METHANE REDIRECTION FOR A NOVEL BIOLOGICAL NITROGEN REMOVAL PROCESS IN A SEQUENTIAL BATCH REACTOR SYSTEM

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ABSTRACT

Excess nutrients in wastewater discharge is leading to eutrophication of surface waters causing environmental harm. Wastewater treatment plants (WWTPs) are shifting towards water reuse and recovery facilities (WRRFs) to improve process sustainability while recovering higher value resources. Fortunately, methanotrophic bacteria can couple methane usage with nitrogen removal. This research investigates methane redirection for methanotrophic-based nitrogen removal enriched from activated sludge. Different phases allowed evaluation of the nitrogen removal capacity of the methanotrophic culture in a sequential batch reactor. This research is the first to maintain stable nitrate accumulation using methanotrophic cultures. The maintenance of nitrification-denitrification activity using the same culture found to be challenging. Advantageously, the methanotrophic culture enriched for nitrogen removal can be employed for biopolymer accumulation to further consolidate the WRRF concept. This study confirms the potential of using methanotrophic bacteria for stable nitrogen removal and biopolymer accumulation, however, further studies are still needed to improve process feasibility.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

Activated Sludge	AS
Aerobic Methane Oxidation Coupled to Denitrification	AME-D
Ammonium Mineral Salts	AMS
Ammonium Monooxygenase	AMO
Ammonium Oxidizing Bacteria	AOB
Ammonium Removal Efficiency	ARE
Anaerobic Ammonium Oxidation	ANAMMOX
Anaerobic Digestion	AD
Anaerobic Fixed Bed Reactor	AFBR
Anaerobic Methane Oxidation Coupled to Denitrification	ANME-D
Biochemical Oxygen Demand	BOD
Biological Nitrogen Removal	BNR
Calvin Benson Bassham	CBB
Chemical Oxygen Demand	COD
Continuous Stir Tank Reactor	CSTR
Dissolved Oxygen	DO
Free Ammonia	FA
Free Nitrous Acid	FNA
Gas Chromatography	GC
Greenhouse Gas	GHG
Hydraulic Retention Time	HRT
Hypoxically Aerobic Oxidation Coupled to Denitrification	HYME-D
Intracytoplasmic Membrane	ICM
Modified Ludzack-Ettinger	MLE
Membrane Biofilm Reactor	MBfR
Methane Loading Rate	MLR
Methane Monooxygenase	MMO
Mineral Salts Medium	MSM
Nitrate Removal Efficiency	NRE
Nitrite Accumulation Rate	NAR
Nitrite Oxidizing Bacteria	NOB
Nitrogen	N

Nitrogen Loading Rate	NLR
Particulate Methane Monooxygenase	pMMO
Polyhydroxyalkenoates	PHA
Polyhydroxybutyrate	PHB
Ribulose Monophosphate Cycle	RuMP
Sequential Batch Reactor	SBR
Shortcut Biological Nitrogen Removal	SBNR
Sludge Volume Index	SVI30
Solid Retention Time	SRT
Soluble Methane Monooxygenase	sMMO
Specific Denitrification Rate	SDNR
Thermal Conductivity Detector	TCD
Total Kjendhal Nitrogen	TKN
Total Nitrogen	TN
Total Solids	TS
Total Suspended Solids	TSS
Tricarboxylic Acid Cycle	TCA
Upflow Anaerobic Sludge Blanket Reactor	UASB
Volatile Suspended Solids	VSS
Waste Activated Sludge	WAS
Wastewater Treatment Plant	WWTP
Water Resource Recovery Facility	WRRF

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

1.1 BACKGROUND

Anthropogenic activities and anaerobic degradation of organic matter in natural environments is impacting the global atmospheric methane concentration [1]. From pre-industrial times there has been an approximate 170% increase in atmospheric methane concentration in part per billion by volume [1–3], making it one of the primary factors affecting climate change [4]. Atmospheric methane concentration is likely to increase further as population size continues to grow. The increase in population size is leading to an increase in wastage, food production, and fossil fuel consumption [5]. Methane is the second most abundant anthropogenic greenhouse gas (GHG), next to carbon dioxide, and can trap 20-25 times more heat in the atmosphere than carbon dioxide [2,5,6]. Moreover, anthropogenic activities account for 63% of methane emissions, with natural emissions from anaerobic degradation making up the remaining 37% [5]. The leading anthropogenic contributions of methane emissions in North America, particularly in the United States, are energy and industry, agriculture, and homes and businesses [7].

Unfortunately, within energy and industry, a major GHG emissions source is wastewater treatment [8]. Wastewater treatment plants (WWTPs) contribute 7% to global anthropogenic GHG emissions. This value is expected to increase an additional 8% from the years 2020 to 2030 [8]. The projected increase can be attributed to an increase in energy consumption and input from a larger loading rate because of population size, and the result of stringent regulations applied to WWTPs adding the need for additional treatment technologies [9]. Because of the projected increase in emissions, WWTPs are shifting towards water resource recovery facilities (WRRFs). These facilities have a primary goal of meeting regulations for discharge standards in a sustainable and economical manner while recovering higher value products.

1.2 WASTEWATER TREATMENT PLANTS EUTROPHICATION CONTRIBUTION

There is growing public concern surrounding the environmental impacts of WWTPs, not only because of the GHG emissions emitted, but also because of the enormous amounts of fossil fuel-based energies consumed [5,10,11]. In addition to GHG emissions and energy consumption, excess nutrients, particularly nitrogen and phosphorus, in treated wastewater discharge is contributing to eutrophication of surface waters. Eutrophication is the process of nutrient enrichment dissolved in run-off, wastewater, and groundwater leading to the excess growth of blue-green algae. Harmful effects of eutrophication include oxygen depletion, fish toxicity, dead-zones, and ground water contamination [12]. Therefore, to improve overall water quality, the nutrients need to be significantly reduced or eliminated before discharge into surface waters.

Nitrogen can be removed from wastewater either by biological or physical-chemical processes. However, because of the low cost and effectiveness of biological treatment processes, they have been widely adapted over physical-chemical processes [13]. The most mature mainstream biological removal process is conventional biological nitrogen removal (BNR), carried out through the process of nitrification and denitrification. Besides conventional BNR, an up-and-coming mainstream nitrogen removal application is shortcut biological nitrogen removal (SBNR). Both processes aim to transform and remove nitrogen from wastewater.

1.3 METHANE AS AN UNEXPLOITED RESOURCE IN WASTEWATER TREATMENT PLANTS

Global changes need to be made to better utilize and mitigate the methane produced from anthropogenic activities to hamper climate change. Fortunately, methane is a natural gas and several methane-driven uses and biotechnologies are emerging. Methane-driven uses and biotechnologies include, methane as an alternative transportation fuel source, methane conversion to liquid fuels such as biodiesel and methanol [5,14], energy generation [5], methane as a feedstock for the development of single-cell proteins, dietary and health supplements, biopolymers production, and as a carbon source replacement for denitrification [15–17].

As mentioned, WWTPs are a contributor to GHG emissions. GHG emissions from WWTPs are largely attributed to biogas production from anaerobic digestion (AD) of organic matter, the release of methane from anaerobic treatment processes because of its poor solubility, and the consumption of fossil fuel-based energies [18]. Biogas produced during the AD process contains 75% methane [6,19], 25% carbon dioxide, and trace fractions of hydrogen sulfide, nitrogen, oxygen, carbon monoxide, ammonia, argon, and other volatile organic compounds [20]. Even with the numerous methane applications, biogas is still underutilized because of a number of associated obstacles, such as: (1) the existence of impurities and moisture; (2) low handling and collecting capabilities; (3) the lower efficiency in combined heat and power (CHP); and (4) the lack of infrastructure for biogas processing [18]. However, biogas generation continues to increase globally, reaching energy values of 1.28 EJ [21]. Therefore, the energy produced should be harvested or used for alternative purposes to create and/or recover higher value products.

Even with the high energy values of biogas production, less than 10% of WWTPs in the United States are incorporating it into their facilities for heating and electricity. The alarming number of WWTPs that do not use the produced biogas for heating and electricity continue to combust or flare the gas into the environment [22,23]. From the aforementioned uses of methane, interest can be placed on the use of methane as a carbon source replacement for biological nitrogen removal processes. Unfortunately, conventional denitrification relies on the addition of external carbon sources, such as methanol, to meet the discharge regulations applied, which can be cost intensive. Therefore, because methane is a widely available and inexpensive resource, it is being considered as a carbon source replacement for denitrification. Besides methane availability, methane used as a carbon source produces less sludge and leads to less organic carbon residual in treated wastewater compared to methanol [24–26]. Thus, methane oxidation can be utilized in engineered bioprocesses, with focus on wastewater treatment.

With increasing awareness surrounding global climate change, methanotrophic bacteria (methanotrophs) are being reviewed for their applications in GHG mitigation [27]. Methanotrophic bacteria are abundant in nature and act as an important biological methane sink [28]. Methanotrophs are a unique bacteria that can utilize methane as their sole carbon and energy source through methane oxidation to carbon dioxide [29]. Moreover, methanotrophs have the capacity to co-oxidize ammonium to gaseous nitrogen, giving them the potential to be readily employed in BNR processes while using methane as the carbon source [30].

Finally, from the aforementioned uses of methane, a promising application is biodegradable biopolymer accumulation in mixed culture microorganisms with high poly-hydroxy-alkenoates (PHA) capacity. Biodegradable biopolymers are increasing in popularity as an alternative to petroleum-based and single-use plastics. As methane can be utilized by the unique methanotrophic bacteria, they are an ideal candidate for biopolymer production. Within methanotrophs, methane is converted to poly-hydroxy-butyrate (PHB), a member of the PHA family, under nutrient deficient conditions. Fortunately, the stored PHB can be extracted from the intracellular granules of the methanotrophs and applied as a material for green bioplastics. The benefits of utilizing methane driven bioplastics are product degradation at their end-life and an increase in biopolymer production sustainability by reducing feedstock consumption and cost by 30% (assuming methane as a replacement feedstock) [31].

1.4 RESEARCH RATIONALE

Changes within WWTPs need to occur to better utilize biogas produced from AD. WWTPs are continuing to flare biogas to the environment instead of utilizing it for resource recovery and higher value products. Furthermore, utilizing AD driven biogas can help to consolidate the WRRF concept of sustainable treatment processes and resource recovery. Fortunately, methanotrophic bacteria are a unique microorganism that have the ability to utilize methane as their sole carbon and energy source. Because of this ability, methanotrophs can be employed in various biotechnologies for single-cell proteins, methanol production, biopolymer accumulation, nitrogen recovery, and biological nitrogen removal processes. Therefore, utilizing biogas produced onsite within WWTPs is appealing to ease GHG emissions, while also recovering resources.

Emphasis can be placed on methanotrophic bacteria involvement in biological nitrogen removal while using methane as the carbon source. Currently, WWTPs rely on conventional biological nitrogen removal to meet discharge regulations. Furthermore, a budding mainstream nitrogen removal process is shortcut biological nitrogen removal. However, this process is still struggling to achieve stable nitrogen removal under mainstream operating conditions. Therefore, there is currently a gap in mainstream nitrogen removal applications and the use of methanotrophic bacteria may help. Methanotrophic-based nitrogen removal is not widely studied but can be advantageous in mainstream wastewater applications. Methanotrophic-based nitrogen removal advantages include onsite methane usage, the ability to alter between ammonium assimilation and ammonium oxidation for nitrogen recovery, the capacity of methanotrophic bacteria to assimilate and/or oxidize different nitrogen sources, and the applicable niche of utilizing dissolved methane in anaerobic treatment processes. Therefore, the aim of this research is to investigate the capacity of methanotrophic bacteria in nitrogen removal processes under mainstream operating conditions while using methane as the sole carbon source.

Furthermore, methanotrophic bacteria can be employed in other biotechnologies while using methane as the carbon source for resource recovery. As stated, these applications include single-cell proteins, methanol production, and biopolymer accumulation. Of these unique applications, biopolymer accumulation was explored using the methanotrophic culture enriched for nitrogen removal. Biopolymer accumulation was evaluated straightaway without prior enrichment for the specific application of biopolymer production. Overall, the abilities of the unique methanotrophic bacteria can be advantageous in mainstream wastewater applications with emphasis on biological nitrogen removal, nitrogen recovery, and biopolymer accumulation.

1.5 RESEARCH OBJECTIVES

The main objective of this research is to investigates the idea of redirecting methane for BNR in municipal WWTPs. This objective was achieved by exploiting the unique abilities of methanotrophic bacteria enriched from activated sludge. Further, methane produced from anthropogenic activities can be used as the sole carbon source by the culture. Emphasis in this research is placed on exploring methanotrophic nitrification and denitrification capacity in relationship to the applied methane and oxygen concentrations. To achieve the overall objective, several specific objectives were completed:

1) The nitrification and denitrification capacity of the methanotrophic mixed culture was evaluated in separate batch experiments under different methane and oxygen flow rates to determine the ideal operating conditions for sequential nitrification-denitrification. To induce nitrification by the methanotrophic culture, methane was supplied in lower flow rates than oxygen to create competition between ammonium and methane for oxidation. Whereas, to induce methanotrophic denitrification, oxygen was applied in lower flow rates to drive methane oxidation and denitrification.
2) From the above objective, different sequential batch reactor cycles were developed and evaluated under different methane and oxygen flow rates.
    a) Initially, sufficient methane and oxygen concentrations were applied to induce methanotrophic-based nitrogen removal. Moreover, the nitrogen loading rate

applied was altered based on the hydraulic retention time to entice competition between ammonium and methane for oxidation.

b) Second, the methane concentration was reduced with the aim of allowing ammonium to outcome methane for oxidation. Furthermore, the dissolved oxygen concentration was altered by changing the setpoint instead of flow rate to further encourage ammonium oxidation.

3) From the nitrifying methanotrophic culture, the denitrification capacity was evaluated in a batch experiment. Different methane and oxygen concentrations were applied to the headspace of serum bottles to evaluate the relationship between the methanotrophic culture and nitrate.

4) Finally, as methanotrophs can have various applications in biotechnologies, the biopolymer production capacity of the nitrogen removing methanotrophic culture was evaluated. This objective reviewed the applicability of using the methanotrophic-based culture for alternative purposes, while eliminating the conventional enrichment period applied in other biopolymer accumulation studies.

This research demonstrates how methanotrophic bacteria can be utilized and/or integrated in mainstream wastewater applications. The research illustrates resource reuse and recovery, such as biogas usage and biodegradable bioplastics production, to help consolidate the WRRF concept and help to fill the gap in mainstream wastewater applications.

1.6 RESEARCH APPROACH AND THESIS LAYOUT

To achieve the aforementioned research objectives, a literature review was completed on methanotrophic bacteria involvement in nitrification and denitrification processes. This section of the literature review focuses on methanotrophic-based ammonium and nitrate oxidation, and aerobic methane oxidation coupled to denitrification (AME-D). Another section in the literature review focuses on emerging mainstream biological nitrogen removal processes with emphasis on shortcut biological nitrogen removal (SBNR). Finally, a brief literature review was completed on the factors impacting PHB formation within methanotrophic bacteria. All information pertaining to the literature review can be found in **Chapter 2: Literature Review**. While completing the literature review, it was discovered that there is almost no focus on complete methanotrophic-based nitrification in wastewater. However, several studies exist related to ammonium and nitrate conversion by methanotrophic bacteria. Besides the lack of methanotrophic-based nitrification research, AME-D studies focus on how methanotrophs can provide organic substances for nitrate and nitrite reduction by denitrifying bacteria.

Chapter 3: Methane Redirection for Novel Biological Nitrogen Removal using the Enrichment of a Methanotrophic Mixed Culture in a Sequential batch Reactor System. This chapter investigates the feasibility of methane redirection for biological nitrogen removal using a methanotrophic mixed culture under mainstream operating conditions. Further, this chapter is broken down into four major sections based on the operating conditions applied to the sequential batch reactor (SBR). Section one evaluates the nitrification and denitrification capacity of the methanotrophic mixed culture under different methane and oxygen flow rates. Section two evaluates the sequential nitrification-denitrification ability of the culture under sufficient methane and oxygen concentrations. Section three focuses on applying a consistent low methane flow rate and a ranging dissolved oxygen concentration to induce nitrification-denitrification under different nitrogen loading rates. Finally, Section four evaluates the ability of the nitrifying methanotrophic culture to perform denitrification in batch activity tests.

Chapter 4: Poly-hydroxy-butyrate (PHB) Accumulation in Methanotrophic Mixed Cultures Enriched for Novel Biological Nitrogen Removal evaluates the PHB accumulation capacity of the methanotrophic mixed culture under various operating conditions. It is noteworthy that the methanotrophic bacteria employed during Chapter 4 was collected from the SBR system that was induced for nitrogen removal. Finally, **Chapter 5: Conclusions and Future Work** consists of process integration within wastewater treatment plants, the role methanotrophic-based nitrogen removal can play, and future work surrounding this research.

Essentially, the thesis is composed of five chapters. Chapter 1: Introduction and Justification, Chapter 2: Literature Review with emphasis on methanotrophic ammonium oxidation and denitrification processes, Chapter 3 and Chapter 4 which consists of the research and experimental work, and Chapter 5: Conclusions and Future Work surrounding methanotrophic-based nitrogen removal. Emphasis should be placed on Chapter 3 as it includes the bulk of the research and experimental work, with particular focus on the SBR evaluation.

CHAPTER 2: LITERATURE REVIEW

2.1 CONVENTIONAL BIOLOGICAL NITROGEN REMOVAL

Understanding the basic fundamentals of conventional biological nitrogen removal (BNR) processes is important to evaluate if there is room for alternative processes in WWTPs. BNR is the process of nitrogen conversion and elimination from wastewater. BNR can be broken down into two main processes, nitrification and denitrification, which are carried out by specific microorganisms under certain environmental conditions.

2.1.1 AN OVERVIEW OF NITRIFICATION

Nitrification is a two-step process carried out under aerobic conditions. The first step involves the chemolithoautotrophic oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), referred to as ammonium oxidation or nitritation. The second step is the chemolithoautotrophic oxidation of nitrite (NO_2^-) to nitrate (NO_3^-), termed nitrite oxidation or nitratation [32]. Ammonium oxidation is completed by autotrophic ammonium oxidizing bacteria (AOB). AOB are part of the beta subdivision of *Proteobacteria* with the most common bacteria being *Nitrosomonas*. Additional bacteria that can partake in ammonium oxidation include *Nitrosococcus, Nitrosopira, Nitrosovibrio,* and *Nitrosolobus* [12]. Ammonium oxidation involves the use of the ammonium monooxygenase (AMO) enzyme and the hydroxylamine oxidoreductase (HAO). In comparison, nitrite oxidation is completed by autotrophic nitrite oxidizing bacteria (NOB). NOB are part of the alpha subdivision of *Proteobacteria* with the most common bacteria being *Nitrobacter*. Similar to ammonium oxidation, other bacteria involved in nitrite oxidation are *Nitrospira, Nitrospina, Nitrococcus*, and *Nitrocystis* [12].

Ammonium oxidation is the rate limiting step in the nitrification process because of the instability of nitrite in natural water environments. Therefore, nitrite accumulation during nitrification is low as it is readily transformed to nitrate. Additionally, carbon dioxide is required as a carbon source for cellular synthesis by the bacteria involved in nitrification. As previously stated, the nitrification process is carried out under aerobic conditions. The theoretical oxygen requirement for the process completion is 4.57 gO_2 consumed per gN transformed [12]. During the nitrification process, it is important to note that ammonium simply changes forms to nitrate and is not removed from the system [12]. Besides ammonium transformation, oxygen demand is eliminated because oxygen is a requirement for both ammonium and nitrite transformation. Oxygen requirement can be divided between the two nitrification processes, ammonium oxidation to nitrite and nitrite oxidation to nitrate. Theoretically, ammonium oxidation consumes 3.43 gO_2/gNH_4-N oxidized, while nitrite oxidation consumes 1.14 gO_2/gNO_2-N oxidized [12].

In addition to oxygen requirement, alkalinity in the form of calcium carbonate ($CaCO_3$) is needed during nitrification. Thus, approximately 7.14 $gCaCO_3$ is needed during nitrification to oxidized 1 gNH_4-N. The energy yielding nitritation reaction is shown in **Equation 2-1**, the energy yielding nitratation reaction is shown in **Equation 2-2**, and the overall biological nitrification reaction is shown in **Equation 2-3**. The energy created during **Equations 2-1** and **2-2** is utilized by the bacteria for cellular growth and maintenance. Furthermore, the ammonium ion (NH_4^+) can be assimilated into the cell tissue of the bacteria, as displayed in **Equation 2-4**, assuming the common bacterial chemical formula of $C_5H_7O_2N$. The overall biomass yield from the nitrification process is 0.16 gbiomass produced per gNH_4-N oxidized.

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (2\text{-}1)$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \qquad (2\text{-}2)$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \qquad (2\text{-}3)$$

$$4CO_2 + HCO_3^- + NH_4^+ + H_2O \rightarrow C_5H_7O_2N + 5O_2 \qquad (2\text{-}4)$$

The above nitrification process uses oxygen as the electron acceptor, hence the theoretical oxygen requirement, and ammonium and nitrite as the electron donors. Factors that can impact or inhibit the rate of nitrification because of sensitivity associated with nitrifying microorganisms include pH, dissolved oxygen (DO) concentration, temperature, organic and inorganic substrates, and free ammonia (FA) and free nitrous acid (FNA) [12]. The DO concentration is important and should remain at concentrations above 1 mgO_2/L. DO concentrations below 1 mgO_2/L will slow or halt nitrification as oxygen becomes the limiting requirement of the process.

2.1.2 AN OVERVIEW OF DENITRIFICATION

Denitrification is the second process in the nitrification-denitrification reaction. Denitrification can be classified as assimilatory or dissimilatory. Assimilatory denitrification is the reduction of nitrate to ammonium-nitrogen (NH_4-N). Assimilatory denitrification occurs when there is a lack of ammonium-nitrogen in the system to be used for cellular synthesis as seen in **Equation 2-4** above. Dissimilatory denitrification is the reduction of nitrate to dinitrogen gas (N_2) with specific intermediates of nitrite, nitric oxide (NO), and nitrous oxide (N_2O), respectively. Nitric oxide and nitrous oxide are gaseous products. Unfortunately, nitrous oxide is a major GHG having the capacity to trap heat in the atmosphere if emitted which is a concern associated with denitrification. The process of nitrate reduction to nitrite is termed denitratation, and the reduction of nitrite to dinitrogen gas is denitritation. **Equation 2-5** demonstrates the basic overall denitrification reaction.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2\text{-}5)$$

The denitrification process is carried out under anoxic conditions, where anoxic means without the need for oxygen requirement. Heterotrophic bacteria, including *Achromobacter, Aerobacter, Alcaligenes, Bacillus, Brevibacterium, Flavobacterium, Lactobacillus, Micrococcus, Proteus, Pseudomonas,* and *Spirillum* are involved in dissimilatory denitrification [12]. One of the major challenges of anoxic denitrification is the need for sufficient electron donors (chemical oxygen demand (COD) or energy) for nitrate and nitrite reduction. Limited electron donors can inhibit or halt the denitrification process.

Electron donors for denitrification can come from two sources, either internal or external to the WWTP. Internal sources are present within the system, such as incoming wastewater as readily biodegradable organics (rbCOD) or slowly biodegradable organics (sbCOD), and through biomass as endogenous decay. The latter, external sources, are applied directly to the anoxic zone. External carbon sources used commonly include methanol (CH_3OH), acetate, and ethanol, however, methanol is the most widely used. Methanol is widely used as an external carbon source because it is readily available, has the lowest cost per kg of nitrates reduced, and has extensive experience in denitrification processes [12]. Often external sources are required when stringent effluent regulations need to be met (6 to 8 mgN/L) or when the biochemical oxygen demand (BOD) to total kjendhal nitrogen (TKN) ratio (BOD:TKN) is low. Methanol has a COD equivalent of 1.5 gCOD/gCH_3OH. Therefore, assuming methanol as the external carbon source for denitrification, theoretically about 2.7 to 3.3 gCH_3OH is required per gNO_3-N removed. The actual value of methanol required in full-scale applications tends to range from 3.3 to 3.8 gCH_3OH consumed per gNO_3-N removed [12]. The increased methanol value is the result of unused methanol in the effluent, methanol consumed from the aerobic zone influent into the anoxic zone, and nitrate reduction from endogenous decay [12]. Interestingly, smaller anoxic tanks with shorter hydraulic retention times (HRT) require greater denitrification rates, leading to higher external carbon doses. The HRT is the amount of time the wastewater spends in the system.

The theoretical carbon requirement for denitratation is 1.14 gCOD/gNO_3-N reduced and for denitritation 1.72 gCOD/gNO_2-N reduced. The electron acceptors in the denitrification process are nitrate and nitrite. During the denitrification process approximately 3.57g of alkalinity is produced as calcium carbonate implying that there is a 50% recovery from the nitrification process. The biomass yield produced during denitrification is higher when compared to nitrification reaching a value of 0.4 gbiomass/gNO_3-N. Similar to nitrification, factors that impact denitrification rates include the DO concentration, pH, and temperature. The DO concentration is of importance because as the DO concentration reaches 1 mgO_2/L, the denitrification rate approaches zero. Conventional nitrification-denitrification processes can achieve ammonium removal efficiencies (ARE) of 95% and a nitrate removal efficiencies (NRE) of 85-90% [12]. From the aforementioned information on nitrification and denitrification, it can be denoted that there is room for sustainable and alternative bioprocesses to remove nitrogen from wastewater. Of the emerging bioprocesses, shortcut biological nitrogen removal (SBNR) is growing in popularity.

2.2 ALTERNATIVE BIOLOGICAL NITROGEN REMOVAL PROCESSES

Two well studied bioprocess for nitrogen removal from wastewater are shortcut biological nitrogen removal (SBNR) and anaerobic ammonium oxidation (ANAMMOX). These processes are growing in popularity as a result of their energy-saving and energy-neutral phenomenon [33]. These processes are deemed this way because they reduced and/or eliminate the need for organic carbon, and they lower operational costs. SBNR and ANAMMOX are well developed as sidestream treatment processes with installations in over 114 WWTPs globally [34]. Because of their success in sidestream treatment, focus has shifted towards research, development, and integration of SBNR and ANAMMOX under mainstream operating conditions.

2.4.1 SHORTCUT BIOLOGICAL NITROGEN REMOVAL

SBNR processes rely on the fact that nitrite is an intermediate during both nitrification and denitrification. Thus, the aim of SBNR is the direct conversion of nitrite to dinitrogen gas by the nitrite pathway. SBNR processes can have energy savings of 60%, because of a 25% decrease in oxygen consumption, by avoiding nitrite oxidation to nitrate during nitrification [35]. Besides energy savings, the external carbon requirement is reduced by 40%, alongside a 20% decrease is carbon dioxide emissions [35]. Finally, SBNR processes reduce sludge production by 35% and 55%, when compared to conventional nitrification and denitrification, respectively [35].

Two well studied SBNR processes are nitrite shunt and deammonficiation [13]. Essentially, nitrite shunt is the process of partial nitrification (ammonium oxidation to nitrite), followed by nitrite conversion directly to dinitrogen gas [13]. This process is carried out by AOB and heterotrophic bacteria, similar to the conventional nitrification and denitrification process. Whereas, deammonification aims to only oxidizes 50% of the ammonium to nitrite, while the remaining ammonium is anaerobically oxidized to dinitrogen gas by anaerobic ammonium oxidizing bacteria (ANAMMOX bacteria) [13]. **Figure 2-1**, from Soliman and Eldyasti [13], demonstrates the nitrite shunt and deammonification processes in comparison to conventional biological nitrogen removal.

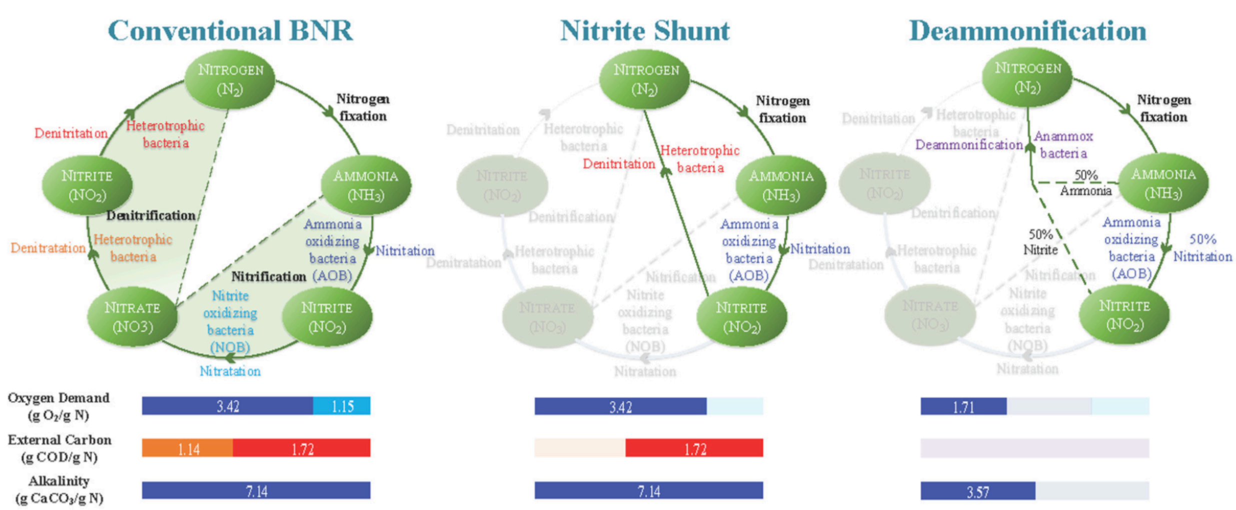

Figure 2-1: Shortcut biological nitrogen removal comparisons (nitrite shunt and deammonification), to conventional biological nitrogen removal (nitrification-denitrification) [13].

2.2.1.1 FACTORS IMPACTING SUCCESSFUL SHORTCUT BIOLOGICAL NITROGEN REMOVAL

Partial nitrification is successful when AOB can accumulate in a system, while simultaneously suppressing NOB. Several factors can play an important role in achieving successful SBNR through AOB accumulation. These factors include the DO concentration, temperature, pH, HRT, solid retention time (SRT), alkalinity, and free ammonia (FA) and free nitrous acid (FNA).

Maintaining a low DO concentration can help to accumulate AOB over NOB because they have a lower Monod oxygen saturation constant than NOB. The Monod oxygen saturation constant for AOB is 0.3 mgO_2/L, compared to 1.1 mgO_2/L for NOB [36]. Maintaining a low DO concentration for nitrite accumulation was confirmed by Wei et al. [37] who observed a nitrite accumulation decrease from ~95% to ~3% when the DO concentration was adjusted from 2 to 4 mgO_2/L, respectively. Moreover, further reducing the DO concentration to 0.8 mgO_2/L showed nitrite accumulation of ~94% [37].

The effect of temperature is evident on AOB accumulation. Several studies illustrated higher ammonium oxidation and nitrite accumulation rates at temperatures between 30 and 35°C [38]. Furthermore, temperature impacts the FA and FNA concentrations. Gabarro et al. [39] demonstrated a change in the FA concentration from 20.76±4.23 to 122.92±27.23 $mgN\text{-}NH_3/L$ under the temperatures of 25 and 35°C, respectively. Consequently, the opposite was observed for the FNA concentration showing a decrease from 0.47±0.09 to 0.12±0.02 $mgN\text{-}HNO_2/L$ under operating temperatures of 25 and 35°C, respectively [39]. AOB are inhibited by FA concentrations ranging between 10 and 150 $mgN\text{-}NH_3/L$, compared to NOB which are inhibited by FA concentrations ranging between 0.1 and 1 $mgN\text{-}NH_3/L$ [40]. Similar trends are observed for FNA having inhibiting concentrations of 0.42 to 1.72 mgN-HNO_2/L on AOB, and lower concentrations of 0.026 to 0.22 mgN-HNO_2/L on NOB [41]. Therefore, maintaining a pH between 7.5 and 8.5, as suggested by literature, is suitable for NOB suppression [38].

Finally, alteration of the HRT and the SRT can promote NOB washout over AOB because of the difference in doubling time. The doubling time of AOB is 10 to 13 hours and the doubling time of NOB is 7 to 8 hours [38]. Wan et al. [42] demonstrated that at a HRT of 7.2 and 12 hours, the ammonium removal and nitrite accumulation was greater than 90%, whereas, a drop in the HRT to 2.4 hours led to an inconsistent ARE ranging between 20% and 56%. Furthermore, Li et al. [43] found that the optimal HRT to treat acrylic fiber wastewater was 20 hours with an corresponding ARE and nitrite accumulation of 97% and 87%, respectively. As for the impact of SRT, Ahn et al. [44] studied a continuous stir tank reactor (CSTR) illustrating that NOB washout was observed when the SRT was 3 days. Furthermore, in an activated sludge process, an SRT of 10 to 13 days led to 95% nitrite accumulation, whereas, increasing the SRT to 16 days led to a 58% decrease in nitrite accumulation [45].

2.4.2 SIDESTREAM SHORTCUT BIOLOGICAL NITROGEN REMOVAL

Factors impacting SBNR processes are well demonstrated in the first installation of a deammonification process. A well-developed deammonificiation technology to treat sidestream wastewater is DEMON®. The first DEMON® installation in North America was at the York River Treatment Plant in 2012 [46]. The ANAMMOX seed was brought over in dormant form from Europe. The DO setpoint was managed in order to convert 50% of the ammonium to nitrite. Excess DO in the system can inhibit ANAMMOX bacteria leading to a reduction in process performance. ANAMMOX inhibition is observed by increases in nitrite concentration within the system. ANAMMOX inhibition by the DO concentration was demonstrated during the initial start-up period. During this period, nitrite concentrations reached 30 mgNO_2-N/L. However, applying anaerobic conditions can reverse the ANAMMOX inhibition.

Deammonificiation is impacted by temperature, thus a decrease in ARE was observed as the environmental temperature decreased over the winter months. Therefore, additional heating can be applied to maintain sufficient ARE. Furthermore, there was a decline in the ARE with a corresponding 13% to 20% increase in nitrate production. Nitrate production increase is attributed to the increased accumulation and activity by the NOB present in the system. Unfortunately, NOB accumulation in deammonification processes leads to a decrease in the ARE because competition for nitrite between NOB and ANAMMOX bacteria increases. Overall, the first DEMON® application achieved a successful ARE of 80%. From this installation there were 63% savings in aerations costs, 100% savings in carbon source addition, 100% savings in alkalinity, and an 80% reduction in biomass production compared to conventional BNR [46]. Several leading outcomes of the start-up and optimization study include robust control through pH and the DO concentration, NOB can be supressed without the addition of external inhibitors, and sidestream wastewater needs to be characterized before start-up to improve the AOB activity [46].

Because sidestream SBNR processes are well studied and have been shown to achieve high ARE and nitrite accumulation rates (NAR), research is now focusing on specific aspects for process improvements. Such processes are focusing on NOB suppression, increased nitrogen loading (NLR), and the DO concentrations. Soliman and Eldyasti [13] reviewed ammonium conversion and nitrite accumulation for high strength wastewater under a novel DO strategy as the first step to the nitrite shunt process in an SBR. The greatest NLR applied was 1.2 kgN/m^3.d and the DO concentration was adjusted based on the phase of operation. During Phase I, complete nitrification of ammonium to nitrate was observed at a NLR of 0.4 kgN/m^3.d. The ARE was high (99$\pm$0.1%) and an effluent nitrate concentration of 236.9 mgNO_3-N/L was observed. Phase II was evaluated under a reduced DO concentration to promote AOB growth over NOB, as demonstrated by literature. The DO range of 0.5 to 0.8 mgO_2/L achieved an ARE of 100%, a NAR of 85%, and the nitrate concentration in the effluent stabilized around 30 mgNO_3-N/L. Once the process was stable, the NLR was increased to 0.675 kgN/m^3.d under Phase II operating conditions. When the NLR was increased, the ARE decreased to 96.2%, however, there was a corresponding increase in the NAR to 93.9%. The effluent nitrite concentration reached 287.2 mgNO_2-N/L and the effluent nitrate concentration was below 25 mgNO_3-N/L. Finally, during Phase III operation, the DO range varied between 0.6 to 1.2 mgO_2/L. The NLR was progressively increased to a maximum value of 1.2 kgN/m^3.d. After an adjustment period by the bacteria, a successful ARE of 98% and a NAR of 94% was achieved [13].

2.4.3 MAINSTREAM SHORTCUT BIOLOGICAL NITROGEN REMOVAL

Mainstream applications of SBNR are becoming increasing popular because of its success in sidestream treatment processes. Wang et al. [47] reviewed a pilot scale step-feed activated sludge system for SBNR. The pilot plant was designed to treat 30 m^3/d of wastewater, at an HRT of 6.4 hours, and an SRT of 3 to 10 days. The influent ammonium concentration was 40 mgNH_4-N/L and the influent nitrate and nitrite concentrations were undetectable. Four phases were performed during process operation to evaluate the different experimental parameters. During Phase I it was determined that the optimal mainstream operating conditions to achieve stable nitrite shunt was a DO concentration of 4 mgO_2/L, an SRT of 4 days, and a sludge return ratio of 2.5 times the inlet flow. Under these conditions, the NAR stabilized at 90% because NOB were suppressed. The ARE achieved was 87.3%. Unfortunately, during Phase II the ammonium concentration in the influent fluctuated significantly, which led to poor nitrite accumulation (below 0.5 $mgNO_2$-N/L) and an increase in nitrate concentration (greater than 10 $mgNO_3$-N/L). The poor nitrite accumulation and the elevated nitrate concentration illustrate that the stable nitrite shunt process seen in Phase I had been lost because of ammonium fluctuation and NOB growth.

Because the stable NAR was lost during Phase II, the DO concentration, SRT, and sludge return ratio were adjusted for Phase III and IV operation. A stable NAR (80%) was achieved in Phase III by reducing the DO concentration from 2.5 to 2 mgO_2/L, and in Phase IV by reducing the DO concentration from 2 to 1.5 mgO_2/L. During Phase IV almost all the ammonium was oxidized to nitrite, NAR of 89.7%, and very little nitrate was observed in the effluent, 0.3 $mgNO_3$-N/L. Additionally, by applying the multiple step-feed strategy, the effluent concentrations of nitrite and nitrate were below 0.3 mgN/L after denitritation in an anoxic reactor [47]. This study is an important demonstration of successful nitrite shunt under mainstream operating conditions without the addition of an external carbon source.

Similarly, Gu et al. [48] developed a novel A-B process configuration for nitrogen removal by nitrite shunt under mainstream operating conditions. The laboratory scale A-B process consisted of an anaerobic fixed bed reactor (AFBR) and an SBR, respectively. The SBR system altered between anoxic and oxic cycles. Aeration was supplied to the SBR with a set DO concentration of 1.5 mgO_2/L during the oxic cycles. The influent synthetic ammonium concentration was 45 mgNH_4-N/L. The experiment was split into two phases: Phase I which did not involve influent bypass flow to the SBR, and Phase II which did involve influent bypass flow directly to the SBR. During Phase I ammonium was oxidized to nitrite as observed by a NAR of 86%. Additionally, 78% of the total nitrogen (TN) was removed during denitritation at an influent carbon to nitrogen ratio of 2.9. Unfortunately, as the carbon to nitrogen ratio dropped below 2.9, there was a corresponding decrease in the NAR and TN removal efficiency, 28% and 42%, respectively. Besides the decrease in removal efficiency, there was also an observed increase in nitrate concentration. Because of the poor nitrite shunt performance during Phase I, 19% of influent wastewater bypassed the AFBR, and instead was mixed with the effluent from the AFBR and fed directly into the SBR, hence Phase II. After stabilization under the new configuration, the effluent nitrate concentration was reduced to 1.5 mgNO_3-N/L, the NAR increased to 97%, and the TN removal improved to 85% in the AFBR. It is speculated that the addition of the influent bypass did not impact AOB activity but did supress NOB activity, which led to the stable nitritation activity [48].

Mainstream deammonification has been studied for over a decade with significant progress happening in the last few years [49]. Han et al. [50] reviewed the effect of carbon and aeration on a deammonification process under mainstream conditions in two SBR systems, SBR-A and SBR-B. The SBR temperatures were maintained at 25°C and cycle times lasted 6 hours. The SBR systems were fed with effluent from a high-rate activated sludge process. The total nitrogen concentration in the effluent was 22±5.9 mgN/L, corrected by the addition of ammonium bicarbonate. The influent total carbon to nitrogen ratio ranged between 1.5 and 6.2 depending on the phase of operation. Intermitted aeration was applied, where the DO concentration was maintained between 0.5 and 1.5 mgO_2/L during the oxic phase. The ANAMMOX bacteria used to seed the SBRs were from Strass WWTP [51], and later from a sidestream bench-scale process [52].

A total of seven phases were completed over the course of 514 days. SBR-A had a high aeration frequency with a DO setpoint of 1.5 mgO_2/L, compared to SBR-B with a low aeration frequency and DO setpoint between 0.3 and 1.5 mgO_2/L. Overall in SBR-A, when the influent soluble COD (sCOD) to nitrogen ratio was increased from 1.4 to 2.7, there was an corresponding increase in total nitrogen removal efficiency from 18% to 27%, respectively. Once the process stabilized in SBR-A, the total nitrogen removal efficiency increased to 66%. Similar trends were observed for nitrogen removal efficiency in SBR-B. However, the lower DO concentration in SBR-B led to a decrease in ammonium removal rates. Therefore, all subsequent phases completed on SBR-A and SBR-B were evaluated under the higher DO concentration profile.

Switching from low to high carbon loading rates resulted in an increase in ammonium concentration in the effluent of both reactors. Interestingly, nitrate accumulation increased in both systems when altering back to the low carbon loading phases, while the ARE remained the same. Therefore, the SRT was reduced to decrease the nitrate production while maintaining the ARE. Reducing the SRT improved the nitrogen removal rates to 10 and 13 mgN/L.d for SBR-A and SBR-B, respectively. During the high carbon loading phases, the contribution breakdown for total nitrogen removal by ANAMMOX bacteria was 50 to 60%, whereas, during the low carbon loading phases the contribution increased to 70%. Unfortunately, a major challenge with mainstream dammonification is NOB out-selection. During this experiment, the NOB contribution to nitrogen removal rate continuously increased and the ANAMMOX bacteria contribution rate continuously decreased [50].

During the high carbon loading phases there was a 20% decrease in deammonification contribution to total nitrogen removal when compared to the low carbon loading phases. However, the ANAMMOX bacteria still contributed up to 57% in removal efficiencies. Furthermore, an overall average of 70% deammonification was seen during the low carbon loading phases for total nitrogen removal. The higher denitrification rates contributed to a higher total nitrogen removal rate of 31 mgN/L.d under the high carbon loading phases, compared to the low carbon loading phases removal rate of 13 mgN/L.d. In conclusion, this research has demonstrated that carbon to nitrogen ratio and aeration frequency impact mainstream deammonficiation processes. Furthermore, the results obtained in the single-stage deammonification process were comparable to other mainstream deammonification applications [53–55]. Additional specific information regarding mainstream deammonification research and studies can be found in the review paper by Cao et al. [49].

Finally, there is still a significant amount of research that needs to be conducted before implementation of SBNR in mainstream applications. Focus can be placed on stabilization of process performance under fluctuating carbon to nitrogen ratios, how to maintain the ANAMMOX bacteria activity under low carbon to nitrogen ratios and lower operating temperatures, continued suppression of NOB, and final process polishing [49]. From the information on conventional nitrification-denitrification and mainstream SBNR, it can be denoted that there is still room for nascent nitrogen removal biotechnologies. In this concern, the unique capabilities of methanotrophic bacteria can play a vital role in these nitrogen removal processes.

2.3 METHANOTROPHIC BACTERIA

Aerobic methanotrophic bacteria, here referred to as methanotrophs, are a group of gram-negative *Proteobacteria* from the methylotrophic family. Methanotrophs have the ability to utilize methane as their sole carbon and energy source [29]. It is noteworthy that methanotrophs are a methane-consuming microorganism, differing from methanogenic bacteria (methanogens), which are a methane-producing microorganism as part of their energy yielding metabolism [56].

Methanotrophs utilize methane or single carbon compounds as their sole carbon and energy source [29]. Methanotrophs are classified into three types, Type I, II, and III, based on their carbon assimilation pathway for their energy requirement. Type I or gamma-proteobacteria utilize the Ribulose Monophosphate (RuMP) cycle, Type II or alpha-proteobacteria use the Serine cycle, and finally, Type III or verrucomicrobia assimilate carbon by the Calvin-Benson-Bassham (CBB) cycle [29]. Type I and Type II are the key methanotrophic groups, differing in membrane arrangements, preferred methane to oxygen ratio, nitrogen fixation, and carbon dioxide assimilation pathways [5,57]. Some general characteristics of Type I and II methanotrophs are highlighted in **Table 2-1**. Finally, methanotrophic bacteria are found in numerous environments including, but not limited to, freshwater, landfills, rice fields, hot springs, drainage water, forest soils, activated sludge, denitrification reactors, and acidic hot springs [58–62].

Table 2-1: Type I and II methanotrophic general characteristic comparison [5], [35].

Characteristic	Type I (gamma-proteobacteria)	Type II (alpha-proteobacteria)
Assimilation pathway	RuMP	Serine
Membrane arrangement	Stacked membranes	Peripheral membrane rings
Methane to oxygen ratio	Lower CH_4 to higher O_2	Higher CH_4 to lower O_2
Nitrogen fixation	Strain specific	All types
CO_2 assimilation	5-15% of biomass	50% of biomass
Colour	Pinkish	Tanish/white

2.2.1 OVERVIEW OF METHANE OXIDATION BY METHANOTROPHIC BACTERIA

All methanotrophs are capable of performing methane oxidization terminally to carbon dioxide in order to meet their energy and cellular requirements [29]. The first step of the methane oxidation process involves the methane monooxygenase (MMO) enzyme to oxidize methane into methanol when oxygen is present. The MMO enzyme is found in both soluble (sMMO) and particulate (pMMO) form. The sMMO enzyme is located in the cytoplasm and the pMMO enzyme is located in the intracytoplasmic member (ICM) [26]. The expression of the MMO enzyme depends on the copper concentration within the feed source provided to the methanotrophs. Thereby, the sMMO is expressed under lower copper concentrations (<1 μm) and the pMMO is expressed under higher copper concentrations (>1 μm) [14,26,29]. It can be noted that methanotrophs expressing the pMMO enzyme tend to have higher affinity towards methane and higher growth yields [63]. Interestingly, all methanotrophs can express the pMMO enzyme, while only certain strains can express the sMMO enzyme.

Besides MMO expression, Type I methanotrophs tend to have higher growth rates than Type II methanotrophs. However, Type II have greater carbon dioxide incorporation than Type I. Type II methanotrophs can incorporate 50% of the carbon dioxide produced for cellular synthesis compared to Type I at only 5 to 15% [64]. Finally, Type II methanotrophs can be advantageous because they have the capacity to accumulate biopolymers under nutrient deficient conditions, whereas, only very limited Type I strains have this same ability [65]. **Figure 2-2** depicts the methane oxidation pathway and the associated enzymes within methanotrophic bacteria [57].

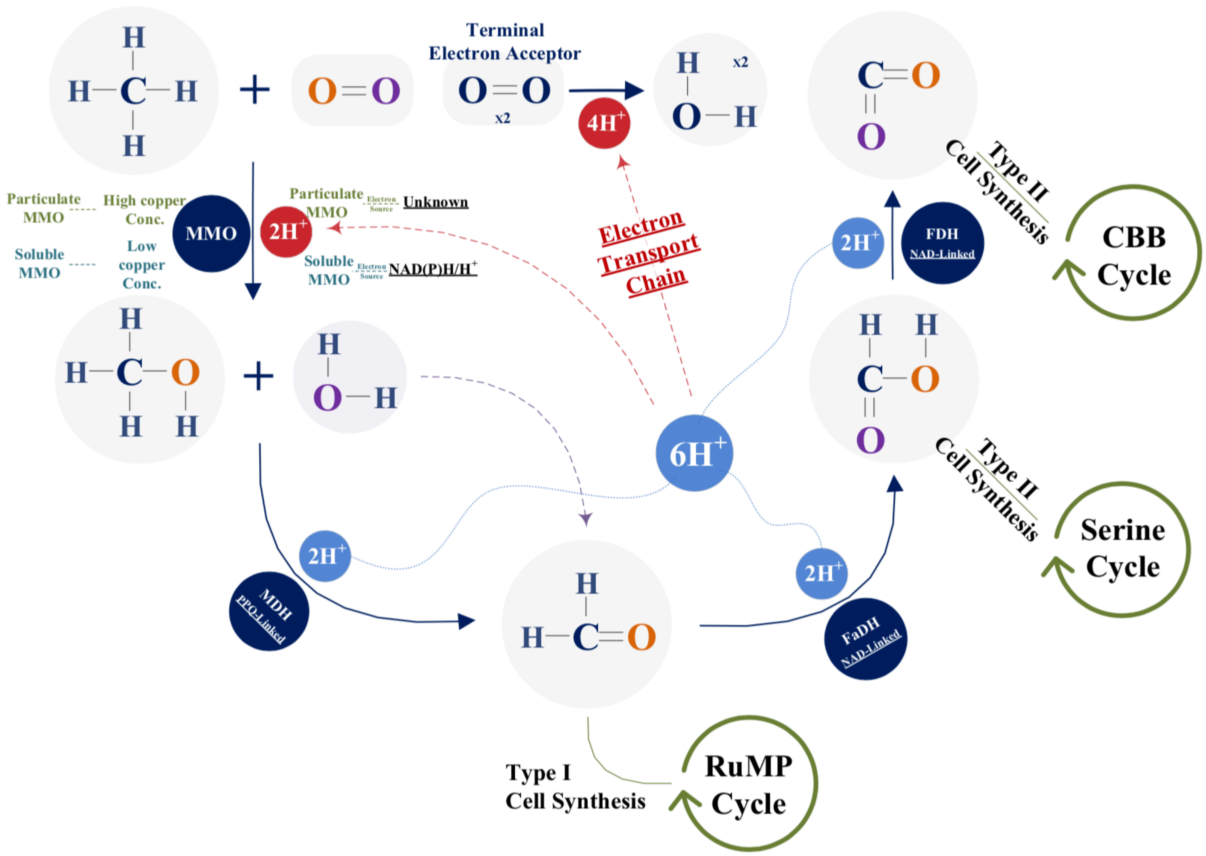

Figure 2-2: Methane oxidation pathway shown for Type I, II and III methanotrophic bacteria. MMO: methane monooxygenase; MDH: methanol dehydrogenase enzyme; FaDH: formaldehyde dehydrogenase enzyme; FDH: formate dehydrogenase enzyme; PQQ: pyrroloquinoline quinone; NAD(P)H: nicotinamide adenine dinucleotide; RuMP: ribulose monophosphate pathway; CBB: calvin-benson-bassaham. Figure from AlSayed (2017) [57].

As stated, methane oxidation to methanol is completed by the MMO enzyme. Besides the MMO enzyme, methanotrophs also secret a series of enzymes which aid in the methane oxidation process [29,60,64,66]. During methane oxidation, the electron acceptor oxygen, is split into two atoms. The first atom is required for methane hydroxylation and the second atom is used for water (H_2O) formation [18]. In a subsequent step, methanol is oxidized to formaldehyde (CHOH), along with the reduction of pyrroloquinoline quinone (PQQ) to $PQQH_2$ [57]. Methanol oxidation utilizes the periplasm located quinoprotien methanol dehydrogenase (MDH) enzyme. Formaldehyde is highly toxic to methanotrophs, thus is quickly oxidized to formate (CHOOH) followed by carbon dioxide for methanotrophic energy requirement. Formaldehyde can also be directed through the different carbon assimilation pathways for cellular synthesis [29,67]. Formaldehyde oxidation to formate is carried out by the formaldehyde dehydrogenase (FaDH) enzyme located in the cytoplasm, while formate oxidation to carbon dioxide is completed by the formate dehydrogenase (FDH) enzyme [68,69].

2.2.2 BASIC NITROGEN METABOLISM

The diversity of nitrogen metabolism is well known in methanotrophs. The majority of methanotrophic strains utilize inorganic nitrogen, specifically ammonium and nitrate, as their cellular nitrogen sources [58–60]. However, certain strains of methanotrophs (all Type II and few Type I) have been shown to fix atmospheric nitrogen gas, giving them the ability to utilize it as their nitrogen source [58,70,71]. Consequently, ammonium utilization as the nitrogen source can inhibit methanotrophic growth in two ways. The first way, is that ammonium oxidation leads to the production of toxic intermediates (hydroxylamine and nitrite) and the second way, is that ammonium creates competition with methane for oxidation by the MMO enzyme. Therefore, using nitrate as the nitrogen source for methanotrophic incorporation has a tendency to produce higher growth rates for Type I and II [67,72]. On the other hand, applying methane to the methanotrophic culture in sufficient concentrations can suppress ammonium oxidation by the

MMO, shifting the culture towards ammonium assimilation for cellular synthesis. In contradiction to the above statement of methanotrophic growth rate on different nitrogen forms, He et al. [73] observed that at the same nitrogen concentrations of ammonium and nitrate, there was greater biomass production when ammonium was used as the nitrogen source. This finding agrees with a study completed by Nyerges and Stein [74] in which methanotrophic growth rate and biomass production was elevated when using ammonium as the nitrogen source. However, it is noteworthy that the energy requirements of methanotrophs cannot solely be fulfilled by nitrogen, rather, methane oxidation is an important requirement for cellular energy generation.

The impact of different nitrogen forms, ammonium, nitrate, or nitrogen gas, on methanotrophs can differ based on the environmental conditions or ecosystem. For example, Hu and Lu [75] demonstrated that in paddy soil, Type I methanotrophs were stimulated with ammonium as the nitrogen source, whereas Type II methanotrophs were not. Moreover, it was observed that when nitrate was used as the nitrogen source both Type I and II were stimulated in the paddy soil [75]. Interestingly, when ammonium inhibition on methanotrophic activity becomes evident, Type II methanotrophs can still form stable dominant communities because of their higher resistances to elevated ammonium concentrations [76,77]. Type I methanotrophs prefer nitrate as the nitrogen source as evident by increased methane oxidation rates and growth rates observed [57]. Furthermore, the nitrogen source applied when cultivating methanotrophic bacteria can be a viable selection parameter for Type I or Type II.

2.4 METHANOTROPHS IN BIOLOGICAL NITROGEN REMOVAL

Ammonium and nitrate are common nitrogen sources for methanotrophic growth. Similar to methane, ammonium is assimilated in Type I methanotrophs by the RuMP pathway and in Type II methanotrophs by the serine pathway. **Equations 2-6** and **2-7** display the ammonium assimilation pathways in Type I and II methanotrophs, respectively. The two equations were initially proposed by Karthikeyan et al. [67] assuming a methanotrophic microorganism formula of $C_4H_8O_2N$ [67].

$$CH_4 + 1.5O_2 + 0.12NH_4^+ \rightarrow 0.12C_4H_8O_2N + 0.53CO_2 + 1.71H_2O + 0.12H^+ \quad (2\text{-}6)$$

$$CH_4 + 1.57O_2 + 0.1NH_4^+ \rightarrow 0.1C_4H_8O_2N + 0.59CO_2 + 1.75H_2O + 0.1H^+ \quad (2\text{-}7)$$

2.3.1 METHANOTROPHIC INVOLVEMENT IN NITRIFICATION

It is widely reported that ammonium oxidizing bacteria (AOB) and methanotrophic bacteria share similarities. Noteworthy similarities between AOB and methanotrophs are the ability to grow on specific substrates, both contain intracellular membrane bound monooxygenase enzymes, and both have the capacity to co-oxidize other substrates [30,78]. Emphasis can be placed on the ability of AOB and methanotrophs to co-oxidized other substrates. Therefore, AOB have the capacity to co-oxidize methane while methanotrophs have the ability to co-oxidize ammonium. Essentially, methanotrophs use and react to different nitrogen sources (ammonium, nitrate, and dinitrogen gas) under different methane and oxygen conditions giving them the ability to partake in nitrogen removal processes [18]. Methanotrophic-based nitrification can occur because of similarities associated with the pMMO enzyme and the ammonium monooxygenase (AMO) enzyme of AOB in the reducing equivalents, inhibitors, and active sites [1].

During the first step of methane oxidation, when both ammonium and methane are available to methanotrophs, there is competition between the two substrates to be oxidized by the pMMO enzyme [1,30]. Assuming ammonium can outcompete methane, ammonium oxidation will occur resulting in the formation of hydroxylamine (NH_2OH). Hydroxylamine is highly toxic to methanotrophic bacteria, thus is quickly oxidized to nitrite by the hydroxylamine oxidoreductase (HAO) enzyme encoded by the *haoAB* gene or by the cytochrome P460 enzyme encoded by the *cyp*(*cypL*) gene in specific strains [18,30,79,80]. Unfortunately, ammonium oxidation by methanotrophs can inhibit their growth in two ways. The first, is that ammonium oxidation compromises methane oxidation because of competition between ammonium and methane to be oxidized by the pMMO enzyme. Ammonium oxidation leads to a decrease in the rate of methane to methanol oxidation and the overall methanotrophic metabolism. The second way methanotrophic growth is inhibited by ammonium oxidation is from the production and accumulation of toxic intermediates, specifically hydroxylamine and nitrite, which inhibit the FDH enzyme affecting the assimilatory and dissimilatory methane oxidation pathways [18,81].

AOB have developed a strategy to incorporate hydroxylamine into energy and cellular growth by relying on electrons from the oxidation of hydroxylamine [30]. However, methanotrophs do not have this same hydroxylamine incorporation strategy. Fortunately, different strains of methanotrophs have developed unique detoxification mechanisms for hydroxylamine. One coping strategy is the reduction of hydroxylamine back to ammonium through the hydroxylamine reductase (HAR) enzyme [71,82]. The second strategy is the oxidation of hydroxylamine to nitrite, and further nitric oxide and nitrous oxide, through the HAO enzyme. Interestingly, the methanotrophic strain SC2, is the only known strain to possesses both hydroxylamine detoxification mechanisms [71]. **Figure 2-3** [18] demonstrates ammonium oxidation, coping strategies, and encompassing enzymes found in methanotrophic bacteria for nitrogen transformation.

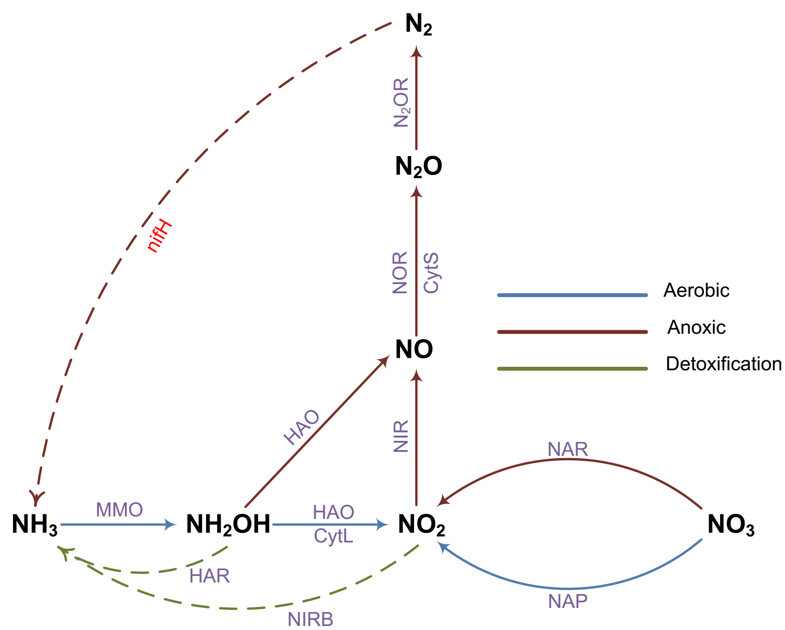

Figure 2-3: Different nitrogen assimilation pathways that have been discovered in methanotrophic bacteria [18]. MMO: methane monooxygenase enzyme; HAO: hydroxylamine oxidoreductase; HAR: hydroxylamine reductase; NIRB: assimilatory nitrite reductase; NIR: nitrite reductase; NOR: nitric oxide reductase; N_2OR: nitrous oxide reductase; NAR: nitrate reductase and; NAP: periplasmic nitrate reductase.

Understanding how methanotrophs and their associated enzymes are involved in the ammonium oxidation process is relatively new and not widely applied for nitrification. Unfortunately, most studies focus on how nitrogen concentration and sources impact methane oxidation because of their natural inhabitant at the interface between oxic and anoxic zones.

Methanotrophic ammonium oxidation research pays little attention to factors impacting ammonium removal rates and efficiencies. Overall, it can be denoted that methanotrophs use and react to different nitrogen sources, i.e. ammonium, nitrate, and nitrogen gas for either cellular growth by assimilation or oxidation to different nitrogen forms.

2.4.1.1 EVIDENCE OF METHANOTROPHIC AMMONIUM OXIDATION

Nyerges and Stein [74] reviewed the effect of ammonium and nitrite on methane oxidation of two Type I (*Methylomicrobium album* and *Methylomonas methanica)* and two Type II (*Methylocystis sporium* and *Methylosinus sporium)* methanotrophic strains. It was illustrated that all strains were able to oxidize ammonium to nitrite except for the strain *M. methanica*. Additionally, the Type II strain *Methylocystis* showed the greatest nitrite production implying increased detoxification capacity of hydroxylamine. Furthermore, they found that as the biomass concentration increased so did the hydroxylamine production, suggesting that this in an enzymatic process [74]. A similar study completed by Hoefman et al. [4] reviewed 16 methanotrophic strains and found that 15 could produce nitrite and 13 could produce nitrous oxide when nitrate was used as the nitrogen source. The study also noted that 14 methanotrophic strains could produce nitrous oxide, but not nitrite, when ammonium was used as the nitrogen source. In contradiction to Nyerges and Stein [74], utilizing ammonium as the nitrogen source did not result in nitrite detection. However, Hoefman et al. [4] attributed the lack of nitrite detection to the immediate conversion of hydroxylamine to gaseous nitrogen, or because peak levels were too small for detection [4].

Moreover, a recent study by Lopez et al. [83] reviewed the optimal ammonium range to achieve high methane oxidation rates. Lopez et al. [83] found that as the ammonium concentration increased, the specific methane oxidation rate and biomass growth decreased. This finding can be attributed to the more apparent competitive substrate inhibition between ammonium and methane. On the other hand, as the ammonium concentration in the prepared ammonium mineral salts (AMS) increased, the ammonium removal rates also increased. The ammonium removal rates increased by 1.7 and 2.4 times at 20 and 80 mmol ammonium concentration, respectively, in comparison to 4 mmol ammonium concentration. The increase in ammonium removal rates can be attributed to the increase in ammonium oxidation rate by the mixed culture. Nitrate production was also greater by 1.2 and 2 times at 20 and 80 mmol, respectively, when compared to 4 mmol ammonium concentration. The increase in nitrate accumulation with increasing ammonium concentration is likely the result of the dominate *Proteobacteria* culture (90.7% relative abundance) at the higher ammonium concentrations. Furthermore, nitrite accumulation was 10 times higher at the 4 mmol ammonium concentration (4 mmol), compared to 80 mmol ammonium concentration, whereas, no nitrite accumulation was observed at 20 mmol [83].

He et al. [73] examined the effect of ammonium conversion on methane oxidation in batch serum bottles using the strain *Methylosinus sporium*. When the culture was evaluated under different ammonium concentrations, methane consumption rates decreased with increased ammonium concentrations. Decreased methane consumption was observed because of increased competition between ammonium and methane for the active sites of pMMO enzyme. The experiment also confirmed that carbon derived from methane oxidation was more biomass driven in methanotrophs when supplied with ammonium over nitrate as the nitrogen source. In agreement with Nyerges and Stein [74], nitrite was present in the AMS driven cultures. Additionally, nitrous oxide was detected in the headspace of the serum bottles for the AMS driven cultures, but not the nitrate mineral salts (NMS) driven cultures. Finally, a pH decrease was observed in the culture when solely grown on ammonium as the nitrogen source. This pH drop can be attributed to methanotrophic metabolism, i.e. by-products of formic and acetic acid, or is largely the result of methanotrophic nitrification as hydrogen ions were present [73].

Besides ammonium conversion to nitrite and/or nitrous oxide, it was reported that methanotrophs can oxidize ammonium to nitrate. He et al. [73] noticed that nitrate was produced under different ammonium concentrations while using a methanotrophic *M. sporium* culture. Nitrate accumulation was observed despite the fact that no activity of the nitrite oxidoreductase was detected. Hence, the metabolic pathway for nitrate production in methanotrophic bacteria has been illustrated but is still unknown [73]. Furthermore, the greatest ammonium conversion to nitrate was at the lowest AMS concentration (182 mgNH_4-N/L).

A recent study completed in 2020 is well worth mentioning for its focus on ammonium removal from reject water (sidestream) in a sequential batch reactor (SBR) using a methane- and methanol- dependent culture [84]. Methanotrophic bacteria were cultivated from activated sludge using AMS and supplied with biogas (55-70% methane) from AD as the carbon source. The methane to oxygen ratio applied to the SBR was 1 to 1.5 based on the biogas concentration and the air flow rate. The total suspended solids (TSS) in the reactor ranged between 3700 to 4400 mg/L throughout the experiment. From a microbial analysis, *Methylophilus* and Type I methanotrophs dominated the mixed culture as result of the feed solution and biogas supplement.

When the ammonium concentration in the reject wastewater was less than 800 mgNH_4-N/L, the ARE was approximately 60%. However, when the ammonium concentration was greater than 800 mgNH_4-N/L, there was a significant reduction in the ARE observed decreasing to approximately 37%. The lower ARE observed under the higher ammonium concentration is speculated to be the result of toxic by-products released during ammonium oxidation, including hydroxylamine and nitrite. The aforementioned removal efficiencies suggest that ammonium removal in the system was the product of both methanotrophs assimilation by the glutamate cycle in methanotrophs, and ammonium oxidation to hydroxylamine and nitrite [84].

Additionally, batch studies were completed on the methanotrophic culture where the feed solution applied was high in ammonium concentration and low in nitrate. A control system filled with only air, was compared to serum bottles filled with 25% biogas. The ARE was 42% and 63.5% after 4 and 6 hours, respectively, when the headspace contained 25% biogas. Interestingly, no real difference was observed in nitrate concentration, 5.2 to 3.2 mgNO_3-N/L, when biogas was used in the headspace. However, the control system with only air showed nitrate accumulation of 5.2 to 31.2 mgNO_3-N/L and an ARE of 16.8% within 4 hours [84]. This study is significant because it demonstrates the potential of ammonium removal by assimilation and ammonium oxidation from reject wastewater using methane- and methanol- dependent microorganisms in an SBR system [84]. Even though several of the above studies do not specifically focus on ammonium removal rates and nitrification ability, they still demonstrate the potential for methanotrophic mixed cultures to be employed in wastewater nitrification processes while utilizing ammonium as the sole nitrogen source.

2.3.2 METHANOTROPHIC INVOLVEMENT IN DENITRIFICATION

Methane utilization as a carbon source for denitrification can be broadly classified into anaerobic methane oxidation coupled to denitrification (ANME-D) and aerobic methane oxidation coupled to denitrification (AME-D), based on the oxygen requirement. The focus of this research is placed on AME-D because of the coexistence of methanotrophic bacteria and denitrifying bacteria (denitrifiers), and because most research under methane redirection is carried out within this particular field [26]. Several AME-D studies have been completed using methane and/or biogas as the carbon source for denitrification in different reactor systems including trickling filter, activated sludge, fluidized bed reactor [17], filter column [16], and membrane biofilm reactor (MBfR) [25]. Aerobic membrane bioreactors have demonstrated achievable denitrification rates while only using methane as the carbon source. Moreover, microaerobic or oxygen-limited conditions have been shown to achieve reasonable denitrification rates through the survival of methanotrophs and denitrifying bacteria [25,85].

Essentially, two literature hypotheses exist under AME-D. The first hypothesis is that methanotrophs produce soluble organics, under low dissolved oxygen (DO) concentrations, that are used by heterotrophic bacteria as electron donors [26,86]. The second hypothesis is that methanotrophs themselves can convert nitrate to nitrous oxide or dinitrogen gas under microaerobic or anoxic conditions [71]. Methanotrophs have been known to complete ammonium oxidation for over a decade, however, in the last few years it was discovered that they can also achieve dissimilatory denitrification [81]. Finally, Alrashed et al. [87] proposed that the aforementioned hypotheses can occur simultaneously in mixed cultures.

The first hypothesis is supported by the observation of soluble organic compounds including methanol [88], acetate [86], citrate [89], polysaccharides, and proteins [90] in AME-D studies. These organic compounds are used as electron donors for nitrate and nitrite reduction by the coexisting denitrifying bacteria. During the AME-D process, methanotrophs not only support denitrifying bacteria by supplying electron donors, but also by consuming oxygen during methane oxidation to create microaerobic or anoxic conditions favoring denitrification rates [72,91]. Zhu et al. [1] estimated the proportions of methanol, which was already secreted by methanotrophs during methane oxidation, used by methanotrophs and denitrifying bacteria present in a system to be 0.4 and 0.6, respectively. These values were calculated assuming minimum energy consumption for methanotrophic cellular maintenance.

When nitrite or nitrate is reduced by pure aerobic methanotrophic cultures, the observed terminal end product is nitrous oxide. Furthermore, nitrous oxide production is also observed when ammonium or nitrate is used as the sole nitrogen source. Unfortunately, nitrous oxide production has limited methanotrophic applications for denitrification in water and wastewater because of the potency associated with nitrous oxide as a GHG [87,92]. However, a study completed by Dam et al. [71] showed that the methanotrophic strain *Methylocystis* sp. SC2 (SC2) could produce dinitrogen gas under anoxic conditions while using nitrate as the nitrogen source and methanol as the electron donor [71].

The SC2 strain has a more diverse nitrogen metabolism pathway compared to other methanotrophic strains. The SC2 strain was found to contain all the required genes for nitrogen fixation, along with the genes needed for ammonium transport, assimilatory nitrate/nitrite reduction, hydroxylamine detoxification, and denitrification. Interestingly, the SC2 strain contains both hydroxylamine detoxification strategies and is the only known methanotroph to do so [71]. Importantly, strain SC2 contains the key genes needed (*nosZ*, *nosR*, and *nosX*) to achieve complete denitrification. A tracer-experiment was completed on strain SC2 under anoxic conditions with nitrate as the sole nitrogen source to evaluate the nitrogen gas production. Nitrogen gas accumulation was observed at a rate of 0.7 nmol N_2/hr/mg dry weight of cells. Additionally, under normal operating conditions a trace amount of nitrous oxide was detected in the headspace. The trace amount of detected nitrous oxide can be attributed to the quick conversion of nitrous oxide to nitrogen gas through the plasmid-borne *nos* operon, evidently demonstrating complete denitrification by the methanotrophic strain [71].

Kits et al. [93] reviewed the effect of different nitrogen sources on methane oxidation and growth of the methanotrophic strain *M. denitrificans* sp. nov. FJG1 (FJG1). The methane and oxygen consumption rates observed were the same in the cultures cultivated on either ammonium or nitrate. However, nitrous oxide was only produced by the culture grown on nitrate. The cultures were placed in a micro-respiratory chamber to further test if the FJG1 strain could consume and/or produce nitrous oxide. It was noted that the addition of nitrate, nitrite and nitric oxide before an anoxic period but after oxygen consumption, led to the production of nitrous oxide. However, nitrous oxide production was only observed when methane was present in the system confirming that nitrous oxide production is highly methane dependent. Because the strain FJG1 could only produce nitrous oxide from NSM, it can be speculated that the production process is dissimilatory in nature in the absence of- or under oxygen limited conditions [93].

Costa et al. [85] reviewed the involvement of methanotrophic bacteria in the denitrification process under microaerobic and anoxic conditions. This experiment was completed in parallel sequential batch reactor (SBR) systems supplied with nitrate as the nitrogen source and methane as the carbon source. Interestingly, methane oxidation and nitrogen consumption rates were higher under microaerobic conditions than anoxic conditions. This finding was confirmed through abiotic batch analysis. Denitrification is palpably dependent on methane oxidation, emphasized by higher methane oxidation rates and denitrification activity under microaerobic conditions compared to anoxic conditions. Similar denitrification activity results were observed by Cao et al. [94] who stressed that microaerobic conditions achieved greater specific denitrification rates (SDNR) and denitrification efficiencies than anoxic conditions. Further, when a control system was used without methane addition, SDNRs and denitrification efficiencies were significantly lower, driving the point that denitrification in AME-D reactors is highly methane driven [94]. Interestingly, nitrite accumulation was seen in the microaerobic reactor during the first five cycles (0.12 to 4.87 mgNO_2-N/L), however, this value gradually decreased to zero mgNO_2-N/L as the process continued [94].

Another AME-D experiment that was completed in 2019 [95] successfully evaluated how methane utilization could help to optimize the denitrification process. Optimization of AME-D biotechnologies is seemingly important because excess methane can remain in the effluent during discharge or can escape to the environment because of its poor solubility. Methane utilization was evaluated by altering the HRT, a key design parameter, in an SBR system. As the HRT increased from 6 to 24 hours there was a corresponding increase in nitrate removal efficiency (NRE) from <40% to 97%, respectively. However, the SDNR increased significantly from a 6 to 12-hour HRT, 0.82 ± 0.05 to 2.54 ± 0.09 mg N/g VSS.hr, respectively, but not from a 12 to 24-hour HRT. Lee et al. [95] speculated that the large difference was attributed to methane oxidation and the DO concentration, where the DO concentration was approximately 55% lower during the 12 hour cycle. Contrary to what was observed by Cao et al. [94], no nitrite was present in the effluent at any time. Overall, as the HRT increased there was a corresponding increase in the carbon to nitrogen ratio (from 1.38 to 2.85) and the NRE (from below 40% to greater than 97%), demonstrating the impact of operational parameters on methane utilization. The reaction to different carbon to nitrogen ratios can be attributed to the methanotrophic culture diversity in performing methane oxidation coupled to denitrification [85]. The theoretical value of methane utilized per nitrate consumed (C/N ratio) is 2 mmolCH_4/mmolNO_3^- [95]. Furthermore, Lee et al. also noted an increase in biomass concentration with time, which is attributed to the quick settling capacity of the methanotrophic sludge floc. The calculated sludge volume index (SVI_{30}) value was less than 75 ml/g [95].

Alrashed et al. [87] completed a study with the objective of proving hypoxically aerobic methane oxidation coupled to denitrification (HYME-D) in a membrane biofilm reactor (MBfR) with mixed cultures. The objective was to identify the methane-based denitrification pathway used. Nitrate reduction in the MBfR was impacted by the methane pressure and the HRT. However, the HRT had a greater impact on nitrate reduction than the methane pressure. Nitrite was present in the effluent at a concentration less than 0.2 mgNO_2-N/L during the evaluation of all HRT and methane concentrations. Furthermore, nitrous oxide was not detected in the headspace of the MBfR implying that there was complete denitrification of nitrate to dinitrogen gas. Complete denitrification by the mixed culture was further confirmed by the metagenome analysis. The metagenome analysis identified the *nos* gene in the mixed culture which is required for nitrous oxide reduction to dinitrogen gas. Other significant findings from the metagenome analysis were the genes required for aerobic methane oxidation and the functional genes required for denitrification. Syntrophic interaction between methanotrophs (*Methylocystaceae)* and denitrifying bacteria were also identified. *Methylocystaceae* dominantly oxidized methane, together with nitrite reduction to nitric oxide, and denitrifiers (*Flavobacteriacea, Comamonandaceae*, and *Brucellaceae*) reduced nitric oxide to dinitrogen gas, thus completing the denitrification process [87].

Unfortunately, the AME-D process still has pressing issues such as the effect of low methane solubility, high methane concentrations found in the effluent, safety hazards associated with supplying methane and oxygen as flammable gases, nitrous oxide emissions, and the economic aspects of AME-D biotechnologies. Importantly, the above studies demonstrate how methanotrophs use and react to different nitrogen sources, i.e. ammonium, nitrate, and nitrogen gas for either cellular growth by assimilation and/or detoxification of ammonium, hydroxylamine, and nitrite by nitrification-denitrification. The key parameter that may control such behaviour is the methane and oxygen concentration. Methane and oxygen concentrations impact competition on the MMO enzyme and the dissolved oxygen (DO) concentration, respectively.

2.5 METHANOTROPHIC BIOPOLYMER PRODUCTION FROM BIOGAS AS A FEEDSTOCK

Besides GHG emissions, another anthropogenic environmental burden is petroleum-based and single-use plastics. Petroleum-based plastics are environmentally burdening because of (a) their reliant on fossil fuels for production [96], (b) difficulties surrounding the identification, sorting, and recycling of these plastics [97], (c) production and release of hazardous emissions if incinerated [98], and (d) the numerous single-use plastics that end up in the environment as litter because of poor disposal and waste management practices [99]. Therefore, bioplastics are growing in popularity as a renewable green plastic alternative to petroleum-based and single-use plastics because they can degrade back to raw material at the end of their life cycle. Biodegradable bioplastics are synthesized naturally by different microorganisms under specific environmental conditions. The majority of bioplastics are in the form of poly-hydroxy-alkenoates (PHAs). Essentially, PHAs are harvested from microorganisms that have a high PHA capacity. PHAs are classified into short- and medium-chain length based on the number of carbon monomers [100]. Currently, over 150 different PHAs, with different monomer chain lengths have been identified [101,102].

Bioplastics still have several drawbacks that need to be addressed including its mechanical properties, the inability to be processed using conventional methods, vulnerability to thermal degradation, and high production costs [103]. Of these drawbacks, emphasis can be placed on the cost intensities associated with conventional bioplastic production, which is five to ten times greater than petroleum-based plastics [104]. Currently, bioplastic production relies on farming practices to cultivate crops as the feedstock carbon source for microorganisms [99].

Furthermore, the cultivated crops need to be converted to suitable carbon sources (feedstock sources) for the microorganisms. Unfortunately, 30% of the costs associated with PHA productions stems from the cost of the feedstock source [105]. Common feedstock sources include vegetable oils, plant oils, fatty acids, and simple sugars [101]. Currently, annual industrial PHB is sold for a market price of $3/kg to compensate for production costs, compared to petroleum-based plastics at $1-2/kg [67,102]. With the current high costs of bioplastic production, research has shifted towards alternative and cheaper feedstock solutions such as organic municipal solid waste, wastewater, carbon dioxide, and methane [31]. Fortunately, utilizing methanotrophic bacteria for PHB production and methane mitigation, can reduce the cost of feedstock from $1/kg to $0.25/kg, assuming methane as the feedstock source [31].

Besides having the capacity to be employed in nitrogen removal processes, methanotrophs have the capacity to accumulate the biodegradable biopolymer poly-hydroxy-butyrate (PHB), a member of the PHA family, under nutrient deficient or unbalanced conditions [65]. Hence, if a major nutrient such as nitrogen or phosphorus is unavailable to the methanotrophs, the culture will switch to utilizing the PHB cycle for its energy requirement instead of the conventional tricarboxylic acid (TCA) cycle [106]. Essentially, PHB production is separated into two phases. During phase one, all the required nutrients are available to the culture to increase the biomass density. While, during phase two, nutrient deficient conditions or unbalanced conditions are applied to promote PHB accumulation as intracellular granules [107]. The stored PHB can be extracted and applied as an alternative raw material for green bioplastics. Therefore, methanotrophs have the ability to aerobically convert methane to PHB under nutrient deficient conditions helping to ease GHG emissions from WWTPs, while simultaneously reducing some of the high cost associated with the current bioplastic production process.

Biopolymer (PHB) production is limited to Type II methanotrophs under nutrient deficient conditions. Unfortunately, it is difficult to maintain Type II dominant cultures as Type I invasion is common because of their higher growth rates and methane affinity [65]. Employing a methanotrophic mixed culture will result in lower biopolymer production compared to pure Type II cultures. However, the stringent operating standards associated with pure cultures do not need to be adhered to which can be beneficial for mainstream integration. In addition to the aforementioned benefit, utilizing excess methanotrophic sludge produced from alternative bioprocesses, i.e. biological nitrogen removal, can help to reduce the time requirement of phase one cultivation for methanotrophic biopolymer accumulation. Additionally, utilizing methanotrophic sludge for biopolymer production can further consolidate the WRRFs concept by recovering higher value products and resources.

Because biopolymer accumulation is mainly limited to Type II methanotrophs, studies are often completed in pure cultures, or Type II dominant cultures, and review the impacts of different environmental conditions on PHB accumulation within these cultures. In literature, different strategies and conditions have been proposed to accumulate a high capacity of PHAs in microorganisms [65,108–110]. Of these strategies, emphasis can be placed on nitrogen concentration and source, phosphorus concentration/limitation, and alternating feast-famine phases. Unfortunately, few studies focus on PHB accumulation within methanotrophic mixed cultures.

2.5.1 FACTORS IMPACTING METHANOTROPHIC PHB PRODUCTION

2.5.1.1 NITROGEN SOURCE AND CONCENTRATION

Nitrogen concentration and source has been a promising contributing parameter to achieve long-term PHB accumulation [111]. Unfortunately, the data available for the selection and accumulation capacity based on nitrogen concentration and source varies significantly in literature. For example, when ammonium was used at the nitrogen source for cultivation before nitrogen elimination, a 9% PHB accumulation capacity was observed in the methanotrophic strain *Methylosinus trichosporium* OB3b (OB3b) [112]. On the other hand, when nitrate was used at the nitrogen source for OB3b a 20% PHB accumulation capacity was observed, and finally utilizing nitrogen gas resulted in a 45% PHB accumulation capacity [112]. Furthermore, Shah et al. [113] demonstrated a 50% PHB accumulation capacity using the same methanotrophic strain OB3b, while using a 20 mmol nitrate mineral salts (NMS) concentration before exposing the culture to nutrient deficient conditions. Whereas, Pieja et al. [114] demonstrated a 38% PHB accumulation capacity utilizing the same methanotrophic strain under an initial NMS concentration of 10 mmol, followed by deficient conditions [114]. Furthermore, the methanotrophic strain *Methylosinus trichosporium* IMV3011 (IMV3011) achieved a PHB accumulation capacity of 7.5% when the ammonium concentration was 50 mg/L and 24.6% when the ammonium concentration was limited to 10 mg/L [109]. On the other hand, Rostkowski et al. [112] saw a 13% PHB accumulation capacity using AMS with a concentration of 10 mmol, but altering the nitrogen source to dinitrogen gas led to an 32% increase in the PHB accumulation capacity. Finally, the methanotrophic strain *Methylocystis parvus* OBBP was grown on ammonium and nitrate, in two separate studies, and a PHB accumulation capacity of 60% [112] and 36% [114] was observed, respectively.

Similarly, dominant Type II mixed cultures have shown a wide variety of PHB accumulation capacities under different nitrogen sources and concentrations. Wendlandt et al. [107] demonstrated a 51% accumulation capacity while using AMS in a mixed culture dominated by the methanotrophic strain *Methlocystis* GB25. Whereas, when a mixed culture was dominated by the methanotrophic strains *Methylocystis* and *Methylosinus* and grown on dinitrogen gas before deficient conditions a 43% accumulation capacity was observed [114]. Furthermore, a 39% PHB accumulation capacity was noted when a 13.5 mmol AMS was used and the culture was dominated by the strain *Methylocystis* [111]. Finally, Fergala et al. [65] demonstrated a 52% PHB accumulation capacity in a Type II dominant culture when cultivated on AMS with a 5 mmol concentration before exposing the culture to deficient conditions.

When nitrate is used as the nitrogen source for PHB accumulation, mixed culture methanotrophs tend to show less PHB production capacity as a result of Type I invasion. This observation was demonstrated in literature [65]. Interestingly, despite having a stable dominant Type II culture, when switching the nitrogen source from ammonium to nitrate, Type I invasion was evident and the PHB accumulation capacity decreased to below the detection limit [65]. Furthermore, Type II methanotrophs are less impacted by ammonium inhibition thus leading to increased PHB accumulation. However, utilizing nitrate as the nitrogen source can lead to greater biomass density [115]. Therefore, Criddle and Sundstrom [110] demonstrated that mixed cultures can first be grown on ammonium, before switching to nitrate to increase their biomass density. This strategy proved successful as a 40% PHB accumulation capacity was observed under a dominant Type II culture [110].

2.5.1.2 PHOSPHORUS

Another common PHB accumulation strategy applied is to limit the phosphorus concentration in the mineral salts medium (MSM). Similar to nitrogen source and concentration, phosphorus concentration can impact methanotrophic growth and PHB accumulation capacity. However, phosphorus is a requirement to maintain MMO enzyme activity (2 to 25 mmol), and eliminating it completely may inhibit culture performance [70]. Thus, research simply limits the phosphorus concentration in the feed source during the limiting periods. This PHB accumulation strategy was demonstrated by Sundstrom and Criddle [116] in the methanotrophic strain *Methylocystis parvus* OBBP (OBBP). When the phosphorus concentration was reduced from 2.9 to 0.12 mmol, there was a concurrent increase in PHB accumulation capacity by 13% (from 18 to 31%). Similarly, in another study using the methanotrophic strain IMV301, when the phosphorus concentration was reduced from 7.3 to 5.7 mmol, there was a concurrent increase in PHB accumulation from 16% to 26.5%, respectively [109]. Furthermore, a mixed culture dominated by the methanotrophic strain *Methlocystis* GB25 showed a 46% PHB accumulation capacity under phosphorus limitations [107]. These studies illustrate that phosphorus limitation is an applicable strategy in accumulating PHB in methanotrophic cultures.

2.5.1.3 METHANE TO OXYGEN RATIO

Besides limiting major nutrients like nitrogen and phosphorus, different methane to oxygen ratios have been shown to impact PHB accumulation capacity. Costa et al. [117] reviewed the impact of oxygen on the methanotrophic metabolism in Type II methanotrophs. Interestingly, a 35% oxygen concentration led to the inhibition of the methanotrophic metabolic reaction. This finding can be attributed to the rapid conversion of methanol to formaldehyde, which is toxic to methanotrophs, thus impacting the PHB accumulation pathway. Conflicting with the previous study, Rostkowski et al. [112] illustrated that at 0.2 atm of oxygen the PHB accumulation capacity was 45% for *Methylosinus trichosporium* OB3b and at 0.3 atm of oxygen the PHB accumulation capacity was 60% for *Methylocystis parvus* OBBP. These findings demonstrate that methanotrophic strains may react differently to different levels of oxygen concentration.

2.5.1.4 CYCLIC FEAST AND FAMINE PHASES

Finally, cycling between phases of feast and famine have proved successful in accumulating a high percentage of PHA in microorganisms (up to 89% [118]). Under this strategy, microorganisms are exposed to alternating periods of excess and deficient nutrient conditions, hence the feast and famine phases [108]. Applying feast and famine conditions have been shown to create mixed cultures which are dominated by microorganisms having robust carbon storage abilities [108]. Under the feast phase, cultures can store fractions of carbon as PHA. This stored carbon provides an advantage because upon exposure to the famine phase, the microorganisms that have high carbon storage as PHA will survive, while those without will not survive. The stored PHA can be used as a carbon source for cellular replication leading to the abundance of high PHA microorganisms [118].

Even though feast-famine conditions have been applied to accumulate PHA in mixed cultures for several decades [119], few studies exist in terms of feast-famine conditions applied while using methane as the carbon source for PHA accumulation. However, a prominent study complete by Pieja et al. [120] reviewed three different cycling parameters to induce PHB accumulation in a mixed methanotrophic culture. The three cycles reviewed were nitrogen limitation, oxygen and nitrogen limitation, and methane and nitrogen limitation in three separate SBR systems. The SBR cycle durations were 24 hours and gas flow rates were altered to maintain the above conditions. As inferred from the available data, after 11 cycles of operation the PHB accumulation capacity was 12%, 10%, and 27% in the reactors effluents for nitrogen limitation, oxygen and nitrogen limitation, and methane and nitrogen limitation as the deficient parameters, respectively [120]. Similarly, Garcia-Perez et al. [121] demonstrated that implementing nitrogen starvation cycles (feast and famine) gradually increased PHB accumulation capacity up to 37%. Finally, Lopez et al. [122] describes the famine phase in a biofilter system as methane limiting and the feast phase as methane abundant.

Furthermore, methane and oxygen concentrations can impact PHB accumulation as a result of methanotrophic metabolism. When utilizing ammonium as the nitrogen source for methanotrophic growth, under famine-like conditions (low methane concentrations), ammonium oxidation will be stimulated producing hydroxylamine as an intermediate. This intermediate forces methanotrophs to utilize stored PHB for methane oxidation, thus leading to an increase in the observed PHB capacity. Similar to applying feast and famine phases, this unexpected result can be attributed towards the survival of methanotrophs with high PHB content, and the death of those with low PHB content. The surviving methanotrophs which contain high PHB percentage, can then be grown on nitrate to increase biomass density, as previously stated [110].

Overall, the nitrogen source and concentration, the phosphorus concentration, and methane and oxygen concentrations (feast and famine cycles) can impact PHB accumulation differently depending on the methanotrophic strain. Furthermore, the majority of PHB studies review Type II pure culture strains or Type II dominant cultures with little focus on methanotrophic mixed cultures. Finally, PHB accumulation involves bacterium enrichment under nutrient sufficient growth conditions utilizing ammonium, nitrate, or dinitrogen gas as the nitrogen source, before exposing the bacteria to nutrient deficient conditions.

CHAPTER 3: REDIRECTING METHANE FOR BIOLOGICAL NITROGEN REMOVAL USING THE ENRICHMENT OF A METHANOTROPHIC MIXED CULTURE IN A SEQUENTIAL BATCH REACTOR SYSTEM[1]

3.1 INTRODUCTION

Anthropogenic activities are affecting global atmospheric methane concentration, playing a role in global climate change [1]. From pre-industrial times, there has been an approximate 170% increase in atmospheric methane concentration in parts per billion [3]. Therefore, global changes need to be made to better utilize and mitigate the methane produced from anthropogenic activities to hamper climate change. Wastewater treatment plants (WWTPs) contribute 7% to GHG emissions with an anticipated 8% increase from the years 2020 to 2030 [8]. This projected emissions increase is largely attributed to greater energy consumption because of the growing population size and stringent regulations applied to WWTPs. As a result of the increased energy consumption, there has been a shift from WWTPs to water resource recovery facilities (WRRFs) [9]. For the aforementioned reasons, alternative, sustainable, and cost-effective treatment strategies are being introduced while still meeting the discharge standards applied. On the other hand, nutrients removal prior to discharge will help ease eutrophication caused by excess nutrients, particularly nitrogen and phosphorus. Eutrophication leads to the growth of blue-

[1] A modified version of this Chapter has been submitted to the *Chemical Engineering* Journal

green algae which causes oxygen depletion, fish toxicity, dead zones, and groundwater contamination [12].

The most common nitrogen removal process within WWTPs is conventional biological nitrogen removal (BNR) completed through the process of nitrification-denitrification. Nitrification-denitrification is the conversion of ammonium to dinitrogen gas under aerobic and anoxic conditions. Nitrification is completed by ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), in the presence of oxygen. Oxygen and alkalinity in the form of calcium carbonate are important requirements in maintaining stable nitrification rates. Denitrification is completed by heterotrophic denitrifying bacteria under anoxic conditions. A parameter of denitrification is the need for sufficient electron donors during nitrate and nitrite reduction to dinitrogen gas. Besides nitrification and denitrification for nitrogen removal from wastewater, shortcut biological nitrogen removal (SBNR) is growing in popularity because of its energy-saving and energy-neutral phenomena. SBNR is beneficial because of the 60% energy savings associated with the 25% reduction in oxygen consumption, the 40% reduction in external carbon requirements, the 20% reduction in GHG emissions, and the 35% and 55% reduction in sludge production when compared to nitrification and denitrification, respectively [35]. SBNR has been successful in sidestream treatment of reject water and/or wastewater containing low carbon to nitrogen ratios. However, making the shift from sidestream treatment to mainstream treatment has proved difficult because of instability in removal efficiencies when loading rates are adjusted [49]. It can be concluded that there is room for alternative removal strategies to consolidate the WRRFs concept.

In WWTPs, the biogas produced on site during anaerobic digestion (AD) of sludge is an unexploited resource that can be used to consolidate the WRRFs concept. It is composed of up to 75% methane, 25% carbon dioxide, and other trace elements [123]. Biogas production from AD has driven various biotechnologies including single cell-proteins, biodiesel, dietary and health supplements, biopolymers, energy generation, and carbon source replacements [5,18]. Interestingly, from the AD driven biogas, methane is a widely available resource which can be used as an alternative electron donor for BNR. In this concern, methanotrophic bacteria (methanotrophs) are of particular interest as they can couple nitrogen removal with methane mitigation, by utilizing methane as their sole carbon and energy source [29].

All methanotrophs can perform methane oxidization terminally to carbon dioxide to meet their energy and growth requirements [29]. The first step in the methane oxidation process involves the methane monooxygenase (MMO) enzyme to oxidize methane into methanol when oxygen is present. The MMO enzyme is found in both soluble (sMMO) and particulate (pMMO) form, where the sMMO is located in the cytoplasm and the pMMO is found in the intracytoplasmic membrane (ICM) [26]. The majority of the methanotrophic strains utilize inorganic nitrogen including ammonium and nitrate as their cellular nitrogen sources [58–60]. However, certain strains have the ability to fix atmospheric nitrogen for their nitrogen source [58,70,71]. It is noteworthy that the energy requirements of methanotrophs cannot solely be fulfilled by nitrogen, rather, methane oxidation is a necessity for cellular energy generation.

It is well understood that methanotrophs share several similarities with ammonium oxidizing bacteria (AOB). An important similarity is the capacity to co-oxidize other substrates

[78]. AOB have the ability to co-oxidize methane and methanotrophs can co-oxidize ammonium. The ability to co-oxidize other substrates gives methanotrophs the capacity to participate in BNR processes. The ammonium monooxygenase (AMO) enzyme of AOB and the MMO enzyme of methanotrophs are similar in reducing equivalents, inhibitors, and active sites [1]. When ammonium and methane are available to methanotrophs in the presence of oxygen, there is competition between them to be oxidized by the MMO enzyme. Assuming ammonium can outcompete methane for oxidation by the MMO enzyme the by-product of hydroxylamine is produced. Consequently, hydroxylamine is toxic to methanotrophs and is thereafter rapidly oxidized to nitrite by the hydroxylamine oxidoreductase [82]. Methanotrophs have developed coping strategies to deal with hydroxylamine toxicity. Two observed strategies within methanotrophs are the conversion of hydroxylamine back to ammonium by the hydroxylamine reductase enzyme [71,82] and further the oxidation of hydroxylamine to nitrite. In the latter copping strategy, nitrite is rapidly reduced to nitric oxide and nitrous oxide [4,74].

The second coping mechanism was demonstrated by Hoefman et al. [4] who found that when ammonium was used as the nitrogen source, 14 out of 16 methanotrophic strains reviewed could produce nitrous oxide but not nitrite. Similarly, Nyerges and Stein [74] reviewed four methanotrophic strains and observed that three out of the four could oxidize ammonium to nitrite. They also found that as the biomass concentration increased so did the hydroxylamine production [74]. This study speculates that different methanotrophic strains have different levels of detoxification capacity which was proven by the increase in nitrite production in one particular strain [74].

Interestingly, besides ammonium conversion to nitrite and/or nitrous oxide, it was reported that methanotrophs can oxidize ammonium to nitrate. He et al. [73] noticed that nitrate was produced under different ammonium concentrations while using the methanotrophic pure culture *Methylosinus sporium*. Nitrate accumulation was observed despite the fact that no activity of the nitrite oxidoreductase was detected. Hence, the metabolic pathway for nitrate production in methanotrophic bacteria has been illustrated but is still unknown [73]. Furthermore, nitrate accumulation was observed by a methanotrophic culture under three different ammonium concentrations, however, very little information is provided in terms of ammonium removal rates and nitrate concentrations [83].

Utilizing methane as an external carbon source for denitrification is characterised as anaerobic- (ANME-D) and (AME-D) aerobic- ammonium oxidation coupled to denitrification. Two hypotheses exist under AME-D. The first hypothesis is that methanotrophs produce soluble organics, under low dissolved oxygen (DO) concentrations, that are used by heterotrophic bacteria as electron donors [26,86]. This hypothesis is supported by the observation of soluble organic compounds including methanol [88], acetate [86], citrate [89], polysaccharides, and proteins [90] in AME-D studies. The second hypothesis is that methanotrophs themselves can convert nitrate and nitrite to nitrous oxide or dinitrogen gas under microaerobic or anoxic conditions [71]. Besides releasing by-products during methane oxidation, methanotrophs can contribute to denitrification by consuming oxygen creating microaerobic or anoxic conditions which favor denitrification rates [72,91]. A commonality observed during AME-D studies is that methane and nitrogen consumption rates tend to be higher under microaerobic conditions instead of the standard anoxic conditions for denitrification [94,95,124].

Overall, it can be denoted that methanotrophs use and react to different nitrogen sources, i.e. ammonium, nitrate, and nitrogen gas for either cellular growth by assimilation and/or detoxification of ammonium, hydroxylamine, and nitrite by nitrification-denitrification. The key parameter that may control such behaviour is the methane and oxygen concentrations. Methane and oxygen concentrations impact competition on the MMO enzyme and the dissolved oxygen (DO) concentration, respectively. Accordingly, this is the first known study that investigates the idea of redirecting methane for BNR in municipal WWTPs using a methanotrophic mixed culture enriched from activated sludge. Emphasis of this research is placed on exploring the nitrification and denitrification capacity in relationship to the applied methane and oxygen as the control parameters in achieving nitrogen removal. Initially, nitrification and denitrification capacity were evaluated separately under different methane and oxygen flow rates. From the obtained results, sequential batch reactor nitrogen removal phases were developed. The first two phases maintained sufficient methane and oxygen concentrations. In the latter two phases, methane and oxygen concentrations, hydraulic retention time, and nitrogen loading rate were altered.

3.2 MATERIALS AND METHODS

3.2.1 SEQUENTIAL BATCH REACTOR DESIGN AND OPERATION

The bench scale sequential batch reactor (SBR) is composed of a 5 L working volume glass reactor (Biostat® A, Sartorius Stedim Biotech GmbH, Germany) and is depicted in **Figure 3-1a** and **b**. Temperature, pH, mixing speed, dissolve oxygen saturation, and gaseous methane and oxygen flow rates were monitored and controlled through the Biostat® Unit A Control System. The SBR temperature was maintained at 27$\pm$2℃ through a heating jacket attached to a cooling device, while the pH was controlled at 6.8$\pm$0.3 by the addition of a 10% sodium hydroxide solution. Mixing speed was kept between 425 and 600 rpm by a mechanical stirrer. Pure gaseous methane and oxygen (Praxair, 99%) were supplied in various flow rates and sparged through the bottom of the SBR into the liquid. The dissolved oxygen (DO) saturation was monitored and adjusted using the Control Unit A based on the saturation setpoint or the oxygen flow rate by a dissolved oxygen electrode. The reactor was fed from a 50 L feeding tank by a peristaltic pump (Masterflex L/S Digital Pump System with Easy-Load II Pump Head, Germany). The effluent was discharged from the reactor into an 80 L holding tank during the decanting phase. Liquid samples were periodically collected from the middle of the reactor using Biostat® Pump A and refrigerated at 4℃ prior to analysis.

Different SBR cycles were operated based on the current experimental objective. The SBR cycles consisted of filling, aerobic or anoxic reaction, settling, and decanting phases. Decanting resulted in the removal 2.5 L of supernatant, replacing the removed liquid with 2.5 L of fresh mineral salts medium (MSM) from the 50 L feed tank unless specified otherwise. Extended solids retention time (SRT) was applied (> 30 days) as the aim was to maintain high biomass concentrations within the system. All of the SBR cycles for the phases of operation (Phase I to VI) are depicted in **Figure 3-2**. Phase I reviewed the ability of the methanotrophic culture to perform nitrification in batch mode. Phase II evaluated the denitrification capacity of the culture in batch mode. Finally, Phases III to VI reviewed the continuous nitrogen removal ability by the methanotrophic mixed culture. Phases III to VI were separated based on cycle duration, nitrogen loading rate (NLR), and cycle configuration. The NLR was adjusted based on the ammonium chloride (NH_4Cl) concentration in the MSM and the HRT for all phases. It is noteworthy that nitrate in the form of sodium nitrate ($NaNO_3$) was used as the sole nitrogen source while evaluating the denitrification capacity. Detailed operating conditions along with targeted outcomes per phase is displayed in **Table 3-1**.

Emphasis is on Phases V and VI which only varied in the NLR applied while maintaining the HRT and other cycle parameters. During Phases V and VI operation three different DO saturation setpoints were utilized to induce aerobic methanotrophic nitrogen removal. Meanwhile, gaseous methane was continuously supplied at a flow rate of 10 $mlCH_4/min$ to maintain the methanotrophic culture.

a)

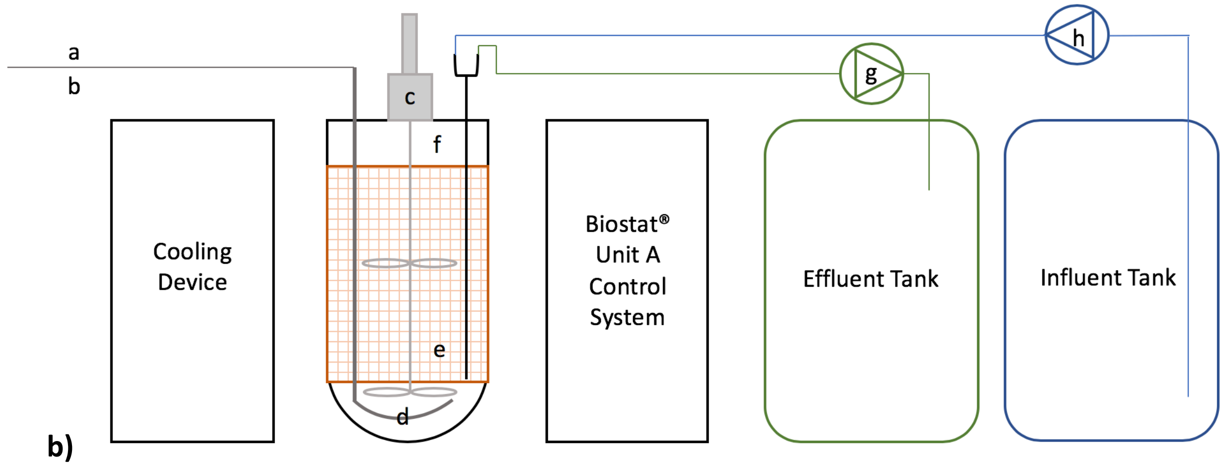

Figure 3-1: a) Actual SBR system used during the experiment. b) Schematic diagram of the SBR system, where a: gaseous methane piping from cylinder; b: gaseous oxygen piping from cylinder; c: mechanical mixer; d: diffuser; e: cooling/heating jacket; f: 5L glass reactor tank; g: effluent pump; and h: influent pump.

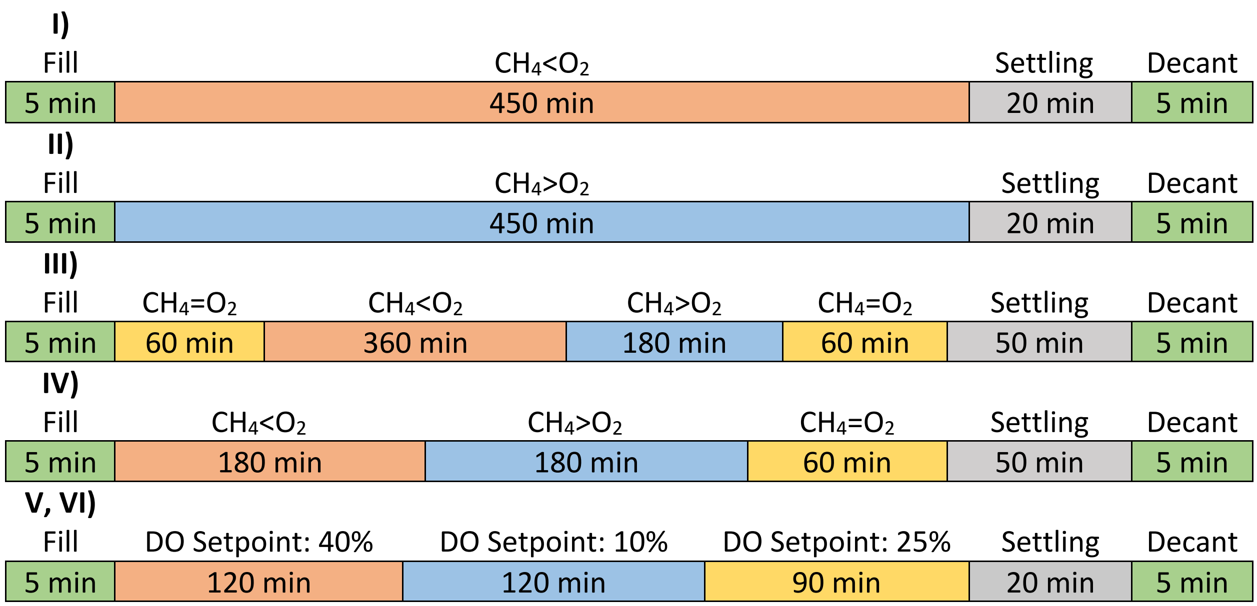

Figure 3-2: All SBR cycle times for operation of phases to achieve the experimental outcomes. Phases V and VI operated on the same cycle time under different nitrogen loading rates.

Table 3-1: Detailed operating conditions applied to the SBR during Phases I to VI of the experiment. Nitrogen loading rate is based on the ammonium-nitrogen concentration. Where N-removal implies methanotrophic-based nitrogen removal.

Phase	HRT (hr)	Target Outcome	NLR (kgN/m^3.d)	Nitrogen[a] (mgNH$_4$-N/L)
I	16	Nitrification	0.15$\pm$0.01	100$\pm$4.0
II	16	Denitrification	0.12	80.0[b]
III	24	N- Removal	0.09$\pm$0.04	92.6$\pm$37.0
IV	16	N- Removal	0.14$\pm$0.04	91.3$\pm$26.0
V	12	Nitrification	0.216$\pm$0.30	108$\pm$12.2
VI	12	Nitrification	0.103$\pm$0.01	51.3$\pm$5.6

[a]based on influent ammonium concentration from feed solution
[b]based on nitrate concentration in MSM

3.2.2 CULTIVATION CONDITIONS

Waste activated sludge (WAS) collected from Humber Wastewater Treatment Plant (Toronto, Canada) was used as seed for the enrichment of the methanotrophic mixed culture. Seed was cultivated in fed-batch mode with the following conditions: HRT (24 hours), temperature (27$\pm$2°C), pH (6.8$\pm$0.3), and methane to oxygen flow ratio (1:1). The following chemical concentrations were used as feed in a mineral salts medium (MSM) [58] (mg/L): 500 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 270 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA, 1 ml of trace metals, and 5 ml of copper stock solution. The trace metal solution contained the following chemical concentrations (mg/L): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.H_2O$, 30 H_3BO_3, 3 $NaMoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$, and 20 $CoCl_2.2H_2O$. The copper stock solution had a concentration of 1000 mg/L of $CuSO_4.5H_2O$. Ammonium chloride (NH_4Cl) was used as the nitrogen source and was added directly to the MSM unless specified otherwise. It is noteworthy that nitrate in the form of sodium nitrate ($NaNO_3$) was used as the nitrogen source while evaluating the denitrification capacity in the SBR (Phase II).

3.2.3 DENITRIFICATION BATCH OPERATING CONDITIONS

Six batch methanotrophic denitrification activity tests were performed at varying incubation times and gaseous methane to oxygen ratios in the headspace as detailed in **Table 3-2**. Methanotrophic bacteria was collected directly from the SBR reactor during Phase V after the DO saturation setpoint of 40% (2 hours after the cycle time started). Experiments were run in duplicates of 25 ml of liquid volume in 125 ml serum bottles capped with butyl rubber stoppers. Gaseous methane, oxygen, and helium (Praxair, 99%) were applied to the bottle headspace with a gas tight syringe. Bottles were placed in an orbital shaker at 180 rpm and the temperature was maintained at $27 \pm 2°C$ to mimic the SBR operating conditions. Headspace samples were withdrawn from the serum bottles at the beginning and end of each of the incubation times. Liquid samples were collected prior to starting each batch experiment and at the end of the incubation times for analysis. The feed solution collected from the SBR and used during the batch denitrification experiment was rich in nitrate and depleted in ammonium, comparable to nitrate mineral salts (NMS). Separate anoxic (0% O_2), microaerobic (10% O_2), and aerobic (50% O_2) conditions were applied to the headspace of the serum bottles based on the batch phase. Anoxic conditions were not evaluated for the 18, 24, and 48 hours incubation periods.

Table 3-2: Detailed operating conditions for the six batch methanotrophic denitrification activity tests. Influent nitrate concentration was taken directly from the SBR. 100ml of helium was applied to the headspace of each batch experiment.

Batch	Incubation Period (hr)	Influent Nitrate ($mgNO_3$-N/L)	Methane Applied (ml)	Oxygen Applied (ml)	Headspace O_2 (%)
I	2	117±14	100 90 50	0 10 50	0 10 50
II	4	117±14	100 90 50	0 10 50	0 10 50
III	6	117±14	100 90 50	0 10 50	0 10 50
IV	18	116	90 50	10 50	10 50
V	24	113±2	90 50	10 50	10 50
VI	48	113±2	90 50	10 50	10 50

3.2.4 ANALYTICAL METHODS

Liquid samples from the SBR and batch experiments were analysed for ammonium-nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), and nitrite-nitrogen (NO_2-N) using ion chromatography (Dionex™ Integrion™ HPIC™ System, Thermo Fisher Scientific, Sunnyvale, California). Volatile suspended solids (VSS) and total suspended solids (TSS) were measured according to standard methods [125,126]. Sludge volume index (SVI_{30}) tests were performed according to Metcalf and Eddy (2014) [12] with a 1 L settleometer. Gas samples were withdrawn from the reactor headspace, periodically or once every hour, using a gas tight syringe and injected into the gas chromatography (GC) equipment (SRI 8610C, SRI Instrumentation, Torrance, USA) to measure methane, oxygen, and nitrogen gas concentrations. A preconstructed calibration curve was utilized for the conversion of peak areas into gas concentrations. The GC used was equipped with a thermal conductivity detector (TCD) and molecular sieve column (Restek, Bellefonte, PA). Helium gas (Praxair, 99%) was used as the carrier gas (15 ml/min) for the injected samples. Temperature for injections were kept constant at the following: injector 60℃, oven 80℃, and TCD 80℃.

3.3 RESULTS AND DISCUSSIONS

3.3.1 NITRIFYING AND DENITRIFYING METHANOTROPHIC CAPACITY

3.3.1.1 METHANOTROPHIC NITRIFICATION CAPACITY

Methanotrophic bacteria and AOB share similarities giving them the ability to co-oxidize other substrates [30,78]. As a result, methanotrophic-based nitrification is possible because of similarities associated with the pMMO and AMO enzyme [1]. When ammonium and methane are both available to methanotrophs there is competition to be oxidized by the pMMO enzyme. To supress ammonium oxidation by the pMMO enzyme, methane can be supplied in excess allowing it to outcompete ammonium for oxidation. Therefore, to induce methanotrophic nitrification, methane flow rate was lowered to allow ammonium to outcompete methane for oxidation while maintaining sufficient oxygen concentration as a methanotrophic requirement. Methanotrophic nitrification capacity was evaluated over three consecutive days, which consisted of applying three different methane to oxygen flow rates. The different methane and oxygen flow rates and other experimental outcomes found while evaluating the nitrification capacity are summarized in **Table 3-3**. The SBR was operated in batch mode while evaluating the nitrification capacity.

After the 8-hours cycle duration the SBR was supplied with methane and oxygen in equal flow rates and fresh AMS to restore the culture activity.

Table 3-3: Experimental outcomes from the methanotrophic mixed culture nitrification and denitrification capacity from the batch tests in the SBR.

Parameter	Phase I			Phase II	
	Day 1	Day 2	Day 3	Day 1	Day 2
Cycle Duration (hr)	8	8	8	8	8
Influent NH_4-N ($mgNH_4$-N/L)	100	104	96	-	-
Influent NO_3-N ($mgNO_3$-N/L)	-	-	-	80	80
Effluent NH_4-N ($mgNH_4$-N/L)	0.8	23	20	-	-
Effluent NO_3-N ($mgNO_3$-N/L)	2	26	35	0.1	0.3
Methane Flow Rate ($mlCH_4$/min)	50	15	10	100	50
Oxygen Flow Rate (mlO_2/min)	100	50	25	50	0
Removal Efficiency (%)	99.2	77.9	79.2	99.9	99.6
Nitrogen Removal Rate (gN/L.d)	0.298	0.243	0.228	0.240	0.239
Ammonium Conversion to Nitrate (%)	0	25	36	-	-
Nitrogen Gas Production (%)	-	-	-	5	10

When evaluating the methanotrophic nitrification capacity, the application of the methane flow rate at 50 $mlCH_4$/min led to complete ammonium removal after 4 hours as demonstrated in **Figure 3-3**. However, ammonium was not removed by nitrification, instead, it was incorporated for cellular growth and synthesis. The inference of ammonium assimilation is emphasized by the absences or trace concentrations of nitrate present during the cycle. Ammonium assimilation can be attributed to the suppression of ammonium oxidation because methane was supplied in excess. Hence, the methane flow rate was reduced for the subsequent days as methanotrophic nitrification was not observed at a methane flow rate of 50 $mlCH_4$/min. The reduction in the methane flow rate to 15 $mlCH_4$/min led to the presences of nitrate during the cycle indicating methanotrophic induced nitrification. Ammonium conversion to nitrate was highest when the methane flow rate was further reduced to 10 $mlCH_4$/min reaching a value of 36% compared to a methane flow rate of 15 $mlCH_4$/min achieving an ammonium conversion value of 25%. The unaccounted-for nitrogen can be assumed to be either reduced to gaseous nitrogen or utilizing for cellular synthesis and maintenance. The findings from the nitrification batch experiment confirms the impact of methane flow rate on methanotrophic-based nitrification.

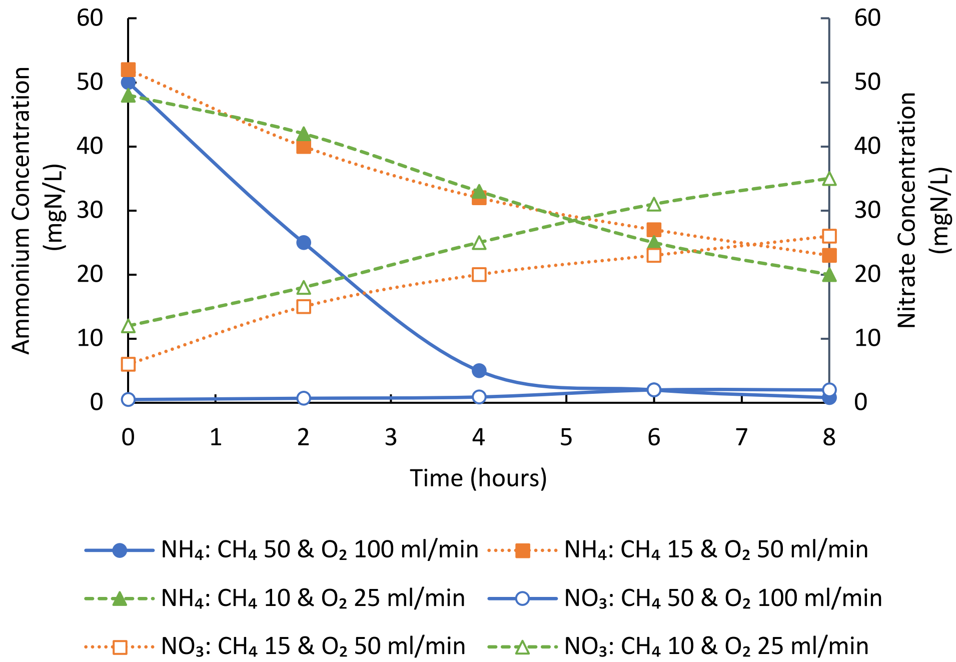

Figure 3-3: Methanotrophic nitrification activity results observed during batch mode.

Unlike this study, He et al. [73] studied the effect of ammonium conversion on methane oxidation by changing the ammonium concentrations in the AMS. The results observed by He et al. [73] are in contradiction with the attained results in the present study. Interestingly, the greatest ammonium conversion to nitrate was at the lowest ammonium concentration of 180 $mgNH_4$-N/L [73]. This finding may imply that ammonium was able to outcompete methane despite of its low concentration. Nonetheless, in the study completed by He et al. [73], significantly high ammonium concentrations were used which led to the complete inhibition of methanotrophic activity because of ammonium toxicity. This statement is supported by the observed reduction in biomass growth during the study at ammonium concentrations greater than 180 $mgNH_4$-N/L [73].

Nitrite was not observed under the three methane flow rates evaluated. The absence of nitrite in the system is contrary to a study completed by Nyerges and Stein [74]. Nyerges and Stein [74] found that three out of the four methanotrophic strains reviewed could produce nitrite from ammonium oxidation as a detoxification strategy to hydroxylamine. In contrary, Hoefman et al. [4] performed a similar study evaluating 16 methanotrophic strains and found that 14 methanotrophic strains produced nitrous oxide but not nitrite when ammonium was used as the nitrogen source. Hoefman et al. [4] speculates that the lack of nitrite was the result of the rapid conversion of hydroxylamine to nitric oxide and nitrous oxide or because peak levels were too low for detection [4].

Interestingly, nitrate was observed with increasing ammonium concentrations in negative correlation to the methane flow rate applied. Similar speculations to Hoefman et al. [4] can be made for the present study hypothesizing that nitrite was rapidly converted into nitrate not nitrous gases. It can be assumed that the sufficient oxygen conditions hampered the denitrifying pathways of the mixed culture. As a result, nitrite was converted to nitrate in absences of the conversion of nitrate to nitrogen gas. However, the microorganism responsible for the nitrite to nitrate conversion activity is unknown. Further investigations are still needed to confirm such speculations.

3.3.1.2 METHANOTROPHIC DENITRIFICATION CAPACITY

Similar to nitrification capacity, the denitrification capacity was evaluated in batch mode. In the case of denitrification capacity, oxygen is the critical parameter to controlling the DO concentration to maintain either anoxic or microaerobic conditions. As previously mentioned, nitrate in the form of sodium nitrate was used as the nitrogen source and added directly to the MSM during Phase II. The two AME-D hypotheses that exists are that methanotrophs secrete by-products during methane oxidation which are used as electron donors by denitrifying bacteria and that methanotrophs themselves directly convert nitrate or nitrite to nitrous oxide and dinitrogen gas. However, the only known methanotrophic strain capable of producing dinitrogen gas under anoxic conditions is *Methylocystis* sp. [71]. Furthermore, the first hypothesis stated is well studied as a means to replace methanol with methane as the external carbon source for denitrification [1,26,127]. Therefore, to induce AME-D anoxic and microaerobic conditions were applied to encourage methane oxidation by methanotrophs and/or nitrate reduction. The different oxygen flow rates and other experimental outcomes found while evaluating the denitrification capacity are summarized in **Table 3-3**.

During Phase II, nitrate removal rates achieved under microaerobic and anoxic conditions were 0.240 and 0.239 gNO_3-N/L.d, respectively. The results obtained appear in contradiction to Cao et al. [94] and Costa et al. [85] whom illustrated that microaerobic conditions resulted in higher specific denitrification rates (SDNR) and denitrification efficiency when compared to anoxic conditions. Interestingly, this finding was not observed while completing this study as microaerobic and anoxic conditions produced similar nitrate removal rates. Furthermore, Lee et al. [95] observed a SDNR increase from 0.82±0.05 to 2.54±0.09 mgN/gVSS.hr from a 6 to 12 hours HRT, respectively. The large difference in SDNR was attributed to the lower dissolved oxygen concentration (55% lower) applied during the 12-hours HRT.

The most noticeable difference between the oxygen flow rates of 50 and 0 mlO_2/min was the dinitrogen gas production as shown by the greater percentage produced in **Figure 3-4**. Anoxic conditions, or an oxygen flow rate of 0 mlO_2/min, resulted in greater dinitrogen gas production suggesting greater denitrification activity. The higher denitrification activity under anoxic conditions is contrary to other AME-D studies where microaerobic conditions proved better denitrification results [85,94]. The contradicting result is likely the product of the greater oxygen flow rate applied to the SBR on Day 1 (50 mlO_2/min) in comparison to Day 2 (0 mlO_2/min). Therefore, it can be concluded that on Day 1 the methanotrophic culture was unable to consume oxygen quick enough to create microaerobic conditions for denitrifiers, thus slowing down the denitrification rate [12,72,91].

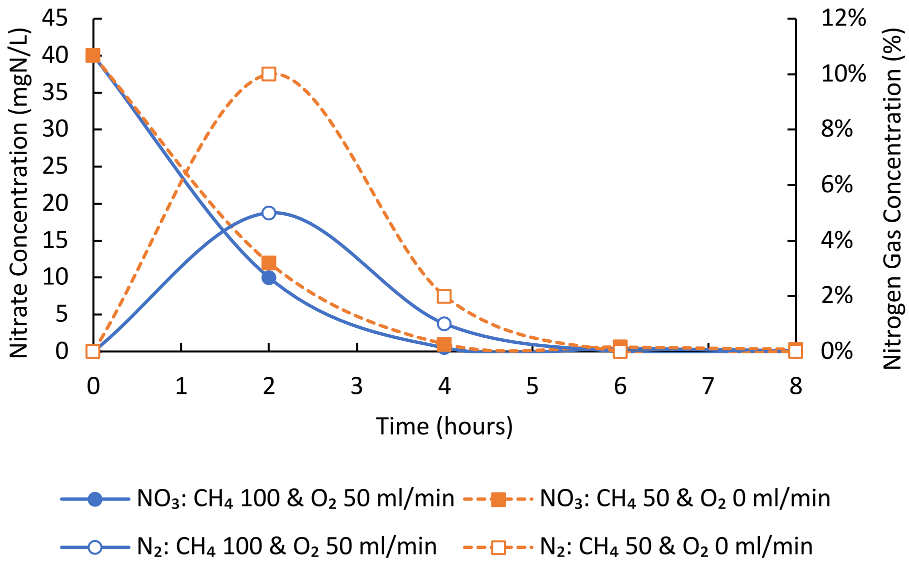

Figure 3-4: Methanotrophic denitrification activity results observed during batch mode.

3.3.2 CONTINUOUS METHANOTROPHIC NITROGEN REMOVAL

Based on the aforementioned nitrification and denitrification batch SBR experiments, a complete methanotrophic driven nitrogen removal cycle was developed using AMS as the feed solution. The original objective was to induce methanotrophic-based nitrification-denitrification instead of assimilation. The initial results obtained during Phase III, as shown in **Figure 3-5**, were promising as ammonium conversion to nitrate was 96% during the nitrification period. Additionally, during the denitrification period there was nitrate reduction to gaseous nitrogen. Unfortunately, these results rapidly deteriorated as the culture shifted towards ammonium consumption for cellular growth and synthesis. This result was further confirmed by the poor nitrate concentration observed over the cycle duration and by the inconsistent gaseous nitrogen production. Ammonium removal by assimilation is further confirmed by the small incremental changes in total nitrogen (TN) concentration in the liquid and by the increase in biomass concentration over the duration of one cycle.

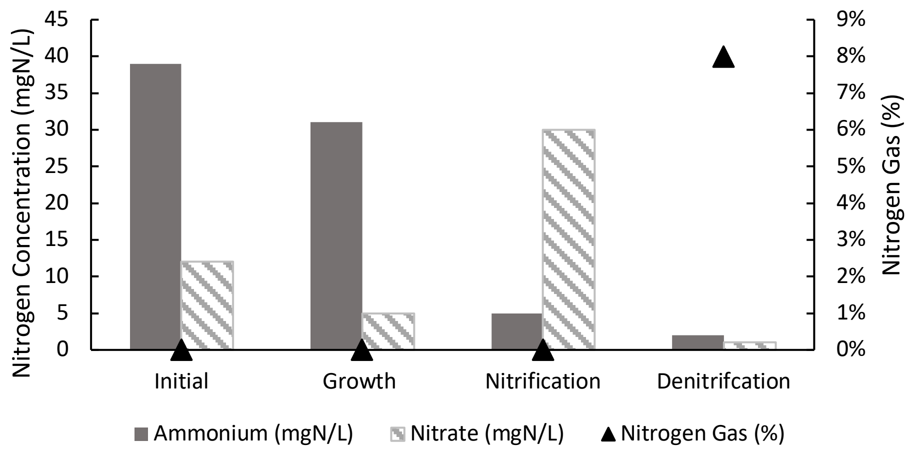

Figure 3-5: Initial nitrogen removal results observed by the methanotrophic culture during Phase III evaluation. Initial ammonium and nitrate concentration are based on values obtained in the reactor 10 minutes after the fill period.

Because of the poor nitrification-denitrification performance during Phase III, the cycle duration was reduced to 8 hours in order to increase the NLR. The aim of increasing the NLR by reducing the HRT was to promote ammonium oxidation by outcompeting methane for the MMO enzyme. Unfortunately, nitrification-denitrification was still not induced using this method. The ammonium removal trend for Phase III and IV can be seen in **Figure 3-6**. Ammonium was consumed for cellular growth and synthesis similar to Day 1 of the results obtained during the nitrification evaluation (Phase I). There was high biomass production within the SBR system because of ammonium removal by assimilation. The ammonium removal efficiencies (ARE) were still 97$\pm$2 and 98$\pm$1% for Phase III and Phase IV, respectively. Ammonium removal by assimilation in the SBR is similar to results observed by Kim et al. [84]. It was found that ammonium was removed by assimilation or oxidation to hydroxylamine and nitrite in reject water [84]. Because nitrite and nitrate were not found in high concentration during Phases III and IV, it is speculated that ammonium assimilation was the primary contributing factor to the high removal efficiencies.

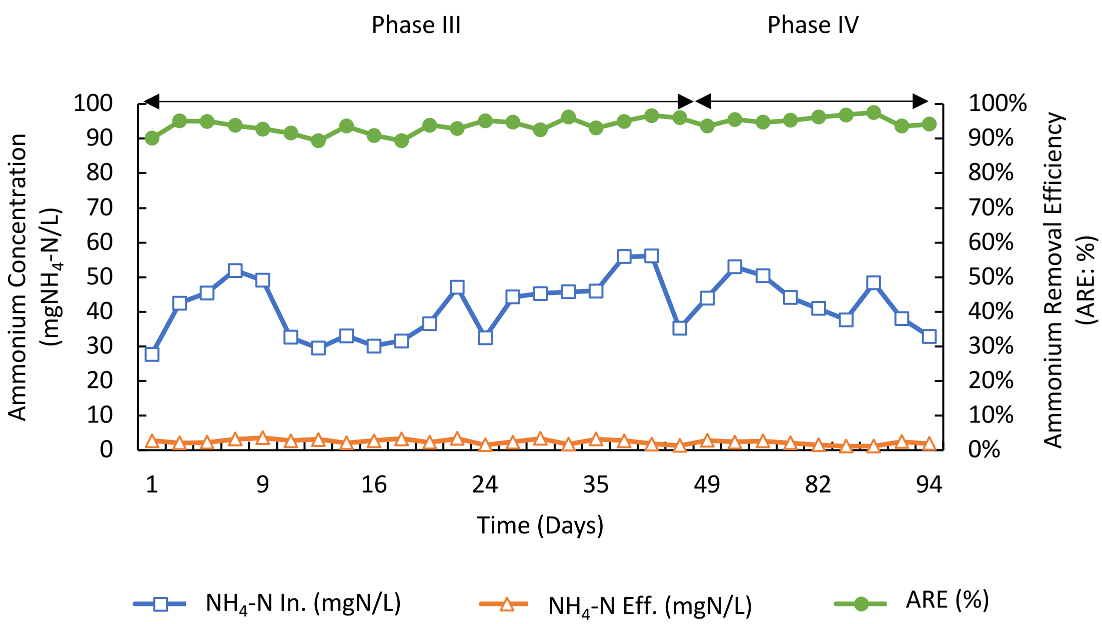

Figure 3-6: The ammonium removal trend observed by assimilation over the 12- and 8-hour cycle periods, where, ARE (%) is ammonium removal efficiency, NH_4-N In. (mg/L) is ammonium concentration observed in the reactor 10 minutes after the fill period.

Even though ammonium was removed by assimilation, removal efficiencies are comparable to the conventional nitrification processes of around 95% [12]. During Phase III, nitrate concentration gradually reduces on average from 3.4 to 1.6 mgNO_3-N/L in the effluent when supplied with methane as the carbon source. Furthermore, the study by Kim et al. [84] validated the concept of nitrogen removal from reject water using a methane- and methanol- dependent culture in a SBR. This study mentions that there was only a minor difference in the nitrate concentration from 5.2 to 3.2 mgNO_3-N/L when supplied with biogas as the carbon source [84]. Similarly, this was observed for the nitrate concentration when evaluating continuous methanotrophic nitrogen removal under Phase III.

Figure 3-7 shows the SBR headspace composition by percentage over the duration of one 8-hour cycle period. The average nitrogen gas concentration by percentage over the duration of one cycle in the SBR headspace was 2.9±0.23%. Throughout the cycle the oxygen concentration by percentage in the headspace was above 40%. DO concentration above 1 mgO_2/L will halt denitrification rates [12]. Therefore, nitrogen gas production is likely low because aerobic conditions were maintained throughout the cycle duration. Besides the high oxygen concentration, there was also a significant amount of methane by percentage observed in the headspace. Because methane concentration by percentage was continuously elevated, methane was able to outcompete ammonium for oxidation explaining why ammonium was assimilated instead of oxidized.

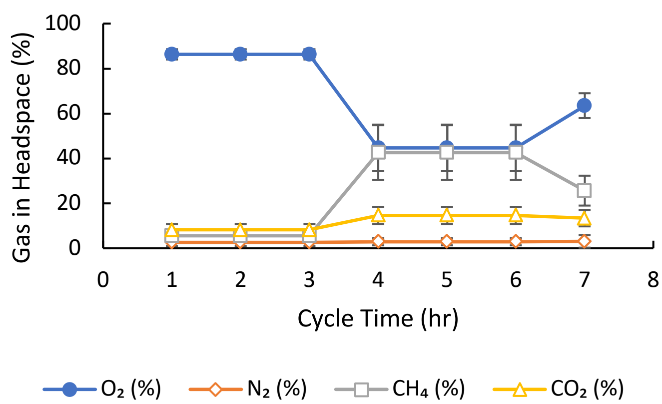

Figure 3-7: Average headspace concentration and standard deviation observed while operating at specific methane and oxygen flow rates and an 8-hour cycle time (headspace samples were only taken at 3, 6, and 7 hours during the 8- hour cycle, extra points were included to emphasize the trend and specific cycle).

An additional benefit of the methanotrophic sludge is the good settling ability. An SVI_{30} value of 53 ml/g was calculated based on total solids (TS) within the SBR. The calculated SVI_{30} value is well below 100 ml/g which is the value considered for good settling sludge [12]. Because of the good settling capacity of the methanotrophic sludge, settling time in the SBR was shortened to 20 minutes for the following two Phases. Similar results were obtained by Kim et al. [84] when comparing the settling capacity of methanotrophic sludge to activated sludge (AS). Complete sedimentation of the methanotrophic sludge was observed in five minutes with a settling retention time of 10 minutes compared to a settling time of 50 minutes for AS [84].

The attained results from Phases I and II and the first week of Phase III imply that nitrification-denitrification can be achieved by a methanotrophic mixed culture as summarized in **Table 3-4**. However, the conditions applied in Phase III and IV did not induce the same nitrification-denitrification behaviour. The strategy followed in Phases III and IV was to alter the methane to oxygen ratio while still maintaining sufficient methane concentrations. Such a strategy did not evaluate the high ratio between methane and ammonium which is a decisive factor for methanotrophic nitrification. The aim of the applied strategy was to maintain more favorable conditions for the methanotrophic mixed culture by providing a sufficient energy and cellular carbon source. In addition to the poor nitrification activity, it was observed that despite the high nitrogen removal maintained (up to 99%), the ratio between the methane applied and the amount of nitrogen removed was impractical. From an alternative perspective, if the SBR process were optimized, and given that methane is considered as a cheap carbon feedstock, the process offers the potential application to capture/recover nitrogen found in the cells instead of removing it and converting it to the gaseous forms. Nitrogen capture and recovery is supported by the high growth rates of methanotrophic bacteria compared to regular heterotrophs [14].

Table 3-4: Summary of results while completing Phases III to VI of the methanotrophic-based nitrogen removal in the SBR.

Phase	III	IV	V	VI
Cycle Duration (hr)	12	8	6	6
Influent NH_4-N (mgNH_4-N/L)	92.6±37	91.3±26	108±12.2	51.3±5.6
Effluent NH_4-N (mgNH_4-N/L)	2.5±1.03	2.0±0.8	12.7±5.4	5.3±1.9
Effluent NO_3-N (mgNO_3-N/L)	1.6±0.8	0.0	96.9±12.8	48.9±6.0
Ammonium Removal Rate (mgNH_4-N/L.hr)	7.5±3.15	11.2±3.23	16.2±2.13	7.0±2.21
Ammonium Removal Efficiency (%)	97±2	98±1	89±5	87±7
Ammonium Conversion to Nitrate (%)	NA	NA	87±11	87±7
Methane Applied (L/d)	73.2	54	14.4	14.4

3.3.3 LOW METHANE AND OXYGEN APPLICATION FOR CONTINUOUS METHANOTROPHIC NITROGEN REMOVAL

Based on the results from Phases III and IV, a reduced 6-hour cycle was developed to increase the NLR while lessen the competition between ammonium and methane for oxidation. Besides lessening the competition with ammonium, the goal of the two new Phases was to avoid excess methane supplied, hence, the low continuous methane flow rate. Therefore, reducing the methane flow rate while maintaining a higher NLR would hypothesize to induce methanotrophic-based nitrification. Furthermore, lowering the methane to nitrogen ratio should create a more sustainable and cost competitive process.

During the first five days of operation in Phase V, nitrite was present in the effluent before tapering to zero mgNO_2-N/L. The result of nitrite reaching zero mgNO_2-N/L is similar to an observation made by Cao et al. [94]. Cao et al [94] found that nitrite accumulated in a microaerobic reactor during the first five cycles (0.12 to 4.87 mg NO_3-N/L). However, as the process continued there was a gradual decrease to zero mgNO_2-N/L in the reactor [94]. On the other hand, after the first five days of this study, the ammonium conversion to nitrate gradually increased as the process continued. The tapering of nitrite concentration and the greater conversion of ammonium to nitrate is illustrated in **Figure 3-8** by the higher nitrate concentration observed in the effluent with time. Nitrate accumulation may be the result of the DO concentration applied of 3.2 mgO_2/L. Higher DO concentration is suitable for nitrification but significantly slows down denitrification rates. The results obtained are promising as the average ARE was 89±5%, the average ammonium conversion to nitrate was 87±11%, and the average nitrate concentration in the effluent was 96.9±12.8 $mgNO_3$-N/L. A pH drop was observed over the duration of one cycle. Similar results were observed by He et al. [73] who noticed a pH decrease when a methanotrophic culture was solely grown on ammonium as the nitrogen source.

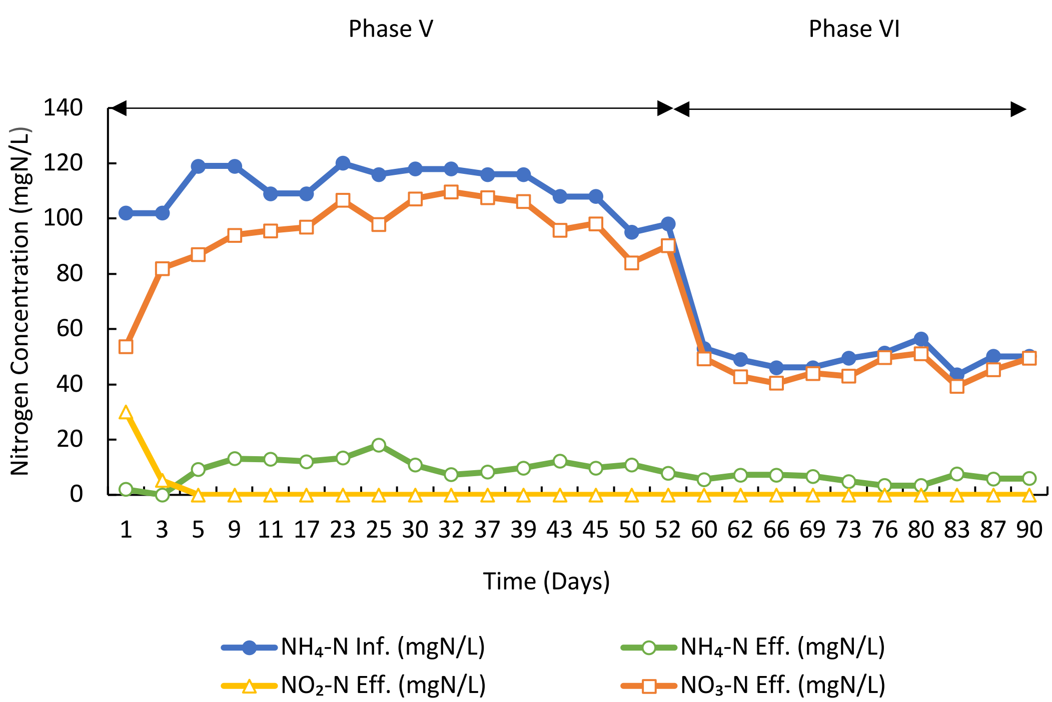

Figure 3-8: SBR mixed methanotrophic nitrogen removal results observed during Phase V and VI separated by the black line arrows at the top. Influent feed ammonium concentration was utilized in the figure.

Ammonium oxidation can inhibit methanotrophic growth by outcompeting methane for oxidation by the pMMO and through the production of toxic intermediates [18,81]. These two statements explain the constant biomass concentration observed within the reactor which is demonstrated in **Figure 3-9**. Over one cycle, the TSS and VSS concentrations were 3261$\pm$55 mg/L and 2737$\pm$29 mg/L, respectively. These values are similar to those found in activated sludge systems ranging from 2500 to 4000 mg/L based on TSS [12]. Finally, after a 20-minute settling time the TSS and VSS in the effluent were approximately 125 and 80 mg/L, respectively. The VSS/TSS ratio throughout the cycle duration ranged between 83% and 85%, which falls within the common range of 80 and 90% [12]. The SVI_{30} of the methanotrophic sludge during this phase was 66 ml/g based on TSS concentration within the system. This SVI_{30} value is similar to the value observed by Lee et al. [95] who illustrating a rapid settling of methanotrophic sludge as a result of floc size. Further, an SVI_{30} value calculated by Kim et al. [84] was less than 75 ml/g. The comparable SVI_{30} value emphasizes the good settling capacity of methanotrophic sludge allowing for shorter settling periods.

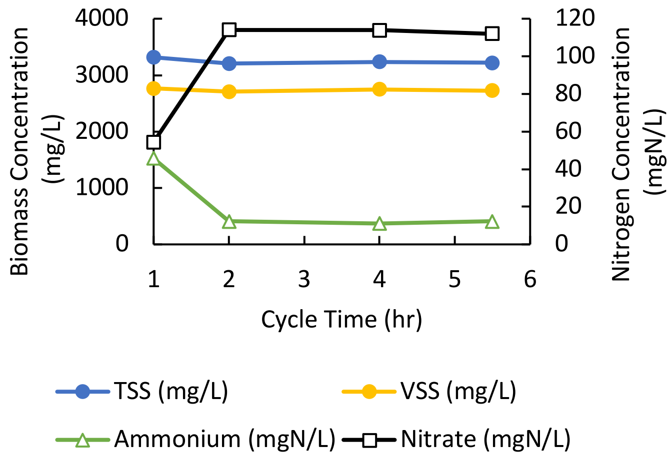

Figure 3-9: Results from one 6- hour cycle in Phase V, with focus on the TSS, VSS, ammonium, and nitrate concentrations.

During Phase VI the influent ammonium concentration was reduced to 51.3±5.6 mgNH_4-N/L to more align with mainstream operating conditions. However, ammonium concentration of mainstream conditions can range anywhere from 12 to 50 mgNH_4-N/L depending on the current environmental factors [12]. Similar results to Phase V were observed during Phase VI operation as shown in **Figure 3-8**. During Phase VI operation, all operating parameters remained the same with the exception of the influent ammonium concentration. Nitrite was not detected in the effluent during Phase VI operation as the same DO concentration was maintained. The ARE achieved was 87±7%, which is only 2% lower than Phase V. The average ammonium conversion to nitrate was 87±7% and the nitrate accumulation observed in the effluent was 48.9±6.0 mgNO_3-N/L. Similar TSS, VSS, ammonium, and nitrate concentrations trends to Phase V were observed over the duration of one cycle. The TSS and VSS biomass concentrations displayed in **Figure 3-10** are 1742±48 mg/L and 1514±28 mg/L, respectively. Consistent nitrate production, along with constant TSS and VSS concentration over the duration of one cycle clearly demonstrate ammonium removal by nitrification instead of assimilation. Lower influent ammonium concentration led to the reduction of TSS and VSS biomass concentration by approximately 60% in comparison to Phase V. Furthermore, VSS/TSS ratio was 85 to 86%, which is slightly higher than the Phase V ratio of 83 to 85%.

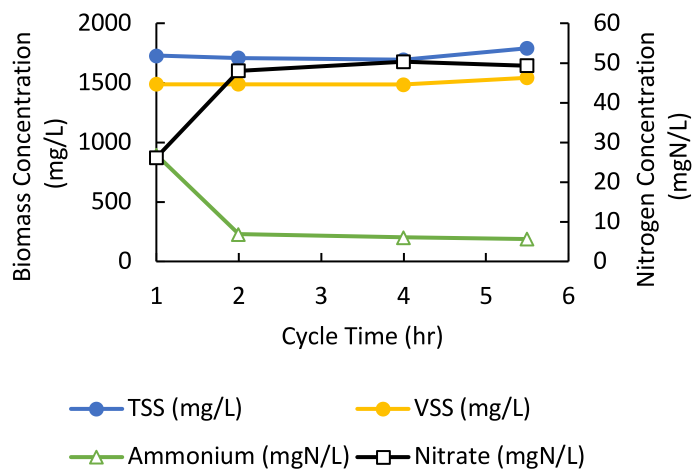

Figure 3-10: Results from one 6- hour cycle in Phase VI, with focus on the TSS, VSS, ammonium, and nitrate concentrations.

Headspace gas concentrations during Phases V and VI follow similar trends to one another. Similar trends are observed because all operational parameters were kept the same with the exception of the influent ammonium concentration. The SBR headspace gas concentrations by percentage for Phases V and VI are shown in **Figure 3-11a** and **3-11b**, respectively. The observed headspace concentrations differ from Phase IV in that oxygen concentration by percentage was always greater than methane concentration by percentage. Besides oxygen concentration, methane concentration by percentage was never greater than 27% during Phases V and VI compared to Phase III where methane concentration reached 43%. When comparing the different SBR headspace concentrations it was assumed that ammonium was able to outcompete methane for oxidation by the pMMO because the methane concentration was lower during Phases V and VI. From this, it can be concluded that methane and oxygen concentrations can drive ammonium removal by assimilation or oxidation.

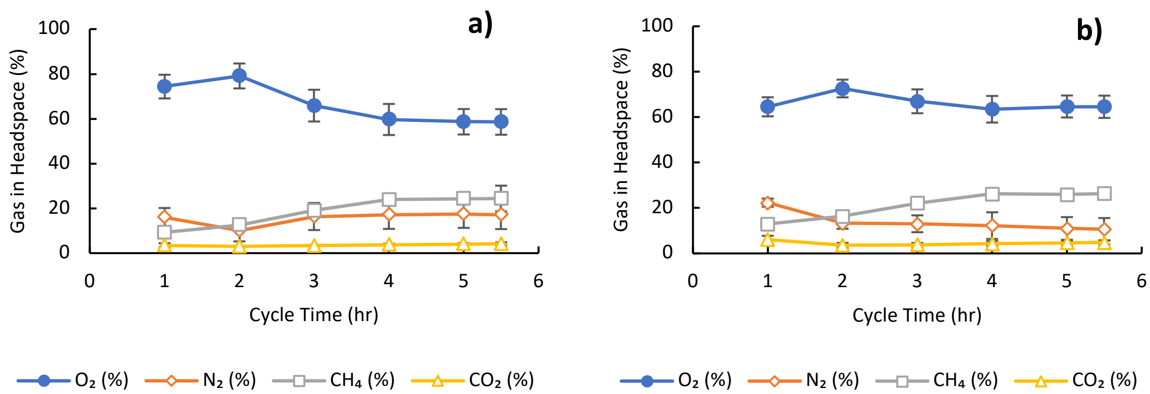

Figure 3-11: a) Average headspace concentration and standard deviation observed while operating under varying DO setpoints and a 6 hour cycle duration, Phase V. b) Average headspace concentration and standard deviation observed while operating under varying DO setpoints and a 6 hour cycle duration, Phase VI.

As shown in **Figure 3-2**, the strategy applied to Phases V and VI was to maintain a constant low methane flow rate while altering the DO setpoint from 40% in first 2 hours to 10% in the subsequent 2 hours, and finally to 25% for the remaining 1.5 hours. The aim of this strategy was to induce nitrification activity followed by denitrification activity of the mixed culture. It is evident, as shown in **Figures 3-9** and **3-10**, that nitrification activity was successfully induced with ARE of 89±5% and 87±7% for Phases V and VI, respectively. It is reported that methanotrophs possess the genes responsible for ammonium oxidation to hydroxylamine and nitrite [30]. However, the nitrate production pathway is unclear and nitrite oxidoreductase expression has not been reported in the literature. Therefore, this study is the first to demonstrate the occurrence of nitrification using a methanotrophic mixed culture. Indeed, further investigation into the microbial structure and metabolic flux are still required.

During Phases V and VI pure gaseous methane applied was significantly reduced to 14.4 L/d compared to Phases III and IV at 73.2 and 54 L/d, respectively. Reducing the methane applied positively impacted the feasibility of the process assuming 40 to 60 L/d of biogas production from lab scale anaerobic treatment [24]. However, biogas production can change significantly upon process scale up and the initial carbon to nitrogen ratio applied to the anaerobic treatment process. The mainstream ammonium concentration applied under Phase VI demonstrates the versatility of the methanotrophic bacteria under different NLR. Furthermore, high ammonium removal rates were achieved under the NLR of 0.216 ± 0.3 and 0.103 ± 0.01 kgN/m^3.d. The nitrogen removal results and applicable findings for Phases III to VI are summarized in **Table 3-4**. Besides the initial promising results, further optimization of the process in terms of methane application rate and DO concentration are required to improve the process feasibility.

On the other hand, no denitrification activity was observed during Phases V and VI despite the low oxygen conditions maintained. Interestingly, it has been widely reported that nitrate is the favorable nitrogen source for methanotrophic growth [67,72]. In accordance, nitrate observed in the SBR should have at least been partially consumed by the methanotrophic culture for cellular growth. However, as shown in **Figure 3-9** and **3-10**, nitrate concentration was maintained throughout the cycle even when the DO setpoint was increased to 25% after 4 hours. To further understand the reason for such observation, two sets of batch tests were performed. The obtained results are discussed in the following section (**Section 3.4**).

Finally, throughout all the SBR experiments evaluated, it was assumed that the microbial culture was dominated by methanotrophic bacteria. This assumption is valid as methane was supplied as the sole carbon source creating unfavorable conditions for AOB and NOB growth. Furthermore, the methanotrophic dominant culture was confirmed when completing a batch experiment in 125 ml serum bottles. The methanotrophic culture used in the batch experiment was inoculated from the SBR. The biomass was centrifuged, followed by decanting of the supernatant, and resuspended in fresh AMS. The headspace was filled with pure oxygen and no methane. During the batch experiment biomass decay was quickly observed because of the lack of methane for cellular growth and maintenance by the methanotrophic culture.

3.4 METHANOTROPHIC DENITRIFICATION BATCH EXPERIMENT

To further investigate the potential to perform denitrification using the same nitrifying culture, batch denitrification tests were performed under different oxygen concentrations (0 to 50%) inoculated with the biomass collected from the SBR during Phase V.

From **Figure 3-12a** and **3-12b**, it can be deduced that under anoxic conditions (0% oxygen concentration), no biomass increase was observed with only a slight increase in the nitrate removal efficiency (NRE). Under anoxic conditions after 6 hours a 12% NRE was reached. Increasing the oxygen concentration to 10%, led to a slight enhancement in the NRE, however, it was correlated with an increase in biomass concentration. After 6 hours under 10% oxygen concentration, the NRE reached 24%, thereafter, a decline in the biomass concentration was observed while the NRE remained constant. High NRE and biomass growth was attained after 24 hours by having sufficient methane and oxygen (50% oxygen concentration) in the headspace of the serum bottles. There was a corresponding increase from 28% to 83% in the NRE when the incubation period increased from 6 to 24 hours. In general, nitrate removal during this experiment can be referred to cellular assimilation rather than denitrification. Such inference is based on the correlation observed between the biomass growth and the NRE as shown in **Figure 3-12a** and **3-12b**.

The highest nitrate removal rate achieved was 5.2$\pm$0.1 mgNO_3-N/L.hr. This result is comparable to Werner and Kayser [17], who noted a nitrate removal rate of 6.25 mgNO_3-N/L.hr in a trickling filter system with leachate as the medium. However, Werner and Kayser's [17] removal rate was achieved by AME-D and assimilation, whereas, the nitrate removal rate achieved during this experiment was the result of assimilation. Another comparable nitrate removal rate was achieved by Waki et al. [128] in an activated sludge process using a synthetic medium. There, the nitrate removal rate was 2.5 mgNO_3-N/L.hr and nitrate was removed by AME-D and assimilation.

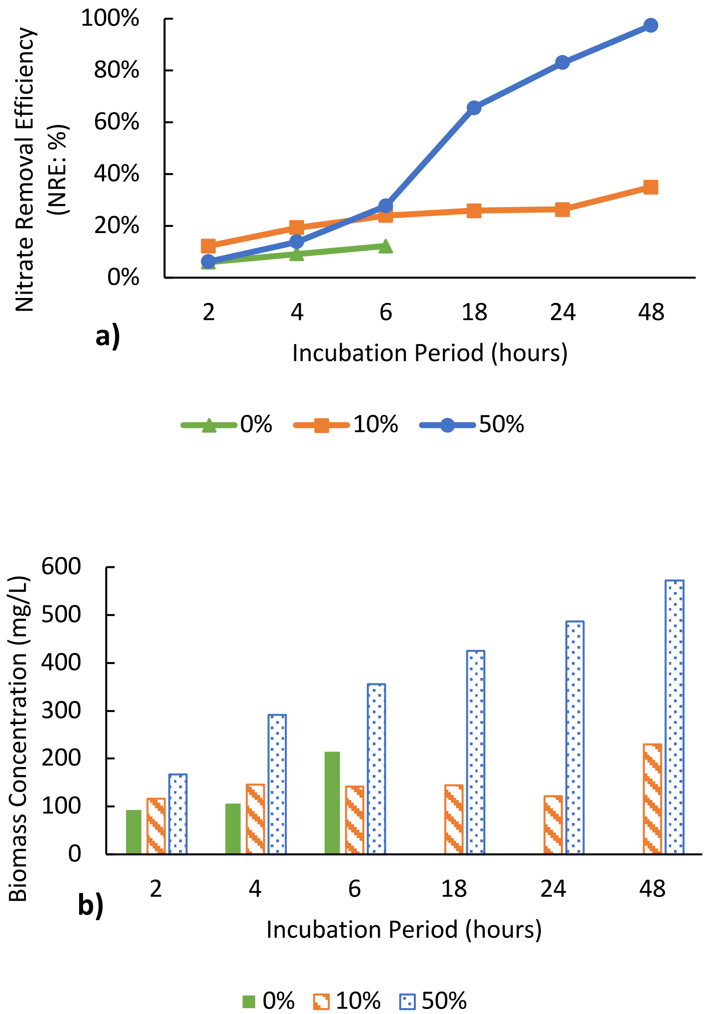

Figure 3-12: a) Nitrate removal efficiency (NRE) observed under the different methane to oxygen ratios applied to the headspace. b) Change in biomass concentration, from initial to final, over the duration of the incubation period.

Two key inferences can be drawn out of the batch denitrification experiment. First, the attained culture does not have the capacity to perform denitrification regardless of the applied oxygen conditions. This inference is in contradiction with the reports showing the denitrification capacity of methanotrophic mixed cultures enriched from activated sludge and anaerobic digested sludge [73,128]. It is unclear why denitrification capacity was not maintained in the selected culture in the SBR. The SRT maintained was long enough not to washout any microbes

(> 30 days), low DO concentration was maintained after two hours, nitrate was abundant following two hours, and constant methane was supplied either to perform methanotrophic denitrification or to produce organic soluble intermediates for other denitrifiers. The second inference is that under sufficient methane and oxygen conditions, the culture needs a lag phase of six hours to utilize nitrate as the nitrogen source. This inference is in contradiction with the reports that methanotrophs can easily switch between different nitrogen sources [110]. Indeed, further investigation is needed to understand such behaviour and how to maintain nitrifying-denitrifying methanotrophs in the mixed culture.

3.5 CONCLUSION AND FINAL REMARKS

This research validates the potential of methane redirection for biological nitrogen removal using a methanotrophic mixed culture enriched from activated sludge. Methane flow rate or concentration has been shown to be the key parameter to switch the methanotrophic culture capacity between assimilation and nitrification. In this research, it was presented for the first time that methanotrophic mixed cultures can perform complete nitrification with high ammonium removal efficiency and ammonium conversion to nitrate. Despite being observed for a limited duration, the maintenance of nitrifying/denitrifying culture was not successfully attained. Even so, AME-D capacity has been widely reported using pure and mixed cultures.

Here, it is laudable that the methanotrophic-based nitrogen removal is a nascent process and not as mature as BNR processes and shortcut BNR processes, as can be deduced from **Table 3-5**. However, it is very prominent for many reasons including:

- Its ability to maintain comparable ammonium removal efficiencies and ammonium conversion to nitrate under mainstream loading rates.
- The capacity of methanotrophic cultures to assimilate and/or oxidize different nitrogen sources (nitrogen gas, nitrate, and ammonium).
- Depending on the ratio between methane and nitrogen, the methanotrophic mixed culture can alter between assimilation or oxidation which allow for nitrogen recovery not removal in the case of methane abundance or low nitrogen concentrations.
- The methanotrophic biomass enriched for nitrogen removal have multiple applications such as bioplastic accumulation, single-cell proteins, and methanol production.
- A very appealing application niche is utilizing the dissolved methane in the anaerobic treatment effluents while simultaneously removing or recovering any remaining nitrogen prior to the discharge. In such application many challenges are resolved including methane poor solubility, methane recovery from anaerobic effluents, and poor nitrogen removal in anaerobic systems.

Nonetheless, there are still many challenges need to be overcome to make the technology competitive with the mature processes for BNR. These challenges are addressed in **Chapter 5**. Current process feasibility based on the obtained results under mainstream operating conditions are compared to conventional and emerging mainstream nitrogen removal processes to further validate and demonstrate the importance of novel nitrogen removal strategies, **Table 3-5**.

Table 3-5: Comparison of current and novel biological nitrogen removal processes with this research.

Process	Reactor Type	Volume (L)	Medium	HRT (hr)	DO (mgO_2/L)	Influent ($mgNH_4$-N/L)	Effluent ($mgNH_4$-N/L)	ARE (%)	References
Conventional BNR	MLE	-	Municipal	4 – 9	3.0	20 – 55[1]	<10[1]	>90	[12]
PNDA (two-stage)	A/O Biofilm	4.2	Synthetic	3.75	-	60	1	98	[129]
Mainstream Deammonification (single-stage)	IFAS	200	Secondary Effluent	-	0.46±005	50	15	70±4	[130]
Mainstream Deammonification (two-stage)	MBBR	200	UASB Effluent	17.4	0.6	39	7.4	81	[131]
Mainstream ANAMMOX (two-stage)	Plug flow granular	4000	Stage-A Effluent	1.5 – 2.0	0 – 2.0	26.8	6.8	74.6	[132]
ANITA Mox Prototype (Mainstream Deammonification)	IFAS	50,000	AS Effluent	2.5 – 3.0	0.2 – 0.8	50	20	60	[133]
Mainstream Nitrite Shunt	SBR	8	Synthetic	24	1.5	40	0.1±0.1	99	[134]
Mainstream Nitrite Shunt	AS+MBR	17400	Primary Settling Tank	6.4	1.5	40	0.63±0.01	98	[47]
Methanotrophic BNR (Phase VI)	SBR	5	Synthetic	12	0.8 – 3.2	51.3±5.6	5.3±1.9	87±7	This research

[1]TN for the influent and effluent
[2]calculated based on TN removal rate and HRT
A/O: Anoxic and oxic

MLE: Modified Ludzack-Ettinger
PNDA: Partial nitrification/denitrification with ANAMMOX
IFAS: Integrated fixed-film activated sludge
UASB: Upflow anaerobic sludge blanket

This research validates the potential of methane redirection for biological nitrogen removal using a methanotrophic mixed culture. It was presented for the first time that methanotrophic mixed cultures can perform complete nitrification with ammonium removal efficiency of 89$\pm$5% and 87$\pm$7% and ammonium to nitrate conversion ratio of 87$\pm$11% and 87$\pm$7% at nitrogen loading rate of 0.216$\pm$0.3 and 0.103$\pm$0.01 kgN/m^3.d, respectively. Methane flow rate or concentration has been shown to be the key parameter to switch the culture capacity between assimilation and nitrification. Despite observed for a limited duration, the maintenance of nitrifying/denitrifying culture has not been successfully attained. Yet, AME-D capacity has been widely reported using pure and mixed cultures. As a nascent technology, methanotrophic-based nitrogen removal has some advantages, but many challenges need to be overcome to make it comparable with the mature BNR processes.

CHAPTER 4: POLY-HYDROXY-BUTYRATE (PHB) ACCUMULATION IN METHANOTROPHIC MIXED CULTURE ENRICHED FOR NOVEL BIOLOGICAL NITROGEN REMOVAL[2]

4.1 INTRODUCTION

Anthropogenic activities are contributing to atmospheric greenhouse gas (GHG) emissions. Furthermore, a significant anthropogenic GHG contributor is wastewater treatment plants (WWTPs). Currently, WWTPs contribute 7% to GHG emissions with an anticipated 8% increase from the years 2020 to 2030 [7]. The projected emissions increase is largely attributed to an increase in energy consumption and input from larger loading rates as population size continues to grow. Therefore, global changes need to be made to better mitigate the release of anthropogenic GHGs, and utilize major pollutants like methane, to hamper climate change. Besides GHG emissions, another anthropogenic environmental burden is petroleum-based and single-use plastics. Petroleum-based and single-use plastics production is consuming enormous amounts of fossil-fuels every year. Additionally, numerous single-use plastics are ending up in natural environments as litter because of poor disposal and waste management practices [99]. Fortunately, bioplastics are an emerging renewable green plastic that can degrade back to raw material (carbon) at the end of their life cycle, hence biodegradable bioplastics/biopolymers [135,136].

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[2] A modified version of this Chapter has been submitted to the *Biochemical Engineering* Journal

Biodegradable biopolymers are increasing in popularity as an alternative to petroleum-based and single-use plastics. Advantageously, biopolymers can be significant in reducing environmental damage caused by petroleum-based and single-use plastics [103]. The majority of bioplastics are in the form of poly-hydroxy-alkenoates (PHAs). Biodegradable biopolymers are harvested from microorganisms that have high PHA capacities. Bioplastic production relies on farming practices to cultivate suitable feedstock sources, i.e. vegetable and plants oils, for PHA accumulating microorganisms [99]. With that said, the current bioplastic production methods are cost intensive and can be five to ten times greater than the standard petroleum-based plastics [104]. With the current high costs of bioplastic production, research has shifted towards alternative and cheaper feedstock solutions such as organic municipal solid waste, wastewater, carbon dioxide, and methane [31]. Fortunately, biogas (composed of up to 75% methane) produced during anaerobic digestion (AD) of organic matter within WWTPs is a promising feedstock source for biodegradable biopolymers.

Methane can be utilized by the unique methanotrophic bacteria (methanotrophs) as its sole carbon and energy source making them an ideal candidate for biopolymer accumulation from methane as a resource, while also mitigating GHG emissions from WWTPs [29]. Furthermore, methanotrophs are classified into three types (Type I, II, and III) based on the carbon assimilation pathway used for their energy requirement. Type I and Type II are more widely studied [14,75,137] and differ in membrane arrangements, their preferred methane to oxygen ratio, the ability to fix atmospheric nitrogen, and their carbon dioxide assimilation pathways [5,57]. Fortunately, Type II methanotrophs have the capacity to accumulate biopolymers under nutrient deficient conditions. Whereas, only very limited strains of Type I methanotrophs have this same ability [65]. In Type II methanotrophs, methane is converted to poly-hydroxy-butyrate (PHB), a member of the PHA family. The process of PHB production using methanotrophs takes place in two phases. During phase one, all the required nutrients are available to the culture to increase the biomass concentration. While, during phase two, unbalanced conditions are applied to promote PHB accumulation as intracellular granules [107]. The stored PHB is then extracted and applied as a material for green bioplastics.

In literature, different strategies and conditions have been proposed to accumulate a high capacity of PHA in microorganisms. Of these strategies, emphasis can be placed on cycling between nutrient sufficient and nutrient deficient conditions. To accumulate a high capacity of PHB in methanotrophs the major nutrients that are often cycled include nitrogen, phosphorus, or a combination of these [65,108–110]. Additionally, oxygen availability has also been reviewed as a successful PHB accumulation parameter [112]. Besides nutrient cycling and oxygen availability, it was found that applying feast and famine like conditions, based on methane availability, can induce PHB accumulation in methanotrophs [120,122]. During the feast phase, there is an abundance of methane available to the culture, while during the famine phase, methane availability is limited [122]. Applying feast and famine conditions have been shown to create mixed cultures which are dominated by microorganism having robust carbon storage abilities [108].

Methanotrophic bacteria have the ability to use different nitrogen sources, i.e. ammonium, nitrate, and nitrogen gas, for cellular growth and maintenance. However, utilizing ammonium as the nitrogen source is prominently used to select a high number of Type II PHB accumulating methanotrophs. Type II methanotrophs have higher resistance to ammonium as the nitrogen source, thus, they are able to form stable dominant cultures [76,77]. This selection tool is demonstrated, where, adding a sufficient amount of ammonium during the growth of mixed culture methanotrophs (Type I and II) leads to the selection of a Type II dominant culture, while also inhibiting the growth or survival of Type I [115]. Similarly, utilizing ammonium as the nitrogen source to select a dominant Type II methanotrophic culture from activated sludge with high PHB accumulation capacity was demonstrated by Fergala et al. [65]. When cycling through continuous nitrogen sufficient and deficient conditions (30 cycles), a high PHB accumulation capacity of 52.9±4% was observed [65]. This study demonstrates that using ammonium as the nitrogen source can select a dominate Type II culture, while cycling through nutrient starvation cycles can induce a high PHB capacity in the culture.

Recently, there have been several reports about the potential of utilizing methanotrophs in WWTPs for nitrogen removal [26,30,84], methane mitigation [18], or both [127,138]. Few studies review the applicability of utilizing the methanotrophic sludge enriched for alternative purposes, i.e. biological nitrogen removal and methane mitigation, to accumulate PHB. Therefore, the aim of the experiment was to investigate the applicability of a methanotrophic mixed culture enriched for biological nitrogen removal to successfully accumulate PHB upon immediate exposure to nutrient deficient conditions. Prior to nutrient deficient conditions, the methanotrophic culture was grown on ammonium as the nitrogen source and exposed to famine-like conditions for almost 50 days in a sequential batch reactor (SBR) system enriched for methanotrophic-based nitrogen removal as expressed in **Chapter 3, Phase V operation**. Famine-like conditions were applied in the SBR based on the lower methane availability, as described by Lopez et al. [122]. Three different operating conditions were applied to the batch experiments to induce PHB accumulation in the culture. This study reviewed the following conditions: (I) continued feast-famine operating conditions, (II) nitrogen limiting conditions, and (III) phosphorus limiting conditions. Furthermore, different methane and oxygen ratios were applied in the batch experiment to provide feast-like conditions (sufficient methane), along with different incubation periods.

4.2 MATERIALS AND METHODS

4.2.1 SBR OPERATION OVERVIEW

The SBR system had a 5 L working volume and was operated for approximately 50 days prior to the evaluation of the PHB accumulation capacity. Synthetic ammonium mineral salts (AMS) medium was used as the sole nitrogen source for the mixed culture. The SBR cycle duration lasted 6 hours, where, after 6 hours, 2.5 L of medium was decanted and replaced with fresh AMS. Pure gaseous methane (99.9% purity, Praxair) was continuously supplied to the reactor creating a headspace concentration of less than 25% methane which can be considered as famine-like conditions. Oxygen gas (99.9% purity, Praxair) was supplied in intervals. Detailed SBR operating conditions can be found in **Chapter 3, Section 3.2**. Biomass collected from the SBR was used as the inoculum for the PHB batch experiment.

4.2.2 PHB ACCUMULATION DESIGN AND OPERATING CONDITIONS

Methanotrophic liquid samples were collected from the process effluent of the SBR. The SBR was operated to achieve methanotrophic-based nitrogen removal. For the present study, three different batch methanotrophic phases were performed to evaluate PHB accumulation under various operational parameters with emphasis on the methane to oxygen ratio applied to create feast-like conditions (excess methane), mineral salts medium (MSM), and incubation period. The three methanotrophic batch experiments are detailed in **Table 4-1**. The process effluent medium (feast-famine conditions) was rich in nitrate (113$\pm$3 mgNO_3-N/L) and depleted in ammonium, the nitrogen free medium (nitrogen-limiting) contained undetectable limits of nitrogen, and the phosphorus free medium (phosphorus-limiting) was rich in ammonium (172$\pm$0.9 mgNH_4-N/L).

During phase I, 25 ml of process effluent medium was collected and directly placed in 125 ml serum bottles capped with butyl rubber stoppers. Whereas, during phases II and III, the process effluent medium was collected from the SBR and centrifuged for 20 minutes at 4000 rpm. The samples were decanted, and the biomass pellets were resuspended in fresh MSM. The samples were then placed in 125 ml serum bottles with 25 ml of MSM and capped with butyl rubber stoppers. Prior to the addition of methane and oxygen, air was removed from the serum bottles using a vacuum pump. In phases I to III, the headspace was filled with methane and oxygen (99.9% purity, Praxair) in different volumetric ratios to create feast-like conditions, aerobic (50% methane and oxygen saturation) and microaerobic (90% methane and 10% oxygen saturation). In every case, 100 ml of helium (99.9% purity, Praxair) was applied to the headspace of the serum bottles to fix the partial pressure. All the experimental phases were evaluated in duplicates at a temperature of 27°C and a mixing speed of 180 rpm using an orbital shaker. Gas samples were withdrawn from the headspace at the beginning and at the end of each incubation period. Finally, the total suspended solids (TSS) and volatile suspended solids (VSS) were measured prior to the start of the experiment and again at the end of the incubation period to evaluate methanotrophic growth.

Table 4-1: Operating conditions and parameters evaluated on methanotrophic sludge from the SBR system for PHB accumulation, where the process effluent is directly from the SBR, nitrogen-limiting is the nitrogen free medium salts, and phosphorus-limiting is the phosphorus free medium salts.

Phase	MSM	Incubation Period (hrs)	Methane Applied (ml)	Oxygen Applied (ml)
I	Process effluent	6, 12, 24, 48	90	10
		6, 12, 24, 48	50	50
II	Nitrogen-limiting	6, 12, 24, 48	90	10
		6, 12, 24, 48	50	50
III	Phosphorus-limiting	6, 12, 24, 48	90	10
		6, 12, 24, 48	50	50

4.2.3 CULTIVATION MEDIUM

The methanotrophic bacteria utilized in the PHB experiment was cultivated on ammonium mineral salts (AMS) within the SBR system as detailed in **Chapter 3, Section 3.2.2**. During the SBR operation a low methane to oxygen ratio was applied providing famine-like conditions. The initial AMS concentration was approximately 108 mgNH_4-N/L, however, during the time of the biomass collection from the SBR the ammonium concentration was depleted to approximately 12.7 mgNH_4-N/L.

The following concentrations were applied in the nitrogen-limiting medium [58] (mg/L): 500 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 270 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA, 1 ml of trace metals, and

5 ml of copper stock solution. While the following concentration were applied in the phosphorus-limiting medium [58] (mg/L): 500 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 400 NH_4CL, 4 Fe-EDTA, 1 ml of trace metals, and 5 ml of copper stock solution. The trace metal solution contained the following chemical concentrations (mg/L): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.H_2O$, 30 H_3BO_3, 3 $NaMoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$, and 20 $CoCl_2.2H_2O$. The copper stock solution had a concentration of 1000 mg/L of $CuSO_4.5H_2O$.

4.2.4 ANALYTICAL METHODS

The liquid samples collected were refrigerated at 4℃ prior to analysis. The liquid samples collected from the feast-famine conditions and phosphorus-limiting conditions were analysed for ammonium-nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), and nitrite-nitrogen (NO_2-N) using ion chromatography (Dionex™ Integrion™ HPIC™ System, Thermo Fisher Scientific, Sunnyvale, California). The VSS and TSS concentrations were measured according to standard methods [125,126]. Gas samples were withdrawn from the serum bottle headspace using a gas tight syringe and injected into the gas chromatography (GC) equipment (SRI 8610C, SRI Instrumentation, Torrance, USA) to measure methane, oxygen, and nitrogen gas concentrations. The GC used was equipped with a thermal conductivity detector (TCD) and molecular sieve column (Restek, Bellefonte, PA). Helium gas (99% purity, Praxair) was used as the carrier gas (15 ml/min) for the injected samples. A preconstructed calibration curve was utilized for the conversion of peak areas into gas concentrations. Temperatures for injections were kept constant at the following: injector 60℃, oven 80℃, and TCD 80℃.

4.2.5 PHB EXTRACTION METHOD

PHB extraction from the methanotrophic mixed culture was performed according to Teeka et al. [139] and Shi et al. [140] with modifications. In all the batches, 10 ml of culture medium was collected at the end of the incubation period and centrifuged for 20 minutes at 4000 rpm and 20°C. The supernatant was decanted, and the biomass pellets were resuspended in 10 ml of 5% sodium hypochlorite solution (Ward's Science, Rochester New York). The resuspended samples were placed in an orbital shaker at 37°C and 110 rpm for a minimum of one hour. The cells were than washed, centrifuged, decanted, and resuspended in 20 ml of distilled water. The above step was repeated three times with 20 ml of acetone, ethanol, and distilled water, respectively. Finally, the PHB samples were resuspended in 50 ml of distilled water and a chemical oxygen demand (COD) analysis using HACH was performed according to standard methods. The COD readings were completed using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA) and converted to a PHB concentration based on a preconstructed calibration curve of known PHB concentration. Furthermore, sodium hypochlorite destroys all the cell components that do not contain PHB creating a reasonable assessment for PHB concentration [141]. Finally, as PHB is essentially composed of carbon monomers, and samples were washed and resuspended in distilled water, duplicate COD readings provide accurate results as only PHB could induce a reaction.

4.3 RESULTS AND DISCUSSIONS

Standard PHB accumulation strategies applied are to cycle between periods of nutrient sufficient and deficient conditions as shown in **Figure 4-1**. Major nutrient cycling includes nitrogen and phosphorus. However, nitrogen cycling has been shown to lead to the highest PHB accumulation capacity in methanotrophic bacteria [65,120,121]. The nutrient deficient periods often last 24 or 48 hours, followed by nutrient sufficient (or growth) periods for another 24 or 48 hours [65,142]. On the other hand, phosphorus limiting cycles have been shown to achieve high PHB accumulation capacity in methanotrophic bacteria. Phosphorus limitation is demonstrated in literature, where a mixed culture dominated by the methanotrophic strain *Methlocystis* GB25 showed a 46% PHB accumulation capacity under phosphorus limitations [107].

Finally, the feast and famine selection strategy has proved successful in accumulating a high percentage of PHA using different microorganism (up to 89% [118]). Even though feast-famine conditions have been applied to accumulate PHA in mixed cultures for several decades [119], few studies exist in terms of feast-famine conditions applied while using methane as the carbon source for PHA accumulation. Because these strategies have all demonstrated successful PHA accumulation capacity, they were employed to accumulate PHB in a methanotrophic mixed culture. It is noteworthy that the culture was not grown to solely select or create a culture dominated by Type II methanotrophs, instead, it was grown to be employed in a nitrogen removal process.

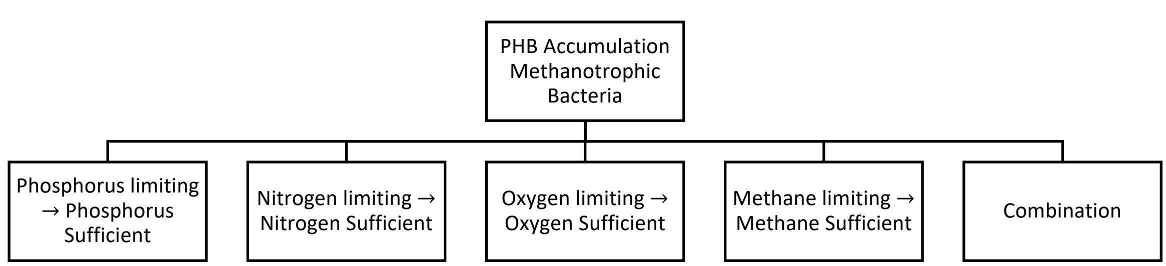

Figure 4-1: Strategies applied to accumulate a high capacity of PHB in methanotrophic bacteria.

4.3.1 CONTINUED FEAST-FAMINE OPERATIONAL CONDITIONS

Famine-like conditions can induce methanotrophic-based nitrogen removal when ammonium is used as the nitrogen source. When ammonium and methane are available to methanotrophs, in the presence of oxygen, there is competition between them to be oxidized by the methane monooxygenase (MMO) enzyme. Therefore, applying famine-like conditions (low methane availability) can stimulate ammonium oxidation/removal by the MMO enzyme. Besides stimulating ammonium oxidation, if coupled with a feast phase, continued famine-like conditions may have the potential to stimulate PHB production in methanotrophs. Pieja et al. [106] demonstrated that short-term methane starvation did not induce PHB production, however, repeated periods of methane and nitrogen limitation did induce PHB production [120]. Therefore, it was assumed that the standard pulse-feeding feast and famine strategy applied may not be applicable for significant methanotrophic PHB accumulation [120]. Unlike the aforementioned study, in the present experiment, the methanotrophic culture was exposed to long-term methane-limited conditions which may contribute to the PHB accumulation capacity. Therefore, the aim of phase I was to expose the culture to feast-like conditions by providing sufficient methane in the headspace of the serum bottles.

Methanotrophic-based ammonium oxidation was stimulated in the SBR, thus the process effluent medium utilized was depleted in ammonium while rich in nitrate. The highest PHB content by percentage of dry weight (mgPHB/mgVSS) observed in the mixed culture was 15±0.67% under aerobic conditions (50% methane and oxygen saturation) and an incubation period of 12 hours. Increasing the incubation time to 24 and 48 hours did not further increase the PHB accumulation capacity. The PHB accumulation capacity at 24 and 48 hours was 12±0.13 and 13±0.56%, respectively. In comparison, the PHB content when the culture was exposed to the same process effluent medium and microaerobic conditions (90% methane and 10% saturation) at a 12-hour incubation period was 11±0.74%. Similarly, the PHB capacity at 24 and 48 hours was 12±0.05 and 15±0.16%, respectively. **Figure 4-2** displays the PHB capacity of the methanotrophic culture when exposed to the process effluent medium. It is important to note that these values are the PHB accumulation capacity after one feast cycle, and it is assumed that applying continued feast-famine cycles would further increase the PHB accumulation capacity.

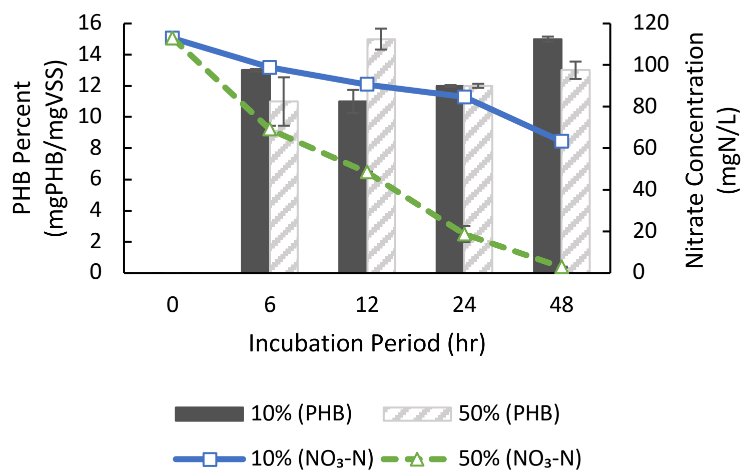

Figure 4-2: PHB percent (mgPHB/mgVSS) and nitrate concentration observed over the different incubation periods and oxygen saturation utilizing the process effluent as the medium.

Interestingly, there was an increase in biomass concentration under aerobic and microaerobic conditions as displayed in **Table 4-2**. This increase may be attributed to the nitrogen availability in the process effluent, as nitrate was consumed for assimilation over time as shown in **Figure 4-2**. Furthermore, the increase in biomass concentration was significantly greater under aerobic conditions as a result of sufficient methane and oxygen availability. Therefore, the greater PHB accumulation capacity under aerobic conditions may be the result of the greater biomass concentration observed. Moreover, under aerobic conditions, increasing the incubation period past 12 hours did not lead to an increase in PHB accumulation capacity. Similarly, applying oxygen limitation in the microaerobic conditions (greater methane availability than aerobic conditions) did not necessarily provide a competitive advantage for PHB accumulation capacity as observed by the lower PHB content.

Table 4-2: Change in biomass concentration (mgVSS/L) observed from the initial to final incubation period under the different mediums evaluated.

Incubation Period (hr)	**Process effluent**		**Nitrogen-limiting**		**Phosphorus-limiting**	
Headspace Conditions	Microaerobic	Aerobic	Microaerobic	Aerobic	Microaerobic	Aerobic
6	65±5	431±21	-112±18	425±17	-5±5	206±8
12	87±7	495±9	-53±3	460±26	-46±4	250±0
24	100±14	531±15	-21±37	609±19	-46±0	539±33
48	134±20	171±3	115±37	575±9	-244±24	452±52

Utilizing the process effluent as the medium source can be considered as normal operating conditions as all the essential nutrients were available to the culture. Assuming this is the case, the PHB accumulation capacity was greater than that obtained by Zhang et al. [109]. The largest PHB capacity in this study after one cycle was 15±0.67% at an initial nitrate concentration of approximately 113 mg/L and cultivated on ammonium as the nitrogen source. Whereas, the PHB accumulation capacity was 9.8% in the methanotrophic strain IMV3011 at a nitrate concentration of 160 mg/L and cultivated on ammonium as the nitrogen source [109]. Similarly, the PHB capacity of the Type II methanotrophic strain *Methylocystis hirsuta* under nutrient sufficient conditions was 7.8±1.0% when cultivate on nitrate as the nitrogen source [121]. The larger PHB content observed during the present study can be attributed to the feast and famine application. As demonstrated by Pieja et al [106] applying short-term famine phases did not induce PHB, however, applying long term feast and famine phases along with nitrogen starvation did [120]. Furthermore, the IMV3011 strain was not exposed to these same feast-famine conditions as the headspace was replenished every 12 hours in a 1:1 v/v ratio of methane and oxygen [109]. Therefore, it can be deduced, that even under normal operating conditions, the long-term famine like conditions attributed to a greater PHB accumulation capacity in the culture. Finally, it is assumed that applying long-term feast and famine conditions to the current culture would further increase the PHB accumulation capacity as demonstrated by Pieja et al. [120].

4.3.2 NITROGEN-LIMITING CONDITIONS

Methanotrophic bacteria have the ability to use different nitrogen sources, i.e. ammonium, nitrate, and nitrogen gas, for cellular growth and maintenance. Therefore, applying cycles of nitrogen starvation is a well-studied and successful parameter in achieving a high PHB accumulation capacity in methanotrophic bacteria. **Figure 4-3** demonstrates the PHB percent by dry weight (mgPHB/mgVSS) under nitrogen absent conditions for aerobic and microaerobic headspace conditions. The maximum PHB percent observed was 21±1.31% at a 12-hour incubation period and aerobic conditions. As the incubation time increased, there was a plateau in PHB accumulation capacity as demonstrated by the PHB capacity of 19±0.02% at 24 hours and 19±0.06% at 48 hours. The small decrease in PHB accumulation capacity may be attributed to PHB usage as a reducing equivalent for methane. In comparison, the PHB accumulation capacity was 14±0.92, 16±0.07, and 14±0.66% under microaerobic conditions and incubation periods of 12, 24, and 48 hours, respectively.

Furthermore, under aerobic conditions, as the incubation period increased there was a concurrent increase in biomass concentration as demonstrated in **Table 4-2**. On the other hand, when microaerobic conditions were applied biomass decay was observed, with the exception of the 48-hour incubation period. Similar to the process effluent medium, feast-like conditions, and sufficient oxygen availability, likely attributed to the greater PHB accumulation capacity. As nitrogen was absent and feast-like conditions were applied, the increase in biomass concentration can be attributed to PHB storage. Additionally, providing longer incubation periods did not attribute to an increase PHB production. Finally, providing significant methane and limited oxygen did not provide any further incentive for PHB production, instead it may have contributed to PHB usage as observed by the decrease in biomass concentration.

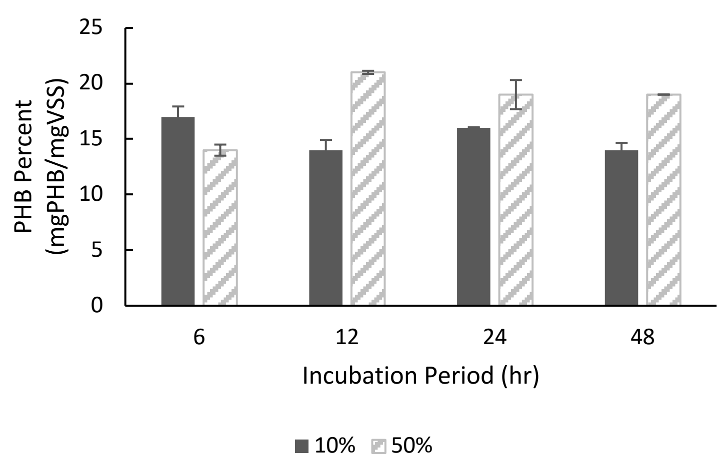

Figure 4-3: PHB percent (mgPHB/mgVSS) and nitrate concentration observed over the different incubation periods and oxygen saturation utilizing the nitrogen limiting medium.

As stated, the maximum PHB accumulation achieved was within a 12-hour incubation time, and after this time the accumulation capacity plateaued. Interestingly, the maximum PHB capacity was observed in a shorter time frame than the standard PHB starvation cycles, which are more commonly 24 or 48 hours [65,142]. This finding implies that the mixed methanotrophic culture may be able to accumulate PHB quicker, as a result of the continued exposure to famine-like conditions. Continued famine-like conditions should lead to a culture with high PHB accumulation capacity because the microorganisms that have the capacity to store PHB as a reducing equivalent will survive, while those that do not have this same ability will decay. Thus, the culture should theoretically be composed of methanotrophs with an initial PHB accumulation capacity. This assumption is demonstrated when comparing to another study. The methanotrophic strain *Methylocystis hirsuta* was cultivated on nitrate and utilized for methane abetment in a gas bubble reactor before exposure to nitrogen starvation conditions to induce PHB accumulation [121]. In the reactor system, the initial PHB accumulation was 0.4±0.0%, whereas, after a 3-day nitrogen starvation period the PHB accumulation capacity increased to 25.7±0.1% [121]. This value is approximately 5% greater than the PHB accumulation capacity achieved after 12-hours in the present study. Similarly, when a methanotrophic culture was exposed to feast-famine and nitrogen starvation, the inferred PHB accumulation capacity was approximately 16% after one 24 hour cycle [120].

Finally, as demonstrated in literature, it is assumed that applying continued feast-famine conditions and nitrogen starvation periods will lead to an increase in PHB accumulation capacity. This assumption is demonstrated by Fergala et al. [65] who, after 30 cycles, was able to achieve a PHB accumulation capacity of 52.9±4.0% when the culture was initially cultivated on ammonium. Another example showing a similar assumption is demonstrated by Garcia-Perez et al. [121], where, after 15 nitrogen sufficient and starvation cycles the PHB accumulation capacity increased to a maximum of 40%, compared to only 25.7±0.1% after one cycle. Finally, Pieja et al. [120] demonstrated a PHB capacity increase from approximately 16% after one cycle to approximately 27% after nine cycles.

4.3.3 PHOSPHORUS-LIMITING CONDITIONS

Figure 4-4 displays the PHB content by percent of dry weight (mgPHB/mgVSS) and the ammonium concentration results obtained under phosphorus limiting conditions. Similar to nitrogen limiting conditions, the highest PHB content observed was after a 12-hour incubation period. After this period the PHB content plateaued demonstrating that additional incubation time did not lead to an increase in PHB production. With that said, the PHB content at 12-hours and aerobic headspace conditions was 11±1.58%. This value is approximately 10% lower than the PHB content observed at 12-hours and nitrogen limiting conditions. Furthermore, the PHB content under aerobic conditions at 24 and 48 hours was 7±0.62 and 8±0.98%, respectively. On the other hand, when microaerobic conditions were applied a lower PHB content was observed, similar to the previous findings. The PHB accumulation capacity under microaerobic conditions was 9±0.08, 7±0.34, and 6±0.58% at 12, 24, and 48 hours, respectively. The lower PHB content in comparison to the other mediums evaluated can be attributed to two major inferences, as detailed below.

The first inference is that the increase in ammonium concentration compared to the SBR system likely inhibited the methanotrophic metabolic pathway. Methanotrophic metabolism can be inhibited by ammonium in two ways. The first, is that when ammonium and methane are available to methanotrophs in the presence of oxygen, there is competition between them to be oxidized by the MMO enzyme, as previously stated. The second, assuming ammonium can outcompete methane for oxidation by the MMO enzyme, the by-product of hydroxylamine is produced. Consequently, hydroxylamine is toxic to methanotrophs and is thereafter rapidly oxidized to nitrite by the hydroxylamine oxidoreductase [82]. Hydroxylamine and nitrite can inhibit the formate dehydrogenase (FDH) enzyme which is required in the assimilatory and dissimilatory methane oxidation pathways for energy generation [18,81]. From **Figure 4-4** it is evident that the ammonium concentration was not reduced with time suggested that it did inhibit the metabolic pathway of the methanotrophic culture. This inference is further confirmed by the decrease in biomass concentration with time, particularly under microaerobic conditions as shown in **Table 4-2**. The methanotrophic culture was likely inactivated by the increase in ammonium concentration of approximately 70 mgNH_4-N/L in comparison to the AMS fed into the SBR system. Additionally, Fergala et al. [31] demonstrated a decrease in biomass growth with increasing ammonium concentration. Similarly, in the methanotrophic strain IMV3011, the PHB content was 24.6% when the ammonium concentration was limited to 10 mg/L, however, increasing the ammonium concentration to 50 mg/L resulted in a concurrent PHB content decrease to 7.5% implying that greater ammonium concentrations can affect PHB accumulation, agreeing with the present study [109].

The second inference contributing to the lower PHB content is that in literature, phosphorus is not completed removed from the medium during the PHB accumulation phase, instead the concentration is reduced. Phosphorus is a requirement to maintain MMO activity (2 to 25 mmol), and eliminating it completely may inhibit culture performance [70]. When phosphorus is used as the deficient condition in literature, the concentration is lowered. This strategy was demonstrated by Sundstrom and Criddle [116], where reducing the phosphorus concentration from 2.9 to 0.12 mmol resulted in a concurrent 18% increase in PHB content. Similarly, another study demonstrated that reducing the phosphorus concentration from 7.3 to 5.7 mmol resulted in a PHB content increase from 16 to 26.5%, respectively [109]. Therefore, the two inferences stated above likely led to the lower PHB content observed in comparison to the two other MSM utilized. However, based on literature, it is hypothesized that applying optimal conditions, while using phosphorus concentration as a PHB accumulation strategy, will results in greater PHB capacity than solely applying feast conditions, similar to phase I of the batch experiment.

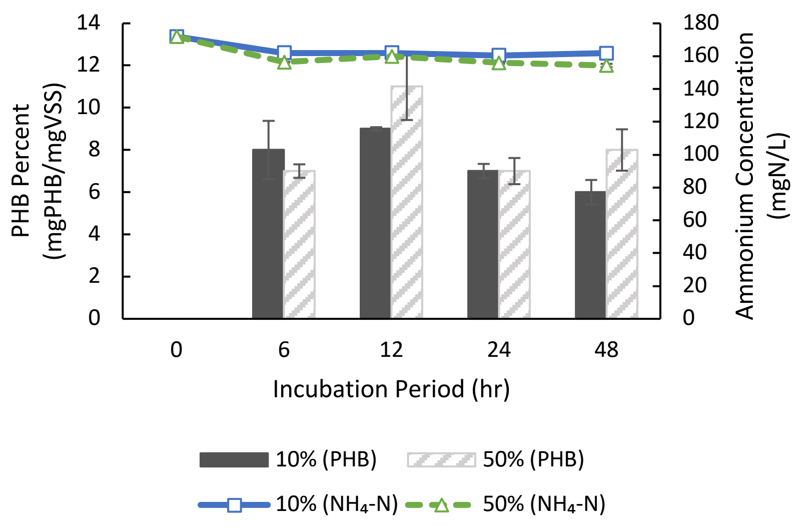

Figure 4-4: PHB percent (mgPHB/mgVSS) and nitrate concentration observed over the different incubation periods and oxygen saturation utilizing phosphorus limiting medium.

4.3.4 GAS CONSUMPTION PER PHB PRODUCTION

Table 4-3 demonstrates the oxygen and methane consumption by percentage in comparison to the PHB produced (mgPHB/mgVSS) under aerobic conditions. The table demonstrates that after 24 hours, the oxygen and methane consumption rates stabilized with PHB production. This finding is the result of oxygen depletion in the headspace after 24 hours as it was not replenished. Furthermore, a greater amount of oxygen and methane was consumed at 12 hours under nitrogen- and phosphorus- limiting mediums in comparison to the process effluent medium. Major nutrients were missing under the nitrogen- and phosphorus- limiting mediums likely leading to the increase in oxygen and methane consumption for energy generation. Additionally, the greater methane and oxygen consumption at the 12-hour period may be attributed to the increase in biomass concentration.

Table 4-3: PHB produced (mgPHB/mgVSS) by percentage per oxygen and methane consumed by percentage, under aerobic headspace conditions.

Incubation Period (hr)	**Process-effluent (%)**		**Nitrogen-limiting (%)**		**Phosphorus-limiting (%)**	
Gas	Oxygen	Methane	Oxygen	Methane	Oxygen	Methane
6	0.88	1.15	1.04	1.70	0.59	0.94
12	0.81	1.10	1.47	2.25	0.94	1.94
24	0.53	0.70	0.81	1.19	0.31	0.52
48	0.55	0.73	0.76	1.12	0.34	0.51

4.4 GENERAL FINDINGS

It was observed that aerobic conditions (sufficient methane and oxygen) led to a greater PHB accumulation capacity in comparison to microaerobic conditions (sufficient methane and limited oxygen). This finding agrees with other PHB accumulation studies as methane and oxygen are usually applied to the headspace in equal volumetric ratios resulting in greater PHB accumulation [65,109,142]. Additionally, biomass growth (TSS) was observed during the starvation periods which is in contradiction with literature. However, often there was a greater increase in the VSS in comparison to the TSS, implying PHB storage under deficient conditions. Therefore, it can be assumed that the increase in PHB production can be attributed to the increase in the biomass concentration observed during the incubation periods. This assumption can be confirmed by the lower PHB content at 48 hours and aerobic conditions for the different medium types. As shown in **Table 4-2**, there was a concurrent decrease in PHB content with biomass concentration. This drop may be attributed to PHB usage, as famine-like conditions (low methane availability) were once again induced. Additionally, it was expected that either the 24- or 48-hour incubation periods would lead to the greatest PHB accumulation capacity as demonstrated by the standard cycle time in literature. However, often the headspace is replenished with methane and oxygen in a ratio of 1:1 v/v every 12 or 24 hours to ensure that there is enough methane and oxygen to sustain the culture [109,142], whereas this did not occur in the present study.

Under all mediums evaluated the PHB content decreased after 12 hours. This decrease can be attributed to PHB usage as a reducing equivalent for methane assimilation as methane and oxygen were not replenished. Corresponding with the results observed by Pieja et al. [106], where even after nitrogen depletion there was still an increase in biomass concentration which can be attributed to the storage of PHB. Finally, it may be possible to accumulate a maximum PHB capacity of approximately 53% when optimal conditions are applied during the PHB accumulation phase similar to another study [65]. A maximum capacity of 53% can be assumed as the biomass seed utilized in the experiment was activated sludge and the nitrogen source applied was solely ammonium, similar to the cultivation conditions applied by Fergala et al. [65].

On a different note, the limited oxygen conditions applied during the microaerobic phase may have inhibited the methanotrophic culture. The methanotrophic culture had previously been exposed to oxygen sufficient conditions in the SBR. Moreover, oxygen is a respiratory requirement by aerobic methanotrophs and the change from high to low oxygen concentration may have deactivated the culture. This inhibition could also explain the decrease in biomass concentration observed under microaerobic conditions as there was still an abundance of methane. Furthermore, agreeing with literature, the greatest PHB capacity was observed under nitrogen limiting conditions confirming that it can be reliable parameter to achieve a high capacity of PHB. **Table 4-4** displays the PHB content summary under microaerobic and aerobic conditions. It is evident, particularly when the process effluent and nitrogen limiting medium was used, that aerobic conditions led to an increase in the PHB accumulation capacity of the culture.

Table 4-4: Summary of PHB percent (mgPHB/mgVSS) for microaerobic and aerobic headspace conditions.

Incubation Period (hr)	**Process effluent**		**Nitrogen-limiting**		**Phosphorus-limiting**	
Headspace Conditions	Microaerobic	Aerobic	Microaerobic	Aerobic	Microaerobic	Aerobic
6	13±0.08	11±1.55	17±0.93	14±0.14	8±1.38	7±0.32
12	11±0.74	15±0.67	14±0.92	21±1.31	9±0.08	11±1.58
24	12±0.05	12±0.13	16±0.07	19±0.02	7±0.34	7±0.62
48	15±0.16	13±0.56	14±0.66	19±0.06	6±0.58	8±0.98

4.5 CONCLUSIONS

Overall, this research validates the applicability of using methanotrophic sludge enriched for alternative purposes to accumulate PHB while using methane as the carbon source. Furthermore, the methanotrophic sludge enriched for nitrogen removal was able to accumulate a greater amount of PHB after one cycle in comparison to methanotrophic sludge enriched for methane abetment. The greater PHB capacity can be attributed to the famine-like conditions applied to the SBR. Despite the lower PHB capacity in comparison with literature, the study has demonstrated that PHB accumulation is feasible as shown by the PHB content after one cycle.

The largest PHB accumulation capacity presented was 21±1.31% under aerobic (feast-like) conditions and nitrogen limitation. Agreeing with literature, nitrogen limitation is a reliable PHB accumulation strategy. Moreover, directly utilizing the process effluent demonstrated a PHB accumulation capacity of 15±0.67%. Unfortunately, the PHB accumulation capacity was significantly lower when phosphorus limiting conditions were applied, however, the lower capacity can be attributed to the inhibition by ammonium and the complete elimination of phosphorus from the medium. Finally, it is assumed that applying continuous feast and famine phases, along with nitrogen starvation, will further increase the PHB capacity.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1 FUTURE WORK

5.1.1 METHANOTROPHIC NITROGEN REMOVAL

Even with the noteworthy results of the methanotrophic-based nitrogen removal process, there are still many challenges that need to be overcome to make the technology competitive with the mature BNR processes. These challenges include:

- Optimizing the system to perform nitrification and denitrification within a single reactor. It was observed that determining the proper cycle configuration and the methane to oxygen to nitrogen ratio is important to achieve sequential nitrification-denitrification within the SBR. An alternative strategy that may be considered in future work is feeding the effluent of the methanotrophic-based SBR into a separate reactor system. The second reactor could utilize an AME-D process for nitrate removal, creating a closed loop process. Ideally, the dissolved methane in the effluent of the first reactor will be used as the sole carbon source in the second reactor reducing the need for applying methane to the AME-D reactor. Finally, the addition of a second reactor could help to lessen the current concerns with the dissolved methane concentration in the effluent.
- There is an associated safety hazard with mixing the flammable gases methane and oxygen [1,26]. This safety hazard can be reduced by supplying separate methane and oxygen gas lines into the bulk liquid of the system [1,127]. Methane can become explosive with air at a volume to volume ratio of 1:10, or with oxygen at a volume to volume ratio of 1:2 [143]. The

handbook of Chemistry and Physics states that limits of methane flammability are 5% (lower) and 15% (upper) by volume in air at room temperature [144]. Additionally, attention needs to be placed on how to handle the nitrous oxide production. Currently, nitrous oxide release and production is being ignored as research is focusing on the removal rates and the interaction between methanotrophs and denitrifiers [1].

- Recirculation of unused gaseous methane and oxygen applied to the system to maintain a closed loop. Methane solubility in water at 25°C is 21.5 mgCH_4/L and decreases with increasing water temperature up to 80°C [145]. As methane solubility is relatively poor, there will be excess methane released from the water which can be recycled. Recycling gases would also reduce the concerns surround biogas production volume onsite from anaerobic digestion. An additional benefit of gas recirculation includes the cost reduction associated with aeration of the process, however, the concern of mixing flammable gases may arise during upscale. The carbon to nitrogen ratio should also be reduced to increase the process sustainability. Besides the carbon to nitrogen ratio, research can also focus on utilizing alternative sources of oxygen for nitrate reduction to eliminate the safety hazard associated with mixing flammable gas.
- Finally, further experimental research should highlight lowering the HRT and gases application rates, the impact of cold weather temperature on the methanotrophic-based nitrogen removal process, further analysis focusing on fluctuating carbon to nitrogen ratios applied, the beneficial uses of the methanotrophic biomass produced, and continued optimization of the methane and oxygen concentrations and system polishing. Finally, a

complete economic and feasibility analysis should be completed on the methanotrophic system providing a full picture of the novel process.

5.1.2 METHANOTROPHIC PHB ACCUMULATION

The PHB accumulation capacity using the methanotrophic biomass enriched for nitrogen removal was lower than other studies, thus, further research can be done to improve the PHB capacity. Focus can be placed on utilizing the PHB accumulation strategy proposed by Criddle and Sundstrom [110] because of the altering nitrogen sources in the SBR system, or cycling between feast-famine periods while using methane and nitrogen limitations [120]. Furthermore, as methanotrophic PHB accumulation capacity was lower than literature values, alternative biotechnologies including single-cell proteins and methanol production should be considered for the production of higher value products.

5.2 RESEARCH SIGNIFICANCE AND MAINSTREAM INTEGRATION

Methanotrophic bacteria are an important microorganism that can help to fill the gap in mainstream nitrogen removal processes. Three solutions have been proposed for mainstream integration. Solutions 1 and 2 are the integration of the methanotrophic-based nitrogen removal process with current activated sludge processes, as depicted in **Figure 5-1** and **Figure 5-2**, respectively. Methanotrophic integration could be successful here, as activated sludge was used as seed for the growth of the methanotrophic culture. The process would use the biogas produced onsite from anaerobic digestion instead of flaring it to the environment. Unfortunately, direct integration within AS processes, **Figure 5-1**, may deem more challenging as the operating parameters (SRT, HRT, NLR, COD, and microorganism interaction) would need to align with the

AS process. On the other hand, depending on the role of the methanotrophic-based system, it could be used for ammonium recovery by assimilation, ammonium conversion to nitrate by oxidation, or PHB cultivation aligning with the WRRF concept. Solution 2, depicted in **Figure 5-2**, is similar to Solution 1 and can have the same added benefits. However, the methanotrophic culture can be utilized to polish any remaining nitrogen, either ammonium or nitrate, before discharge into surface waters. Solution 2 is applicable because the methanotrophic sludge has a better settling capacity than activated sludge, meeting discharge regulations on biomass concentrations. Additionally, placement here would be easier as the process will no longer need to closely align with the AS processes.

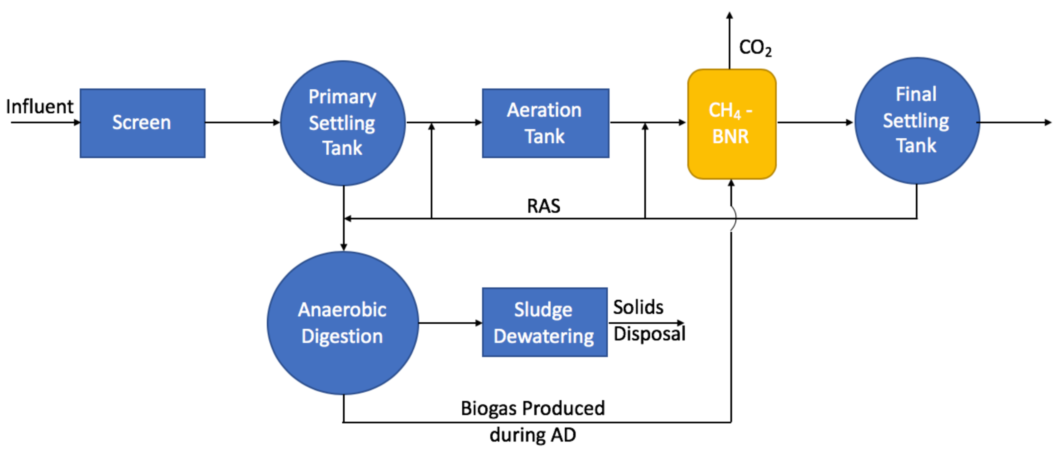

Figure 5-1: Methanotrophic BNR integration with mainstream removal strategies.

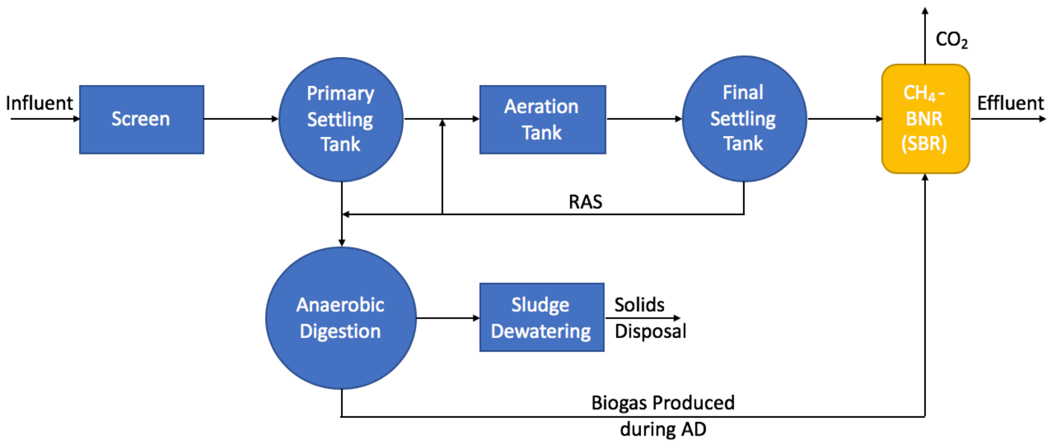

Figure 5-2: Methanotrophic BNR integration as a polishing stage post activated sludge treatment.

Finally, the methanotrophic-based nitrogen removal process can be utilized as a post anaerobic treatment process as shown in **Figure 5-3**. During anaerobic treatment, methanogenic bacteria produce methane as part of their energy metabolism. Based on the solubility of methane a portion of the produced methane will remain dissolved in the supernatant. The supernatant discharged from anaerobic treatment can be used as the influent to the methanotrophic-based SBR in a closed system to minimize the escape of methane. The dissolved methane in the supernatant can be used as the sole carbon source by the methanotrophic bacteria while polishing any remaining nitrogen prior to discharge. Process integration with anaerobic treatment may alleviate the aforementioned concern with mixing the flammable gases methane and oxygen.

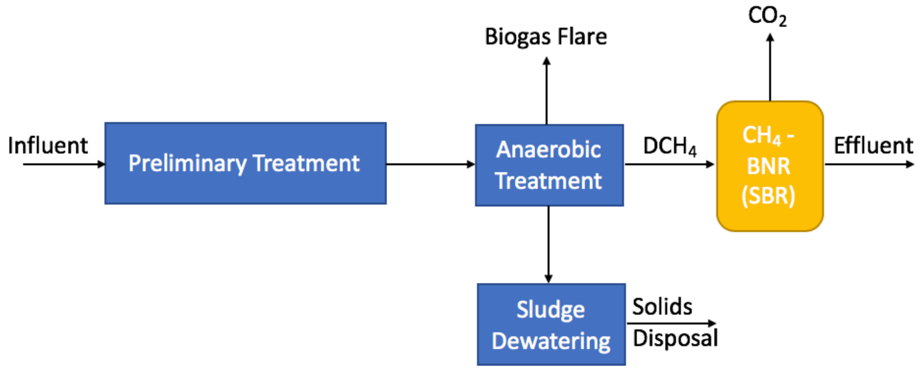

Figure 5-3: Methanotrophic BNR utilized as post anaerobic treatment.

5.3 CONCLUSIONS

Methanotrophic bacteria are a vital microorganism which can be applied to various biotechnologies. This research focused on the use of methanotrophic bacteria in nitrogen removal processes and PHB production using methane as the carbon source. The concluding highlights from **Chapters 3** and **4** are denoted below.

Chapter 3 focused on methane redirection for methanotrophic-based nitrogen removal in an SBR system. Different phases were evaluated within the SBR with the aim of validating the nitrification and denitrification ability by altering the methane and oxygen flow rate as a key parameter. Despite being observed for a limited duration, the maintenance of the nitrifying/denitrifying culture was not successfully attained. Even so, AME-D capacity has been widely reported using pure and mixed cultures. This study is significant as it was the first to maintain stable nitrate accumulation using methanotrophic mixed cultures. When ammonium was removed by assimilation, the ammonium removal efficiency (ARE) was 98±1%. Under elevated ammonium concentrations and mainstream conditions, the ARE was 89±5% and 87±7% and the ammonium conversion to nitrate was 87±11% and 87±7%, respectively. This study confirms the potential of using methanotrophic bacteria for nitrogen removal, yet, further studies are still needed to improve process feasibility and address the mentioned challenges.

The objective of **Chapter 4** was to evaluate the applicability of methanotrophic bacteria enriched for nitrogen removal to accumulate biopolymers. Biopolymers are growing in popularity as green plastic alternatives to petroleum-based and single-use plastics. PHB in microorganisms is essentially accumulated in two phases, the first being nutrient sufficient and the second being nutrient deficient conditions. The PHB accumulation capacity was evaluated because the culture was exposed to famine-like conditions within the SBR (Phase V). The methanotrophic culture was immediately exposed to nutrient deficient conditions upon removal from the SBR system. The highest PHB accumulation capacity within the methanotrophic mixed culture was 21±1.31% under a nitrogen-limiting medium and aerobic conditions. Interestingly, utilizing direct effluent from the SBR led to the PHB accumulation capacity of 15±0.67% compared to a phosphorus-limiting medium of 11±1.58%. The poor PHB accumulation capacity in the phosphorus-limiting medium is attributed to the ammonium concentration, thus inhibiting methanotrophic growth. Finally, microaerobic conditions slowed methane oxidation which consequently led to a decrease in PHB production.

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APPENDICIES

APPENDIX A: EQUATIONS

Appendix A consists of specific equations used in Chapters 3 and 4.

Sludge Volume Index (SVI_{30}): "volume occupied by 1 g of sludge after 30 minutes of settling [12]"

$$SVI\ \left(\frac{ml}{g}\right) = \frac{\left(settled\ volume\ of\ sludge, \frac{ml}{L}\right)\left(\frac{10^3 mg}{1g}\right)}{TSS\ \left(\frac{mg}{L}\right)} \tag{3-1}$$

Nitrogen Loading Rate (NLR): amount of nitrogen present in the influent and applied to the system process over the duration of a day

$$NLR\ \left(\frac{kgN}{m^3 \cdot d}\right) = \frac{(NH_4\text{-}N)_{feed}\left(\frac{kgN}{m^3}\right)}{HRT\ (d)} \tag{3-2}$$

Nitrogen Removal Rate (NRR): amount of nitrate removed in relation to time

$$NRR\ \left(\frac{mgNO_3\text{-}N}{L \cdot hr}\right) = \frac{(NO_3\text{-}N)_{feed} - (NO_3\text{-}N)_{final}\left(\frac{kgN}{m^3}\right)}{HRT\ (hr)} \tag{3-3}$$

Ammonium Removal Efficiency (ARE): amount of ammonium-nitrogen removed expressed, as a percentage

$$ARE\ (\%) = \frac{(NH_4\text{-}N)_{feed} - (NH_4\text{-}N)_{eff}}{(NH_4\text{-}N)_{feed}} \times 100\% \tag{3-4}$$

Nitrate Removal Efficiency (NRE): amount of nitrate-nitrogen removed, expressed as a percentage

$$NRE\ (\%) = \frac{(NO_3\text{-}N)_{feed} - (NO_3\text{-}N)_{final}}{(NO_3\text{-}N)_{feed}} \times 100\% \tag{3-5}$$

Total Suspended Solids (TSS): solids in wastewater that can be trapped by a filter

$$TSS\ \left(\frac{mg}{L}\right) = \frac{W_2(mg) - W_1(mg)}{V(L)} \tag{3-6}$$

Volatile Suspended Solids (VSS): the fraction of volatile solids present in the TSS sample

$$VSS\ \left(\frac{mg}{L}\right) = \frac{W_3(mg) - W_2(mg)}{V(L)} \tag{3-7}$$

APPENDIX B: ANALYTICAL EQUIPMENT DESCRIPTION

Gas Chromatography (GC): The GC operates by injecting a small amount of gas from an airtight syringe into the GC equipment. The injected sample is heated and quickly vaporized. A carrier gas, i.e. helium or hydrogen, is utilized to help the vaporized gas sample travel to the column. The column is usually a thin metal or glass tube filled with a substance that has a high boiling point. The sample travels through the column, and as it does it is separated out into its respective components by adsorption. Finally, as the sample approaches the end of the column it moves past a detector which identifies peaks, which can later be converted to mass concentrations for analysis.

Ion Chromatography (IC): The IC is utilized to measure the anions and cations in liquid samples. The IC works by separating ionic species based on interactions with resin, depending on the species type and size. Liquid samples pass through a pressurized column where ions are absorbed. The retention time of the species through the column determines the ion concentration of the sample. The key anions measured during this process are nitrite, nitrate, chlorine, fluoride, and sulfate, while the key cations measured are ammonium, potassium, calcium, and magnesium. Liquid samples should be filtered prior to use with the IC as organic matter and sediments can cause damage to the equipment and lead to inaccurate peak concentrations.

APPENDIX C: DATA

Chapter 3: Data

Influent, feed, and effluent ammonium concentrations observed during Phase III and IV of SBR operation:

Day	NH_4-N In (mgN/L)	NH_4-N Feed (mgN/L)	NH_4-N Eff (mgN/L)	ARE (%)
1	27.7	55.4	2.7	95.0
2	42.5	85.0	2.0	97.5
6	45.4	90.9	2.2	97.5
7	51.9	103.8	3.2	96.9
9	49.0	98.1	3.5	96.3
13	32.5	65.1	2.7	95.8
14	29.4	58.8	3.1	94.6
15	33.0	66.1	2.1	96.8
16	30.1	60.2	2.7	95.4
17	31.5	63.1	3.3	94.6
20	36.6	73.2	2.2	96.9
21	47.0	94.1	3.3	96.4
23	63.8	127.6	1.4	98.9
24	32.5	65.0	1.5	97.5
29	44.2	88.5	2.3	97.3
30	45.2	90.5	3.4	96.2
31	45.7	91.4	1.7	98.0
35	46.0	92.0	3.2	96.5
36	55.8	111.7	2.7	97.5
37	64.8	129.7	1.4	98.8
38	56.0	112.1	1.8	98.3
42	35.2	70.4	1.4	98.0

Day	NH_4-N In (mgN/L)	NH_4-N Feed (mgN/L)	NH_4-N Eff (mgN/L)	ARE (%)
44	61.5	123.1	1.6	98.6
49	43.9	87.9	2.8	96.8
50	52.9	105.8	2.3	97.7
51	50.4	100.8	2.6	97.3
52	58.3	116.7	1.2	98.9
81	44.0	88.1	2.0	97.6
82	40.9	81.9	1.5	98.1
83	37.6	75.3	1.2	98.4
87	47.0	94.0	2.1	97.7
89	48.3	96.6	1.1	98.7
93	38.0	76.1	2.4	96.8
94	32.8	65.7	1.9	97.1

Influent and effluent ammonium, nitrite, and nitrate concentrations observed during Phase V and VI SBR operation:

Day	NH_4-N Inf. (mgN/L)	NH_4-N Eff. (mgN/L)	NO_2-N Eff. (mgN/L)	NO_3-N Eff. (mgN/L)
1	102.0	2.0	30.0	53.7
3	102.0	0.0	5.3	82.0
5	119.0	9.2	0.0	87.0
9	119.0	13.1	0.0	94.0
11	109.0	12.9	0.0	95.6
17	109.0	12.1	0.0	96.9
23	120.0	13.4	0.0	106.6
25	116.0	18.1	0.0	97.9
30	118.0	10.8	0.0	107.2
32	118.0	7.3	0.0	109.7
37	116.0	8.3	0.0	107.7

Day	NH_4-N Inf. (mgN/L)	NH_4-N Eff. (mgN/L)	NO_2-N Eff. (mgN/L)	NO_3-N Eff. (mgN/L)
39	116.0	9.8	0.0	106.2
43	108.0	12.2	0.0	95.8
45	108.0	9.8	0.0	98.2
50	95.0	10.9	0.0	84.1
52	98.0	7.9	0.0	90.2
1	53.0	5.6	0.0	49.3
3	49.0	7.2	0.0	42.9
7	46.1	7.2	0.0	40.4
10	46.1	6.8	0.0	44.0
14	49.5	4.8	0.0	43.0
17	51.5	3.4	0.0	49.7
21	56.5	3.4	0.0	51.2
24	43.4	7.6	0.0	39.3
28	50.2	5.9	0.0	45.3
31	50.2	5.9	0.0	49.5

Gas composition by percentage in the headspace of the SBR for Phase III, Phase V, and Phase VI, respectively:

Hours	O_2 (%)	N_2 (%)	CH_4 (%)	CO_2 (%)	O_2 (Std)	N_2 (Std)	CH_4 (Std)	CO_2 (Std)
1	86.31	2.64	5.58	8.26	2.28	1.61	2.17	2.53
2	86.31	2.64	5.58	8.26	2.28	1.61	2.17	2.53
3	86.31	2.64	5.58	8.26	2.28	1.61	2.17	2.53
4	44.69	2.92	42.59	14.62	10.37	1.61	12.19	3.81
5	44.69	2.92	42.59	14.62	10.37	1.61	12.19	3.81
6	44.69	2.92	42.59	14.62	10.37	1.61	12.19	3.81
7	63.47	3.10	25.68	13.38	5.54	2.80	6.65	3.61

Hours	O_2 (%)	N_2 (%)	CH_4 (%)	CO_2 (%)	O_2 (Std)	N_2 (Std)	CH_4 (Std)	CO_2 (Std)
1	74.34	16.07	9.27	3.52	5.28	4.08	1.19	0.86
2	79.09	10.05	12.61	3.03	5.56	4.80	1.26	0.22
3	65.84	16.35	18.99	3.38	7.09	6.04	2.06	0.29
4	59.66	17.18	23.85	3.83	6.93	6.36	2.15	0.37
5	58.64	17.43	24.25	4.11	5.68	6.15	1.78	0.35
6	58.56	17.22	24.39	4.20	5.71	6.48	5.74	0.63

Hours	O_2 (%)	N_2 (%)	CH_4 (%)	CO_2 (%)	O_2 (Std)	N_2 (Std)	CH_4 (Std)	CO_2 (Std)
1	64.56	22.33	12.94	6.08	4.20	1.80	2.36	1.67
2	72.61	13.27	16.30	3.61	3.90	2.42	1.55	1.01
3	66.97	13.05	22.09	3.79	5.25	3.71	1.35	0.81
4	63.47	12.24	26.16	4.31	5.85	5.86	0.68	1.10
5	64.69	10.98	25.90	4.74	4.84	5.00	0.79	0.86
5.5	64.56	10.63	26.31	4.84	4.90	4.94	0.86	0.89

Batch denitrification activity results obtained:

Batch	Time (hr)	O_2 Target (%)	NRE (%)	Change in Biomass (mg/L)
I	2	0	4%	95
		10	10%	116
		50	6%	167
II	4	0	5%	108
		10	16%	146
		50	14%	291
III	6	0	8%	217
		10	17%	142
		50	28%	356
IV	18	10	23%	144
		50	66%	425
V	24	10	26%	122
		50	83%	486
VI	48	10	45%	230
		50	97%	572

Chapter 4: Data

Methanotrophic PHB accumulation (mgPHB/mgVSS × 100) under 50% oxygen saturation:

Incubation Period (hrs)	Effluent (50%)	Nitrogen Limiting (50%)	Phosphorus-Limiting (50%)	Eff. Std (50%)	N-Limiting Std (50%)	P-Limiting Std (50%)
6	11%	14%	7%	1.55%	0.14%	0.32%
12	15%	21%	11%	0.67%	1.31%	1.58%
24	12%	19%	7%	0.13%	0.02%	0.62%
48	13%	19%	8%	0.56%	0.06%	0.98%

Methanotrophic PHB accumulation (mgPHB/mgVSS × 100) under 10% oxygen saturation:

Incubation Period (hr)	Effluent (10%)	Nitrogen Limiting (10%)	Phosphorus-Limiting (10%)	Eff. Std (10%)	N-Limiting Std (10%)	P-Limiting Std (10%)
6	13%	17%	8%	0.08%	0.93%	1.38%
12	11%	14%	9%	0.74%	0.92%	0.08%
24	12%	16%	7%	0.05%	0.07%	0.34%
48	15%	14%	6%	0.16%	0.66%	0.58%

Nitrate concentration (mgN/L) observed while utilizing the process effluent medium:

Incubation Period (hr)	Eff. NO_3-N (mgN/L) 10%	Eff. NO_3-N (mgN/L) 50%	Eff. NO_3-N Std (mgN/L) 10%	Eff. NO_3-N Std 50%
0	112.9	112.9	2.0	2.0
6	98.8	69.1	0.8	1.8
12	90.7	48.4	2.0	0.4
24	84.6	18.7	2.3	3.9
48	63.3	2.8	2.5	0.3

Ammonium concentration (mgN/L) observed while utilizing the phosphorus limiting medium:

Incubation Period (hr)	Eff. NH4-N (mgN/L) 10%	Eff. NH4-N (mgN/L) 50%	Eff. NH4-N Std (mgN/L) 10%	Eff. NH4-N Std (mgN/L) 50%
0	171.96	171.96	0.86	0.86
6	162.00	156.39	0.72	0.34
12	161.94	159.93	1.81	0.12
24	160.38	155.93	1.15	0.58
48	161.84	154.33	1.51	1.00