2%. The trend of elevated loading rate resulting in the reduction of TAN oxidation efficiency as the kinetics transitions from first order to mixed order and zero-order with respect to bulk TAN concentration is expected in a biofilm technology (Andreottola et al., 2000). The observed trend in TAN oxidation efficiency with elevated loading rate, although identical to previous studies by Schopf et al., (2019), at similar elevated TAN SALR. The TAN oxidation efficiency is not comparable to the values Schopf et al., reported, as the operation conditions, such as the influent TAN concentration and airflow rate, were different. Furthermore, the reduction of TAN oxidation efficiency with increasing TAN SALRs of 5 to 7 g TAN/$m^{2}\cdot$d is possibly an indication that the system remained DO concentration mass transfer limited while performing stable PN as the TAN SALR increases above 5 g TAN/$m^{2}\cdot$d.

The percent NO_x as nitrite did not statistically significantly vary between TAN SALRs of 4 and 5 g TAN/$m^{2}\cdot$d with average values of 88.1 ± 2.1 and 90.3 ± 2.4%, respectively (Figure 3.1B). Comparing TAN SALR of 5 to 7 g TAN/$m^{2}\cdot$d, the percent NO_x as nitrite shows a statistically significant but slight increase ($p=0.009$) to an average value of 94.8 ± 1.4%. Previous studies have inferred that operating at an elevated TAN SALR can potentially suppress the NOB populations or activity due to the preferential uptake of oxygen by the AOB populations, resulting in high nitrite accumulation in the effluent (Daalkhaijav and Nemati, 2014). Moreover, the strong positive correlation (Spearman's correlation coefficient (R_S) $=0.862$) between the elevated TAN SALR and percent NO_x as nitrite in this study is likely an indication that elevated TAN SALR can be an effective and efficient design strategy for achieving PN in an MBBR system under mainstream municipal conditions. These results are comparable to the findings of Schopf et al., (2019), that reported a strong linear relationship between TAN SALR and percent NO_x as nitrite in an MBBR system operated at a high influent TAN concentration. A relation that was used as a design curve for SALR to achieve stable PN in an MBBR system. In contrast, these results are different from the findings of Choi et al., (2018), who operated MBBR systems with sidestream wastewater, in which elevated TAN SALR was reported to be ineffective at maintaining adequate NOB populations and/or activity suppression. The discrepancy in these findings is likely due to varying influent TAN concentrations and operational control strategies which could potentially have various impact on embedded AOB and NOB communities due to diffusion gradients intrinsic to biofilms in MBBR systems. Moreover, investigation of the microbial communities structure in the biofilm across the elevated TAN SALRs under the conventional TAN concentration is recommended to identify the mechanism of PN and to identify whether the high percent NO_x as nitrite measured occurs due to NOB activity suppression and/or NOB community suppression in the microbiome.

Overall assessment of the elevated loaded PN MBBR system shows that the design TAN SALR of 5 g TAN/m^2·d demonstrates stable performance with a TAN SARR of 2.2 ± 0.1 g TAN/m^2·d and percent NO_x as nitrite of 90.3 ± 2.4%. The performance of the elevated loaded mainstream PN MBBR system is comparable to those reported in previous, less stable or unstable mainstream PN MBBR systems that required elevated operation intensity to maintain PN performance (Gu et al., 2012; Kowalski et al., 2019b, 2019a; Piculell et al., 2016a). PN was achieved in these studies with a combination of DO/TAN ratio control and

FA inhibition (Kowalski et al., 2019a, 2019b) and a combination of alternate feeding between mainstream and sidestream with limited biofilm thickness (Piculell et al., 2016a), control strategies of which are operationally intensive. Whereas elevated TAN SALR, a simple and passive design strategy, used in this study, has been herein shown to achieve robust and stable PN performance without operational intervention. This is possible by designing the system as an elevated TAN SALR, with a carrier fill fraction that creates elevated loading operation, hence likely providing multiple morphological benefits to the biofilms that impact the embedded AOB and NOB communities, as shown in Schopf et al. (Schopf et al., 2021, 2019). Ultimately the elevated loading design strategy results in a stable performance of the mainstream PN system. Therefore, it is believed that elevated TAN SALR is a promising design approach to provide high and stable effluent nitrite for subsequent anammox treatment under mainstream municipal conditions.

3.5.2 Optimal HRT

The PN MBBR, PN_2 was operated at distinct HRTs of 1.6, 2.0, 2.2, and 2.5h, at now identified optimal average TAN SALR of 5 g TAN/m^2·d, temperature maintained at 19.5 ± 0.2°C, airflow rate at 1.5 ± 0.1 L/min, DO at 7.0 ± 0.1 mg O_2/L, and pH 7.8 ± 0.2 to verify optimal HRT that maintains optimum TAN SARR and percent NO_x as nitrite (Figure 3.2). An HRT of 1.6h shows the statistically lowest TAN SARR of 1.5 ± 0.1 g TAN/m^2·d and TAN oxidation efficiency of 27.5 ± 0.1% compared to other HRTs of 2.0, 2.2, and 2.5h investigated in this study (Figure 3.2A). The low TAN SARR and TAN oxidation efficiency are likely due to the decrease in AOB populations and/or activity at this short HRT of 1.6h, specifically compared to the typical HRTs of 4h (Schopf et al., 2019) and 12h (Kowalski et al., 2019a) that have been reported in PN MBBR systems.

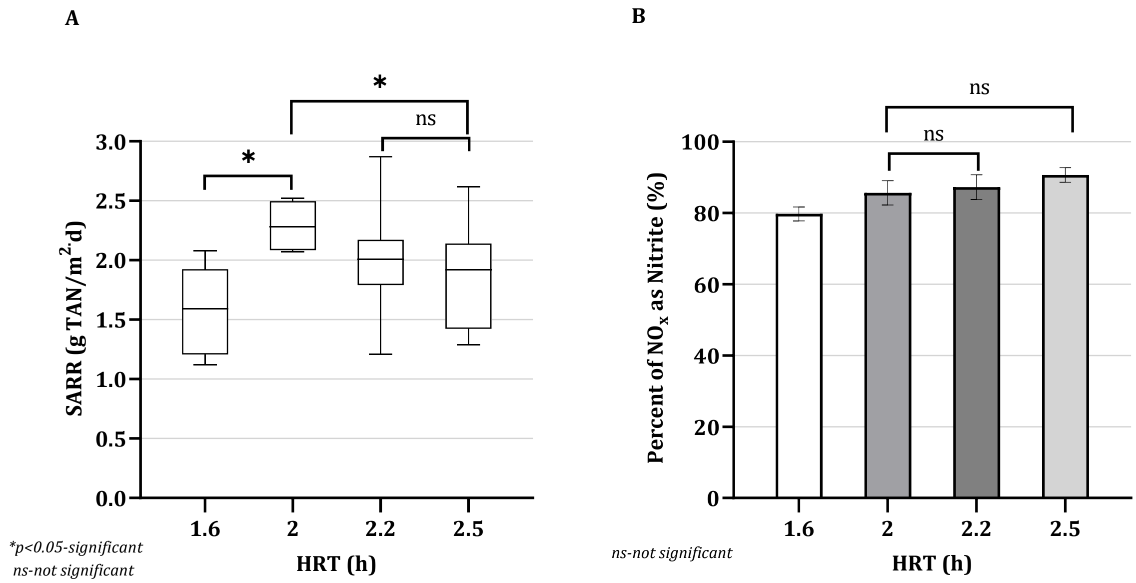


Figure 3.2 PN MBBR performance: **(A)** SARR across HRTs, (**B)** Percent NO_x as nitrite across HRTs, average ± 95% confidence interval.

On the other hand, with the increase in HRT from 1.6 to 2.0h, the TAN SARR and TAN oxidation efficiency significantly increases to 2.4 ± 0.1 g TAN/m^{2}·d and 45.1 ± 0.5%, respectively. Meanwhile, a further shift in HRT to 2.2 and 2.5h shows unstable performance, which is indicated by the large variations in TAN SARR (Figure 3.2A) and TAN oxidation efficiency measurements of 41.4 ± 6.1% and 40.2 ± 7.4%. Comparing the TAN SARR and TAN oxidation efficiency measured values at all investigated HRTs, the HRT of 2h, is observed to be optimal and likely supports a high, stable AOB population or activity in the elevated loaded PN MBBR system (Chen et al., 2019b).

Furthermore, with an increase in HRT from 1.6 to 2h, the percent NO_x as nitrite demonstrates a statistically significant but slight increase (*p=0.03*) from 79.8 ± 1.8 to 85 .7 ± 3.0% (Figure 3.2B). At HRTs of 2, 2.2, and 2.5h, percent NO_x as nitrite did not vary statistically significantly (*$p>0.05$*) from each other, and at these HRT values, the systems demonstrate an average percent NO_x as nitrite of 86.6 ± 1.8%. The statistical similarity and moderate correlation ($R_s=0.463$, $p=0.08$) between increasing HRT and percent NO_x as nitrite for HRTs of 2.0, 2.2, and 2.5h may indicate that operating the MBBR system beyond HRT of 2h does not likely support increased NOB populations or activity suppression (Chen et al., 2018); or the PN MBBR system has reached its limit of NOB suppression achieving an average percent NO_x as nitrite of 88.4 ± 1.3%. Finally, it is important to note that a short HRT of 2h is beneficial with respect to the sizing of the system (resulting in a smaller-sized reactor) and would potentially offer savings on the system's capital cost (De Clippeleir et al., 2011). Therefore, the stable TAN SARR of 2.4 ± 0.1 g TAN/m^2·d and high percent NO_x as nitrite of 85 .7± 3.0% at an HRT of 2h shows that a PN MBBR system can be operated within a small tank volume to achieve stable PN performance.

3.5.3 Optimal airflow rate

The PN MBBR reactor PN_3 based on the optimal isolated TAN SALR and HRT, was operated at distinct varying airflow rates of 1, 1.5, 2, and 4 L/min, measured TAN SALR at 5 g TAN/m^2·d, HRT 2h, temperature 19.8 ± 0.1°C, and pH 7.8 ± 0.1 to validate the optimum airflow rate of the mainstream elevated loaded PN MBBR system (Figure 3.3). As the airflow rate increases from 1.0 to 1.5 and to 2.0 L/min, the average TAN SARR & TAN oxidation efficiency were 1.9 ± 0.2 g TAN/m^2·d & 37.4 ± 0.2%, 2.3 ± 0.1 g TAN/m^2·d & 43.6 ± 0.6% , and 2.2 ± 0.1 g TAN/m^2·d & 40.2 ± 0.2%, respectively (Figure 3.3A). As the steady incremental airflow rate of 0.5 L/min did not show an observable statistically significant difference, the airflow rate was increased further to 4 L/min. With the change in airflow rate from 2 to 4 L/min, the TAN SARR significantly increases to 3.2 ± 0.2 g TAN/m^2·d, corresponding to a TAN oxidation efficiency of 63.8 ± 0.2%. Furthermore, the increase in airflow rate strongly correlates with an increase in TAN SARR and TAN oxidation efficiency (*R_s=0.9532*), highlighting the sensitivity of nitrifying communities to changes in DO concentration on the biofilm surface, driven by increased oxygen penetration depths leading to a higher rate of oxygen consumption within the biofilm (Rusten et al., 1995; WEF, 2011).

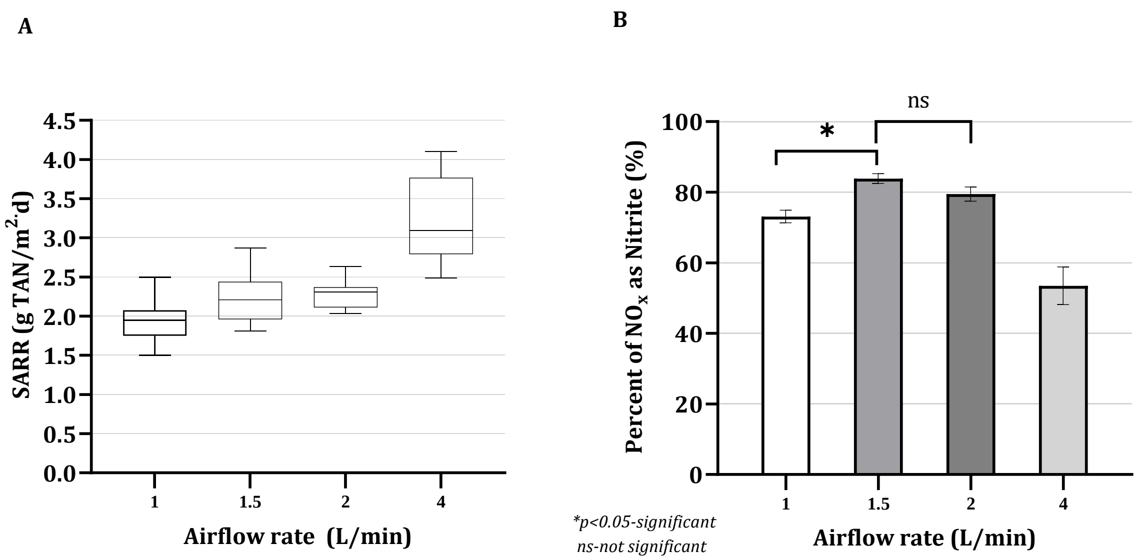


Figure 3.3 PN MBBR performance: **(A)** SARR across airflow rates, **(B)** percent NO_x as nitrite across airflow rates, average ± 95% confidence interval.

The percent NO_x as nitrite shows a statistically significant increase from 73.1 ± 1.3 to 84.3 ± 1.4%, with a change in airflow rate from 1 to 1.5 L/min. However, as the airflow rate increases from 1.5 to 2 and 4 L/min, the percent NO_x as nitrite decreases from 84.3 ± 1.4 to 79.5 ± 1.9 and 53.5 ± 5.0% (Figure 3.3B). On the other hand, the bulk DO concentration in the reactor across the airflow rates of 1.5, 2.0, and 4.0 L/min did not statistically differ, demonstrating an average DO concentration value of 6.9 ± 0.1 mg O_2/L. It is expected that with the increase in airflow rate above 1.5 L/min, the system is gradually transitioning from PN to complete nitrification. Moreover, with an increased airflow rate, the mass transfer of oxygen from the bulk solution to the biofilm surface improves, subsequently promoting deeper DO penetration through the biofilm to the embedded biomass (Rittmann and McCarty, 2001; Taherzadeh et al., 2012). Therefore, it is possible that the microbial community has shifted, and a significant quantity of NOB cells has accumulated in the system with the shift in airflow rate from 2 to 4 L/min, initiating greater oxidation of nitrite to nitrate (~49.5 ± 1.4%).

Generally, it is believed that NOB populations are more sensitive to DO than AOB as they have a higher oxygen half-saturation coefficient (K_o) than AOB (Picioreanu et al., 1997). Hence, high DO concentrations or oxygen-enriched conditions can provide NOB with a competitive advantage over AOB and possibly promote their proliferation or activity in the system. While Chen et al., (2019b), attributed an unchanged bulk DO concentration with an increased airflow rate to a likely increase in utilization by AOB populations, there was still a possible NOB role, as *Nitrospira* genera were detected in the reactors with an approximate relative abundance of 1.9% (Chen et al., 2019b). Therefore, although the bulk liquid DO concentration remained unchanged in this study, the increase in oxygen supply with an increased airflow rate greater than 1.5 L/min possibly results in a greater oxygen uptake rate of the reactor, thus accelerating the activity and/or population of NOB.

This study hence identifies the optimal design TAN SALR, HRT, and airflow rates of 5 g TAN/m^2·d, 2h, and 1.5 L/min, respectively, for the mainstream elevated loaded PN MBBR system. The overall performance of the optimized low-operational intensive PN MBBR system operated at a temperature of 19.8 ± 0.3°C, DO of 6.9 ± 0.4 mg O_2/L, and pH of 7.7 ± 0.2 is shown in Table 3.2. This study shows that PN is feasible using an elevated TAN SALR as a design strategy under mainstream conditions. Elevated TAN SALR as a passive and simple design strategy provides a stable PN system with no additional operational control measures. The successful implementation of elevated TAN SALR, a design strategy could advance current efforts to identify low operational intensity and effective PN control strategies in mainstream municipal wastewaters where NOB populations and/or activity suppression have been reported with multiple and complex control strategies resulting in high operational intensive systems (Delgado Vela et al., 2015). Therefore, this result provides insight into the design of a promising robust, reliable, and low operational intensity PN MBBR system for the treatment of mainstream municipal wastewaters.

Table 3.2 Performance of the mainstream elevated loaded PN MBBR system (average ± 95% confidence interval).

	Influent	Effluent
TAN concentration (mg TAN/L)	32.5 ± 2.2	18.1 ± 0.3
Nitrite concentration (mg NO_2^--N/L)	0.2 ± 0.1	10.9 ± 0.3
Nitrate concentration (mg NO_3^--N /L)	B/LOQ	1.4 ± 0.4
COD (mg/L)	27.6 ± 1.8	18.7 ± 0.8
Alkalinity (mg/L $CaCO_3$)	311.3 ± 13.8	213.5 ± 7.5

**B/LOQ*-Below the limit of quantification

3.6 Conclusions

This study identifies optimal TAN SALR, HRT, and airflow rates that achieve optimum TAN removal kinetics and percent NO_x as nitrite for stable PN performance of the mainstream elevated loaded PN MBBR system. The increase in TAN SALR significantly affects the TAN SARR and percent NO_x as nitrite of the mainstream elevated loaded PN MBBR system. The optimal TAN SALR of 5 g TAN/m^2·d results in a TAN SARR of 2.2 ± 0.1 g TAN/m^2·d and percent NO_x as nitrite of 90.3 ± 2.3%. Also, a change in HRT from 1.6 to 2h shows to significantly affect SARR and percent NO_x as nitrite. At the same time, a further increase in HRT from 2.2 to 2.5h demonstrated an unstable TAN SARR with no significant observable change in percent NO_x as nitrite of the elevated loaded PN MBBR system. The optimal and short HRT of 2.0h shows a stable PN performance with an average TAN SARR of 2.4 ± 0.1 g TAN/m^2·d and a percent of NO_x as nitrite of 85.7 ± 3.0%. With an increase in airflow rate from 1.0 to 4.0 L/min, the TAN SARR increases from 1.9 ± 0.2 to 3.2 ± 0.2 g TAN/m^2·d while the percent of NO_x as nitrite decreases from 84.8 ± 1.4 to 53.5 ± 5.0%, as the systems gradually transitions from PN to complete nitrification. The MBBR system demonstrates PN at an airflow rate of 1.5 L/min with TAN SARR of 2.3 ± 0.3 g TAN/m^2·d and a percent of NO_x as nitrite of 84.3 ± 1.4%. The optimal isolated design parameters, TAN SALR of 5 g TAN/m^2·d, HRT 2h and airflow rates of 1.5 L/min demonstrate a stable PN performance with TAN SARR of 2.3 ± 0.3 g TAN/m^2·d, and a percent of NO_x as nitrite of 84.8 ± 1.2%. The results herein present the optimal design parameters of the mainstream elevated loaded PN MBBR system. The study provides a possible direct design pathway for implementing a low operational intensity PN control strategy that would provide stable effluent quality, small footprint, robust and high rate PN at mainstream municipal wastewaters.

3.7 Supplementary Information

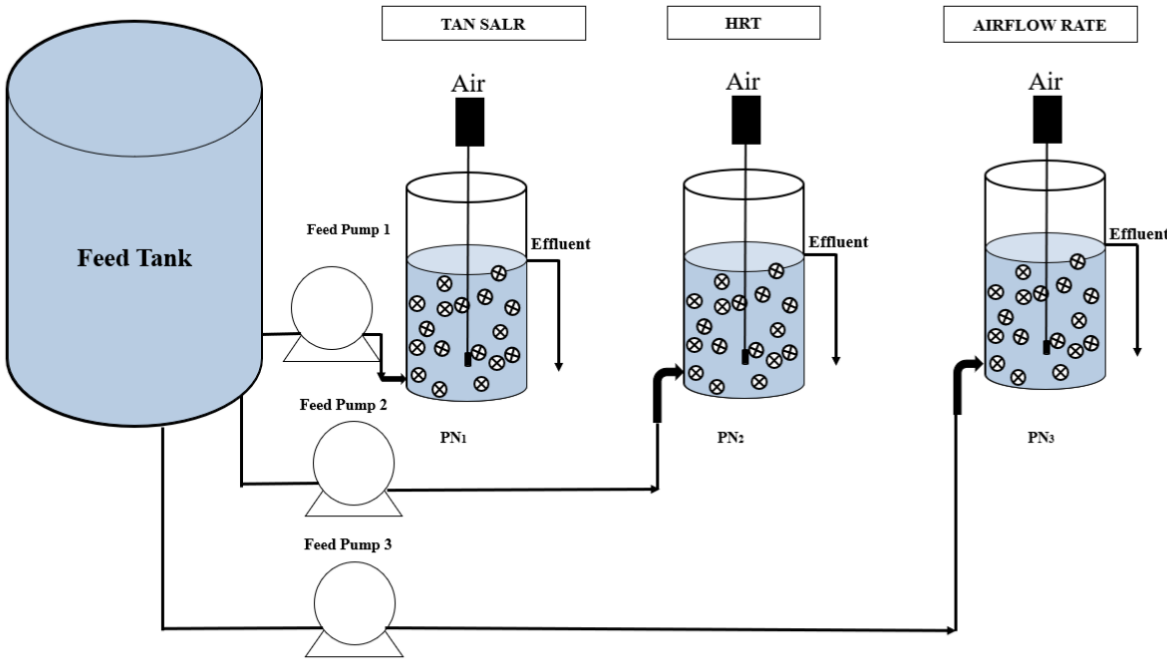
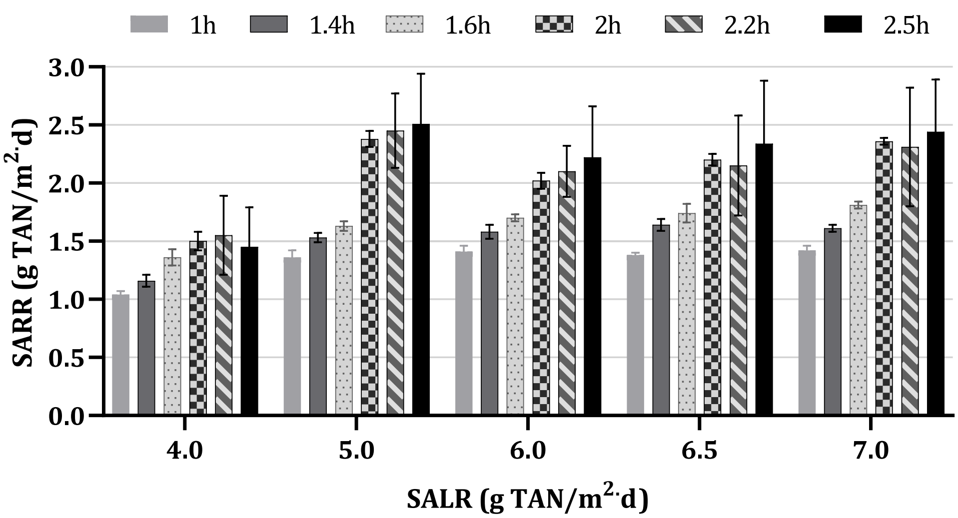


Figure S3.1 Schematic of a low operational intensity MBBR PN system. Each reactor: PN_1, PN_2 and PN_3 testing and isolating one primary condition: TAN SALR, HRT, & airflow, respectively.

A



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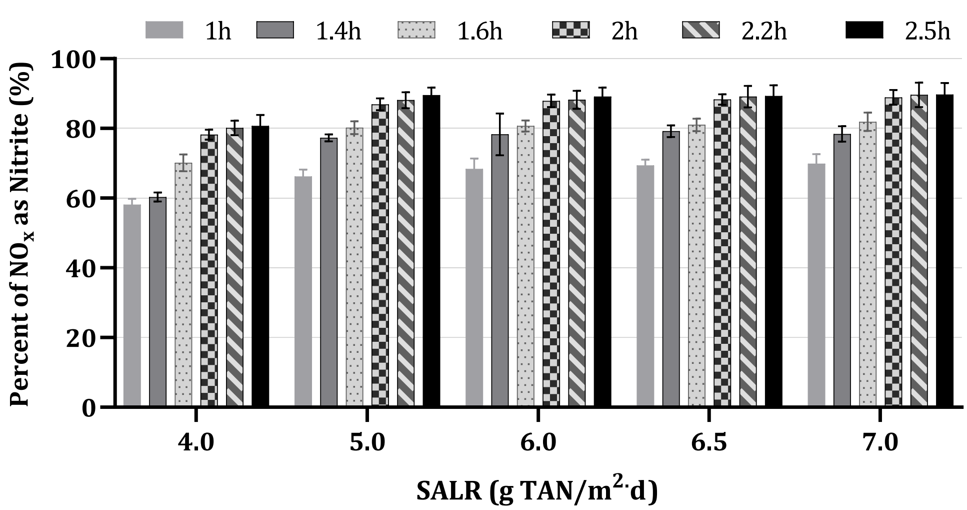
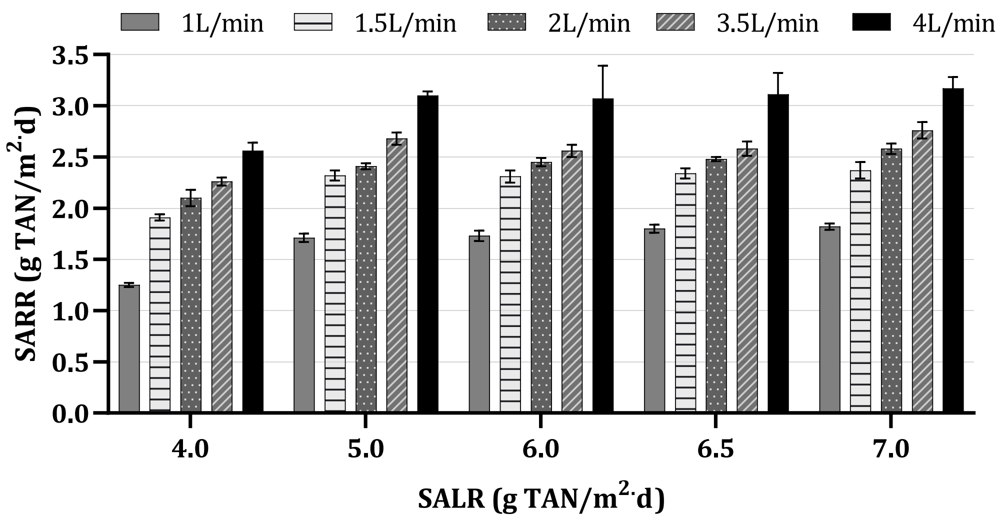


Figure S3.2 PN MBBR performance; **(A)** TAN SALRs across SARR, **(B)** TAN SALRs across percent NO_x as nitrite, at HRT of 1h (Light grey), 1.4h (dark grey), 1.6 (dotted pattern), 2 (dark pattern) 2.2 (diagonal shading) and 2.5 (black solid fill) (average ± 95% confidence interval.

A



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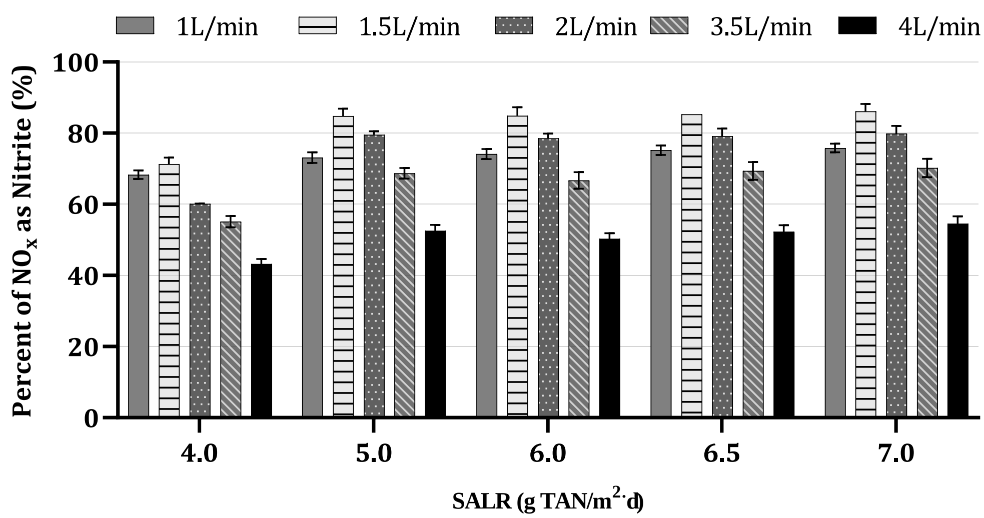

Figure S3.3 PN MBBR performance; **(A)** TAN SALRs across SARR, **(B)** TAN SALRs across percent NO_x as nitrite, at airflow rates of 1L/min (Light grey), 1.5L/min (horizontal shading), 2L/min (dotted pattern), 3.5L/min (diagonal shading) and 4L/min (black solid fill) (average ± 95% confidence interval).

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CHAPTER 4 - DESIGN STRATEGY AND MECHANISM OF NITRITE OXIDATION SUPPRESSION OF ELEVATED LOADING RATE PARTIAL NITRITATION SYSTEM

4.1 Context

Chapter 4 presents the published article titled *design strategy and mechanism of nitrite oxidation suppression of elevated loading rate partial nitritation system by* Ikem, J., Chen, H. and Delatolla, R. (Frontiers in Microbiology, 2023 14:1142570). This study provides a new design configuration of the mainstream elevated loaded partial nitritation MBBR system to achieve appropriate effluent stoichiometric ratio for subsequent downstream anammox treatment. The study further identifies the mechanism of nitrite oxidation suppression of mainstream elevated loaded partial nitritation MBBR systems.

4.2 Abstract

There is a current need for a low operational intensity, effective, and small footprint system to achieve stable partial nitritation for subsequent anammox treatment at mainstream municipal wastewaters. This research identifies a unique design strategy using an elevated total ammonia nitrogen (TAN) surface area loading rate (SALR) of 5 g TAN/m^2·d to achieve cost-effective, stable, and elevated rates of partial nitritation in a moving bed biofilm reactor (MBBR) system under mainstream conditions. The elevated loaded partial nitritation MBBR system achieves a TAN surface area removal rate (SARR) of 2.01 ± 0.07 g TAN/m^2·d and NO_2^--N: NH_4^+-N stoichiometric ratio of 1.15:1, which is appropriate for downstream anammox treatment. The elevated TAN SALR design strategy promotes nitrite-oxidizing bacteria (NOB) activity suppression rather than a reduction in NOB population as the reason for the suppression of nitrite oxidation in the mainstream elevated loaded partial

nitritation MBBR system. NOB activity is limited at an elevated TAN SALR likely due to thick biofilm embedding the NOB population and competition for dissolved oxygen (DO) with ammonia-oxidizing bacteria for TAN oxidation to nitrite within the biofilm structure, which ultimately limits the uptake of DO by NOB in the system. Therefore, this design strategy offers a cost-effective and efficient alternative for mainstream partial nitritation MBBR systems at water resource recovery facilities.

4.3 Introduction

Partial nitritation and anaerobic ammonia oxidation (anammox), known as the PN/A process, is an autotrophic nitrogen removal process based on two consecutive processes: ammonia-oxidizing bacteria (AOB) oxidizing about 55% of the total ammonia nitrogen (TAN) to nitrite, and subsequently, anammox bacteria (AnAOB) converting the produced nitrite and residual TAN to nitrogen gas with limited nitrate production. Compared to conventional nitrification and denitrification, PN/A is more cost-effective and energy-efficient and could significantly reduce the energy consumption of water resource recovery facilities (WRRFs) by 20-40% (Agrawal et al., 2018; Pedrouso et al., 2019). Over the past decade, the PN/A process has been successfully implemented to treat sludge digester centrate, also termed "sidestream wastewaters," within municipal WRRFs (Lackner et al., 2014; Lotti et al., 2015b). In recent years, there has been renewed interest in exploring the PN/A process at conditions more typical for mainstream municipal wastewater. However, mainstream municipal wastewaters are characterized by low TAN concentrations (20-40 mg TAN/L), high C/N ratios (7-12 g COD/g-N), variable TAN loading rates, and low temperatures (<10^{o}C), with these characteristics limiting the successful implementation of the PN/A process at WRRFs. These mainstream wastewater characteristics have been demonstrated to limit AOB and AnAOB growth rates and also increase the challenge of achieving effective nitrite oxidation suppression; which is necessary to maintain ideal effluent quality and long-term process stability in mainstream PN/A systems (Gilbert et al., 2015; Hoekstra et al., 2019; Li et al., 2018).

Long-term nitrite oxidation suppression in mainstream PN/A systems has been investigated using a one-stage system, where partial nitritation and anammox occur in the same reactor, or a two-stage system with partial nitritation and anammox occurring in separate reactors (Chen et al., 2018; Choi and Jung, 2022; Gu et al., 2018a; Gustavsson et al., 2020; Laureni et al., 2016; Schraa et al., 2020; Trojanowicz et al., 2021). While the one-stage configured systems are mostly studied due to the lower cost of infrastructure than two-stage configured systems, the latter offers the opportunity to optimize the stable partial nitritation and anammox process independently and maintain a balanced ratio between functional bacteria groups (AOB and AnAOB) to achieve higher volumetric nitrogen removal rates.

In a two-stage configured system, optimization of the partial nitritation process requires effective operational control strategies to ensure that the nitrite-oxidizing bacteria (NOB) population or activity is continuously suppressed within the system (Choi and Jung, 2022; Gu et al., 2018a; Piculell et al., 2016a). In this regard, several operational control strategies have been employed in a two-stage configured system involving either suspended growth or attached growth systems to achieve stable partial nitritation. Recently, research has focused on hybrid system where attached growth biofilms and flocs coexist, also known as integrated fixed-film activated sludge (IFAS) systems, and biofilm technologies such as the moving bed biofilm reactor (MBBR) system (Gu et al., 2018a; Gustavsson et al., 2020;

Kowalski et al., 2019a; Laureni et al., 2016, 2019; Trojanowicz et al., 2021). Recent interest in biofilm technologies is due to their ability to retain highly specialized microbial populations and also because biofilm structure results in substrate gradients that can promote suppression of the growth of the NOB population or suppression of the NOB activity (Brockmann and Morgenroth, 2008; Gilbert et al., 2014; Lotti et al., 2015a; Pérez et al., 2014; Piculell et al., 2016a).

Several operational control strategies have been applied using the MBBR technology to selectively inhibit NOB population or activity. Some of the operational control strategies that have been reported in mainstream partial nitritation two-stage MBBR systems include: alternating feed from sidestream to mainstream; bioaugmentating with AOB biomass; controlling biofilm thickness; exposure of biomass to toxic sidestream effluent or conditions favorable for AOB growth; employing a combination of DO/TAN ratio control and FA inhibition; and alternating anoxic and aerobic condition through intermittent aeration. Notwithstanding the potential of NOB suppression using these operational control strategies, these strategies are all operationally intensive and have demonstrated difficulty in achieving long-term suppression of NOB population or activity, overall process stability, and stable effluent quality.

In recent studies, an elevated TAN loading rate, as a means of a passive and low operational design strategy, has been shown to achieve stable partial nitritation rates within a two-stage configured MBBR system (Ikem et al., 2023; Schopf et al., 2021, 2019). Schopf et al., achieved stable and robust partial nitritation at an elevated TAN loading rate of 6.5 g TAN/m^2·d in an MBBR system fed with TAN concentration of 125 mg TAN/L, simulating TAN concentrations observed in industrial wastewaters. In this system, stable partial nitritation was attributed to the morphological impacts resulting from operating at an elevated TAN loading rate that could allow for NOB activity to be effectively suppressed (Schopf et al., 2021). Consequently, the feasibility of using this design strategy to achieve stable partial nitritation has been evaluated at conditions and TAN concentrations between 25 to 44 mg TAN/L typical for mainstream municipal wastewater (Ikem et al., 2023). Ikem et al. identified critical optimum design parameters, a TAN surface area loading rate (SALR), a hydraulic retention time (HRT), and an airflow rate optimized for stable partial nitritation of a mainstream elevated loaded MBBR system. However, this system did not achieve the ideal effluent NO_2^--N:NH_4^+-N stoichiometric ratio of 1.31:1 for subsequent anammox treatment. Specifically, the average NO_2^--N:NH_4^+-N stoichiometric ratio reported in this study was at 0.70:1, which is not comparable to the optimized ratio proposed by Strous et al., (1998) for anammox operation. The operation of anammox system with inappropriate effluent stoichiometric ratio from the partial nitritation system has been demonstrated to impact the attachment and enrichment of anammox cells in a full-scale MBBR, leading to process instability (Stefansdottir et al., 2015). Hence, further optimization of the elevated loaded partial nitritation MBBR system is required to improve effluent quality for downstream anammox operation. Moreover, further investigation of biofilm characteristics, embedded cells, and microbiome response is needed to understand nitrite oxidation suppression mechanism responsible for stable partial nitritation of mainstream elevated loaded MBBR systems. Although previous work has investigated the mechanism and method of nitrite oxidation suppression caused by employing elevated TAN loading rate for partial nitritation control, these studies were performed at higher influent TAN concentrations not typically observed in mainstream municipal wastewater (Schopf et al., 2021, 2019). Thus, there is a significant knowledge gap and a need to investigate further the mechanism of nitrite oxidation suppression in the elevated loaded partial nitritation system that would ensure high performance and long-term operational stability under mainstream conditions.

Therefore, this study aims to identify the mechanism of nitrite oxidation suppression of mainstream elevated loaded partial nitritation MBBR systems. The specific objectives were to (i) characterize the performance of a two-reactor in series designed mainstream elevated loaded partial nitritation MBBR system; (ii) determine the effects of elevated TAN loading rate on biofilm thickness, biofilm mass, biofilm density, and embedded cells, and how these characteristics influence the performance of the mainstream partial nitritation MBBR system; and (iii) quantitate the AOB and NOB population counts within the attached growth, biofilm community and identify whether NOB population suppression or NOB activity suppression is responsible for partial nitritation.

4.4 Materials and methods

4.4.1 Reactor configuration and operation

Two reactors in series partial nitritation MBBR system configuration were identified in the preliminary work of this study based on findings from Ikem *et al.,* to optimize the effluent concentration from the partial nitritation MBBR system for subsequent downstream anammox treatment (Ikem et al., 2023). The experimental set-up consisted of two identical lab-scale MBBR reactors (2 L each) operated in series (Figure 1). The reactors were filled with high-density polyethylene AnoxK^{TM}5 carriers (AnoxKaldnes, Lund, Sweden) with a diameter of 25 mm, height of 4 mm, and bulk surface area of 800 m^2/m^3. The AnoxK™5 were seeded carriers harvested from a biological oxygen demand (BOD) removal municipal integrated film-activated sludge (IFAS) wastewater treatment system located in Hawkesbury, Ontario, Canada, and were operated in a single bench lab partial nitritation MBBR system operated at elevated TAN concentrations prior to this study. The reactors were designed at a target TAN SALR of 5 g TAN/m^2·d, recommended as the optimum for the partial nitritation MBBR system based on the previous findings, as it demonstrated stable and steady performance as well as high partial nitritation rate (Ikem et al., 2023; Schopf et al., 2019). The TAN SALR in the nitrifying MBBR systems at ambient temperature are conventionally designed typically between 0.45 to 1.0 g TAN/m^2·d with respect to target effluent TAN concentrations (Hem et al., 1994; Odegaard, 1999; Young et al., 2017b); thus, the TAN SALR present in this study was significantly elevated (referred to as elevated TAN SALR) than conventional TAN SALR which would potentially allow operating high-rate partial nitritation and small land footprint system. The elevated TAN SALR of 5 g TAN/m^2·d in the partial nitritation reactors was achieved at mainstream concentrations through operation at relatively short target HRT of 2h and low carrier fill fraction, at 6% and 9.5%. The first reactor, denoted P_{N1}, contained 39 carriers, and the second reactor P_{N2}, contained 28 carriers. The carriers accounted for 9.5% and 6% of the total reactor volume, respectively. These different fractions were necessary to operate both reactors at similar elevated TAN SALR values of 5 g TAN/m^2·d, as P_{N2} was fed with the effluent of P_{N1} and hence required fewer carriers to achieve the same SALR as P_{N1}. For clarity and easy understanding of this manuscript, the reactors in a series of P_{N1} and P_{N2} will be referred to as $P_{N1\text{-}N2}$.

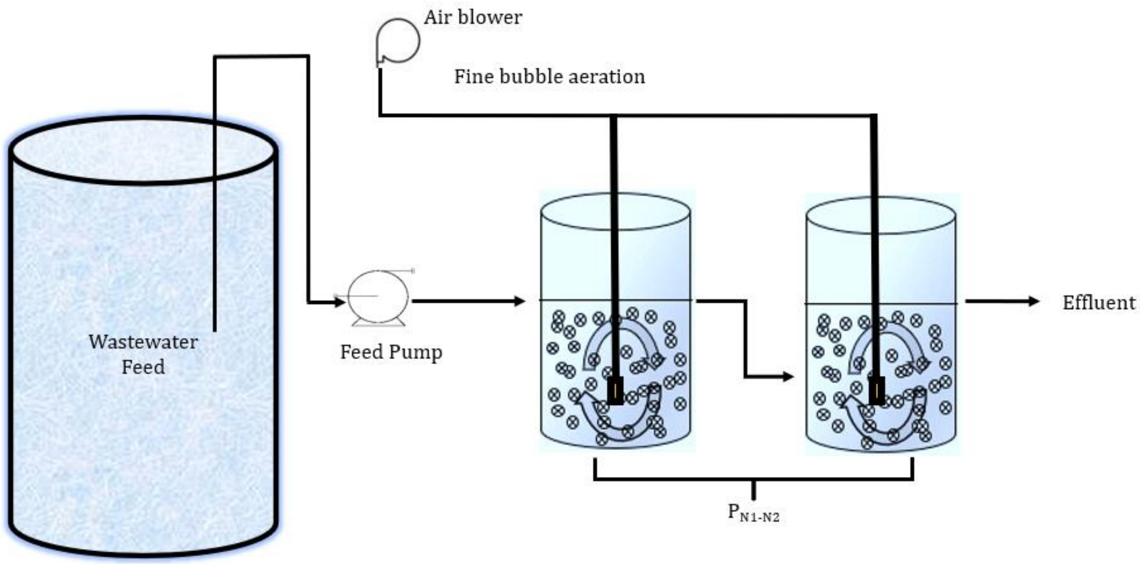

Figure 4.1 Experimental set-up.

P_{N1} and P_{N2} were both equipped with a coarse bubble aeration system, with diffusers positioned at the base of the reactors to provide adequate DO in the bulk solution and allow sufficient mixing of the carriers. The airflow rates were maintained at 1.5 and 1 L/min for P_{N1} and P_{N2}, respectively, as previous findings demonstrated that adjusting airflow above 1.5 L/min resulted in increased nitrite oxidation to nitrate (Ikem et al., 2023). Moreover, P_{N2} had lower influent TAN concentration and fewer carriers than P_{N1}; as such would necessitate a reduced airflow rate to limit nitrite oxidation. P_{N1} was fed with a peristaltic pump with influent from a wastewater feed tank, and P_{N2} was fed via gravitational flow with the effluent from P_{N1}. The target (measured) influent TAN concentrations were 30 (32.16 ± 0.46) mg TAN/L for P_{N1} and 20 (20.4 ± 1.54) mg TAN/L for P_{N2}, all within the conventional limits of mainstream municipal TAN concentrations. The operational conditions for both reactors were the same with the following target (measured) values: SALR of 5 (5.07 ± 0.22) g TAN/m^2·d, HRT of 2 (2.02) h, DO of 6.5 (6.84 ± 0.05) mg O_2 /L, pH of 7.5 (7.76 ± 0.05), and temperature 20 (19.9 ± 0.10)^{o}C.

4.4.2 Wastewater feed

Synthetic wastewater (SWW) was prepared based on a recipe from previous studies (Delatolla et al., 2009; Schopf et al., 2019; Tian et al., 2019). The SWW simulated wastewater effluent from a carbon removal process without TAN removal and was composed as follows (per L of SWW): 0.14 g $(NH_4^+)_2SO_4$ (corresponding to a concentration of approximately 30 mg NH_4^+-N/L), 0.39 g $NaHCO_3$, 0.06 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.06 g KH_2PO_4, and 0.004 g $FeSO_4 \cdot 7H_2O$. Trace nutrients (per L of SWW) included: $MnCl_2 \cdot 4H_2O$: 0.10 mg, $Na_2MoO_4 \cdot 2H_2O$: 0.03 mg, $CuSO_4 \cdot 5H_2O$: 0.10 mg, $CoCl_2 \cdot 6H_2O$: 0.001 mg, and $ZnSO_4 \cdot 7H_2O$: 0.03 mg. The carbon source composition (per L of synthetic wastewater): glucose 4.86 mg, sodium acetate 2.59 mg, and peptone 4.86 mg resulted in a sCOD concentration of 25 mg sCOD/L, thereby mimicking post-carbon effluent (Schopf et al., 2019).

4.4.3 Constituent analyses

Wastewater constituent analyses were performed on each of the two MBBR reactors in series, P_{N1} and P_{N2}. Wastewater samples were collected three times a week from both reactors for analyses, and the samples were tested in triplicate for the following parameters: TAN, nitrite, nitrate, DO, sCOD, pH, temperature, alkalinity, total suspended solids (TSS), and volatile suspended solids (VSS). To quantify the kinetics of the reactors, the following standard methods were used: Nessler-4500C-NH_3, 4500B- NO_2^- and nitrate 4500A- NO_3^- for TAN, nitrite, and nitrate, respectively. DO and temperature measurements were acquired using a HACH Flexi HQ30d DO/temperature probe (HACH, Loveland, CO, USA) and pH was measured using a SympHony VWR pH probe (VWR, Ontario, Canada). sCOD was quantified using a HACH 8000 (HACH, Loveland, CO, USA), and TSS and VSS were measured using standard methods 2540D (Clesceri et al., 1998). In addition, the aeration rates were monitored and measured using a Dwyer VFA-24 Visi-Float® acrylic airflow meter (DWYER, Michigan City, IN, USA).

4.4.4 Biofilm morphology, thickness, and mass

Stereomicroscopy was used to acquire images of in-situ biofilm thickness and morphology. Stereomicroscopy was used because it does not require any sample preparation as such maximizes the integrity of the sample and artifact creation. Four carriers per reactor were extracted and analyzed within four hours of being harvested. Five images per carrier were acquired at ×2 magnification and were used to determine biofilm thickness. Also, one image per carrier was acquired and analyzed at ×4 magnification to assess the biofilm surface morphology. All of these images were taken at random locations across the carrier surface to avoid bias. Finally, two images per carrier were obtained at ×0.8 magnification to verify biofilm attachment across the carriers. The biofilm thicknesses were quantified using ImageJ 1.52a image processing software (Wayne Rasband, USA).

Biofilm mass was measured using a protocol described and modified by (Arabgol et al., 2020; Delatolla et al., 2008; Ren et al., 2016; Schopf et al., 2018). Biofilm carriers were harvested from MBBR reactors P_{N1} and P_{N2} and dried at 105ºC overnight. Dried carriers were cooled in a desiccator for a minimum of 30 minutes, after which their weights were recorded as W_1. The dried carriers were then thoroughly cleaned with a stiff-bristled brush and warm water and were dried again at 105ºC overnight. The dried carriers were cooled for another

30 minutes in a desiccator, and their weights were recorded as W_2. The biofilm mass was calculated as the difference between W_1 and W_2. Prior testing was done to ensure that biofilm removal and heating did not cause any significant change in the mass of the biofilm carrier.

4.4.5 Cell viability

Biofilm carriers were harvested from each reactor P_{N1} and P_{N2} and cut into sections to expose the inner biofilm surfaces. The sections were stained using a Film Tracer LIVE/DEAD biofilm viability kit (Life Technologies Ontario, Canada), which comprises SYTO 9 stain (a green nucleic acid stain that illuminates the intact cell membrane) and propidium iodide, which only stains cells with damaged cell membranes (i.e., non-viable cells). Calcofluor white stain (Sigma-Aldrich, MO, USA) was used to fluoresce biofilm extracellular polymeric substances (EPS) (Chen et al., 2007). Viable and non-viable embedded cells in the biofilm were observed using a 510/AxioImager confocal laser scanning microscope (CLSM) (Zeiss, VA, USA) equipped with argon and helium-neon lasers with a variety of wavelengths. A minimum of 20 images were acquired at various depths across the entire biofilm support media. Cell viability based on the CLSM images was determined using NI Vision Assistant 8.0 software (National Instruments, LabView, 2018). The biofilm area was determined and outlined by tracing the calcofluor white stain. The image colour threshold on the CLSM images was used to calibrate the area of an identifiable single cell. The standardized images were then used to quantify the biofilm area and the area of viable & non-viable cells (Ahmed and Delatolla, 2021; Delatolla et al., 2009; Ren et al., 2016).

4.4.6 Quantification of AOB and NOB

DNA extraction

AOB and NOB cell counts were determined using droplet digital PCR (ddPCR). Two carriers each, from reactors P_{N1} and $P_{N2,}$ were collected, and genomic DNA from their biofilms was extracted using a FastDNA*Spin Kit for Soil (MP Biomedicals, CA, USA). The DNA concentrations were analyzed using an Invitrogen Qubit™ 3.0 Fluorometer. Extracted DNA was stored at -80°C until ddPCR analysis.

ddPCR analyses

AOB cell counts were quantified by targeting ammonia monooxygenase subunit A (amoA). The set of amoA primers (*amoA*-1f/ *amoA*-2r) targeted a stretch of conserved regions of the known amoA gene sequence of *Nitrosomonas europaea*. Also, two coupled primers, FGPS872f/FGPS1269r and NSR1113f/NSR1264r, were used to amplify the NOB, *Nitrobacter,* and *Nitrospria,* respectively (Table 1) (Degrange and Bardin, 1995; Geets et al., 2007; Rotthauwe et al., 1997). The ddPCR mixture was constituted using 5 µL of sample and 11 µL of QX200™ ddPCR™ EvaGreen*Supermix (Bio-Ras, Hercules, CA), including 0.23 µL of each forward and reverse primer (10mol/L) and 6.04 µL of nuclease-free water. Each ddPCR analysis was performed using a 20 µL reaction mixture combined with 65 µL of droplet generation oil for EvaGreen to form droplets using a droplet generator. Using a multi-channelled pipette, 40 µL of generated droplets were carefully transferred into a 96 well ddPCR plate (Eppendorf, Hamburg, Germany). The plate was sealed using a plate sealer machine and transferred into a T100™ thermal cycler (Bio-Rad, Hercules, CA) for DNA amplification. The amplification program consisted of the following steps: denaturation at 95°C for 5 minutes, followed by 50 cycles of 30 seconds at 95°C, 60 seconds at the corresponding primer annealing temperature (Table 4.1), and 30 seconds at 72°C, followed by a 5 min cooldown at 4°C, 5 minutes at 90°C for droplet stabilization and a hold at 12°C. The QX200 droplet reader (Bio-Rad, Hercules, CA) was applied, and the results were analyzed using QuantaSoft Software (Bio-Rad, version 1.7.4, Hercules. CA). The software measures the number of positive and negative droplets per fluorophore per sample, with each positive counted as a 1 and each negative counted as a 0. The quality control measure ensured that the total droplets quantified were > 10,000, and < 5 positive droplets were identified as negative controls (Tian et al., 2020).

Table 4.1. Primer details and annealing temperatures.

Target	Primer	Sequence (5'-3')	Annealing Temperature (°C)	Reference
AOB	amoA-1f	GGGGTTTCTACTGGTGGT	53	(Rotthauwe et al. 1997)
	amoA-2r	CCCCTCKGSAAAGCCTTCTTC		
NOB - *Nitrobacter*	FGPS872f	CTAAAACTCAAAGGAATTGA	56	(Degrange and Bardin, 1995)
	FGPS1269r	TTTTTTGAGATTTGCTAG		
NOB - *Nitrospira*	NSR1113f	CCTGCTTTCAGTTGCTACCG	53	((Bao et al., 2017)
	NSR1264r	GTTTGCAGCGCTTTGTACCG		

4.4.7 Statistical analyses

Statistical analyses of wastewater constituents, biofilm thickness, mass and viable/non-viable cell percentages were determined using the student's t-test, with a *p*-value less than 0.05 indicating a significant difference. In addition, the student's t-test was used to ascertain statistical significance for the bacterial gene copies with a *p*-value of less than 0.10 considered to indicate a statistically significant difference. Graphical charts and bars were prepared using GraphPad Prism 8.4.1, with error bars in figures being plotted at the 95% confidence intervals.

4.5 Results and discussion

4.5.1 Performance of a two-reactor in series partial nitritation MBBR system

The partial nitritation MBBR system, both P_{N1} and P_{N2}, was operated at a target elevated TAN SALR of 5 g TAN/m^{2}·d and showed steady and stable performance over a 90-day period of operation (Figure 4.2), with measured SALR values for P_{N1} and P_{N2} 5.07 ± 0.22 and 5.05 ± 0.12 g TAN/m^{2}·d respectively. The average TAN surface area removal rate (SARR) of the first reactor, $P_{N1,}$ is 2.25 ± 0.08 g TAN/m^{2}·d, corresponding to a TAN oxidation efficiency of 45.3 ± 1.1%. The resultant TAN SARR of 2.01 ± 0.07 g TAN/m^{2}·d from the complete partial nitritation MBBR in series system, $P_{N1\text{-}N2}$ (Figure 4.2A), corresponds to a TAN oxidation efficiency of 59.7 ± 1.3%. These values are comparable to the ideal TAN oxidation efficiency of 57%, based on the stoichiometric NO_2^--N: NH_4^+-N molar ratio of 1.32:1 for subsequent anammox treatment (Strous et al., 1997; Van Dongen et al., 2001). The observed TAN oxidation efficiency from the partial nitritation MBBR system, $P_{N1\text{-}N2,}$ is also similar to the TAN oxidation efficiency of 60% previously reported in the mainstream partial nitritation MBBR system while utilizing a combination of DO/TAN ratio control and FA inhibition as control strategies (Kowalski et al., 2019b). However, the current elevated loading, two-reactor in series design strategy herein studied does not require operational control while demonstrating robust and steady performance.

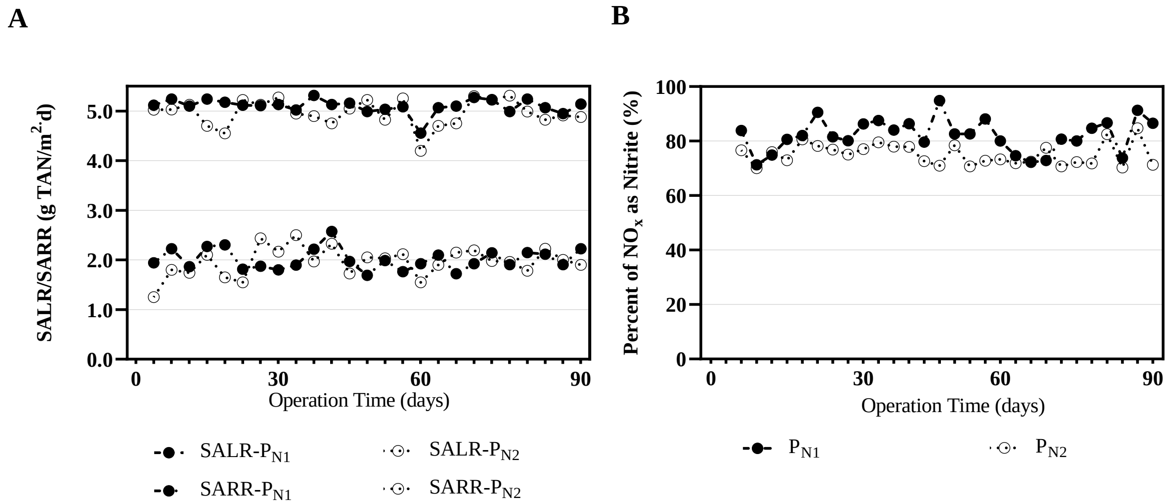

Figure 4.2 Performance of partial nitritation MBBR system **(A)** SARR/ SALR over time for P_{N1} and P_{N2} **(B)** percent NO_x as nitrite over time for P_{N1} and P_{N2}.

The percent NO_x as nitrite was monitored in the partial nitritation MBBR system over a period of 90 days and demonstrates stable and steady performance (Figure 4.2B). The P_{N1} and P_{N2} reactors show an average percent NO_x as nitrite of 81.2 ± 2.1 and 74.7 ± 0.9%, respectively. The average percent NO_x as nitrite of the total oxidized TAN from the partial nitritation MBBR system, $P_{N1\text{-}N2}$, indicates that partial nitritation was achieved, i.e., TAN was oxidized to nitrite and oxidation of nitrite to nitrate was largely mitigated. The relative nitrate production varied between 13 and 16% in the reactors P_{N1} and P_{N2}. Similar levels of relative nitrate production have also been reported in mainstream partial nitritation and single-stage PN/A MBBR systems (Gilbert et al., 2014; Gu et al., 2018b; Gustavsson et al., 2020; Persson et al., 2017). Therefore, the minimal levels of nitrate build-up in the elevated loaded partial nitritation MBBR system suggest that the NOB population in the biofilm or the NOB activity of the embedded population are possibly suppressed.

Finally, the average effluent TAN, nitrite, and nitrate concentrations from reactor P_{N1} of 20.1 ± 1.30 mg TAN/L, 7.14 ± 0.81 mg NO_2^--N/L, and 4.02 ± 0.21 mg NO_3^--N/L demonstrate a NO_2^--N: NH_4^+-N stoichiometric ratio of 0.36:1 (Figure 4.3). This stoichiometric ratio from reactor P_{N1} is below the ideal metabolic ratio of 1.32:1 for partial nitritation systems upstream of anammox systems. From a practical standpoint, a stoichiometric ratio near 1:1 has also been reported sufficient for mainstream anammox operation in an MBBR system (Gu et al., 2018a). Therefore, with the introduction of the second reactor (P_{N2}) in series to the first reactor (P_{N1}) in this study, the average effluent TAN, nitrite, and nitrate concentrations of 12.9 ± 1.84 mg TAN/L, 13.9 ± 1.56 mg NO_2^--N/L and 3.11 ± 0.29 mg NO_3^--N/L, were measured respectively. These P_{N2} effluent concentrations correspond to a metabolic ratio of 1.15:1. As such, this ratio provides suitable nitrogen speciation for the subsequent downstream anammox treatment, and the two reactors in series partial nitritation MBBR system configuration in this study herein provides a stable effluent quality for subsequent downstream anammox operation.

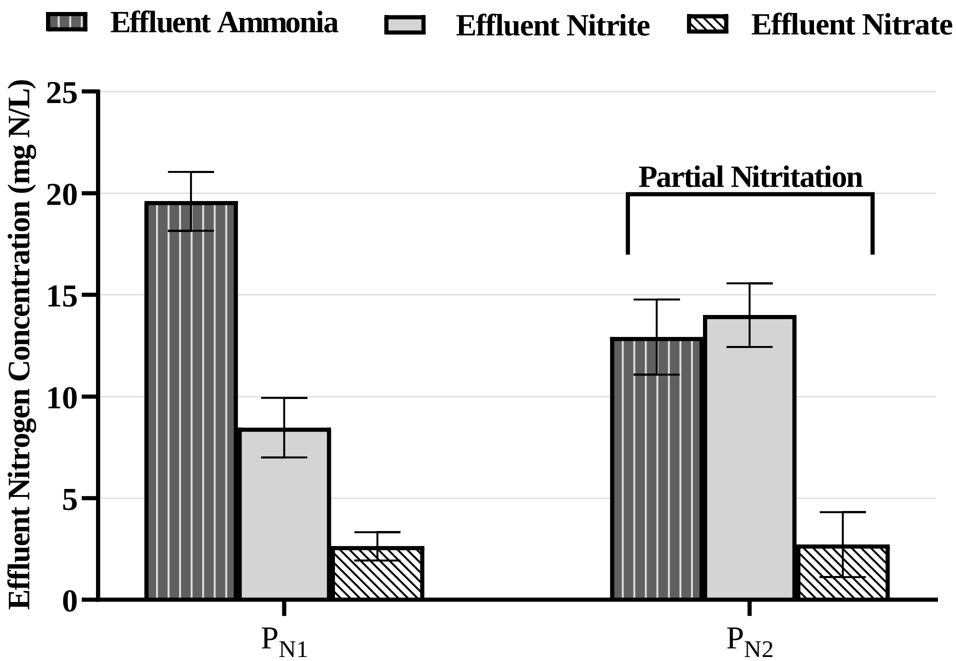

Figure 4.3 Effluent TAN (vertical shading), nitrite (solid light gray fill), and nitrate (diagonal shading) concentrations in the partial nitritation MBBR system, P_{N1}, and P_{N2} (average ± 95% CI).

4.5.2 Biofilm characteristics and morphology

The meso-scale effects of the elevated TAN SALR on the biofilm were quantified through the analysis of biofilm characteristics described as biofilm thickness, biofilm mass, biofilm density, and morphology (Figure 4.4). The biofilm thickness is 577 ± 21 and 517 ± 43 µm; the biofilm mass is 38.8 ± 1.2 and 35.5 ± 3.1 mg/carrier corresponding to biofilm density (calculated based on biofilm thickness and biofilm mass) of 29.9 ± 9.2 and 33.7 ± 8.7 kg/m^3 in the P_{N1} and P_{N2} reactors, respectively. The average biofilm thickness of 547 ± 65 µm in the partial nitritation MBBR system (P_{N1} and P_{N2}) is higher than the 300 µm reported in the mainstream partial nitritation MBBR system (Piculell et al., 2016c, 2016b). The difference in biofilm thickness could be expected as the carrier type and control strategy utilized in both studies are different and would result in varying biofilm composition or characteristics. However, the biofilm thickness, mass, and density are within the range reported in a previous study on elevated loaded partial nitritation MBBR systems with comparable operational conditions (Schopf et al., 2021).

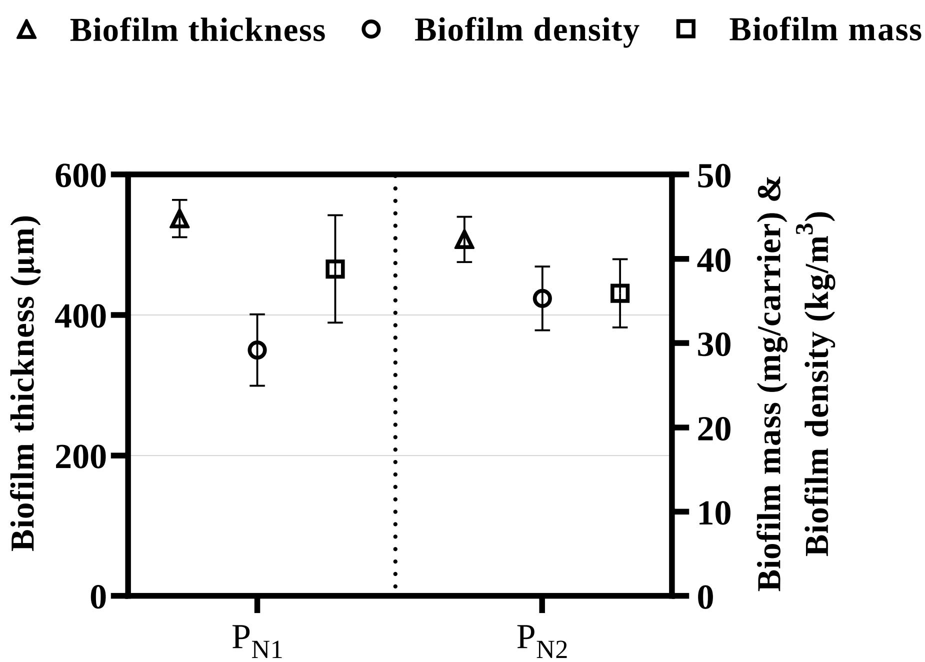

Figure 4.4 Biofilm thickness (triangle), biofilm mass (square), and biofilm density (circle), for partial nitritation MBBR system, P_{N1}, and P_{N2} (average ± 95% CI).

On the other hand, the qualitative assessment of the biofilm shape and morphology shows a rough, irregular, and thick biofilm. This finding is consistent with previous studies showing thick biofilms at similar elevated TAN SALR of 5 g TAN/m^2·d in a partial nitritation MBBR system (Schopf et al., 2019). The thick biofilm is possible as elevated loading conditions and high substrate load allow for a deeper substrate penetration into the biofilm, supporting high cell growth. Furthermore, previous studies have demonstrated that increased biofilm thickness leads to steep substrate gradients and stratification of the metabolic process throughout the biofilm, likely resulting in a more biodiverse and heterogeneous biofilm, thus potentially influencing the overall performance of the system (Suarez et al., 2019; Torresi et al., 2016). Therefore, the stable and steady partial nitritation performance observed in this study at elevated TAN SALR could be explained by stratification that occurs in thick biofilms with AOB population dominating the upper layers of the biofilm and NOB population at deeper layers as the system operates under DO mass transfer rate limited conditions (Malovanyy et al., 2015; Pérez et al., 2014). As such, NOB population or activity is likely suppressed due to the limited diffusion of DO in the biofilm of elevated loading partial nitritation MBBR system of the herein study.

4.5.3 Cell viability

In an MBBR system, the higher substrate removal rate can be related to live cells or biomass activities on the carrier (Ødegaard, 2006), and as such, the biomass embedded in the biofilm controls the reactor performance. Biofilm structure, in general, and within the MBBR system, typically consists of viable cells and non-viable bacterial cells (Figure 4.5) along with numerous other substances secreted by the cells and attached substances to the extracellular matrix of the biofilm. The total cell viability (%) ranges from 58.5 to 99.9% in P_{N1} and from 45.8 to 97.1% in the P_{N2} reactor (Figure 4.5A). Although total cell viability is likely affected by residual TAN concentration and specific embedded microbial communities, the live fraction of total cells, which is the ratio of live cells and the total number of cells (viable + non-viable cells), is stable (Figure 4.5B-C) and within conventional range in a nitrifying MBBR system (Young et al., 2017).

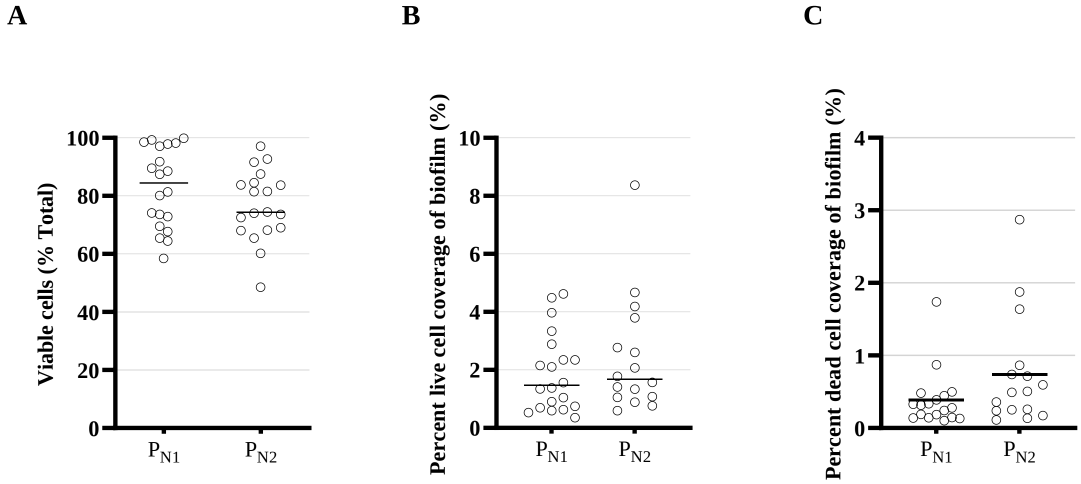

Figure 4.5 CLSM viability of embedded cells for partial nitritation MBBR system, P_{N1} and P_{N2}: **(A)** viability as a fraction of total cells (%) **(B)** percent live-cell coverage of biofilm, and **(C)** percent dead cell coverage (average ± 95% CI).

Hence, the viability of the cells does not appear to constrain the performance of the elevated loaded partial nitritation MBBR reactors, and the mainstream elevated loaded partial nitritation MBBR system retained carriers with viable embedded biomass to maintain sufficient microbial activities for stable and steady partial nitritation performance.

4.5.4 Quantification of AOB and NOB

The copies of AOB and NOB were quantified in the partial nitritation MBBR reactors P_{N1} and P_{N2} using ddPCR (Figure 4.6). In mainstream partial nitritation reactors, the low bulk nitrite concentration would likely select for *Nitrospira* rather than *Nitrobacter* due to its high substrate affinity (Wang et al., 2019). As such, *Nitrospira* was the only NOB detected in the reactors P_{N1} and P_{N2}. During the stable and steady partial nitritation, the average gene copy numbers of AOB are $1.92 \pm 3.40 \times 10^9$ and $2.02 \pm 2.51 \times 10^9$ copies/carrier, and the average gene copy numbers of NOB are 6.20 ± 1.55 x 10^8 and 1.15 ± 1.20 x 10^9 copies/carrier, in reactor P_{N1} and P_{N2}, respectively.

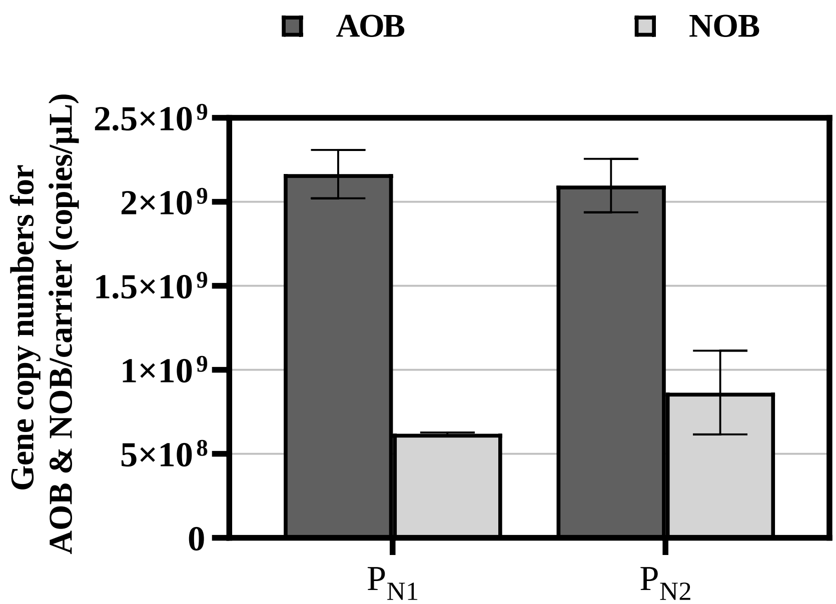

Figure 4.6 ddPCR data for partial nitritation MBBR system P_{N1} and P_{N2}, showing AOB (dark grey fill) and NOB (light grey fill) gene copies (average ± 95% CI).

These average AOB and NOB, gene copy numbers are statistically similar and correspond to AOB to NOB ratios of 3.21:1 and 2.12:1 in reactors P_{N1} and P_{N2}, respectively. These low ratios are comparable to a single mainstream PN/A MBBR system (Gilbert et al., 2015). Also, these values are higher than those published from full nitrification conventional suspended growth systems by Limpiyakorn et al., (2005); Yao and Peng, (2017), and full nitrification MBBR system by Zhang et al., (2019). However, these AOB to NOB ratios are lower than those reported in partial nitritation, conventional suspended growth, and MBBR systems operated under elevated TAN concentrations (Abzazou et al., 2016; Zhang et al., 2020). This distinction in AOB to NOB ratios in full nitrification and partial nitritation systems is possible due to varying reactor types and experimental and operational conditions. Therefore, in the study, the elevated loaded partial nitritation MBBR system demonstrates that NOB activity suppression is the likely dominant mechanism responsible for stable and steady partial nitritation performance under mainstream conditions.

4.5.5 The mechanism of nitrite oxidation suppression in the mainstream partial nitritation MBBR system

To determine the mechanism of nitrite oxidation suppression caused by the low operational intensity partial nitritation design strategy, the driving hypothesis is that elevated TAN SALR and the resulting impact on the biofilm morphology will play a key role in suppressing NOB population or activity in the partial nitritation MBBR system. Previous studies have suggested that the steep substrate gradient present in the biofilm and competition for DO between AOB and NOB, acts as the main mechanism for the control of NOB population or activity in a biofilm-based system (Brockmann and Morgenroth, 2008; Isanta et al., 2015; Laureni et al., 2016; Pérez et al., 2014). This is possible as external mass transfer boundary layer controls the movement of the substrate (TAN) or the electron acceptor (DO) and determines the actual TAN or DO concentration at the biofilm surface (Brockmann and Morgenroth, 2008; Isanta et al., 2015; Laureni et al., 2016; Pérez et al., 2014). The resultant mass transfer limitations (DO mass transfer limited condition) have been numerically demonstrated to promote the suppression of NOB in a partial nitritation MBBR system (Pérez et al., 2020).

Therefore, in this study, elevated TAN SALR and resulting morphological impact on biofilm is the main factor that could be possibly controlling NOB activity suppression rather than NOB population suppression. At elevated TAN SALR, AOB possibly has a higher competitive advantage over NOB due to the high TAN concentration in the bulk solution resulting from elevated TAN SALR, which results in DO being preferentially consumed by AOB compared to NOB. Moreover, elevated TAN SALR, in addition to the resultant biofilm thickness and morphology (roughness), builds an increased mass transfer resistance that possibly limits the mass transfer of oxygen from the bulk solution into the biofilm, resulting in limited DO uptake by NOB and subsequently suppressing their activity. Hence, by maintaining significant AOB activity and suppressing NOB activity, the MBBR system achieves 59.7% TAN oxidation efficiency and, thus, a NO_2^--N:NH_4^+-N stoichiometric ratio of 1.15:1 with 83.2 ± 1.2% percent of NO_x as nitrite.

4.6 The Implications of the study

The study shows that an elevated loading design strategy, as opposed to operational control strategies, is able to achieve stable, steady, and robust partial nitritation in an MBBR system under mainstream municipal conditions. The finding from this study provides a feasible design strategy that does not require multiple and complex operational control measures or monitoring, to achieve stable and steady partial nitritation. The operation at elevated TAN SALR and the resulting impact on biofilm morphology essentially restricts DO uptake by NOB, which is beneficial to the long-term suppression of their activity. Therefore, elevated TAN SALR allows for effective NOB suppression that could result in long-term process stability and stable effluent quality for the subsequent downstream anammox treatment at mainstream municipal wastewater. The design strategy is herein demonstrated at the laboratory scale with synthetic wastewater; hence, the elevated TAN SALR strategy requires further investigation at the pilot scale using real wastewater with the potential to guide future implementation of a high-rate, compact, and low-operational intensive partial nitritation system at WRRFs.

In addition, regarding the future of the elevated loaded partial nitritation system following an anammox system as a two-stage configuration for effective nitrogen removal at mainstream municipal wastewater. Successful anammox operation as a two-stage PN/A system is achievable if operated with stable and steady partial nitritation effluent as well as an appropriate NO_2^--N:NH_4^+-N metabolic ratio. According to the results from this study, the elevated loaded partial nitritation MBBR system is robust, effective, stable, steady and can maintain appropriate effluent ratios. As such, anammox enrichment and operation under mainstream conditions is feasible with the possibility of achieving high nitrogen removal rates and long-term process stability. Also, designing the partial nitritation MBBR system at an elevated TAN loading rate allows for high rate partial nitritation and, consequently, a small land footprint. Therefore, the introduction of the combined two-stage PN/A system can be achieved where insufficient land availability exists. On this note, further studies are proposed exploring the elevated loaded partial nitritation MBBR system as a two-stage PN/A system for enhanced nitrogen removal from mainstream municipal wastewaters.

4.7 Conclusions

The two-reactor in series elevated loaded mainstream partial nitritation MBBR system achieves stable and steady partial nitritation with an effluent NO_2^--N:NH_4^+-N concentration corresponding to a molar stoichiometric ratio of 1.15:1. Hence, demonstrating possible suitable nitrogen speciation for the subsequent downstream anammox treatment. The biofilm thickness and morphology in the reactors show thick biofilms with the embedded biomass showing no viability constraint, as the embedded cells remained viable, supporting the observed stable and steady partial nitritation performance. The thick biofilm morphology likely reduced the diffusive transport of DO into the biofilm, that limits the DO uptake by NOB population. The AOB and NOB gene copy numbers of the MBBR biofilm show that rather than NOB population suppression, NOB activity suppression is the likely mechanism responsible for the stable and steady partial nitritation performance in the mainstream elevated loaded partial nitritation MBBR system.

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CHAPTER 5 - ASSESSMENT OF MIXING AND AERATION STRATEGIES ON ELEVATED LOADED MOVING BED BIOFILM REACTOR FOR MAINSTREAM PARTIAL NITRITATION

5.1 Context

Chapter 5 presents the research article titled *Assessment of mixing and aeration strategies on elevated loaded moving bed biofilm reactor for mainstream partial nitritation*. This article is in preparation for submission for publication. This study investigates three distinct mixing and aeration conditions employed to operate the partial nitritation MBBR system. The study compares the conventional mixing and aeration condition, continuous aeration with mechanical paddle & aeration, and recirculation pump & aeration utilized to optimize the partial nitritation MBBR system to achieve low dissolved oxygen effluent concentrations for optimal downstream anammox treatment.

5.2 Abstract

The study investigates the kinetics, biofilm characteristics, and embedded biomass of three distinct mixing and aeration conditions, continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration, employed to operate the mainstream elevated loaded partial nitritation (PN) moving bed biofilm reactor (MBBR) system. The study compares the conventional mixing and aeration condition, continuous aeration with mechanical paddle & aeration, and recirculation pump & aeration utilized to optimize the PN MBBR system to achieve low dissolved oxygen effluent concentrations for optimal downstream anammox treatment. The results show that maintaining mixing and aeration in the elevated loaded PN MBBR system with recirculation pump & aeration achieves lower effluent dissolved oxygen concentration and stable partial nitritation with appropriate NO_2^-

-N:NH_4^+-N stoichiometry ratio of 1.09:1 for subsequent anammox treatment compared to operation with continuous aeration. Although operation with mechanical paddle & aeration results in lower bulk dissolved oxygen concentration, the performance of the PN MBBR system was significantly affected as 87% of the influent total ammonia nitrogen remained unoxidized to nitrite or nitrate. The study identifies a recirculation pump with reduced aeration as a possible appropriate mixing and aeration strategy for the operation of the mainstream elevated PN MBBR system.

5.3 Introduction

Partial nitritation and anammox (PN/A) process is a cost-effective alternative to the conventional nitrification/denitrification process for treating low-strength ammonia waste streams as a mainstream treatment system within municipal wastewater. The application of PN/A process in the mainstream municipal wastewater offers substantial economic advantages, including, reduced aeration demand and elimination of the need for external organic carbon source, decreased sludge production, and reduced contribution to N_2O emission (Jetten et al., 1997; Mulder et al., 1995; Wett et al., 2013). Although the PN/A process has demonstrated these significant advantages, it encounters challenges attributed to the specific characteristics of mainstream municipal wastewater. These include low total ammonia concentration (TAN) concentrations of 20-40 mg TAN/L, seasonal temperature fluctuations ranging from 10-25°C, and low-temperature values (< 10°C) found in many countries. These conditions limit the growth rates of ammonia-oxidizing bacteria (AOB) and anammox bacteria (AnAOB) posing a significant challenge in achieving effective nitrite oxidation suppression (Agrawal et al., 2018; Bunse et al., 2020; Gu et al., 2018a; Hoekstra et al., 2019; Malovanyy et al., 2015; Regmi et al., 2015; Thomson et al., 2016; Trojanowicz et al., 2016; Wett et al., 2013). The activity of nitrite-oxidizing bacteria (NOB) or increased population is detrimental in PN/A systems, as NOB can compete with AOB for available dissolved oxygen and AnAOB for available nitrite, ultimately affecting the performance of subsequent anammox operation in mainstream municipal wastewater (Malovanyy et al., 2015; Van Tendeloo et al., 2021).

Mainstream PN/A can be performed in a single reactor system, with a combination of PN and anammox process in one reactor or two-reactor system, splitting PN and anammox process into two different reactors (Chen et al., 2018; Choi and Jung, 2022; Gu et al., 2018b; Gustavsson et al., 2020; Laureni et al., 2016; Schraa et al., 2020; Trojanowicz et al., 2021). While a single reactor system has been extensively studied, few studies exist with respect to the optimization of partial nitritation (PN) and anammox process independently to achieve higher nitritation and anammox rates (Gu et al., 2018b; Liu et al., 2018). In mainstream PN systems, several NOB activity or population control strategies, such as pH control, dissolved oxygen control through aeration mode, and oxygen limiting conditions, have been employed to optimize the process (Bian et al., 2017; Chen et al., 2018; Laureni et al., 2016). However, between these control strategies, aeration mode and dissolved oxygen supplied to the reactor have been demonstrated as the key design and operational parameters for PN process control because they directly affect and impact key functional microorganisms (AOB and NOB) involved in the process (Bunse et al., 2020; Chen et al., 2020; Seuntjens et al., 2020).

The dissolved oxygen concentration plays a vital role in balancing the key functional bacteria (AOB and NOB) in the PN process. It is generally believed that AOB has a higher affinity for oxygen than NOB. Therefore, low dissolved oxygen concentration or oxygen-limited conditions can provide AOB with a competitive advantage over NOB. On this note, low dissolved oxygen concentration under 0.5 mg O_2/L has been employed to suppress nitrite oxidation (Chen et al., 2019; Laureni et al., 2016; Yang et al., 2017). Although, other studies, such as Gilbert et al., and Regmi et al., could not achieve nitrite oxidation suppression at similar low dissolved oxygen concentrations (Gilbert et al., 2014b; Regmi et al., 2014). This was attributed to the proliferation of the *Nitrospira*-like NOB with a high affinity for oxygen that can adapt to low dissolved oxygen concentrations over time. As a result, long-term NOB suppression was identified as a significant challenge (Van Tendeloo et al., 2021). Moreover, in recent studies, the $K_{O,AOB}$ of AOB (0.41 mg O_2/L) have been reported higher than NOB (0.05 mg O_2/L); as such, high dissolved oxygen concentration was utilized to encourage AOB dominance over NOB, with emphasis on suppression of *Nitrospira* like NOB (Chen et al., 2019; Malovanyy et al., 2015; Regmi et al., 2014).

On the other hand, rather than controlling dissolved oxygen concentration through continuous aeration, it is also believed that intermittent aeration under low (< 0.05 mg O_2/L) or high (> 1.5 mg O_2/L) dissolved oxygen concentration can be applied to suppress NOB population or activity (Bunse et al., 2020; Gilbert et al., 2014a; Gustavsson et al., 2020; Miao et al., 2016). This is possible as NOB is shown to have a longer lag time than AOB while transitioning from anoxic to aerobic due to the possible inhibition by intermediate products such as hydroxylamine phases (Ge et al., 2015; Ma et al., 2016). Therefore, with intermittent aeration, the systems usually have a more extended non-aerated period to facilitate nitrite oxidation suppression (Bunse et al., 2020). Although, other studies have suggested longer aerated times than non-aerated times to promote AOB dominance over NOB in the PN system (Miao et al., 2022; Yang et al., 2015). While these contradictory findings could be attributed to varying experimental conditions and reactor configurations (single or two-reactor system), aeration strategies still require optimization to consolidate functional organisms further and enhance PN process stability for subsequent anammox treatment at mainstream municipal wastewaters (Li et al., 2018).

Few studies and limited data exist that have compared different mixing and aeration conditions to achieve stable PN in a two-reactor system, using attached growth technologies such as the moving bed biofilm reactor (MBBR) in mainstream municipal wastewaters (Chen et al., 2018; Laureni et al., 2016; Schraa et al., 2020; Trojanowicz et al., 2021). An MBBR system operating under a novel design strategy using elevated total ammonia nitrogen (TAN) loading rate has shown promise to achieve robust and stable mainstream PN and the oxidation of TAN with limited oxidation of nitrite (Ikem et al., 2023; Schopf et al., 2021, 2019). In these previous studies, stable PN was demonstrated at continuous aeration under elevated dissolved oxygen concentrations between 4 and 6.8 mg O_2/L, as operating at an elevated TAN loading rate would possibly necessitate higher consumption by AOB and NOB communities. However, little is known about applying other distinct mixing and aeration conditions rather than the conventional continuous or intermittent aeration patterns to control bulk dissolved oxygen concentrations in the mainstream elevated loaded PN MBBR systems. Therefore, the study aims to investigate the kinetics, biofilm characteristics and embedded biomass of three distinct mixing and aeration conditions, continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration, employed to operate the elevated loaded PN MBBR systems. Specifically, the study compares, the conventional mixing and aeration condition, continuous aeration with mechanical paddle &

aeration, and recirculation pump & aeration, utilized to optimize the PN MBBR system to achieve low dissolved oxygen effluent concentrations for optimal downstream anammox operation.

5.4 Materials and methods

5.4.1 Experimental setup

The experimental setup consisted of two 2L cylindrical MBBRs (denoted as P_{N1} and P_{N2}) operated in series at elevated TAN surface area loading rate (SALR) for a period of 15 months. Each reactor was packed with AnoxK™5 carriers (Anoxkaldnes, Lund, Sweden) obtained from a bench scale PN MBBR system operated at elevated TAN concentrations (Schopf et al., 2019). Prior to use in this study, the carriers in the bench scale PN MBBR system were previously seeded from a municipal biological oxygen demand removal integrated fixed activated sludge system at the Hawkesbury, Ontario, Canada wastewater facility. The reactors P_{N1} and P_{N2} containing the AnoxK™5 carriers were at a fill fraction of 9.5 and 6%, of the bulk liquid volume. The influent TAN concentration flowing into the P_{N1} was at an average of 44.5 ± 0.6 mg TAN/L, selected as it is within the conventional limits of mainstream municipal TAN concentrations (Ikem et al., 2023).

5.4.2 Reactor design approach

The reactor operation was grouped into three distinct design approaches to investigate and compare the elevated loaded PN MBBR performance. In particular, three distinct mixing and aeration conditions, continuous aeration, mechanical paddle & reduced airflow rate, and recirculation pump & reduced airflow rate, were used to operate the PN MBBRs, P_{N1}, and P_{N2} in series (Figure 5.1). In the first design approach (control PN system), the reactors P_{N1}, and P_{N2}, were equipped with air diffusers, and air continuously supplied at a constant airflow rate of 1.5 L/min in both reactors, corresponding to an elevated dissolved oxygen concentration, average value at 6.78 ± 0.1 mg O_2/L (Figure 5.1A).

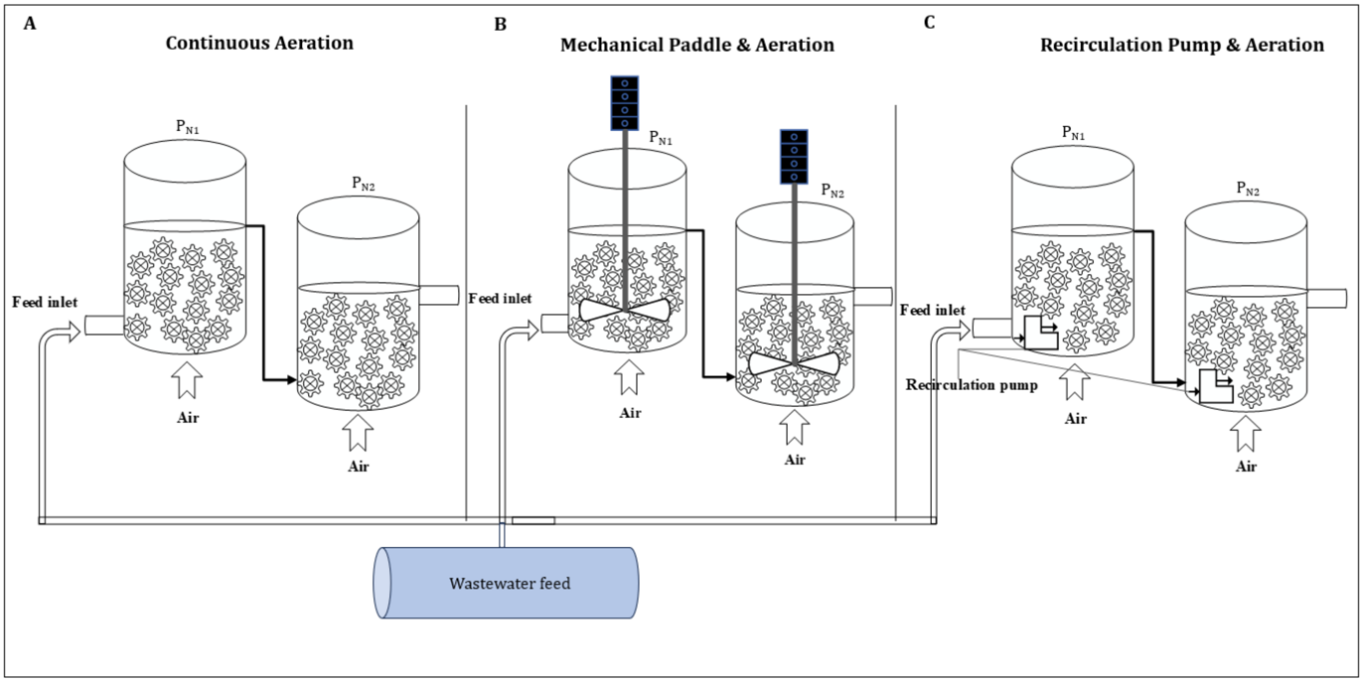

Figure 5.1 Schematics of the reactor operation at distinct mixing and aeration conditions: **(A)** continuous aeration**, (B)** mechanical paddle & aeration and **(C)** recirculation pump & aeration**.**

Subsequently, the second and third design approach utilized two novel mixing and aeration conditions (mechanical paddle & aeration and recirculation pump & aeration) to reduce bulk dissolved oxygen concentration in reactors P_{N1} and P_{N2}. Lowering the bulk dissolved oxygen concentration in the reactors P_{N1} and P_{N2} is important to optimize the PN MBBR system to achieve low dissolved oxygen effluent concentrations for optimal downstream anammox treatment. Therefore, in the second design approach, the reactors P_{N1} and P_{N2} were fitted with a mechanical paddle and a coarse bubble aeration system, facilitating efficient oxygen mass transfer and adequate carrier mixing (Figure 5.1B). The airflow rate was reduced to 0.15 L/min corresponding to an average dissolved oxygen concentration of 3.51 ± 0.1 mg O_2/L. This second mixing and aeration condition will be referred to in this manuscript as mechanical paddle & aeration.

In the third design approach, efficient mixing and aeration were achieved through the installation of a recirculation pump and a coarse bubble aeration system, with diffusers positioned at the base of the reactor at a constant airflow rate of 0.15 L/min corresponding to an average dissolved oxygen concentration of 3.39 ± 0.2 mg O_2/L (Figure 5.1C). For clarity and ease of discussion, the third mixing and aeration condition will be subsequently referred to as a recirculation pump & aeration.

5.4.3 Reactor operation

During mixing and aeration condition at continuous aeration, (control condition), the PN MBBR system was operated for a period of four months. After four months, triplicate samples were analyzed for each reactor to ensure that data points were acquired at a steady state. In this study, a triplicate consisted of three independent samples obtained from the reactors P_{N1} and P_{N2} (PN MBBR system) at a single time point. Therefore, over a minimum of three consecutive days, a set of triplicated data points was obtained per day to act as a representative sample of steady state. At the end of this condition, the mixing and aeration in the PN MBBR system was changed to mechanical paddle & aeration and operated for another three months. Three triplicated data points across three days were taken at a steady state. After three months of mixing and aeration of the PN MBBR system with a mechanical paddle & aeration, it was subsequently switched to a recirculation pump &

aeration. Three triplicated data points across three days were taken after the first three months of operation once a steady state was established. At this mixing and aeration condition, the PN MBBR system was operated for additional five months to ensure that after steady state had been established and adaptation occurred, the MBBR system would continuously achieve stable and high PN rates with mixing and aeration maintained using a recirculation pump & aeration. Throughout the PN MBBR system operation at these distinct mixing and aeration conditions, the HRT was at 2hrs; pH, and temperature were not regulated but were measured with average values at 7.67±0.3 and 20.7±0.1°C, respectively.

5.4.4 Wastewater feed

The PN MBBR system was fed with synthetic wastewater feed prepared and modified based on the recipe employed in previous studies (Delatolla et al., 2009; Young et al., 2017). Synthetic wastewater simulating post-carbon removal municipal wastewater treatment was used in this study. The specific composition of synthetic wastewater (per L of synthetic wastewater) in reactor P_{N1} consisted of 0.21 g $(NH_4^+)_2SO_4$ (corresponding in a concentration of approximately 44 mg NH_4^+-N/L), 0.57 g $NaHCO_3$, 0.08 g $MgSO_4 \cdot 7H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, 0.08 g KH_2PO_4, and 0.006 g $FeSO_4 \cdot 7H_2O$. Trace nutrients (per L of synthetic wastewater): $MnCl_2 \cdot 4H_2O$: 0.10 mg, $Na_2MoO_4 \cdot 2H_2O$: 0.03 mg, $CuSO_4 \cdot 5H_2O$: 0.10 mg, $CoCl_2 \cdot 6H_2O$: 0.001 mg, $ZnSO_4 \cdot 7H_2O$: 0.03 mg. The carbon source composition (per L of synthetic wastewater): glucose 4.86 mg, sodium acetate 2.59 mg, and peptone 4.86 mg resulting in a sCOD concentration of 25 mg sCOD/L mimicking post-carbon effluent (Schopf et al., 2019).

5.4.5 Constituent analyses

The kinetics study included analyses of total ammonia nitrogen (TAN), N-NO_2^- and N-NO_3^- in the PN MBBR system, in accordance with standard methods, methods 4500-NH_3, 4500B-NO_2^- and 4500A-NO_3^-. sCOD was analyzed after filtration with a 0.45um Millipore G filter using a DR 5000 spectrophotometer (Loveland, CO, USA). Alkalinity was determined using a HACH method 8000 in DR 5000 spectrophotometer (Loveland, CO, USA). The temperature and pH were not adjusted, but daily measurements were taken throughout the experimental design approaches. The temperature and dissolved oxygen in the reactors were quantified using the HACH Flexi HQ30d dissolved oxygen probe (Loveland, CO, USA). The pH measurements were recorded with a SympHony pH probe (Radnor, PA, USA). The airflow rate was measured using the Dwyer VFA-24 Visi-Float ® air flow meter (Michigan City, IN, USA).

5.4.6 Biofilm characterization

The biofilm characteristics, including biofilm thickness, mass, and density, were analyzed at each distinct mixing and aeration condition. The biofilm images for biofilm thickness and morphology were acquired using stereoscope imaging a Zeiss Stemi 305 stereoscope (Carl Zeiss Canada Ltd., Toronto, Canada), at each mixing and aeration condition. Twenty stereoscopy images were acquired from triplicate carriers per mixing and aeration condition within 4h of harvesting from the reactors. Five images per carrier at each mixing and aeration condition were acquired at 2x magnification to determine biofilm thickness. Two images per carrier (16 images in total) were obtained at 0.8x magnification to provide an overall carrier image. One image per carrier (8 images) was analyzed at 4x magnification for biofilm surface morphology. All the images at these magnifications were taken randomly across the carrier surface to avoid bias. Under each mixing and aeration condition investigated in this study, a minimum of 1000 thickness measurements were acquired. The biofilm thickness was measured and quantified using the MedCalc Digimizer Image analysis software V.5.6.0 (Ostend, Belgium).

The biofilm mass was measured using the protocol described and modified by previous studies (Delatolla et al., 2008; Tian et al., 2020; Young et al., 2017). Two carriers per mixing and aeration condition were harvested from the PN MBBR and dried overnight at 105°C. The dried carriers were placed inside a desiccator to cool for thirty minutes, and their weight was recorded as W_1. Next, the carrier was thoroughly cleaned with a stiff-bristled brush and water. The clean carriers were dried at 105°C overnight and allowed to cool in a desiccator for thirty minutes, and their weight was recorded as W_2. The biofilm mass was calculated as the difference between W_1 and W_2. Prior testing was done to ensure that the biofilm removal and heating method did not cause any significant change in the biofilm carriers' mass.

5.4.7 Droplet digital PCR analyses

Three carriers per condition were harvested (three biological replicates) and analyzed using the droplet digital polymerase chain reaction (ddPCR). ddPCR analyses were carried out using a 20μL reaction mixture, consisting of 5μL of sample and 11 μL of QX200™ ddPCR™ EvaGreen*Supermix (Bio-Ras, Hercules, CA), including 0.23 μL of each forward and reverse primer (10 μmol/L) and 6.04 μL nuclease-free water (Ikem et al., 2023). A 20 μL of the reaction mixture was mixed with 60 μL of droplet generation oil, and droplets formed using the droplet generator (Bio-Rad). 40 μL of the generated droplets were transferred into a 96-well ddPCR plates (Eppendorf). The plates were sealed and transferred into the T100™ Thermal Cycler machine (Bio-Rad) for amplification. The amplification program consisted of the following steps: denaturation at 95°C for 5 min, followed by 50 cycles of 30 s at 95°C, 60 s at the corresponding primer annealing temperature, and 30 s at 72°C, followed by 5 min cooldown at 4°C, 5 min at 90°C for droplet stabilization and hold at 12°C (Ikem et al., 2023). At the end of the program, the plate was transferred to a QX200 droplet reader (Bio-Rad, Hercules, CA). The results were analyzed using the QuantaSoft Software (Bio-Rad, version 1.7.4, Hercules. CA). The software measures the number of positive and negative droplets per fluorophore per sample, with each positive counted as one and negatives counted as 0. The quality control measure ensured that the total droplets quantified were > 10,000, and < 5 positive droplets were identified as negative controls (Tian et al., 2020).

5.4.8 Statistical analyses

Statistical analyses of wastewater constituents, biofilm characteristics (biofilm thickness, biofilm mass, and biofilm density) and AOB & NOB gene copies and correlation between investigated distinct mixing and aeration conditions were tested using a student t-test and Spearman's rank correlation test, respectively, with *p-value* less than *0.05* indicating a significant difference.

5.5 Results and discussion

5.5.1 The PN MBBR system performance

The observed variations in TAN surface area removal rate (SARR) measurements at distinct mixing and aeration conditions investigated in this study correspond to possible responses to the fluctuations in dissolved oxygen concentration in bulk solution and fluid shear stress on bio carriers resulting from changes in mixing and aeration patterns (Wang et al., 2019), that are utilized to optimize mainstream PN system for downstream anammox treatment (Table 5.1).

Table 5.1 Measured average TAN SALR, TAN SARR, and dissolved oxygen concentration for various mixing and aeration conditions (average ± 95 confidence interval).

	TAN SALR ($g\ TAN/m^2 \cdot d$)	TAN SARR ($g\ TAN/m^2 \cdot d$)	Dissolved oxygen ($mg\ O_2/L$)
Continuous Aeration	7.07 ± 0.2	3.14 ± 0.2	6.78 ± 0.1
Mechanical Paddle & Aeration	7.05 ± 0.1	1.46 ± 0.0	3.51 ± 0.1
Recirculation Pump & Aeration	7.04 ± 0.2	3.01 ± 0.1	3.39 ± 0.2

The kinetic assay rates show that the TAN SARR statistically significantly decreases as the mixing and aeration condition in the PN MBBR system is switched from continuous aeration to mechanical paddle & aeration, with average bulk dissolved oxygen concentration value at 6.78 ± 0.1 and 3.51 ± 0.1 mg O_2/L respectively. Specifically, the TAN SARR measurements with PN MBBR system operation at continuous aeration are 2.2 times higher than when mixing and aeration condition is changed to mechanical paddle & aeration. However, the TAN SARR statistically increases as the mixing and aeration condition in the PN MBBR system shifts from mechanical paddle & aeration to recirculation pump & aeration at a similar average bulk dissolved oxygen concentration of 3.31 ± 0.3 mg O_2/L. During PN MBBR operation with a recirculation pump & aeration, the TAN SARR is 2.1 times greater compared with operation using a mechanical paddle & aeration. On the other hand, despite the variations in dissolved oxygen concentrations with changes in mixing and aeration conditions in the PN MBBR system, the TAN SARR measurements during operation at continuous aeration are comparable to operation using a recirculation pump & aeration, as there is no significant statistical difference, even at an approximately 50% less bulk dissolved oxygen concentration.

The % TAN oxidation efficiency are measured for the three mixing and aeration conditions investigated, with an approximate design target value indicated based on the anammox theoretical NH_4^+:NO_2^- stoichiometric ratio of 1:1.32 (Figure 5.2A). The average % TAN oxidation efficiency of 45.5 ± 1.4% during PN MBBR system operation at continuous aeration is 3.5 times significantly greater than 13.4 ± 1.1% while operating with a mechanical paddle & aeration (Figure 5.2A). The higher % TAN oxidation efficiency achieved through continuous aeration compared to mechanical paddle and aeration can be attributed to the elevated dissolved oxygen supply rate, which enhances oxygen mass transfer efficiency and substrate penetration. This, in turn, promotes increased activity of AOB, leading to higher TAN oxidation efficiency (Cole et al., 2004).

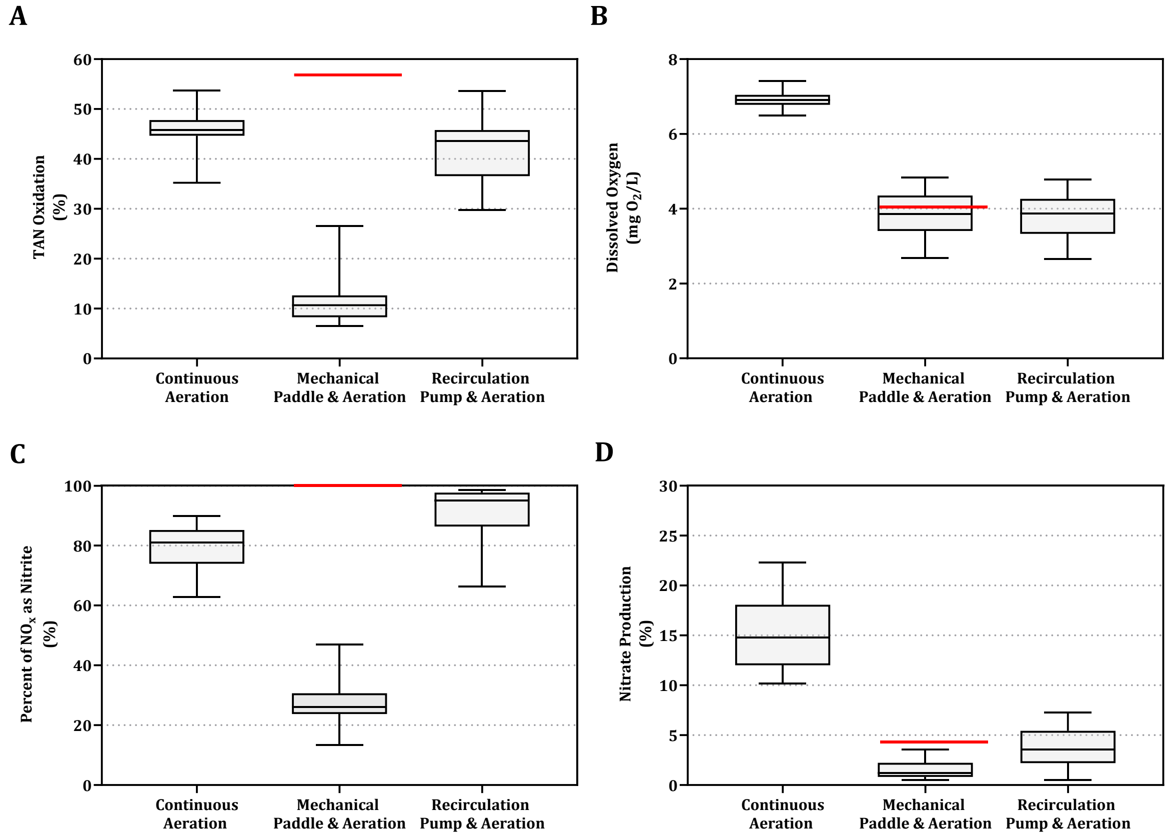

Figure 5.2 TAN oxidation % **(A)**, dissolved oxygen concentration (**B**), percent NO_x as Nitrite **(C)**, and nitrate production % **(D)**, across each mixing and aeration condition, continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration; with horizontal red line indicating estimated target design value.

The average % TAN oxidation efficiency in the PN MBBR system with operation using the recirculation pump & aeration is statistically significantly higher (43.7 ± 1.2%) than during operation with mechanical paddle & aeration (13.4 ± 1.1%). The result corresponds to 3.1 times increase in % TAN oxidation efficiency with the mixing and aeration condition switched from mechanical paddle & aeration to recirculation pump & aeration, and yet the bulk dissolved oxygen concentration in the PN MBBR system remains statistically similar at 3.31 ± 0.3 mg O_2/L (Figure 5.2B). Comparing the % TAN oxidation efficiency during operation at continuous aeration (45.5 ± 1.4%) to recirculation pump & aeration (43.7 ± 1.2%) while considering the variations in the bulk dissolved oxygen concentration (6.78 ± 0.1 and 3.39 ± 0.2 mg O_2/L) at these mixing and aeration conditions, although both values are close to design target value, the result shows no significant statistically difference. It is expected to observe a distinct difference in % TAN oxidation efficiency, primarily attributable to the increased dissolved oxygen concentration in the bulk solution during continuous aeration with a higher airflow rate. This condition is expected to promote a greater proliferation or activity of AOB compared to the operation of the PN MBBR system with a recirculation pump and aeration at lower dissolved oxygen concentration. (Chen et al., 2018). However, the lack of distinction indicates that the operation of the elevated loaded PN MBBR system with a recirculation pump & aeration, yet under a lower bulk dissolved oxygen concentration, possibly maintains adequate AOB population or activity leading to a high and stable TAN oxidation efficiency.

The percent NO_x as nitrite varied with the three distinct mixing and aeration conditions (Figure 5.2C). The average percent NO_x as nitrite from the PN MBBR system is at 85.7 ± 1.2% during operation at continuous aeration and statistically significantly decreased to an average of 26.2 ± 1.3% with operation using the mechanical paddle & aeration. On the other hand, switching the mixing and aeration condition in the PN MBBR system from mechanical paddle & aeration to recirculation pump & aeration, the percent NO_x as nitrite increases from 26.2 ± 1.3 to 97.1 ± 1.1%, demonstrating an ideal design target percent NO_x as nitrite value (Schopf et al., 2019). This observation clearly indicates how distinct mixing and aeration patterns in the elevated loaded PN MBBR could possibly affect nitrite build-up in the system due to possible effects on the biofilm and embedded microbial biomass.

In addition, the three distinct mixing and aeration conditions investigated in this study show various percentages of nitrate build-up in the PN MBBR system (Figure 5.2D). Specifically, the % nitrate production is highest, with an average of 18.8 ± 0.8% during PN MBBR system operation at continuous aeration. However, the % nitrate production is relatively minimal and stable at less than 2 and 5% during PN MBBR system operation with mechanical paddle & aeration and recirculation pump & aeration. Although minimal nitrate production is observed in the MBBR system during operation with mechanical paddle & aeration, the overall PN performance was low at this mixing and aeration condition. Meanwhile, the distinctive low % nitrate production, within design target value and overall stable performance of the PN system during operation with recirculation pump & aeration compared to mechanical paddle & aeration, suggests that a high degree of NOB populations or NOB activity is possibly suppressed in the biofilm at this mixing and aeration condition.

Finally, the effluent nitrogen concentrations are distinct across the three mixings and aeration conditions; continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration investigated in this study (Figure 5.3). Employing continuous aeration for PN MBRR system operation results in effluent TAN concentration of approximately 19.8 ± 0.2 mg TAN/L, nitrite of 16.5 ± 0.4 mg NO_2^--N/L, and nitrate of 10.1 ± 0.2 mg NO_3^-- N/L; thus, the MBBR system yielding towards PN.

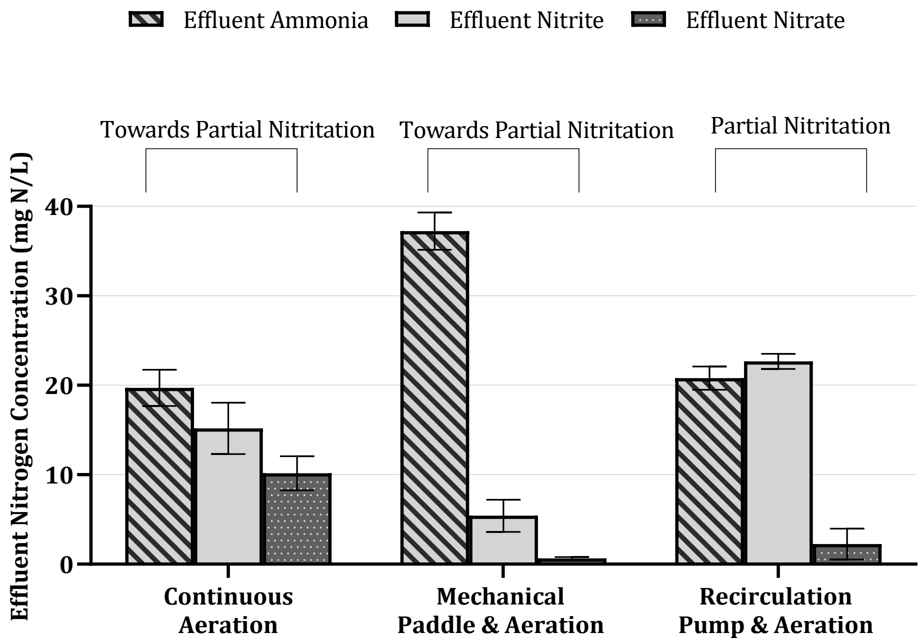

Figure 5.3 Effluent TAN (diagonal bar shading), nitrite (solid gray fill), and nitrate (dotted shading) concentrations at each mixing and aeration condition. Variance indicates 95% confidence intervals.

On the other hand, the operation of the PN MBBR system with a mechanical paddle & aeration did not result in a significant change in TAN concentrations, as 86.7% of TAN in the bulk solution remains unoxidized to nitrate or nitrite. However, the effluent TAN of 20.8 ± 0.3 mg TAN/L, nitrite of 22.7 ± 0.5 mg NO_2^--N/L, and nitrate concentrations of 2.24 ± 0.3 mg NO_3^--N/L equivalent to NO_2^--N:NH_4^+-N stoichiometry ratio of 1.09:1 appropriate for downstream anammox treatment, is achieved through the combination of recirculation pump & aeration, mixing and aeration condition. These findings indicate that compared to operation with continuous aeration or mechanical paddle & aeration, recirculation pump & aeration provide efficient mixing and aeration in the elevated loaded PN MBBR system resulting in a stable and appropriate effluent quality based on the optimized NO_2^--N:NH_4^+-N stoichiometry ratio by Strous et al., (1998) for subsequent downstream anammox operation.

5.5.2 Biofilm characteristics

The biofilm characteristics, thickness, mass, and density are quantified at three distinct mixings and aeration conditions: continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration in this study (Figure 5.4). The biofilm thickness significantly decreases from 561.5 ± 17.2μm to 192.1 ± 15.3μm, with a change in PN MBBR operation from continuous aeration to mechanical paddle & aeration. The result coincides with the rapid decrease in PN MBBR performance observed during operation with mechanical paddle & aeration. Potentially the observed rapid decrease is possible as the fluid turbulence and mechanical collision among moving carriers can increase shear forces leading to observable biofilm detachment (biofilm thickness was less than 200 μm) (Chen et al., 2018; Kanematsu and Barry, 2020; Li et al., 2021). Besides, nitrifying communities have been shown to differ with biofilm thickness resulting from response to operating conditions such as mixing and aeration pattern and loading rate, thus influencing overall reactor performance (De Beer et al., 1994; Piculell et al., 2016).

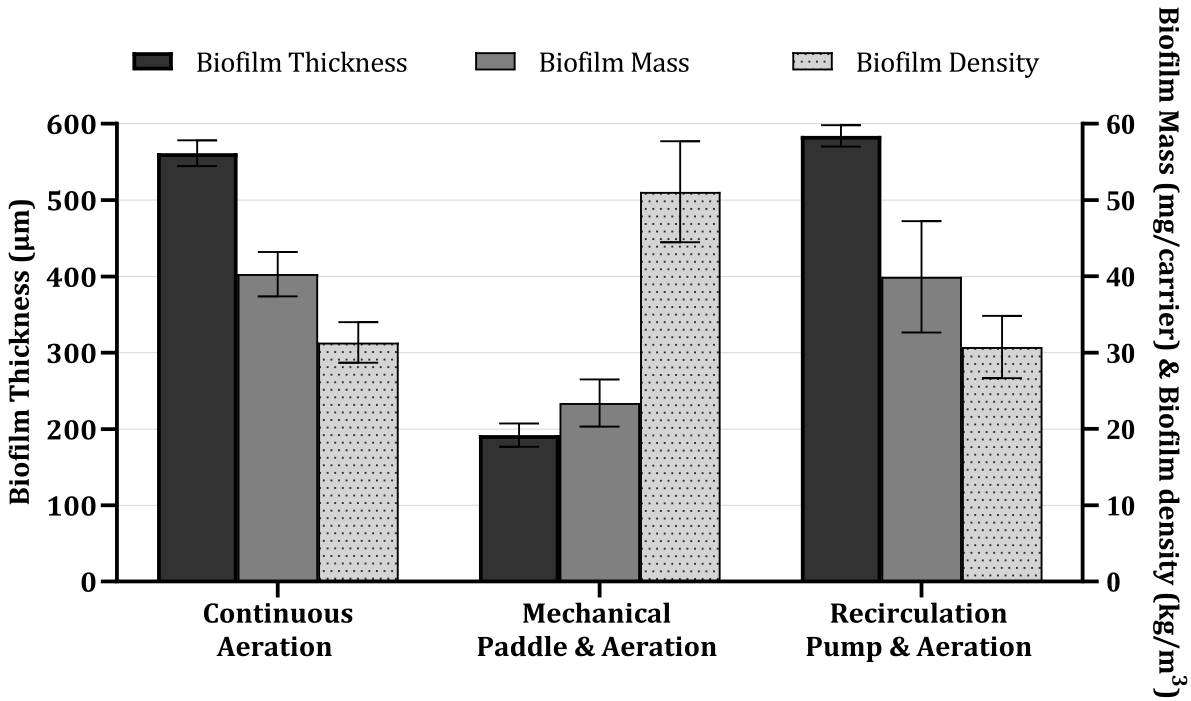

Figure 5.4 Biofilm thickness (black fill), biofilm mass (gray fill), and biofilm density (dotted shading) at each mixing and aeration condition. Variance indicates 95% confidence intervals.

Despite the significant decrease in biofilm thickness during operation with mechanical paddle & aeration, the biofilm thickness significantly increases from 192.1 ± 15.3 µm to 596.3 ± 18.6 µm with the switch of mixing and aeration condition to recirculation pump & aeration. Consistent with the elevated loaded PN MBBR performance, the rapid increase in biofilm thickness corresponds with the observed increase in TAN SAAR, TAN oxidation efficiency, and percent NO_x as nitrite, with operation using a recirculation pump & aeration. This is likely, as increased biofilm thickness creates a partial oxygen penetration due to reduced biofilm surface area, as such, resulting in the available substrate in the bulk liquid phase (TAN and dissolved oxygen) likely preferentially utilized by AOB and consequently limiting NOB population/or activity in the system (Cole et al., 2004; Pérez et al., 2014; Piculell et al., 2016).

There is no significant difference in biofilm mass with operation at continuous aeration and recirculation pump & aeration with average values at 33.4 ± 2.2 and 34.6 ± 5.2 mg/carrier, respectively. The significant change in biofilm mass occurs with operation using a mechanical paddle & aeration when the biofilm did not experience a significant difference in bulk TAN concentration, as 86.7% of TAN in the bulk solution remains unoxidized to nitrate or nitrite. The biofilm density also shows a similar trend, as there is no significant difference between the biofilm density (30.4 ± 2.6 kg/m^3) with operation at continuous aeration and recirculation pump & aeration (31.1 ± 3.1 kg/m^3). However, the biofilm density of 51.1 ± 4.2 kg/m^3 is greater in the PN MBBR system, with operation using a mechanical paddle & aeration. The increased biofilm detachment from the carrier observed with operation with a mechanical paddle may result in the exposure of deeper biofilm that is dense and could potentially affect the performance of the MBBR system (Jaafari et al., 2014; Kanematsu and Barry, 2020; Li et al., 2021).

5.5.3 Quantification of AOB and NOB

The ddPCR was used to quantitate the AOB and NOB population within the biofilm during operation at continuous aeration and recirculation pump & aeration (Figure 5.5). Quantification of AOB and NOB in the PN MBBR system during operation with mechanical paddle & aeration was excluded due to the reduced PN performance, as 86.7% of TAN in the bulk solution remains unoxidized to nitrate or nitrite. A slight but statistically significant increase in AOB gene copy numbers is observed from 2.13 ± 0.3 × 10^8 copies/carrier during operation at continuous aeration to 1.56 ± 0.5 × 10^9 copies/carrier with recirculation pump & aeration. During the operation at continuous aeration, the average NOB gene counts at 2.74 ± 0.3 × 10^8 copies/carrier in the PN MBBR system is statistically significantly higher than 7.71 ± 0.5 × 10^7 copies/carrier observed while operating using a recirculation pump & aeration. This result will likely explain the increased nitrate build-up (>18%) during operation at continuous aeration compared to < 5% with a recirculation pump & aeration. Moreover, under this mixing and aeration condition (continuous aeration), the oxygen mass transfer from the bulk solution to the biofilm surface may improve, consequently promoting deeper dissolved oxygen penetration to the embedded biomass and, as such, encouraging NOB proliferation and activity in the system (Rittmann and McCarty, 2001; Taherzadeh et al., 2012).

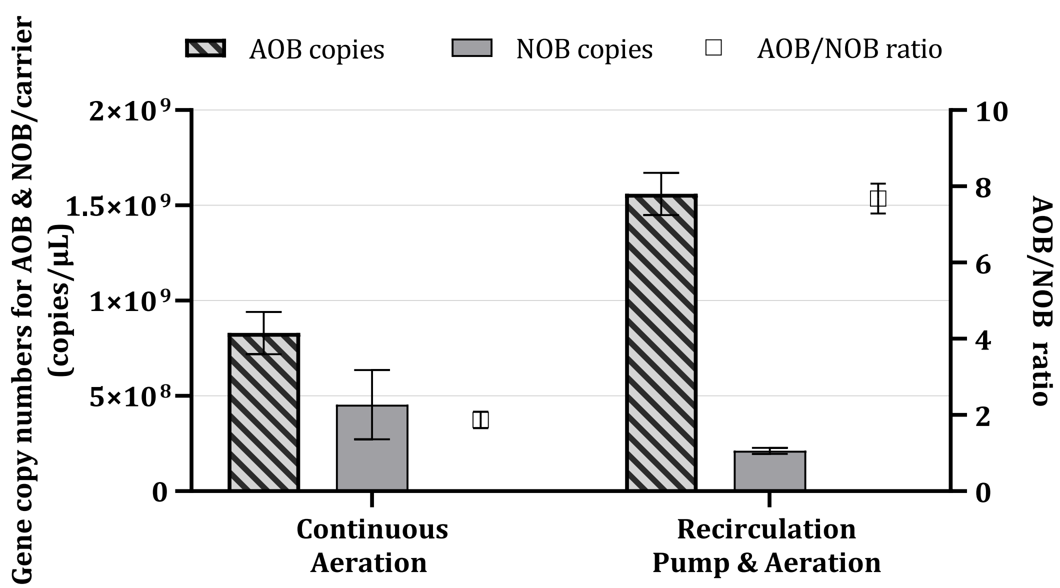

Figure 5.5 ddPCR results for the two distinct mixing and aeration conditions: continuous aeration and recirculation pump & aeration. Variance indicates 95% confidence intervals.

Finally, the AOB to NOB ratios significantly increase from 2.05:1 during operation at continuous aeration to 9.91:1 with operation using a recirculation pump & aeration. The results indicate 4 times higher AOB than NOB abundance in the PN MBBR system during operation with a recirculation pump & aeration compared to operation at continuous aeration. This suggests that higher NOB activity suppression is possibly observed during the operation of the elevated loaded PN MBBR system using a recirculation pump & aeration, also corresponding to the negligible oxidation of TAN to nitrate (i.e., resulting in an ideal NO_2^--N: NH_4^+-N ratio); thus, identifying the mixing and aeration with a recirculation pump & aeration appropriate for the stable operation of the PN MBBR system.

5.6 Conclusions

The findings of this study show that maintaining mixing and aeration in the elevated loaded PN MBBR system using a recirculation pump & aeration has the potential to achieve low dissolved oxygen concentration and optimal effluent quality for downstream anammox treatment at municipal wastewater. Comparing other distinct mixing and aeration conditions, continuous aeration, and mechanical paddle & aeration, operation with recirculation pump & aeration shows an appropriate NO_2^--N:NH_4^+-N metabolic ratio of 1.09:1 and approximately 5 times higher AOB to NOB ratio, thus demonstrating possible higher nitrite oxidation suppression within the mainstream elevated loaded PN MBBR system. Therefore, the study identifies a recirculation pump with reduced aeration as a possible effective and suitable mixing and aeration strategy for the operation of the mainstream elevated PN MBBR system.

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CHAPTER 6 - ELEVATED LOADED COMBINED TWO-STAGE PARTIAL NITRITATION/ANAMMOX FOR MAINSTREAM MUNICIPAL WASTEWATER TREATMENT

6.1 Context

Chapter 6 presents the research article titled *Elevated loaded combined two-stage partial nitritation/anammox for mainstream municipal wastewater treatment*. This article is in preparation for submission for publication. This article discusses the successful operation of the elevated loaded partial nitritation system following an anammox unit as a complete two-stage system for nitrogen removal under mainstream conditions.

6.2 Abstract

This study investigates the feasibility of operating the elevated loaded partial nitritation moving bed biofilm reactor (MBBR) system following the anammox unit as a two-stage configured system for nitrogen removal at mainstream municipal wastewaters. The elevated loaded PN MBBR system provides optimal NH_4^+-N:NO_2^--N stoichiometric effluent ratio of 1:1.17, resulting in the successful operation of a downstream anammox unit with a total nitrogen removal rate at 0.22 ± 0.2 g N/m^2/d and total nitrogen removal efficiency at 74.1 ± 0.7%. The average NO_2^--N to NH_4^+-N molar removal ratio is 1.05 ± 0.1 from the anammox unit. Also, the anammox bacteria (AnAOB) gene copies are at 3.28 ± 0.7 × 10^8, a value significantly higher than the ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) gene copies at 9.17 ± 1.1 × 10^4 and 6.23 ± 1.0, respectively. This confirms that anammox activity is established and nitrogen removal is primarily through the anammox process. Finally, the results and overall system performance demonstrate that the combined two-stage mainstream elevated loaded partial nitritation/anammox MBBR

system has shown promise and offers great insights for further advancement anammox of process at mainstream municipal wastewaters.

6.3 Introduction

Over the past decade, partial nitritation/anammox has been successfully implemented to treat sludge digester centrate, as a sidestream treatment system, within municipal water resource recovery facilities (WRRF). Municipal sidestream partial nitritation/anammox treatment is well established, with over 200 full-scale installations worldwide (Lackner et al., 2014; Malovanyy et al., 2015; Van der Star et al., 2007; Wett, 2006). Despite the demonstrated capacity of the partial nitritation/anammox process for sidestream treatment, the feasibility of partial nitritation/anammox for mainstream municipal wastewater treatment remains a challenge, with no full-scale installations to date (Bunse et al., 2020; Li et al., 2018). The major challenge in the mainstream remains the effective suppression of nitrite-oxidizing bacteria (NOB). Since when NOB enriches in the system, it increases the risk of nitrate accumulation due to competition between key functional bacteria, ammonia-oxidizing bacteria (AOB) and anammox bacteria (AnAOB), potentially leading to a decrease in nitrogen removal efficiency and process instability (De Clippeleir et al., 2013; Xu et al., 2020).

At mainstream municipal wastewaters, several operational control strategies have been employed to optimize the partial nitritation process via selective suppression of NOB populations or activity to ensure the successful operation of downstream anammox unit (Gu et al., 2018; Kowalski et al., 2019; Liu et al., 2018). These operational control strategies have been applied either using the suspended growth or biofilm-based system. The biofilm-based system, such as the moving bed biofilm reactor (MBBR), is advantageous due to the biofilm structure that allows microbial community stratification within the biofilm, creating deeper dissolved oxygen-limited layers for selective suppression of NOB populations or activity. Likewise, the MBBR system has been reported to retain elevated anammox biomass within the biofilm, leading to high process stability (Gilbert et al., 2014; Guo et al., 2016; Gustavsson et al., 2020; Kowalski et al., 2019). The partial nitritation/anammox process has been applied in an MBBR system concomitantly in a single reactor (one-stage system) or two separate reactors (two-stage system) (Gilbert et al., 2014; Gu et al., 2018a; Gustavsson et al., 2020; Iannacone et al., 2021; Laureni et al., 2016). The lower capital cost of the one-stage MBBR system compared to the two-stage system has made the former frequently explored for partial nitritation/anammox application at mainstream municipal wastewaters. However, recent studies have shown that the two-stage partial nitritation/anammox process using the MBBR technology is a favourable alternative towards efficient nitrogen removal in mainstream municipal wastewater treatment (Cao et al., 2023; Gu et al., 2018a).

In recent studies, elevated NH_4^+-N loading rate operational design strategy have been shown to achieve stable partial nitritation with appropriate effluent nitrogen ratio for successful anammox operation within a two-stage configured MBBR system (Ikem et al., 2023b, 2023a; Schopf et al., 2021, 2019). However, despite these findings from the previous studies, little is known about operating the elevated partial nitritation/anammox MBBR system as a combined two-stage system. To the best of the authors' knowledge, no previous studies have evaluated the potential of applying the combined two-stage elevated loaded partial nitritation/anammox MBBR system for enhanced nitrogen removal under mainstream conditions. Hence this study, for the first time, aims to investigate the performance of the promising elevated loaded partial nitritation/anammox configured system for nitrogen removal under mainstream conditions.

6.4 Material and methods

6.4.1 Reactor configuration and operation

The experimental setup consisted of a lab-scale two-stage partial nitritation/anammox configuration (Figure 6.1). The partial nitritation system comprised of two 2L cylindrical MBBR denoted as P_{N1} and P_{N2}, operated in series for 22 months at elevated loading rate. Each reactor in series housed AnoxK™5 carriers (Anoxkaldnes, Lund, Sweden), with a protected surface area of 800m^2/m^3, at 9.5% and 5% fill fraction. The AnoxK™5 carriers were harvested from a single bench MBBR system with previously seeded carriers obtained from a biological oxygen demand (BOD) removal municipal integrated film-activated sludge (IFAS) wastewater treatment system (Hawkesbury, Ontario, Canada). P_{N1} was fed with a peristaltic pump with influent from a wastewater feed tank, and P_{N2} was fed via gravitational flow with the effluent from P_{N1}. The target (measured) influent NH_4^+-N concentrations were 44 (44.5 ± 0.3) mg NH_4^+-N /L for P_{N1} and 30 (29.4 ± 0.7) mg NH_4^+-N/L for P_{N2}, all within the conventional limits of mainstream municipal NH_4^+-N concentrations. The reactor P_{N1} was fed using a peristaltic pump at an influent flow rate of 1 L/h of feed which resulted in a hydraulic retention time (HRT) of 2 h. The mixing and aeration in the reactors were achieved through the installation of a recirculation pump and coarse bubble aeration system, with an airflow rate set at 0.15 L/min. This mixing and aeration resulted in an average dissolved oxygen of 3.25 mg O_2/L in the reactors. The temperature and pH were not controlled but monitored at 20.3 ± 0.1°C and 7.3 ± 0.1, respectively. The effluent from the partial nitritation MBBR, P_{N2} was directed to a 2L holding tank to allow optimization of the HRT in the anammox unit.

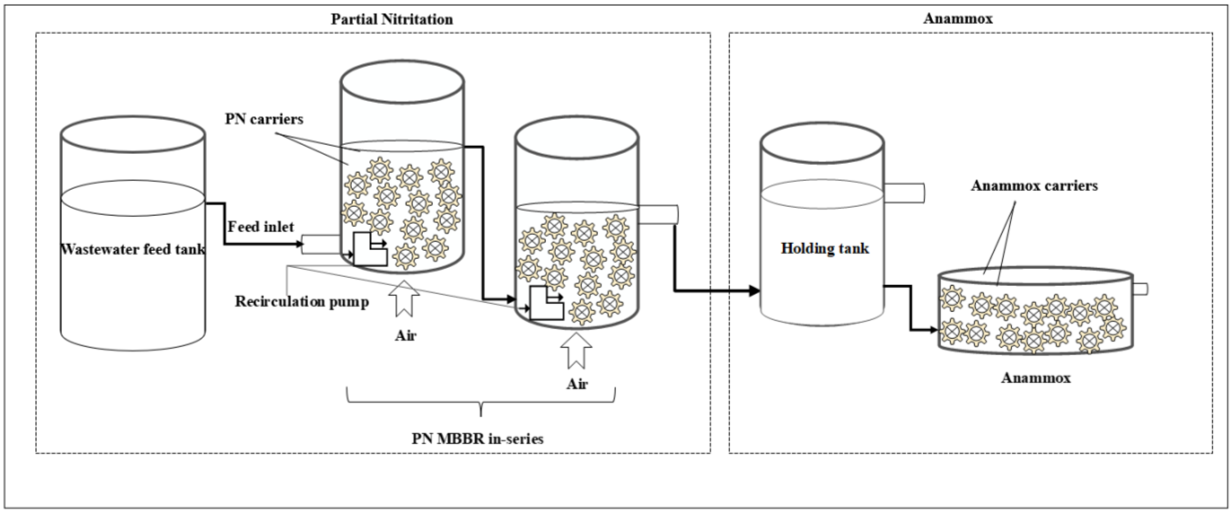

Figure 6.1 Experimental setup.

The effluent from the holding tank was channeled to an anammox MBBR with a working volume of 2L. The reactor was filled to 20% with AnoxK™5 carriers (Anoxkaldnes, Lund, Sweden) with a protected surface area of $800 m^2/m^3$. The carriers were previously seeded from a full-scale, sidestream anammox ANITAMox system (South Durham WRRF, Durham, NC, United States). The anammox reactor was fed with a peristaltic pump with influent from the holding tank and the average optimized HRT of the reactor was at 24h. The mixing of the carriers was achieved by a magnetic stirrer operated at a fixed speed of 30 rpm. The reactor was sealed, and dissolved oxygen was frequently removed from the bulk solution by purging it with nitrogen gas at a fixed flow rate of 0.8 L/min through a gas inlet opening on the reactor top. The average dissolved oxygen concentration in the reactor was between 0.06 to 0.1 ±

0.2 mg O_2/L. The pH was not controlled; however, the temperature was kept at 29 to 30 ± 0.3°C with a temperature control plate wrapped around the reactor.

6.4.2 Anammox activity batch test

A batch test was performed to quantify the contribution and activity of anammox in nitrogen removal. At the beginning of the batch test, 30 carriers were harvested from the anammox reactors and gently rinsed with distilled water. The cleaned carriers were placed in a 1 L reactor containing 0.5 L of target (measured) synthetic medium consisting of 40 (40.2 ± 0.06) mg NH_4^+-N/L as $(NH_4^+)_2SO_4$ and 46 (46.3 ± 0.1) NO_2^- -N/L as $NaNO_2$. During the batch test, the reactor was utterly sealed, and dissolved oxygen was kept below 0.02 ± 0.1 mg O_2/L through continuous purging with nitrogen gas at a constant flow rate of 1.5 L/min. pH was monitored continuously and gradually adjusted using 0.05 M of H_2SO_4. The batch test lasted for 24h with an intermittent sampling of the reactors every 6h, and NH_4^+-N and NO_2^--N concentrations were measured in triplicates to determine volumetric rates (Dapena-Mora et al., 2007).

6.4.3 Wastewater characteristics

The wastewater feed for the partial nitritation MBBR system was prepared based on the recipe developed and modified in previous studies (Hoang et al., 2014; Schopf et al., 2019; Tian and Delatolla, 2019). The specific composition of the wastewater feed at target NH_4^+-N SALR of 7 g NH_4^+-N/m^2/d was as follows: $(NH_4^+)_2SO_4$: 210 mg/L, $NaHCO_3$: 570 mg/L, $MgSO_4 \cdot 7H_2O$: 80 mg/L, $CaCl_2 \cdot 2H_2O$: 30 mg/L, KH_2PO_4: 80 mg/L, $FeSO_4 \cdot 7H_2O$: 6 mg/L. Trace nutrients: $MnCl_2 \cdot 4H_2O$: 0.10 mg/L, $Na_2MoO_4 \cdot 2H_2O$: 0.03 mg/L, $CuSO_4 \cdot 5H_2O$: 0.10 mg/L,

$CoCl_2\cdot 6H_2O$: 0.001 mg/L, $ZnSO_4\cdot 7H_2O$: 0.03 mg/L and carbon source: glucose 4.86 mg/L, sodium acetate 2.59 mg,/L and peptone 4.86 mg/L.

6.4.4 Constituent analyses

The nitrogen constituents, $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, and $N\text{-}NO_3^-$ were analyzed three times a week in accordance with the standard methods (APHA, 2005): methods 4500-NH_3, 4500B-NO_2^- and 4500A-NO_3^-, respectively. Total nitrogen (TN) was calculated as the sum of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, and $N\text{-}NO_3^-$. sCOD was analyzed using a DR 5000 spectrophotometer (HACH, Loveland, CO, USA) after filtration using 0.45 µm, Millipore, G filter. Alkalinity was determined using a HACH method 8000 in DR 5000 spectrophotometer (HACH, Loveland, CO USA). Dissolved oxygen, pH, and temperature were measured using a symphony Multi-Parameter Meter with attached temperature & dissolved oxygen probe and pH probe (VWR, Ontario, Canada).

6.4.5 Biofilm characteristics

Biofilm thickness and morphology

The biofilm thickness and morphology were characterized using the variable pressure scanning electron microscope (VPSEM) imaging at a pressure of 40 Pa using the Tescan USA Inc., Vega II-XMU SEM (Cranberry, PA, USA). Four carriers from the partial nitritation and anammox reactor were harvested and analyzed. Five images per carrier from each reactor were taken at random locations across the surface at ×60 magnification for biofilm thickness. The biofilm thickness was measured and quantified using the MedCalc Digimizer Image analysis software V.5.6.0 (Ostend, Belgium), and for each carrier image, a minimum of 1000 thickness measurements were acquired. Finally, one image per carrier was taken at a randomized location at ×600 magnification to visualize biofilm morphology (Young et al., 2017).

Droplet digital PCR analyses

The droplet digital polymerase chain reaction (ddPCR) was used to quantitate the AOB, NOB, and AnAOB population within the biofilm. The protocol adopted and modified was based on Tian et al., (Tian et al., 2020). The DNA sample was extracted from three biological replicates using the FastDNA*Spin Kit for soil (MP Biomedicals, CA, USA), after which the samples were stored at -80°C. The ddPCR mixture contained 5µL of sample and 11 µL of QX200™ ddPCR™ EvaGreen*Supermix (Bio-Ras, Hercules, CA), including 6.04 µL nuclease-free water and 0.23 µL of each forward and reverse primer (10 µmol/L), the used in this study are listed in Table 1. The set of amoA primers (*amoA*-1f/*amoA*-2r) targeted a stretch of conserved regions of the known amoA gene sequence of *Nitrosomonas europaea*. Also, two coupled primers, FGPS872f/FGPS1269r, and NSR1113f/NSR1264r, were used to amplify the NOB, *Nitrobacter,* and *Nitrospria,* respectively. Hydrazine oxidoreductase (hzo) mRNA was identified as a candidate biomarker of anammox bacteria activity, targeted the conserved regions of known hzo sequence in three different anammox bacterial strains KSU-1, *Candidatus* "*Brocadia anammoxidans*", and *Candidatus "Kuenenia stuttgartiensis*" (Park et al., 2010). The droplets were generated and amplified in a T100™ Thermal Cycler machine (Bio-Rad). The amplification program consisted of the following steps: denaturation at 95 °C for 5 min, followed by 50 cycles of 30 s at 95 °C, 60 s at corresponding primer annealing temperature (Table 6.1), and 30 s at 72 °C, followed by 5 min cooldown at 4 °C, 5 min at 90 °C for droplet stabilization and hold at 12 °C. the plates were read using a QX200 droplet reader (Bio-Rad, Hercules, CA), and data analyzed using the QuantaSoft Software (Bio-Rad, version 1.7.4, Hercules. CA).

Table 6.1 Primer set and annealing temperatures.

Target	Primer	Sequence (5'-3')	Annealing Temperature (°C)	Reference
AOB	amoA-1f	GGGGTTTCTACTGGTGGT		(Rotthauwe et al. 1997)
	amoA-2r	CCCCTCKGSAAAGCCTTCTTC	53	
NOB - *Nitrobacter*	FGPS872f	CTAAAACTCAAAGGAATTGA	56	(Degrange and Bardin, 1995)
	FGPS1269r	TTTTTTGAGATTTGCTAG		
NOB - *Nitrospira*	NSR1113f	CCTGCTTTCAGTTGCTACCG	53	(Bao et al. 2017)
	NSR1264r	GTTTGCAGCGCTTTGTACCG		
AnAOB	hzoF	CATGGTCAATTGAAAGRCCACC	57	(Park et al., 2010)
	hzoR	GCCATCGACATACCCATACTS		

6.4.6 Statistical analyses

Statistical analysis of nitrogen constituents, biofilm characteristics (biofilm thickness), and bacterial gene copies were carried out using the student *t*-test, were a *p*-value less than 0.05 is considered a significant difference.

6.5 Results and discussion

6.5.1 The performance of the elevated loaded partial nitritation and anammox system

The elevated loaded partial nitritation system following the anammox system as a combined two-stage configuration successfully operates, treating an average influent total ammonia nitrogen concentration of 44.5 ± 0.1 mg NH_4^+-N/L, within mainstream municipal concentration (Figure. 6.2).

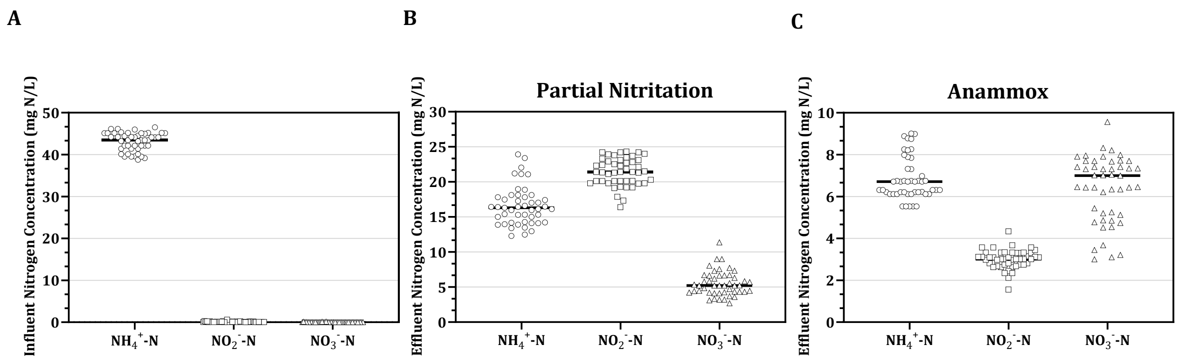

Figure 6.2 Performance of the partial nitritation and anammox system. **(A)** influent nitrogen concentration, **(B)** partial nitritation, and **(C)** anammox, effluent nitrogen concentration. Black horizontal line indicating mean with 95% confidence interval.

The average effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations from the elevated loaded partial nitritation MBBR system are 17.1 ± 0.2 mg NH_4^+-N/L , 20.9 ± 0.1 mg NO_2^--N/L, and 5.5 ± 0.2 mg NO_3^--N/L, respectively (Figure 6.2B), and corresponds to an average NH_4^+-N:NO_2^--N stoichiometric ratio of 1:1.17, which is a suitable metabolic ratio for subsequent downstream anammox treatment considering the theoretical anammox stoichiometry ratio of 1:1.32 (Strous et al., 1997; Van Dongen et al., 2001). This stoichiometric ratio also fits well within the ranges of optimized ratios reported by Gu et al., (2018) and Kowalski et al., (2019) in mainstream MBBR systems while employing distinct operationally intensive control strategies. Meanwhile, in this study, maintaining the ideal effluent molar ratio for subsequent anammox operation in the MBBR system, as previously demonstrated by Ikem et al., is possible as employing elevated NH_4^+-N loading rate as a design strategy offers multiple morphological benefits to embedded biofilms which consequently allows for effective suppression of NOB activity in the biofilm (Ikem et al., 2023a).

The effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentration from the anammox unit is 6.33 ± 0.6 mg NH_4^+-N/L, 2.23 ± 0.2 mg NO_2^- -N/L and 6.58 ± 0.5 mg NO_3^--N/L (Figure 6.2C). The sum of these values corresponds to a TN concentration of 15.2 ± 0.8 mg N/L, representing the final effluent from the combined two-stage elevated loaded partial nitritation and anammox system. This value is within the range of values Gustavsson et al. (2020) reported from an anammox MBBR system. Also, this value can be compared to an average total inorganic nitrogen concentration of 11 mg/L reported from an anammox-integrated fixed activated sludge (IFAS) system (Kowalski et al., 2019). Although Liu et al., (2018) and Laureni et al., (2016) have reported significantly lower TN effluent concentrations between 5.2 and 6 mg N/L from a mainstream partial nitritation/anammox system. It is, however, important to highlight that the final effluent quality presented in this study could be significantly improved through further optimization of the anammox system, which is beyond the scope of this work, but feasible and could form the basis for future work.

Furthermore, the anammox stoichiometric coefficients were estimated from the experimental data. The anammox stoichiometric coefficients are important as they provide insights into the anammox process contribution to TN removal. The average NO_2^--N to NH_4^+-N molar removal ratio is 1.05 ± 0.1. This estimated ratio, close to the optimized value reported by Strous et al., (1998), implies that anammox process was established in the reactor. Accordingly, the yield of NO_3^--N produced per NH_4^+-N consumed molar ratio is at 0.43 ± 0.2, corresponding to 14.8 ± 1.1% relative nitrate production, suggesting that NOB activity was suppressed in the anammox system. Although this relative nitrate production value exceeded the stoichiometric value of 11% for partial nitritation/anammox proposed by Strous et al., (1998), similar trends have been reported in several mainstream partial nitritation/anammox studies (Laureni et al., 2016; Lotti et al., 2015). Specifically, comparable nitrate levels have been reported, with even more significant variations up to 40% relative nitrate level leading to process instability and reduced performance in the system, since nitrate production during anammox reaction often inhibits AnAOB growth and subsequently limits effective removal of nitrogen from the system (Gustavsson et al., 2020). Irrespective of these findings, the results from this present study have shown the possibility of maintaining an appropriate anammox stoichiometric coefficient with minimal relative nitrate production under mainstream conditions.

6.5.2 The partial nitritation and anammox removal kinetics and nitrogen removal efficiency

The NH_4^+-N and NO_2^--N oxidation kinetics of the elevated loaded partial nitritation MBBR system are quantified at NH_4^+-N SALR of 7.03 ± 0.6 g NH_4^+-N/m^2/d. The average AOB nitritation kinetics rate (NH_4^+-N SARR) is at 3.09 ± 0.6 g NH_4^+-N /m^2/d. The average percent of the total oxidized NH_4^+-N that remains as NO_2^--N (NO_x as NO_2^-) is at 98.1 ± 0.3%, corresponding to NOB nitratation kinetics rate (NO_2^--N SARR) of 0.0005 ± 0.2 g NO_2^--N/m^2/d. The NH_4^+-N and NO_2^--N SARR kinetics observed in this study show that a high partial nitritation rate is achieved while utilizing an elevated NH_4^+-N surface loading rate, a simple and passive design strategy.

The appropriate effluent NH_4^+-N:NO_2^- -N metabolic ratio from the mainstream elevated loaded partial nitritation MBBR system was utilized to operate the anammox MBBR system. Stable anammox performance was successfully maintained for over 100 days, and the total nitrogen loading rate (TNLR), total nitrogen removal rate (TNRR), and total nitrogen removal efficiency are presented in Figure 6.3.

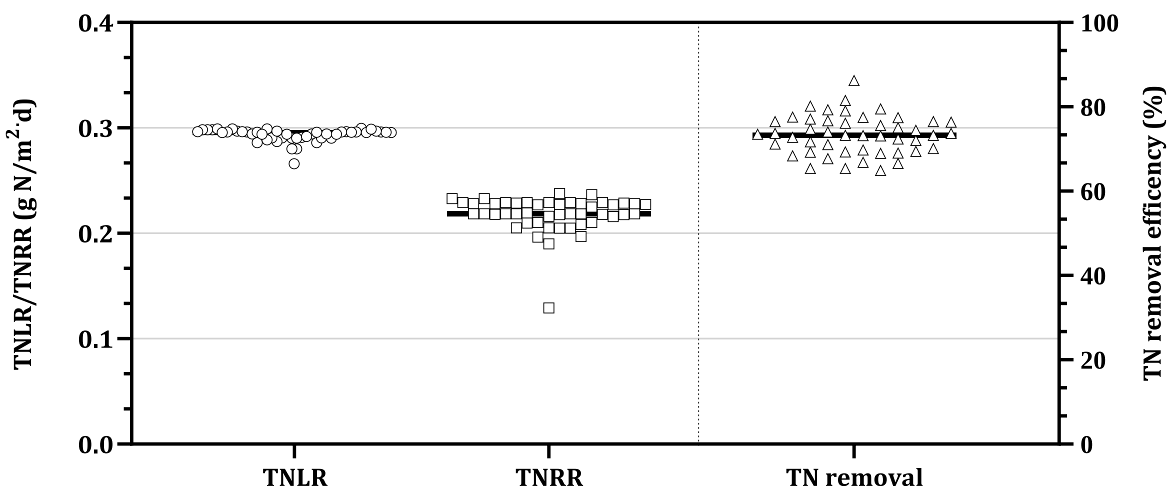

Figure 6.3 The two-stage partial nitritation/anammox, showing the TNLR (circle), TNRR (square), and TN removal efficiency (triangle). Horizontal line indicating mean with 95% confidence interval.

The average TNLR in the anammox reactor is at 0.30 ± 0.1 g N/m^2/d with a resultant TNRR of 0.22 ± 0.2 g N/m^2/d corresponding to a TN removal efficiency of 74.1 ± 0.7%. These values are comparable to the typical values achieved in conventional wastewater treatment systems that employ simultaneous nitrification and denitrification process (Chai et al., 2019). Further investigation also showed that these values are significantly higher than those reported by Regmi et al., (2015) from an anammox MBBR system under mainstream conditions. In contrast, Dosta et al., (2015) and Lotti et al., (2019) have reported higher TN removal efficiency of 88.1% and 85%, respectively, from an anammox system while operating at higher influent nitrogen concentrations between 200-500 mg N/L. A higher TN removal efficiency is expected, as elevated NH_4^+-N in liquid medium from operating NH_4^+-N rich anammox systems promotes higher NOB activity suppression with limited NO_2^--N competition or depletion promoting increased anammox activity in the system (Lackner et al., 2014; Zhao et al., 2022).

To further understand the role of the anammox process in nitrogen removal, batch test kinetics was used to isolate the anammox activity of the biofilm (Figure S6.1). The anammox activity of biofilm is 6.08 ± 1.4 g $N/m^2/d$; which further validates the previous statement that anammox process was completely established in the MBBR system and nitrogen removal was possible, primarily through this pathway. These findings could also explain the high TNRR and TN removal efficiency observed in this study. This is supported by previous research that has demonstrated sufficient anammox attachment and a promising TN removal rate under mainstream conditions with specific surface anammox activity of 0.85 g $N/m^2/d$ (Kowalski et al., 2019). Herein, these observation shows that it is possible to maintain sufficient anammox activity leading to high TNRR and TN removal efficiency, with stable effluent from the elevated loaded partial nitritation system under mainstream conditions.

6.5.3 Biofilm characteristics

The biofilm thickness of carriers in partial nitritation and anammox systems is measured using the VPSEM (Figure 6.4). In the partial nitritation system, the biofilm thickness of the carrier ranges from 546.3 ± 19.3 μm to 601.5 ± 30.6 μm with an average value of 581.7 ± 25.6 μm. The biofilm thickness of the carriers in the anammox system ranges from 150.7 ± 11.4 μm to 250.1 ± 23.3μm, with an average value of 204 .2 ± 14.3 μm. Notably, the average biofilm thickness of carriers in the partial nitritation system is significantly higher than those in the anammox system. The thicker biofilm in the partial nitritation system is potentially due to operating at an elevated loading rate that allows deeper substrate penetration into the biofilm resulting in higher cell growth. Regardless, the observed biofilm thickness in the anammox system is similar to what was seen in other mainstream biofilm-based two-stage partial nitritation/anammox systems (Kowalski et al., 2019).

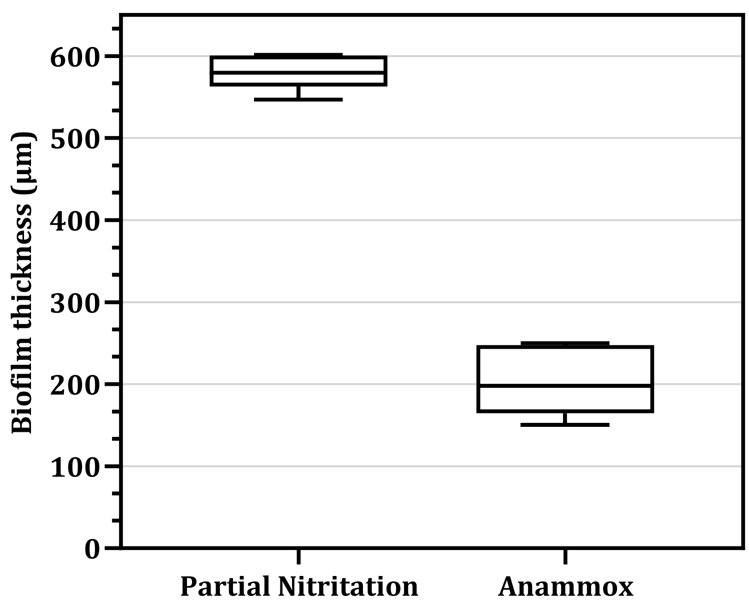

Figure 6.4 Biofilm thickness on partial nitritation and anammox carrier. Error bars indicate the 95% confidence intervals.

Furthermore, the VPSEM was employed to visualize the morphological characteristics of the biofilm in the partial nitritation and anammox system (Figure S6.2). Short rod-shaped bacteria dominated the surface of the partial nitritation biofilm (Figure S6.2A), which are presumably nitrifying microorganisms and are consistent with the findings from Zhang et al., (2013). Generally, as expected, the images of biofilms from the anammox system were distinctive from the partial nitritation system. The biofilm images from the anammox system are more globular-shaped (spherical-shaped bacterium) scattered on the biofilm surface (Figure S6.2B). It is well established that the AnAOB are majorly gram-negative spherical bacteria with a diameter of approximately 1μm (Zeng et al., 2016). Therefore, it is likely that the spherical-shaped bacteria observed in this study are AnAOB, similar to previous observations reported by Liu et al., (2020) and Trigo et al., (2006).

The relative quantification of AOB, NOB (*Nitrospira* and *Nitrobacter*), and AnAOB are measured via the ddPCR to study the nitrifying and anammox bacteria populations within the partial nitritation and anammox system (Table 6.2).

Table 6.2 Relative quantification of AOB, NOB, and AnAOB in the partial nitritation and anammox system determined by ddPCR.

	AOB copies (gene copies/carrier)	NOB *(Nitrospira)*copies (gene copies/carrier)	AnAOB copies (gene copies/carrier)
Partial nitritation	$3.16 \pm 0.8 \times 10^7$	$2.32 \pm 0.6 \times 10^6$	Not detected
Anammox	$9.17 \pm 1.1 \times 10^4$	6.23 ± 1.0	$3.28 \pm 0.7 \times 10^8$

As expected, the average AOB gene copy number of $3.16 \pm 0.8 \times 10^7$ copies/carrier in the partial nitritation system is significantly higher compared to $9.17 \pm 1.1 \times 10^4$ copies/carrier observed in the anammox system. Regarding the NOB population, *Nitrobacter* was not reported as it was not detected in both partial nitritation and anammox systems. The average NOB (*Nitrospira*) gene copy numbers in the partial nitritation system are $2.32 \pm 0.6 \times 10^6$ and 6.23 ± 1.0 in the anammox system. AnAOB was not detected in the partial nitritation system. The average AnAOB gene copies in the anammox system are at $3.28 \pm 0.7 \times 10^8$. The higher AnAOB gene copies compared to AOB and NOB in the anammox system supports the reported anammox stoichiometric coefficient from the experimental data close to the anammox theoretical values and further confirms that anammox process was established in the MBBR system under mainstream conditions.

6.5.4 The feasibility of the combined two-stage elevated loaded partial nitritation and anammox system

The study presents a low-operational intensive, robust, and high-rate partial nitritation system following an anammox system, operating as a combined two-stage elevated loaded partial nitritation/anammox system for enhanced nitrogen removal from municipal wastewater treatment. The findings from this study show the feasibility of using the combined two-stage elevated loaded partial nitritation/anammox system to achieve low effluent nitrogen concentrations when applied under mainstream conditions. The elevated loaded partial nitritation system, high-rate systems, provides an appropriate effluent nitrogen ratio leading to the successful operation of the anammox reactor. Although the result from this study demonstrates a need for further optimization of the anammox system and further investigation of this configuration at a pilot scale using real wastewater, the introduction of the two-stage elevated loaded partial nitritation/anammox system MBBR system has shown promise. As such, it could be integrated into an existing municipal wastewater treatment infrastructure without remodeling or reconstruction.

6.6 Conclusions

The study shows the successful operation of the elevated loaded partial nitritation following the anammox system as a combined two-stage configuration for nitrogen removal under mainstream conditions. The result from the study shows a promising TNRR of 0.22 ± 0.2 g N/m^2/d and TN removal efficiency of 74.1 ± 0.7% from the combined two-stage elevated loaded partial nitritation/anammox MBBR system. The anammox process is identified as the possible main pathway for the observed high removal rate and removal efficiency. This knowledge base can be expanded by further investigation at the pilot scale to advance the implementation of the partial nitritation/anammox process at mainstream municipal wastewater for effective nitrogen removal.

6.7 Supplemental Information

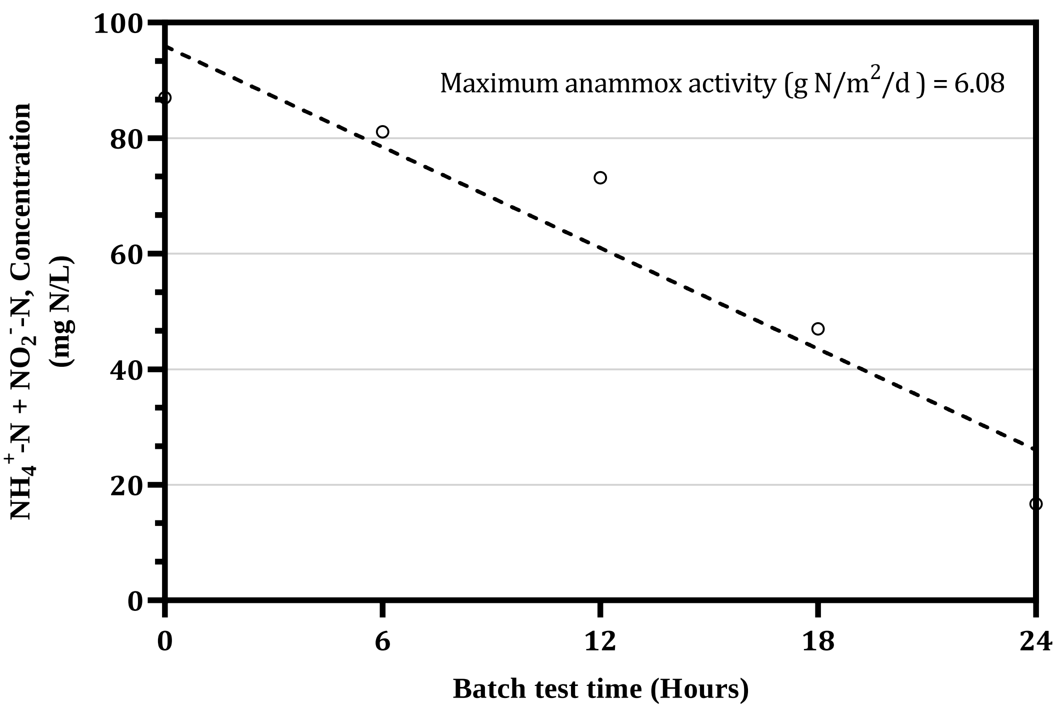

Figure S6.1 Measured NH_4^+-N and NO_2^- -N consumption rate in anammox biofilm (Graphical plots) and maximum anammox activity (box).

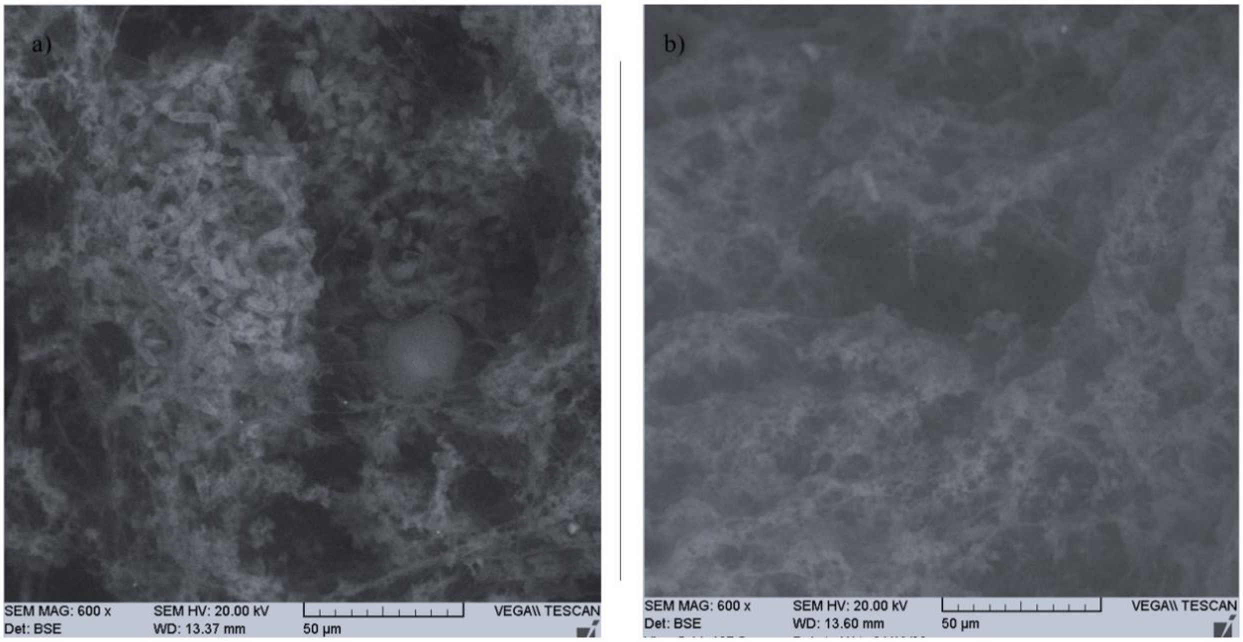

Figure S6.2. VPSEM images of carrier surface conditions at 600x magnification: **(A)** partial nitritation carrier **(B)** anammox carrier.

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CHAPTER 7 - DISCUSSION AND CONCLUSIONS

This dissertation progresses current knowledge of the application of cost-effective biological nitrogen removal process for mainstream municipal wastewater through the identification of design strategy and the subsequent optimization of the partial nitritation (PN) process and the translation of this optimized PN process into a full partial nitritation/anammox (PN/A) system. Specifically, this research addresses critical challenges facing mainstream municipal wastewater that have inhibited the successful implementation of the PN/A process within this wastewater stream using the attached growth, moving bed biofilm reactor (MBBR) technology. In particular, the research isolates the optimal design parameters, identifies a new design configuration and the mechanism of nitrite oxidation responsible for stable PN, and characterizes the effect of mixing and aeration conditions of the mainstream elevated loaded PN MBBR system. Finally, the elevated loaded PN/A MBBR system was successfully operated as a combined two-stage PN/A MBBR technology for enhanced nitrogen removal at mainstream municipal wastewater.

7.1 Isolation of optimal design parameters

Chapter 3 provides findings on the feasibility of using the elevated total ammonia nitrogen (TAN) loading rate, design strategy to achieve stable PN under mainstream conditions. This work identifies critical optimum design parameters, a TAN surface area loading rate (SALR), a hydraulic retention time (HRT), and an airflow rate optimized for stable partial nitritation of a mainstream elevated loaded MBBR system. to isolate these parameters, three PN MBBRs were operated in parallel, with each investigating distinct design parameters (SALR, HRT and airflow rate).

The SALR conditions that achieve optimal TAN removal kinetics and percent NO_x as nitrite are isolated at TAN SALR values 4, 5, 6, and 7 g TAN/m^2·d, with resultant TAN SARR of 1.71, 2.21, 2.23, and 2.44 g TAN/m^2·d and percent NO_x as nitrite of 88.1, 90.3,91.5 and 94.2%. The TAN SARR, 1.71 g TAN/m^2·d of the system loaded at a TAN SALR of 4 g TAN/m^2·d is statistically significantly lower than the TAN SARR of 2.21 TAN/m^2·d measured at a TAN SALR of 5 g TAN/m^2·d. This lower TAN SARR at TAN SALR of 4 g TAN/m^2·d, indicates that the kinetics were likely operated at first order with respect to the bulk liquid TAN concentration, as the system is operating at TAN substrate concentration mass transfer rate limited condition. Meanwhile, the TAN SARRs values of 2.21, 2.23, and 2.44 g TAN/m^2·d measured at TAN SALRs of 5, 6, and 7 g TAN/m^2·d, respectively, show no statistically significant difference. The lack of distinction between the kinetics at these varying TAN SALRs of 5, 6, and 7 g TAN/m^2·d indicates that the reactions are zero-order, as the reaction rate no longer increases in relation to TAN SALR values. The overall assessment shows that the optimum performance of the elevated PN MBBR system is observed at TAN SALR of 5 g TAN/m^2·d with a TAN SARR of 2.21 g TAN/m^2·d and percent NO_x as nitrite of 90.3%.

With the isolated optimum TAN SALR and at varying distinct HRTs of 1.6, 2, 2.2, and 2.5h, the optimal HRT is identified. An HRT of 1.6h shows the statistically lowest TAN SARR of 1.57 g TAN/m^2·d. The shift in HRT from 1.6 to 2h shows a statistically significant increase from 1.57 to 2.39 g TAN/m^2·d. However, a further change in HRT to 2.2 and 2.5h shows unstable performance, indicated by the large variations in TAN SARR. Similarly, with an increase in HRT from 1.6 to 2h, the percent NO_x as nitrite demonstrates a statistically significant but slight increase from 79.8 to 85.7%. At HRTs of 2, 2.2, and 2.5h, the percent NO_x as nitrite did not statistically significantly vary, demonstrating an average percent NO_x

as nitrite of 86.6 %. Comparing the TAN SARR and percent NO_x as nitrite-measured values at all investigated HRTs, the HRT of 2h is observed to be optimal and likely supports a high, stable ammonia-oxidizing bacteria (AOB) population or activity in the PN MBBR system. A short HRT of 2h is beneficial with respect to the sizing of the system, and hence the capital cost and energy savings due to the aeration of a smaller reactor and an overall smaller land footprint. Therefore, the stable TAN SARR of 2.39 g TAN/m^2·d and high percent NO_x as nitrite of 85.7% at an HRT of 2h shows that a PN MBBR system can be operated within a small tank volume to achieve stable PN performance.

The PN MBBR reactor, based on the optimal isolated TAN SALR and HRT, was operated at distinct varying airflow rates of 1, 1.5, 2, and 4 L/min to isolate the optimum airflow rate for the mainstream elevated loaded PN MBBR system. As the airflow rate increases from 1 to 1.5 and 2 L/min, the average TAN SARR are 1.95, 2.32, and 2.18 g TAN/m^2·d, respectively. As the steady incremental airflow rate of 0.5 L/min did not show an observable statistically significant difference, the airflow rate was increased further to 4 L/min. With the change in airflow rate from 2 to 4 L/min, the TAN SARR significantly increases to 3.19 g TAN/m^2·d. On the hand, the percent NO_x as nitrite shows a statistically significant increase from 73.1 to 84.8%, with a change in airflow rate from 1 to 1.5 L/min. However, as the airflow rate increases from 1.5 to 2 and 4 L/min, the percent NO_x as nitrite decreases from 84.8 to 79.5 and 53.5%, as the systems gradually transition from PN to complete nitrification. The findings from the study show that operating the PN MBBR system at an airflow rate of 1.5 L/min demonstrates optimal performance with TAN SARR of 2.30 g TAN/m^2·d and a percent of NO_x as nitrite of 84.8%.

7.2 PN design and mechanism of nitrite oxidation suppression

Chapter 4 provides a new PN design configuration to achieve ideal effluent stoichiometric ratio for subsequent anammox treatment and findings on the possible mechanism of nitrite oxidation suppression responsible for stable PN in the mainstream elevated PN MBBR system. In this study, two identical MBBRs were operated in series at similar elevated loading rates. This study further identifies a new attached growth (MBBR) system configuration (i.e., two reactor in-series) utilizing elevated TAN loading rate as a design strategy to achieve appropriate effluent NO_2^--N:NH_4^+-N stoichiometric ratio for subsequent downstream anammox treatment. The work investigates the effect of the elevated loading design strategy on the mainstream PN MBBR systems through the evaluation of biofilm characteristics, cell viability, and microbiome within the biofilm.

At the macro scale, the operation of the two PN MBBRs in series demonstrates stable and steady PN performance. The average percent NO_x as nitrite and relative nitrate from the PN MBBR system is 77.9% and 14.5%. The high value of percent NO_x as nitrite and minimal levels of nitrate build-up in the elevated loaded partial nitritation MBBR system suggest that the nitrite-oxidizing bacteria (NOB) population in the biofilm or the NOB activity of the embedded population is possibly suppressed. The average effluent TAN, nitrite, and nitrate concentrations from the PN MBBR in-series are 12.9 ± 1.84 mg TAN/L, 13.9 ± 1.56 mg NO_2^--N/L and 3.11 ± 0.29 mg NO_3^--N/L, corresponding to a NO_2^--N:NH_4^+-N stoichiometric ratio of 1.15. This ratio offers possible suitable nitrogen speciation for the subsequent downstream anammox treatment, and the two reactors in series partial nitritation MBBR system configuration in this study herein provides a stable and steady effluent quality for subsequent downstream anammox operation.

The mesoscale and micro-scale effects of the elevated TAN SALR on the biofilm were quantified by analyzing biofilm characteristics described as biofilm thickness, biofilm mass, biofilm density, morphology, and cell viability. The biofilm thickness and morphology in the reactors show thick biofilms with the embedded biomass showing no viability constraint, as the embedded cells remained viable, supporting the observed stable and steady partial nitritation performance. The thick biofilm morphology likely reduced the diffusive transport of dissolved oxygen into the biofilm, which limits the dissolved oxygen uptake by the NOB population. At the molecular scale, the AOB and NOB gene copies in PN reactors were statistically similar. The results of the AOB and NOB gene copy numbers of the MBBR biofilm show that rather than NOB population suppression, NOB activity suppression is the likely mechanism responsible for the stable and steady partial nitritation performance in the mainstream elevated loaded partial nitritation MBBR system.

7.3 Impact of mixing and aeration conditions to promote PN/A configuration

Chapter 5 provides new knowledge on the effects of three distinct mixing and aeration conditions (continuous aeration, mechanical paddle & aeration, and recirculation pump & aeration) on the mainstream elevated loaded PN MBBR system. Specifically, the study compares the conventional mixing and aeration condition, continuous aeration, to mechanical paddle & aeration, and recirculation pump & aeration, utilized to optimize the PN MBBR system to achieve low dissolved oxygen effluent concentrations for optimal downstream anammox. The results demonstrate significantly higher effluent quality corresponding to an appropriate effluent NO_2^--N: NH_4^+-N metabolic ratio of 1.09:1, close to the proposed optimized ratio for the successful anammox treatment during operation with recirculation pump & aeration than continuous aeration and/or mechanical paddle & air.

Also, maintaining mixing and aeration with a recirculation pump & aeration shows 5 times significantly higher AOB to NOB ratio than continuous aeration, suggesting a possible higher nitrite oxidation suppression within the mainstream elevated loaded PN MBBR system. Therefore, the study identifies a recirculation pump with reduced aeration as a possible effective and suitable mixing and aeration strategy for operating the mainstream elevated PN MBBR system.

7.4 Desing of two-stage elevated loaded PN/A MBBR system

Chapter 6 provides findings on the feasibility of operating the elevated loaded partial nitritation system following an anammox system as a combined two-stage system for enhanced nitrogen removal from municipal wastewater treatment. In this study, the appropriate effluent NO_2^--N: NH_4^+-N stoichiometric from the fully optimized PN system was channeled to anammox for enhanced nitrogen removal under mainstream conditions. The result from the study shows a promising total nitrogen removal rate of 0.22 g N/m^2·d and total nitrogen removal efficiency of 74.1% from the combined two-stage elevated loaded PN/A MBBR system. In addition, the anammox activity of biofilm is 6.08 g N/m^2·d, suggesting that anammox process was completely established in the MBBR system and nitrogen removal was primarily through this pathway. This finding is supported by the significantly higher anammox bacteria (AnAOB) than AOB and NOB gene copies in the anammox MBBR system. Finally, the results demonstrate that a combined two-stage elevated loaded PN/A system is feasible under mainstream conditions and provides a knowledge base for further pilot-scale investigation.

7.5 Cost-benefit analyses of the promising two-stage elevated loaded PN/A system

Cost-benefit analyses of the promising two-stage elevated loaded PN/A process investigated in this study were conducted primarily to provide insight into the cost-effectiveness of the treatment process compared with the conventional biological nitrification/denitrification process for nitrogen removal under mainstream conditions (Table 7.1). For ease of comparison, the cost estimation was based on three key operational costs, associated with energy demand due to aeration, chemical demand due to external carbon source, and sludge disposal, all of which account for 40-60% of the total operational cost for biological nitrogen removal in wastewater resource recovery facilities (WRRF). While the economic evaluation of these treatment processes was presented as a like-for-like comparison; however, there are other key differences, such as startup/infrastructure costs that are part of a full-scale design that was excluded that may offset or amplify the treatment cost difference among these two treatment processes. The cost estimate was based on data from a wastewater treatment facility of a Canadian city with a population of 1.05 million, an average daily influent flow rate of 390 million litres per day (MLD), and an average daily influent NH_4^+-N concentration of 42 mg NH_4^+-N/L and average daily influent COD of 308 mg/L. All assumptions and calculation steps are provided in the supplementary information.

Table 7.1 Operational cost of conventional biological nitrification/denitrification system and the two-stage PN/A system in terms of energy demand, chemical demand, and sludge disposal.

	Energy demand CAD	Chemical demand CAD	Sludge disposal CAD	Total CAD
Nitrification/denitrification system	5,352,360	518,665	167,170	6,038,195
Two-stage PN/A system	2,267,380	0	52,195	2,319,575
Annual cost savings	3,084,980	518,665	114,975	3,718,620

*CAD-Canadian dollars

The result shows that compared to the conventional biological nitrification/denitrification process for nitrogen removal, the two-stage elevated loaded PN/A system offers a 57.6% savings on energy cost, 100% savings on chemical cost, and 68.7% savings on the cost of sludge disposal. Therefore, the two-stage configured elevated loaded PN/A system, in addition to high nitrogen removal efficiency, reduced footprint, and ease of operation, is also economically favorable and reduces the overall operational cost of wastewater treatment system by 61.6%, thus saving up to 3.7 million CAD every year.

7.6 Novel contribution, practical application, and future direction

This research provides novel information and develops new fundamental knowledge on the optimization of the mainstream elevated loaded PN MBBR system for successful operation of downstream anammox unit for enhanced nitrogen removal from municipal wastewater. This is the first study to employ the elevated TAN loading rate, design strategy for attached growth technologies to achieve stable and steady PN under mainstream conditions. The study further presents the optimal design, i.e., two reactors in series under specified SALR, HRT, and airflow rate of the mainstream elevated loaded PN MBBR technology to achieve optimal effluent for the anammox process. The research uses select microbial and molecular methods to elucidate elevated loading rate as a PN design strategy that significantly suppresses NOB activity for stable PN performance under mainstream conditions. In addition, this study identifies distinct mixing and aeration condition to achieve low DO effluent and appropriate effluent NO_2^--N:NH_4^+-N stoichiometric ratio for the successful operation of the subsequent anammox system. Finally, the optimized elevated loaded PN/A MBBR system is demonstrated as a combined two-stage configured system for nitrogen removal at mainstream municipal concentrations.

The study provides comprehensive information and new knowledge necessary to design, operate and optimize the PN/A system for the advancement of mainstream municipal treatment. The study isolates critical optimum design parameters of the mainstream elevated loaded PN system. This finding guided the new design, two in-series reactor configuration that provides the ideal effluent NO_2^--N: NH_4^+-N metabolic ratio for subsequent anammox treatment. The findings from this study show that mainstream design configuration is not only novel but also demonstrates advancement of the design strategy and enables this design strategy to have a more significant impact by being applied under mainstream conditions. This study also shows that low DO effluent, stable performance, and ideal effluent quality can be achieved by maintaining mixing and aeration using a recirculation pump and reduced airflow rate in the elevated loaded PN MBBR system. This study demonstrates that with an optimized PN system, operation of the anammox unit as a combined two-stage configured system for mainstream municipal treatment is feasibly resulting in a high nitrogen removal rate, removal efficiency, and stable effluent quality.

The study focused on the optimization of the elevated PN for anammox unit operation under mainstream conditions using a synthetic feed medium. However, to expand the knowledge base and access the impact of scalability and variation in wastewater flows and loads, further study on the elevated loaded PN/A system should be performed at a pilot scale using real wastewater, as it would progress successful implementation at full scale. In addition, while the successful operation of the elevated loaded PN system following the anammox system has shown promise, there is a need for further optimization of the anammox unit to achieve lower effluent nitrogen within set municipal regulations. Also, further investigation of functional bacteria in the elevated loaded PN and anammox system through high-throughput sequencing is recommended as it would provide interesting knowledge of the microbiome and its evolution across operational time.

7.7 Supplementary Information

Description of the cost estimation

The cost estimation compares the two-stage partial nitritation/anammox system investigated in this study with the conventional biological nitrification/denitrification system using the Modified Ludzak-Ettinger (MLE) process (U.S. EPA, 1993), the most common process used for nitrogen removal in municipal wastewater treatment (Metcalf & Eddy, 2014). The cost estimation was stoichiometry-based, allowing easy comparison of the two-treatment process. Sample calculations are presented below.

Key process assumptions for cost estimation are as follows:

- The nitrification/denitrification and partial nitritation/anammox process achieved 100% NH_4^+-N removal.
- That 100% of the COD was utilized and removed in the nitrification/denitrification process.
- The partial nitritation/anammox system was operated post-carbon removal; as such, COD estimation was not considered for this process.
- The sludge disposal cost was calculated with the assumption that all produced biosolids are deposited in the landfill.
- The stoichiometric coefficients, and biomass yield used to estimate oxygen demand and sludge produced are presented in Table S7.3
- All costs of electrical demand on a per kWh basis, landfilling sludge on a per kgVSS basis, and external COD (methanol) addition on a per kgO_2 basis applied in this study

were calculated from literature and industry-provided values and are presented in Table S7.4.

- The following information is provided for a wastewater treatment plant in a Canadian city with a population of 1.05 million.

Flow:

Influent average daily = 390 MLD

NH_4^+-N:

Influent average daily = 42 mg NH_4^+-N /L

COD:

Influent average daily = 308 mg/L

Mass flux

a. Influent NH_4^+-N mass flux calculated as presented in equation S7.1.

$$NH_4^+ - N_{mass\ flux} = Q.C = 42\ \frac{mgNH_4^+ - \mathrm{N}}{L} \times 390\frac{ML}{D} = 16450\frac{kgN}{d} \qquad \text{S7.1}$$

b. Influent COD mass flux calculated as presented in equation S7.2:

$$308\ \frac{mgCOD}{L} \times 390\frac{ML}{D} = 120120\frac{kgCOD}{d} \qquad \text{S7.2}$$

Nitrification /denitrification process

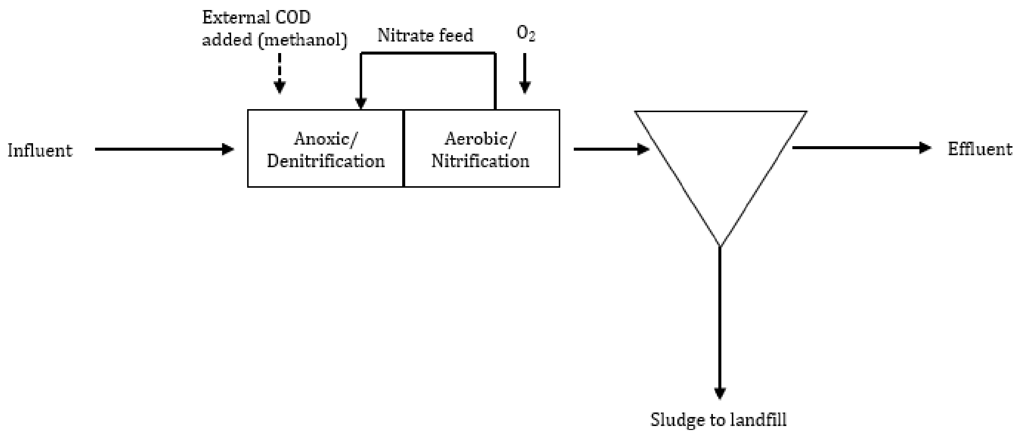


Figure S7.1 Flow diagram of the modified Ludzak-Ettinger (MLE) process for the nitrification/denitrification system.

Nitrification

Table S7.2 Fractional conversion (f_c) for N/DN process

Organism (Y)	AOB	NOB	Denitrifying heterotrophs
Nitrogen	1	1	1

c. Oxygen demand by AOB calculated as presented in equation S7.3.

$$O_{2AOB} = f_{cAOB}.total\ NH_4^+ - N_{massflux}.S_{cAOB}.\frac{MW_{O_2}}{MW_N} = \qquad S7.3$$

$$1 \times 16450\frac{kgN}{d} \times 1.5\frac{kmol_{O_2}}{kmol_N} \times \frac{32kg/kmol}{14kg/kmol} = 56400\frac{kgO_2}{d}$$

d. Oxygen demand by NOB is calculated as presented in equation S7.4.

$$O_{2NOB} = f_{cNOB}.total\ NH_4^+ - N_{massflux}.S_{cNOB}.\frac{MW_{O_2}}{MW_N} = \qquad S7.4$$

$$1 \times 16450\frac{kgN}{d} \times 0.5\frac{kmol_{O_2}}{kmol_N} \times \frac{32kg/kmol}{14kg/kmol} = 18802\frac{kgO_2}{d}$$

e. Sludge production by AOB is calculated as presented in equation S7.5.

$$P_{xAOB} = f_{cAOB}.total\ NH_4^+ - N_{massflux}.Y_{AOB} = \tag{S7.5}$$

$$1 \times 16450 \frac{kgN}{d} \times 0.12 \frac{gVSS}{gN} = 1974 \frac{kgVSS}{d}$$

f. Sludge production by NOB is calculated as presented in equation S7.6.

$$P_{xNOB} = f_{cNOB}.total\ NH_4^+ - N_{massflux}.Y_{NOB} = \tag{S7.6}$$

$$1 \times 16450 \frac{kgN}{d} \times 0.05 \frac{gVSS}{gN} = 823 \frac{kgVSS}{d}$$

Denitrification

g. To remove nitrogen, COD was required in a 1:7.6 N/COD ratio for the denitrification process. Total COD required is calculated as presented in equation S7.7.

$$COD_{required} = f_{cHET}.total\ NH_4^+ - N_{massflux}.S_{cHET} = \tag{S7.7}$$

$$1 \times 16450 \frac{kgN}{d} \times 7.6 \frac{gCOD}{gN} = 125020 \frac{kgCOD}{d}$$

h. The biomass yield of denitrifying heterotrophs was calculated as shown in equation S7.8.

$$P_{xHET} = COD_{required}.Y_{HET} = \tag{S7.8}$$

$$125020 \frac{kgCOD}{d} \times 0.3 \frac{gVSS}{gCOD} = 3751 \frac{kgVSS}{d}$$

i. To estimate if an additional external COD is supplied to remove nitrogen in the influent a COD balance was performed as shown in equation S7.9.

$$COD_{balance} = COD_{required} - COD_{massflux} = \tag{S7.9}$$

$$125020 \frac{kgCOD}{d} - 120120 \frac{kgCOD}{d} = 4900 \frac{kgCOD}{d}$$

Since $COD_{balance}$ $is\ greater\ than$ 0, external carbon source will be added.
Therefore COD_{added} is calculated, shown in equation S7.10.

$$COD_{added} = COD_{balance} = 4900 \frac{kgCOD}{d} \qquad \text{S7.10}$$

Cost estimation for Nitrification/denitrification system

a. Energy

The energy demand for aeration was based on the total oxygen demand of the system (equations S7.3- S7.4), which is represented in equation S7.11.

$$O_{2total} = \Sigma \mathrm{O}_{2\mathrm{demand}} = \qquad \text{S7.11}$$

$$56400 \frac{kgO_2}{d} + 18802 \frac{kgO_2}{d} = 75202 \frac{kgO_2}{d}$$

Therefore, energy demand for aeration can be estimated as shown in equation 7.12.

$$Energy_{demand} = O_{2total} \cdot \frac{1.5kWh}{kgO_2} = 112803 \frac{kWh}{d} \qquad \text{S7.12}$$

$$Energy_{cost} = Energy_{demand} \frac{kWh}{d} .Electricity\ rate \frac{CAD}{kWh} = \qquad \text{S7.13}$$

$$112803 \frac{kWh}{d} \times 0.13 \frac{CAD}{kWh} = 14664 \frac{CAD}{d}$$

$$Annual_{cost} = Energy_{cost} \frac{CAD}{d} .365$$

$$14664 \frac{CAD}{d} \times 365 = 5{,}352{,}360 \frac{CAD}{yr}$$

b. Sludge

Total sludge was the sum of all biomass yields from all organisms (equations S7.5, S7.6, and S7.8), which is represented in equation S7.14.

$$P_{xtotal} = \Sigma Y_{AOB,NOB\&HET=}$$

S7.14

$$1974 + 823 + 3751 = 6548 \frac{kgVSS}{d}$$

Assuming all produced biosolids (P_{xtotal}) are deposited to the landfill, the cost of sludge disposal to the landfill is shown in equation S7.15.

$$Sludge\ disposal\ to\ landfill_{cost} = P_{xtotal} \,.\, Landfill\ rate \frac{CAD}{kgVSS} = \qquad \text{S7.15}$$

$$6548 \frac{kgVSS}{d} \times 0.07 \frac{CAD}{kgVSS} = 458 \frac{CAD}{d}$$

$$Annual_{cost =} Sludge\ disposal\ to\ landfill_{cost} \frac{CAD}{d} \,.\, 365$$

$$458 \frac{CAD}{d} \times 365 = 167{,}170 \frac{CAD}{yr}$$

c. Chemical

The chemical cost was based on the external carbon source (methanol) demand (COD_{added}) for the denitrification process.

$$COD_{added} = COD_{balance} = 4900 \frac{kgCOD}{d}$$

S7.16

$$Chemical_{cost} = COD_{added} = COD_{balance} \,.\, 0.29 \frac{CAD}{kgCOD} =$$

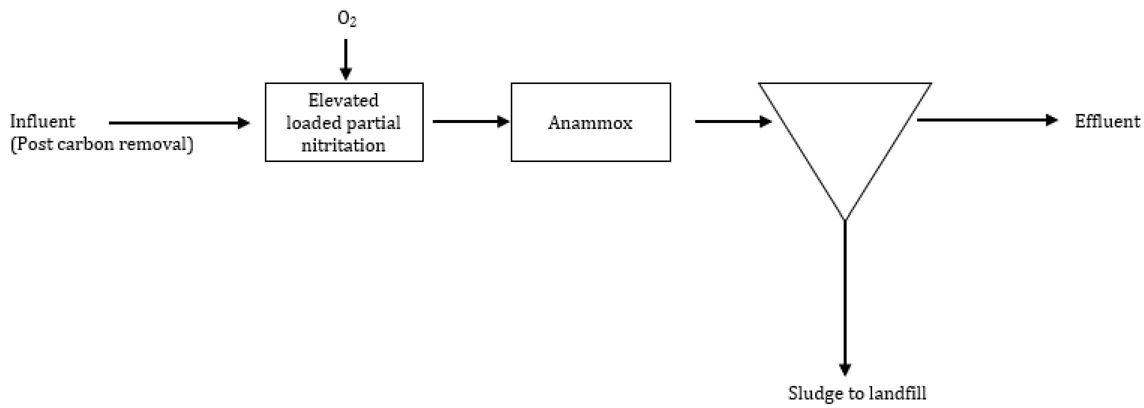
S7.17

$$4900 \frac{kgCOD}{d} \times 0.29 \frac{CAD}{kgCOD} = 1421 \frac{CAD}{d}$$

$$Annual_{cost} = Chemical_{cost} \frac{CAD}{d} \,.\, 365$$

$$1421\frac{CAD}{d} \times 365 = 518{,}665\frac{CAD}{yr}$$

Two-stage partial nitritation/anammox system



Partial nitritation

Based on partial nitritation metabolic reaction, the fraction of influent NH_4^+-N that is partially oxidized by AOB can be calculated as presented in equation S7.18.

$$f_{cPN} = \frac{1.3kgNO_2^- - \mathrm{N}}{1kgNH_4^+ - \mathrm{N} + 1.3\mathrm{kg}NO_2^- - \mathrm{N}} = 0.565 \qquad \text{S7.18}$$

The remaining influent NH_4^+-N is then anaerobically oxidized by anammox, as shown in equation S7.19.

$$f_{cANAMMOX} = 1 - f_{cPN} =$$

$$1 - 0.565 = 0.435 \qquad \text{S7.19}$$

Table S7.2 Fractional conversion (f_c) for PN/A process

Organism (Y)	**AOB**	**NOB**	**AnAOB**
Nitrogen	0.565	0	0.435

a. Oxygen demand was calculated using equation S7.20

$$O_{2AOB} = f_{cAOB}.total\ NH_4^+ - N_{massflux}.S_{cAOB}.\frac{MW_{O_2}}{MW_N} =$$

$$0.565 \times 16450 \frac{kgN}{d} \times 1.5 \frac{kmol_{O_2}}{kmol_N} \times \frac{32kg/kmol}{14kg/kmol} = 31857 \frac{kgO_2}{d} \tag{S7.20}$$

b. Sludge production by AOB is calculated as presented in equation S7.21.

$$P_{xAOB} = f_{cAOB}.total\ NH_4^+ - N_{massflux}.Y_{AOB} =$$

S7.21

$$0.565 \times 16450 \frac{kgN}{d} \times 0.12 \frac{gVSS}{gN} = 1115 \frac{kgVSS}{d}$$

c. It is assumed that NOB activity is suppressed in the PN system.

$$O_{2NOB} = 0;\ P_{xAOB} = 0$$

Anammox

d. Sludge production by AOB calculated as presented in equation S7.22.

$$P_{xANAOB} = f_{cANAOB}.total\ NH_4^+ - N_{massflux}.Y_{ANAOB} =$$

S7.22

$$0.435 \times 16450 \frac{kgN}{d} \times 0.13 \frac{gVSS}{gN} = 930 \frac{kgVSS}{d}$$

Cost estimation for two-stage partial nitritation/anammox system

a. Energy

Energy demand for aeration can be estimated as shown equation S7.23

$$31857\frac{kgO_2}{d}\cdot\frac{1.5kWh}{kgO_2} = 47786\frac{kWh}{d} \qquad S7.23$$

$$Energy_{cost} = Energy_{demand}\frac{kWh}{d}\,.Electricity\ rate\frac{CAD}{kWh} = \qquad S7.24$$

$$47786\frac{kWh}{d}\times 0.13\frac{CAD}{kWh} = 6212\frac{CAD}{d}$$

$$Annual_{cost} = Energy_{cost}\frac{CAD}{d}\,.365$$

$$6212\frac{CAD}{d}\times 365 = 2{,}267{,}380\ \frac{CAD}{yr}$$

b. Sludge

$$P_{xtotal} = \Sigma Y_{AOB,ANAOB=} \qquad S7.25$$

$$1115 + 930 = 2045\frac{kgVSS}{d}$$

$$Sludge\ disposal\ to\ landfill_{cost} = P_{xtotal}\,.Landfill\ rate\frac{CAD}{kgVSS} = \qquad S7.26$$

$$2045\frac{kgVSS}{d}\times 0.07\frac{CAD}{kgVSS} = 143\frac{CAD}{d}$$

$$Annual_{cost} = Sludge\ disposal\ to\ landfill_{cost}\ \frac{CAD}{d}\,.365$$

$$143\frac{CAD}{d}\times 365 = 52{,}195\ \frac{CAD}{yr}$$

Table S7.3 Metabolic process, stoichiometric coefficients, and biomass yield

Process	Stoichiometric coefficients (S_c)	Biomass yield (Y)	Reference
NH_4^+-N oxidation	1.5 molO_2/mol NH_4^+	0.12gVSS/g NH_4^+-N	(Metcalf & Eddy, 2014)
NO_2^--N oxidation	0.5 molO_2/mol NO_2^-	0.05gVSS/g NO_2^--N	(Metcalf & Eddy, 2014)
Heterotrophic oxidation	7.6gCOD/gN	0.30gVSS/gCOD	(Sobieszuk and Szewczyk, 2006)
Anammox	1.3mol NO_2^-/mol NH_4^+	0.13gVSS/g NH_4^+-N	(Metcalf & Eddy, 2014)

Table S7.4 Breakdown of unit cost estimation

		Value	Units	Reference
Energy	Energy demand of aeration	1.50	kWh/kgO_2 dissolved	(Tchobanoglous et al., 2014)
	Electricity rate	0.13	CAD/kWh	(Government of Canada, 2023).
	Cost of aeration*	0.20	CAD/ kgO_2	-
Chemical	External carbon source(Methanol)	0.29	CAD/kgCOD	(Cogert et al., 2019)
Sludge	Mass of sludge to landfill*	4.65	Kg wet sludge to landfill	(Rashwan et al., 2018)
	Cost to landfill sludge	14.5	CAD/mg wet	(Rashwan et al., 2018)
	Cost to landfill/kg biomass produced*	0.07	CAD/kgVSS produced	-

Note:

* cost of aeration = energy demand of aeration × electricity rate
Mass of sludge to landfill = 1% solids content
Cost to landfill per kg biomass produced = (land filled wet sludge × cost per mg to landfill) /1000

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