Anaerobic Ammonia Oxidation (ANAMMOX) processes for Ammonium-rich Lagoon Supernatant Treatment

by

Sen Yang

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Abstract

Ammonium-N and phosphate-P-rich anaerobic digester sludge liquor recovered during sewage sludge dewatering contributes more than 30% of the total nitrogen loading when returned to the municipal wastewater plant. Hence, side stream treatment could offer an economic alternative given its relatively low flow and high concentration of nutrients. However, traditional biological nitrification/denitrification requires considerable energy for nitrification and organic carbon for denitrification. During the past decade, considerable work has described the mechanism of the partial nitrification (nitritation) and anaerobic ammonia oxidation (anammox). The nitritation-anammox processes have been demonstrated to be efficient for nitrogen reduction from high ammonia content waste streams. However, no comprehensive study has compared different reactor configurations, operational conditions and their application for sludge liquor treatment.

Hence, the objectives of this PhD dissertation were to assess different reactor design and operational strategies, investigate microbial population diversity, density and functional stability to optimize ammonium reduction processes. In order to assess reactor design, one and two-stage nitritation-anammox reactor configurations, MBBR (moving bed biofilm reactor) and IFAS (integrated fixed film activated sludge) were compared under varying operation conditions (e.g., various aeration, seeding, feeding and control strategies), along with the need of pre-treatment to reduce P-inhibition.

Under best operation conditions, average NH_4^+-N removal efficiency was 89-91% with an NH_4^+-N loading rate of 0.62-0.68 kg N/($m^3 \cdot d$). Intermittent aeration, maintaining a low DO concentration (0.18-0.28 mg/L), sludge recycling for keeping AOB active and specific anammox activity (SAA) monitoring were important strategies for successfully operation of the nitritation-anammox reactor. Microbial analysis showed that nitritation-anammox biomass harbored a high microbial diversity

when feed raw lagoon supernatant. High throughput sequencing indicated that the dominant nitrifiers in the IFAS-SBR, *Nitrosomonas*, facilitated nitritation; and that *Candidatus Brocadia* was the dominate bacteria responsible for the anaerobic ammonia oxidation observed. Overall the present study showed that application for lagoon supernatant treatment is achievable by applying nitritation-anammox processes, either by two-stage or one-stage operation, but that the one-stage configuration provided considerable simplification and reduced nitrite inhibition with sequential aeration strategy provided.

Preface

This thesis is an original work conducted by Sen Yang. All the research work performed in this thesis was designed and planned by me and supervised by Professors Yang Liu and Nicholas Ashbolt at the University of Alberta. I conducted all experiments, collected and analyzed the data and contributed to data interpretation and manuscript preparation. Professor Yang Liu contributed to the research planning, securing funding for research, research discussion and data interpretation, and manuscript edits review and editing of the manuscripts. Mr. Abdul Mohammed and Professor Nicolas Ashbolt contributed to the research discussion, provided research ideas, and they helped to review manuscripts. Some colleagues also contributed to the manuscripts and their contributions are listed as following:

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intermittent aeration in nitritation-anammox systems: Understanding reactor optimization for lagoon supernatant treatment. International Biodeterioration & Biodegradation, 149, 104938.

Dr. Shengnan Xu contributed to the manuscript edits. Dr. Yun Zhou helped to review the response letter during the manuscript revision process.

Chapter 8:

Dr. Riccardo Boiocchi and Dr. Shengnan Xu contributed to the manuscript edits.

DEDICATION

To my beloved wife

Shiwei Li

To my little one

Miles Yang

And To my dear parents

Taolin Yang

Xiangqin Li

ACKNOWLEDGEMENT

To begin with, I would like to express my deepest gratitude and appreciation to my supervisor Prof. Yang Liu. A big thank-you to you for letting me join your research group and always giving me the guidance and directions for my research study. Your enthusiasm on research produces significant effects on myself and other colleagues in my research group. I am greatly thankful to my co-supervisor Prof. Nicholas Ashbolt and Mr. Abdul Mohammed. They always provide me great support and valuable suggestions during my research study.

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LIST OF ABBREVIATIONS

AMO: Ammonia mono-oxygenase

amoA: Ammonia monooxygenase subunit A gene

Anammox: Anaerobic ammonium oxidizers

ANOVA: Analysis of variance

AOB: Ammonia oxidizing bacteria

BNR: Biological nutrient removal

BOD: Biological oxygen demand

C/N: Carbon/nitrogen ratio

CANDO: Coupled aerobic-anoxic nitrous decomposition operation

CANON: Completely autotrophic nitrogen removal over nitrite

CAS: Conventional activated sludge

COD: Chemical oxygen demand

DEMON: De-ammonification

DNRA: Dissimilatory nitrate reduction to ammonia

DO: Dissolved oxygen

FA: Fee ammonia

FNA: Free nitrous acid

HAO: Hydroxylamine oxidoreductase

HRT: Hydraulic retention time

IFAS: Integrated fixed film activated sludge

MBBR: Moving bed biofilm reactor

MLSS: Mixed liquor suspended solids

MLVSS: Mixed liquor volatile suspended solids

N: Nitrogen

NOB: Nitrite oxidizing bacteria

NOR: Nitrite oxidoreductase

OLAND: Oxygen-Limited Autotrophic Nitrification–Denitrification

qPCR: Quantitative polymerase chain reaction

SBR: Sequencing batch reactor

SHARON: Single reactor system for high ammonia removal over nitrite process

SRT: Solids retention time

SVI: Settling volume index

TSS: Total suspended solids

VFA: Volatile fatty acid

WWTP: Wastewater treatment plant

CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1. Background and motivations

Discharge of insufficiently treated municipal wastewater into the natural water bodies can cause eutrophication in the receiving water bodies because municipal wastewater generally contains excessive nutrients (nitrogen and phosphorus). In Canada, sludge generated from municipal wastewater treatment plants is often treated via anaerobic digestion to stabilized the sludge, reduce the sludge volume and to recover energy. Digested sludge liquor separated from the solids through the dewatering processes commonly contains high concentrations of ammonia, and requires further treatment to reduce the ammonia content. As the trend towards greater energy efficiency and higher effluent standards increases (e.g. < 5 mg/L of ammonium concentration in summer; < 10 mg/L of ammonium concentration in winter from Alberta's ammonia discharge limits), more careful attention of the digested sludge liquor treatment have become necessary. The conventional nitrification/denitrification process which requires significant aeration efforts is energy intensive. Newer biological processes, such as the nitritation/denitritation and nitritation/anammox processes require low oxygen demand are emerging, but information on the implementation of these processes at the full-scale is limited, especially for operations under cold Canadian conditions.

1.2. Objectives

The objectives of this study included:

(1) To study the feasibility of applying anammox IFAS configuration for the treatment of digester sludge thickening lagoon supernatant.

(2) To identify key reactor operation conditions to improve the treatment efficiency of one-stage and two-stage nitritation-anammox processes when treating digester sludge thickening lagoon supernatant.

(3) To compare the nitrogen reduction performance of three reactor configurations, including (i) two-stage nitritation-anammox sequencing batch reactor (SBR), (ii) one-stage nitritation-anammox sequencing batch reactor (SBR), and (iii) continuous operating one-stage nitritation-anammox.

1.3. Thesis organization

The structure of this thesis is listed below:

Chapter 1 introduces the motivations of this study and states objectives.

Chapter 2 provides a comprehensive literature review of the principles and applications of nitritation-anammox process either in one stage or two stage.

Chapter 3 presents research results on the feasibility of applying the anammox IFAS configuration (2^{nd} stage of two stage nitritation-anammox) for the treatment of lagoon supernatant.

Chapter 4 demonstrates the overall treatment efficiency and microbial population dynamics of anammox IFAS reactor, under different HRTs and feeding strategies were investigated.

Chapter 5 demonstrates the impacts of Ostara® pre-treatment on anammox bioreactor treatment efficiency. Batch study on the impact of phosphate on the anammox bacteria was also examined.

Chapter 6 evaluated key factors that affect the nitrogen removal efficiency of one-stage nitritation-anammox under IFAS SBR configuration.

Chapter 7 compared (a) MBBR and IFAS configurations, and (b) continuous and intermittent aeration strategies were compared in one-stage nitritation-anammox CSTR configuration.

Chapter 8 investigates the impact of varied DO concentrations on the overall removal performance, AOB and anammox activities in one-stage nitritation-anammox CSTR configuration treating lagoon supernatant.

Chapter 9 summarizes these published studies and compare the nitrogen removal treatment performance and microbial communities between bioreactors operating under various configurations and operational conditions.

Chapter 10 presents main findings of this study and recommendations for further research.

The appendix includes supporting information.

1.4. References

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Szatkowska, B., Plaza, E., Trela, J., Hultman, B., Bosander, J., 2007. Combined partial nitritation and Anammox biofilm system as a sustainable solution for supernatant treatment. Water Practice and Technology 2.

CHAPTER 2. LITERATURE REVIEW

2.1. Research needs on nitrogen reduction

Efficient removal of nutrients, mainly nitrogen and phosphorus, from municipal wastewater has become an important concern because their accumulation in receiving waters can cause eutrophication. Algal and cyanobacterial growth in eutrophied rivers and lakes eventually leads to anoxic conditions that kill oxygen respiring fish and other animals, and release of cyanotoxins that impact on human and animal health. Untreated ammonium is also a major toxin of aquatic biota. Canadian municipal wastewater treatment plant sludge (generated from primary and secondary treatment processes), is typically treated by anaerobic digesters to reduce the sludge volume and to recover bioenergy. Digested sludge liquor can then be separated from solids by dewatering processes. Residual sludge liquor, which contains high ammonia concentrations (often > 1000 mg/L), needs to be managed before disposal.

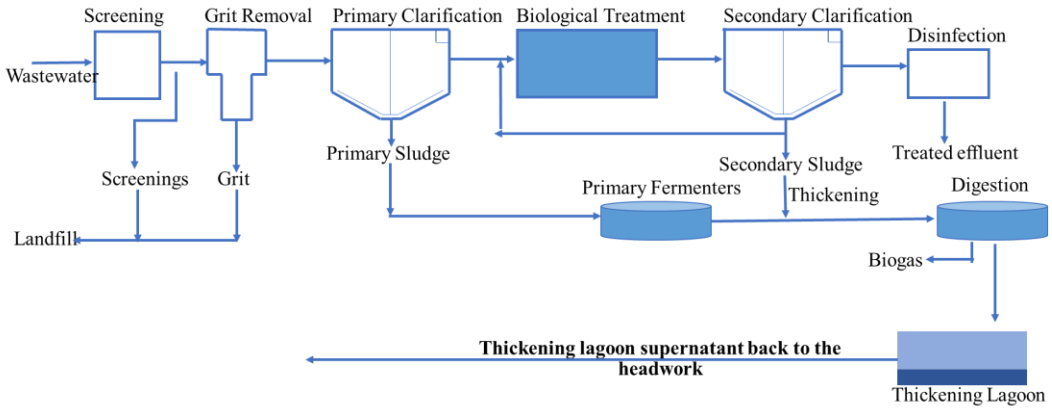

Figure 2.1. A schematic of wastewater treatment processes

Lagoon supernatant is often pumped back to the main wastewater treatment plant for treatment where it contributes about 30% of the plant's incoming nitrogen load but only 1%

to the total wastewater flow, or even lower during high flow conditions (Shao et al., 2018). A schematic of wastewater treatment processes can be found in Figure 2.1. Processes for the treatment of high ammonia lagoon supernatant is known as side-stream treatment.

2.2 Biological nitritation treatment technologies

Compared to chemical nitrogen removal, e.g., struvite (magnesium ammonium phosphate) precipitation, coagulant-aided precipitation, ammonia stripping, ion exchange in selective resins, and breakpoint chlorination, biological treatment is much more economical, energy efficient, and environmentally sound (Gavrilescu and Chisti, 2005). Biological treatment of ammonia-rich wastewater utilizes slow-growing lithoautotrophic bacteria such as *aerobic ammonia-oxidising bacteria* (*AOB*), *nitrite-oxidising bacteria* (*NOB*), *anaerobic anammox bacteria (Anammox)*, and fast-growing *heterotrophic denitrifying bacteria*.

2.2.1 Conventional nitrification/denitrification processes

Nitrification/denitrification processes utilize AOB (which convert ammonia to nitrite), NOB (which convert nitrite to nitrate), and denitrifiers (which convert nitrate to nitrite, nitrite to nitrous oxide, nitrous oxide to nitric oxide, and nitric oxide to nitrogen gas) as shown in Figure 2.2.Nitrification occurs as two-step process. The complete reactions for nitrification, which included biomass production, are as follows (equation 2.1, equation 2.2 and equation 2.3) (Focht and Chang, 1975; Hauck, 1984):

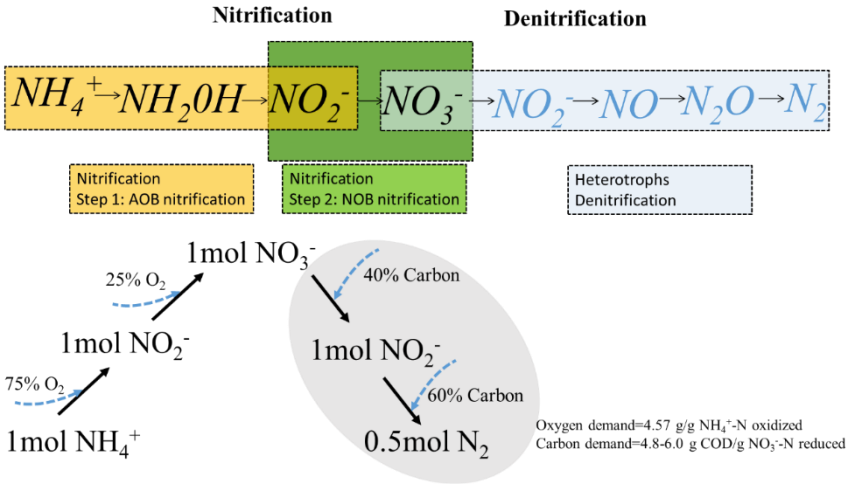

Figure 2.2. Nitrogen transformation, oxygen and carbon requirements for conventional nitrification/denitrification processes

Synthesis and Energy Reaction for AOB (step 1):

$$NH_4^+ + 1.382O_2 + 1.982HCO_3^- \rightarrow 1.891H_2CO_3 + 0.982NO_2^- + 0.018C_5H_7O_2N + 1.04H_2O \quad \text{Equation 2.1}$$

Synthesis and Energy Reaction for NOB (step 2):

$$NO_2^- + 0.0025NH_4^+ + 0.01H_2CO_3 + 0.488O_2 + 0.0025HCO_3^- \rightarrow NO_3^- + 0.025C_5H_7O_2N + 0.0075H_2O \quad \text{Equation 2.2}$$

Overall Synthesis and Energy Reaction:

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 1.88H_2CO_3 + 0.98NO_3^- + 0.021C_5H_7O_2N + 1.041H_2O \quad \text{Equation 2.3}$$

The metabolism reaction for heterotrophic denitrification (acetic acid as the carbon source) is illustrated in equation 2.4:

$$5CH_3COOH + 8NO_3^- + 8H^+ \rightarrow 4N_2 + 10CO_2 + 140H_2O$$ Equation 2.4

Five genera of AOB have been reported: *Nitrosococcus*, *Nitrososmonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus (Jiang and Bakken, 1999)*. Seven genera of NOB have been reported: *Nitrobacter*, *Nitrospina*, *Nitrospira*, *Nitrotoga*, *Nitrolancea*, *Nitrococcus*, and *Candidatus Nitromaritima* (De Boer and Kowalchuk, 2001). *Achromobacter, Thiobacillus*, *Micrococcus* and some species of *Pseudomonas* are identified as denitrifiers (Coyne et al., 1989).

Oxygen and carbon requirements for nitrification/denitrification process are illustrated in Figure 2.2. Based on the above reaction, 4.57 g O_2 is needed per g of NH_4^+-N oxidized and 4.8-6.0 g COD is needed per g of NO_3^--N denitrified (Dalentoft and Jenson, 1994). Usually, nitrite oxidation by nitrite oxidation proceeds more rapidly than ammonia oxidation, and nitrite rarely builds up in the reactors. This process can be effectively utilized for municipal wastewater treatment. However, the treatment of ammonia-rich sludge liquor using this process can be costly due to a high oxygen demand for nitrification and a high carbon source demand for denitrification. It is noted that sludge liquor often contains low COD/N ratios (COD/N < 0.5) due to the extended COD degradation and ammonia dissolution in the digesters.

2.2.2 Nitritation/denitritation processes

Nitritation/denitritation processes utilize AOB which convert ammonia to nitrite (nitritation) and denitrifiers which convert nitrite to nitrous oxide, nitrous oxide to nitric oxide, and nitric oxide to nitrogen gas (denitritation), and eliminate the growth of NOB in the bioreactors (Hellinga et al., 1998). Nitritation step is divided into two steps with

hydroxylamine (NH_2OH) as the intermediate product. In the first step (equation 2.5), NH_3 was oxidized to NH_2OH. In the second step (equation 2.6), NH_2OH was further oxidized to NO_2^-. Equation 2.8 is overall reaction for nitritation carrier by AOB. The autotrophic AOB utilizes NH_4^+ for cell growth where the CO_2 is the carbon sources as shown in equation 2.9. The metabolism reaction for denitritation (acetic acid as the carbon source) is illustrated in equation 2.10.

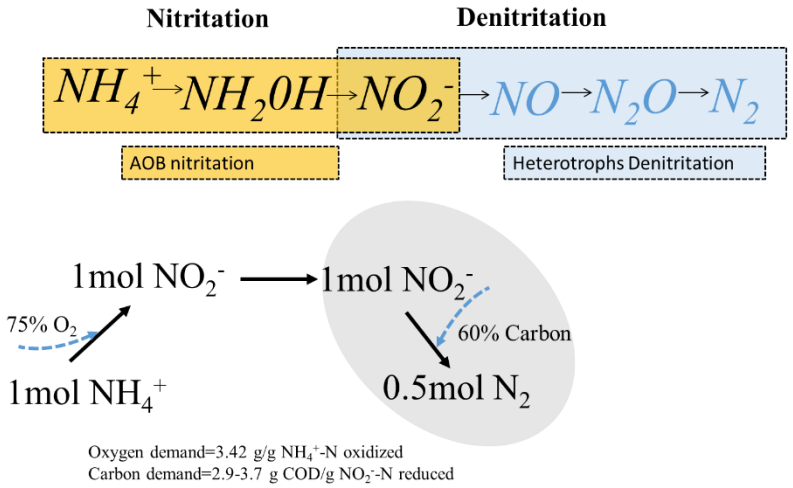

Figure 2.3. Nitrogen transformation, oxygen and carbon requirements for nitritation/denitritation processes

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad \text{Equation 2.5}$$

$$NH_2OH + H_2O \rightarrow NO_2 + 5H^+ + 4e^- \quad \text{Equation 2.6}$$

$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad \text{Equation 2.7}$$

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O \quad \text{Equation 2.8}$$

$$4CO_2 + 4H_2O + NH_4^+ + HCO_3^- + 0.13H^+ \rightarrow 5O_2 + C_5H_7O_2N + 3H_2O \quad \text{Equation 2.9}$$

$$3CH_3COOH + 8NO_3^- + 8H^+ \rightarrow 4N_2 + 6CO_2 + 10H_2O \quad \text{Equation 2.10}$$

Oxygen and carbon requirements for nitritation/denitritation process are illustrated in Figure 2.3. Based on the above reaction, 3.42 g O_2 is needed per g of NH_4^+-N oxidized and 2.9-3.7 g COD is needed per g of NO_2^--N denitrified (Balmelle et al., 1992). This process can save up to 20% of the oxygen demand and up to 40% of the carbon source requirements compared to a conventional nitrification/denitrification process.

2.2.3 Nitritation/anammox processes

Further, the nitritation process may be combined with the anammox (anaerobic ammonium oxidation) as the ***nitritation/anammox*** processes (Figure 2.4). This process has been implemented in full-scale treatment plants over the past 15 years. Anammox bacteria anaerobically oxidize ammonia using nitrite as an electron acceptor, a process that produces nitrogen gas (Strous, 1997). The anammox bacteria is autotrophic bacteria. The anammox bacteria utilizes CO_2 as the carbon source and the energy is obtained via oxidizing NO_2^- to NO_3^-. Anammox bacteria are classified as Planctomycetes phylum (Jetten et al., 1998; Schmid et al., 2000).

The nitritation/anammox process can be either two-stage system or one-stage system (Van der Star et al., 2007; Van Hulle et al., 2010). In two-stage system, nitritation and anammox process are separated into two reactors. The reactions for the 1st stage of nitritation reaction is shown in the equation 2.1.

The overall stoichiometry for the 2nd stage of anammox reaction is shown in equation 2.11. In one-stage system, AOB and anammox bacteria are co-existed. Equation 2.12 shows the combined reactions for one-stage nitritation-anammox process.

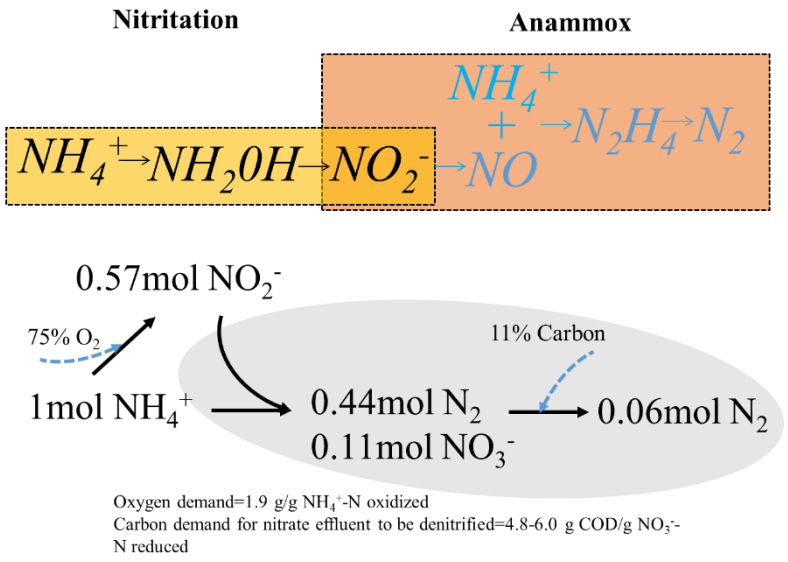

Figure 2.4. Nitrogen transformation, oxygen and carbon requirements for nitritation/anammox processes

Oxygen and carbon requirements for nitritation/anammox process are illustrated in Figure 2.4. Based on the above reaction, 1.9 g O_2 is needed per g of NH_4^+-N oxidized and there is no need to add external carbon sources for nitritation/anammox process.

$$NH_4^+ + 1.382O_2 + 1.982HCO_3^- \rightarrow 1.891H_2CO_3 + 0.982NO_2^- + 0.018C_5H_7O_2N + 1.04H_2O \quad \text{Equation 2.1}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad \text{Equation 2.11}$$

$$NH_4^+ + 1.5O_2 + 0.028HCO_3^- \rightarrow 0.43N_2 + 0.11NO_3^- + 0.028CH_2O_{0.5}N_{0.15} + 1.44H_2O + 0.51H^+ \quad \text{Equation 2.12}$$

Except that the amount of NO_3^--N produced accounts for 11% of the NH_4^+-N and NO_2^--N metabolized during nitritation/anammox process and it requires addition 4.8-6.0 g COD

per g of NO_3^--N denitrified in the final effluent if needed. Overall, oxygen requirements for nitritation/anammox is 57% less than those for conventional nitrification/denitrification because only half of the ammonia is converted to nitrite ion aerobically, while the rest is converted to N_2 anaerobically via anammox.

2.3 Strategies for achieving and maintaining nitritation process

2.3.1 Free ammonia (FA) and free nitrous acid (FNA)

Both of free ammonia (FA) and free nitrous acid (FNA) can impact nitrification reactions. High levels of FA is considered as an inhibitor that can inhibit the activity of nitrite oxidoreductase of NOB (Yang and Alleman, 1992). The FNA inhibition mechanism is that HNO_2 is the toxicity form that inhibits nitrification/denitrification process. In other words, HNO_2 acts as an uncoupler by donating a proton which further interferes with the transmembrane pH gradient (Glass et al., 1997). Free ammonia (FA) and free nitrous acid (FNA) are determined by pH, temperature, NH_4^+ or NO_2^- (equation 2.13 and 2.14) (Anthonisen et al., 1976).

$$\text{FA as } NH_3 \left(\frac{\text{mg}}{\text{L}}\right) = \frac{17}{14} \times \frac{total\ ammonia\ as\ N(\frac{mg}{L}) \times 10^{pH}}{K_b/K_w + 10^{pH}} \qquad \text{Equation 2.13}$$

$$\text{FNA as } HNO_2 \left(\frac{\text{mg}}{\text{L}}\right) = \frac{46}{14} \times \frac{NO_2^- - N(\frac{mg}{L})}{K_a \times 10^{pH}} \qquad \text{Equation 2.14}$$

Where $K_b/K_w = e^{(6344/273+℃)}$ and $K_a = e^{(-2300/273+℃)}$

The reported FA threshold concentrations for inhibiting NOB varies. Rongsayamanont et al. (2010) found that NOB activity was affected when FA reached 5 mg L^{-1}. In the study of Kim et al. (2003), the activity of NOB in a membrane bioreactor was completely

inhibited at FA of 0.2 mg L^{-1} and NOB activity recovered when FA was lower than 0.2 mg L^{-1}. FNA is also considered as an important parameter for inhibiting the activity of NOB especially at low pH (<7.5) (Zhou et al., 2011). The inhibition of FNA on AOB and NOB was reported at 2.8 and 0.06 mg L^{-1}, respectively (Sudarno et al., 2011). In the study of Wang et al. (2017a), it was found that 1.9 mg N L^{-1} of FNA caused over 60% of NO_2^- accumulation in the nitritation bioreactor suggesting successful repression of NOB activity.

2.3.2 Dissolved oxygen (DO)

The DO half saturation coefficient of AOB (0.2-0.4 mg O_2 L^{-1}) is much lower than that of NOB (1.2-1.5 mg O_2 L^{-1}) (Wilczak et al., 1996). The difference allows AOB to outcompete NOB when dissolved oxygen concentration in bioreactor keeps low because of a higher affinity for AOB. Hanaki et al. (1990) found that nitrite oxidation rate carrier out by NOB was significantly affected at 0.5 mg L^{-1} of DO condition in flocs system. In the membrane bioreactor, NO_3^- concentration in the effluent increased at DO of 5.0 mg L^{-1} suggesting that complete nitrification occurred. Furthermore, NO_2^- began to accumulate when DO was decreased from 5 mg L^{-1} to 0.5 mg L^{-1} suggesting that NOB activity was inhibited after conditions change (Bernet et al., 2001). In the study of Ruiz et al. (2003), the optimal DO concentration for achieving stable nitritation process was 0.7 - 1.5 mg L^{-1}. Chuang et al. (2007) concluded that the optimal DO was 0.2 mg L^{-1} where the maximum ammonia oxidation rate achieved 1.46 kg N m^{-3} d^{-1}.

2.3.3 pH

The optimal pH range for AOB growth is 7.0 - 8.5 and for NOB is 6.0 - 7.5 (Anthonisen et al., 1976). Kumar and Nicholas (1983) found that the activity of AOB was adversely

affected when pH is under 6.5 - 7.0. It is suggested that pH should keep at 7.5 - 8.5. In the study of Peng and Zhu (2006), the changes of pH affected the concentration of FA and FNA which will further influence the activities of AOB and NOB.

2.3.4 Temperature

At temperatures between 10 and 20 ºC, the specific growth rate of NOB is significantly higher than that of AOB in the bioreactor (Hynes and Knowles, 1983). Previous study found that the growth rate of NOB-*Nitrobacter* was higher than AOB-*Nitrosomonas* when the temperature was lower than 12 ºC (Helder and De Vries, 1983). Increasing temperature ensures successful enrichment of AOB population while repressing the activity of NOB in the bioreactor. When temperature was higher than 25 ºC, results showed a higher ammonia oxidation rate than nitrite oxidation rate in flocs system (Hellinga et al., 1998). The optimal temperature for maintaining high activity of AOB is 25 - 35 ºC. Moreover, it should be noted that the temperature also affects the chemical equilibriums of FA and FNA (Gabarró et al., 2012).

2.3.5 Sludge retention time (SRT)

The minimum doubling time for AOB and NOB are 7 h and 13 h (Pérez et al., 2014), indicating that AOB can outcompete NOB by adjusting sludge retention time (SRT) in the bioreactor. In the study of Pollice et al. (2002), the effects of SRT on the nitritation performance was investigated. Results showed that the maximum nitrite accumulation was increased from 0 to 500 mg L^{-1} when SRT was reduced from 40 day to 10 day suggesting that by reducing SRT, the AOB activity became dominate.

2.4. Strategies for achieving and maintaining anammox process

2.4.1 Substrates

NH_4^+ and NO_2^- are essential substrates for the growth of anammox bacteria. However, high levels of substrates are toxic to anammox bacteria. Previous study found that at the 600 mg N L^{-1} of NH_4^+ concentration in the batch study, the inhibition on anammox bacteria was obviously observed (Carvajal-Arroyo et al., 2013). The mechanisms of the FA impact on anammox bacteria are discussed in the following part 2.3. Similarly like the FA inhibition mechanism on anammox bacteria activity, FNA (free nitrous acid) has been reported in various studies to be the cause of inhibition (Zhou et al., 2011). In the study of Carvajal-Arroyo et al. (2014), the activity of anammox bacteria was completely inhibited when the feed NO_2^- concentration was higher than 100 mg L^{-1}. Similar levels of NO_2^- inhibition impacts on the anammox reactions were also reported, and the nitrite inhibition on the anammox reactions was believed to be reversible (Fux et al., 2004; López et al., 2008).

2.4.2 Temperature

Based on previous research study, the optimum temperature range for anammox reaction is 30–40 ºC (Strous et al., 1999). At high temperature condition, the membrane proteins are denaturized which causes of release of intracellular organelles and further cell lysis for the anammox reaction (Boleij et al., 2019). Low temperature condition decreases the bacteria growth rate, enzyme activities, and therefore affecting the activity of anammox bacteria (Van Loosdrecht and Jetten, 1998). In the study of Strous et al. (1997), the optimal temperature range for the anammox bacteria growth was between 30 ºC and 37 ºC. Isaka et al. (2008) evaluated the impact of temperature on NH_4^+ removal efficiency. Results showed that NH_4^+ removal efficiency reached 11.5 kg N m^{-3} d^{-1} when the temperature was kept at 37 ºC while the NH_4^+ removal efficiency decreased to 8.1 kg N m^{-3} $d^{-1}$and 0.36 kg

N m^{-3} d^{-1}, respectively when the temperature dropped to 20-22 ºC and 6 ºC. The tolerance ability for anammox bacteria to varied temperatures were reported to be dependent on the different species of the anammox bacteria (Park et al., 2017; Wu et al., 2018). Previous studies reported that the activity of the anammox genus *Candidatus Scalindua* was observed at 20 and 30 °C, but not at 10 °C in a up-flow fixed-bed column reactor (Awata et al., 2012). In the study of Narita et al. (2017), the maximum nitrogen removal rates were 6.7 and 1.1 g N L^{-1} d^{-1} at 35 °C and 15 °C, respectively where *Candidatus Brocadia sinica* was enriched in membrane bioreactor.

2.4.3 Free ammonia (FA) and free nitrous acid (FNA)

The FA and FNA inside the cell change the pH and neutralizes the transmembrane potential which will cause cell death during anammox reactions (Zhang et al., 2016). It was reported that activity of anammox bacteria was significantly affected when FA was higher than 14 mg L^{-1} (Waki et al., 2007). The lowest of FA inhibition threshold on anammox bacteria was reported at concentration of 2.0 mg L^{-1} (Jung et al., 2007). In the study of Strous et al. (1999), FNA at level of 6.0 μg HNO_2-N L^{-1} caused complete inhibition on the activity of anammox bacteria. Anammox bacteria was inhibited when the concentration of FNA was increased to 0.04 mg HNO_2-N L^{-1} (Egli et al., 2001). Therefore, the control of FA and FNA is important for maintaining a stabilized operation system.

2.4.4 Organic carbon sources

Organic carbon source can adversely impact anammox reactions through the following two different proposed mechanisms among different studies: in the first reported mechanism, inhibition on anammox bacteria occurs due to the competition between the anammox

bacteria and the heterotrophs especially under high concentrations of organic matters since the growth rate of heterotrophs is much higher than anammox bacteria (Lawson et al., 2017). In the study Güven et al. (2005), the growth rate of anammox bacteria was affected because of the competition with heterotrophs in one reactor system when influent ratio of C/N was high than 1. In the study of Chamchoi et al. (2008), the activity of anammox bacteria was completely inhibited when COD concentration exceeded 200 mg L^{-1}. The activity of anammox bacteria was reduced to 30% when ethanol concentration in the influent was higher than 50 mM. The application of anammox bacteria is suitable for the treatment of wastewater with low C/N ratio (Gao et al., 2012). In the second reported mechanism, the anammox bacteria utilizes organic matter instead of using NH_4^+ and NO_2^- as substrates (Giustinianovich et al., 2016). As a result, the nitrogen removal efficiency performed by anammox bacteria is adversely affected.

2.4.5 Other reported influencing factors

High salinity results in high osmotic pressure, and the anammox bacteria in high salt concentrations will die or become dormant (Kartal et al., 2006). In the study of Liu et al. (2009), the nitrogen removal efficiency carried out by anammox bacteria under anoxic condition was sharply reduced when salt concentration was increased to 30 g L^{-1}. Phosphate is also reported as inhibitor on the anammox reactions. The enzyme activity of anammox bacteria was affected under high PO_4^{3-} feed condition, through forming complex structures which causes a lower enzyme activity (Zhang et al., 2017). Previous study reported that 150 mg P L^{-1} of PO_4^{3-} caused a loss of the anammox activity (Van de Graaf et al., 1996). The activity of anammox bacteria was found 1.6-times lower at 60 mg P L^{-1} of PO_4^{3-} and 5-times lower at 120 mg P L^{-1} of PO_4^{3-} feed conditions (Pynaert et al., 2003). Since

anammox bacteria are light-sensitive microorganisms, the activity of anammox bacteria can be inhibited with light. Previous study reported that long term exposure of light had adversely impact on the growth of anammox bacteria (Jin et al., 2012).

2.5 Nitritation-anammox process applications

2.5.1 One-stage nitritation-anammox

Depending on the aeration strategies, dominant microbial community, suspended or biofilm growth and reactor control strategies, one stage nitritation-anammox process include: DEMON (Deammonification) (Wett et al., 2015), OLAND (oxygen limited autotrophic nitrification denitrification) (Schaubroeck et al., 2012), CANON (completely autotrophic nitrogen removal over nitrite) (Vázquez-Padín et al., 2009), SNAP (single-stage nitrogen removal using anammox and nitritation) (Zhang et al., 2014).

In this process, NH_4^+ is partially oxidized to NO_2^- carried out by AOB. Under oxygen limited conditions, the activity of NOB is successfully repressed due to a lower affinity of NOB to oxygen compared to AOB. In the meantime, the oxidized NO_2^- and the remaining NH_4^+ in the bioreactor is further converted to N_2 carried out by anammox bacteria either simultaneously (Corbalá-Robles et al., 2016) or intermittently (Yang et al., 2015) depending on the operation strategy and its activity performance. Equation 2.12 shows the overall reaction for one stage nitritation-anammox process. As discussed in part 2.3 and 2.4, useful strategies can be applied for maintain a stabilized operation of nitritation and anammox process. Those control parameters include substrates loading, temperature, dissolved oxygen concentration, SRT, FA and FNA. Yang et al. (2016) investigated the different process control strategies for one stage nitritation-anammox operation. Results

showed that the optimal DO concentration was 0.70 - 0.80 mg L^{-1}. The activity of anammox bacteria began to decrease at a higher DO (1.0 - 1.2 mg L^{-1}) condition. In the study of Lackner and Horn (2013), moving-bed biofilm reactor (MBBR) operation and SBR operation for one stage nitritation-anammox were compared. Results showed that MBBR system achieved higher nitrogen removal rates than SBR operation because of a higher biomass and less aeration limitation. Various configurations of one stage nitritation-anammox process have been established for the pilot-scale and full-scale operation. The configurations include the moving bed biofilm reactor (MBBR), integrated fixed-film activated sludge (IFAS), granular sludge and SBR (Castro-Barros et al., 2015; Malovanyy et al., 2015a; Malovanyy et al., 2015b). Among those configurations, SBR is the most commonly applied reactor type for nitritation-anammox process and over 80% of configurations are operated as one stage nitritation-anammox process (Lackner et al., 2014).

2.5.2 Two-stage nitritation-anammox

This two stage nitritation-anammox is also called Sharon®-Anammox® (single reactor high activity ammonia removal over nitrite-anaerobic ammonium oxidation) processes (Hwang et al., 2005). Equation 2.1 and 2.11 show the separated reactions in two reactors for the two stage nitritation-anammox. In the first aerobic reactor, NH_4^+ is partially oxidized to NO_2^- by AOB. Ideally, almost no NO_3^- is produced in this reactor under specific operation conditions (i.e. higher temperature, high levels of FA and low dissolved oxygen concentration) where the activity of NOB is inhibited. In the second anaerobic reactor, the nitritation effluent containing the remaining NH_4^+ and produced NO_2^- is further treated carried out by anammox bacteria (Gut et al., 2006). Unlike one stage nitritation-anammox process, high levels of NO_2^- concentration in the bioreactor should be prevented in case the

activity of anammox bacteria is inhibited due to the accumulation of NO_2^- (Kimura et al., 2010). In two-stage nitritation-anammox process, two reactors are separated where the effluent from nitritation process is used as the influent for the 2nd stage of anammox process. For nitritation process (1st stage), SHARON is most intensively applied technologies for the treatment of high ammonia rich wastewater. The operation temperature was 30 - 40 ºC which enabled successful repression of NOB activity. For anammox process (2nd stage), SBR operation has been reported as most reliable reactor for the anammox enrichment. In the study of Fux et al. (2002), a pilot scale SBR reactor for two-stage system was investigated. Over 90% of the total influent nitrogen was removed. A continuously stirred tank reactor (CSTR) was successfully applied for anammox process (Wang et al., 2017b). The results suggested that the addition of hydroxylamine could be applied for fast reactivation of anammox bacteria. Two-stage process has no risks for oxygen inhibition on the anammox bacteria. Also, two-stage has lower risks for anammox to be out competed by heterotrophs since most of carbon sources were utilized in the 1st stage of nitritation reactor. One stage operation has lower risk for NO_2^- inhibition. However, the interactions between different groups of bacteria (i.e. AOB, NOB, heterotrophic bacteria and anammox bacteria) are needed to be monitored and analyzed (Jaroszynski and Oleszkiewicz, 2011).

2.5.3 Operational challenges for nitritation-anammox process

The operation for nitritation-anammox process either in one stage or two stage have been a challenge. Control for NOB growth, adjustments for dissolved oxygen concentrations, NO_3^- build up, interferences with heterotrophs and anammox seed sludge souses are considered as operational challenges especially due to the sensitivity of anammox bacteria during the start-up and optimization of this process. Since the final product of nitritation

process is NO_2^-, the NO_2^- from the effluent in nitritation process are needed to be treated (Ge et al., 2015). Either anammox process or denitrification process can be the secondary treatment options. Moreover, for the side stream treatment, biggest of concern by applying nitritation process is the production of high level of N_2O (Okabe et al., 2011). Incomplete AOB denitrification is believed the main pathway and the high NO_2^- accumulation triggers the N_2O emissions (Pan et al., 2016). While in the nitritation reactor, AOB is dominant and the NO_2^- is the final product. Many of researchers now are focusing on how to mitigate the N_2O emissions for the treatment of side stream wastewater.

For the anammox bacteria enrichment, there is a shortage of the anammox seed sludge. In nature, no anammox bacteria activity can be detected because of its low abundance. It also takes a long time to start up the reactors (Van der Star et al., 2007). The start-up time for the first full-scale anammox reactor lasted for 3.5 years in Rotterdam (Lackner et al., 2014). Studies will be needed to investigate different strategies to speed up the start-up time. High levels of NH_4^+ and NO_2^- have toxicity to anammox bacteria (Tang et al., 2010). The inhibition caused by substrates results in instability of the anammox process, especially for the high influent concentrations of the NO_2^- for two-stage reactor. Dissolved oxygen (DO) concentration control for one stage system is important since anammox bacteria is sensitive to oxygen. A high DO will result in complete nitrification while low DO will affect the NH_4^+ oxidation rate which will impact the overall nitrogen removal efficiency.

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CHAPTER 3 THE VALUE OF FLOCS AND BIOFILM BACTERIA FOR ANAMMOX STABILITY WHEN TREATING AMMONIA-RICH DIGESTER SLUDGE THICKENING LAGOON SUPERNATANT [1]

3.1. Introduction

Biological treatment of ammonia-rich wastewater by conventional nitrification-denitrification processes requires considerable energy (to deliver aeration) and external organic carbon source (for denitrification), which results in a high solids production and solids management requirement, and often faces problems of poor performance and instability (Khin and Annachhatre, 2004). Anaerobic ammonium oxidation (anammox) provides an energy/cost-effective alternative for ammonium removal with wastewaters high in nitrogen (Jetten et al., 2001). The anammox bacteria utilize ammonium and nitrite directly under anoxic conditions to form dinitrogen gas (Strous et al., 1998). Recently, the feasibility of the anammox in various reactor configurations and modes of operation to treat ammonia-rich wastewaters generated from anaerobic digestion of sludge have been demonstrated (Jeanningros et al., 2010; Gonzalez-Martinez et al., 2011; Schaubroeck et al., 2012; Pereira et al., 2017). In particular, attached biomass (*i.e.*, biofilm) and granular sludge have been utilized to have a longer retention time, reducing wash out and promoting the growth of slow growing bacteria (*i.e.*, anammox bacteria). Previous studies have also shown that as compared to flocs, biofilm and granular sludge biomass better tolerate stress conditions (Huang et al., 2015; Shi et al., 2015). However, study also showed that flocs

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with a loose structure have advantages in substrates transportation and diffusion from the liquid phase into the flocs. And it can enhance the growth of microbes and the removal of contaminant (Pérez et al., 2005). Hence, attached biomass and flocs may differ in their physical structural properties, but it is unreported how this may impact on their functional microbial diversity.

Bioreactors that operate with both biofilm (i.e. MBBR) and flocs are called integrated fixed-film activated sludge (IFAS) reactors. In this IFAS configuration, biofilm could enrich the slow growing anammox bacteria and their detachment may lead to "seeding" of the mixed liquor flocs. Thus, the IFAS configuration combines the advantages of biofilm on a carrier and suspended growth systems, and biomass in attached and suspended phases may work synergistically to enhance ammonia removal.

Previous studies have demonstrated that hybrid IFAS systems which have segregated microbial communities and functions between biofilm and flocs could improve the one stage partial nitrification and anammox (PN/A) process (Laureni et al., 2019); however, no studies have been reported on the impact of IFAS on the second stage (*i.e.*, anammox only stage) of a two-stage PN/A process. Our current study addresses that research gap, and was conducted to (1) investigate the feasibility of applying the anammox IFAS configuration (second stage of two stage PN/A) for the treatment of digester sludge thickening lagoon supernatant, and (2) investigate the responses of biofilm and flocs in the hybrid system under high organic loading conditions. Initially, a synthetic nitrite source was used, followed by switching the reactor feed to a nitritation effluent to demonstrate the second stage of a PN/A system. Biofilm and flocs in the IFAS reactor were assessed, comparing the variations of the microbial community in the reactor.

3.2. Methods and materials

3.2.1. Raw lagoon supernatant feeding

Ammonia-rich supernatant was collected from a digester sludge thickening lagoon in the City of Edmonton once a month. The lagoon supernatant was pretreated on-site in an Ostara® facility for phosphorus removal. Pretreated lagoon supernatant was then transported to the University of Alberta Campus and used as laboratory bioreactor feed. To minimize the microbial activity, the collected lagoon supernatant was stored in a 4 °C cold room prior to use.

3.2.2 Feed water quality

The water chemistry of the reactor feed water in three different operation phases, the lagoon supernatant and the nitritation reactor effluent (used as part of phase III feed) is shown in Table 3.1. Reactor feed water in Phase I and phase II was supplemented with $NaNO_2$. As shown in Table 3.1, NH_4^+-N in the feed was 250 ± 20 in phase I and increased to 420 ± 35 mg-N/L in phase II. NO_2^--N was supplemented to maintain a molar ratio of NH_4^+-N to NO_2^--N at 1:1.32 required for anammox reaction; measured concentrations were 325 ± 35 and 525 ± 45 mg-N/L in phase I and phase II, respectively. The COD concentration increased from 145 ± 25 mg/L in phase I to 305 ± 55 mg/L in phase II.

In phase III, the effluent from a laboratory nitritation reactor treating lagoon supernatant was used to provide NO_2^--N and mimic the complexity of real wastewater. Design, operation conditions, and reactor performance of the nitritation reactor are available in our previously published literature (Shao et al., 2019). The nitritation reactor converted over 95% of NH_4^+-N to NO_2^--N, and the average effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations were 10 ± 5 mg-N/L, 965 ± 55 mg-N/L, and 22 ± 3.5 mg-N/L, respectively.

After mixing, phase III feed water NH_4^+-N and NO_2^--N concentrations were 435 ± 45 mg-N/L and 535 ± 65 mg-N/L, respectively, comparable to the Phase II feed water condition ($P > 0.05$). The COD concentration in phase III increased significantly to 583 ± 75 mg/L, as compared to phase II ($P < 0.05$).

3.2.3. Anammox reactor operation

A 6 L working volume of bench scale integrated fixed film activated sludge (IFAS) reactor was operated in a sequencing batch mode for 259 days. The reactor was initially seeded with AnoxK™5 biofilm carriers, kindly provided by Veolia (Montreal, Canada), with a 38% volume fill ratio (*i.e.*, volume ratio of biocarriers and total reactor volume).

Reactor operation can be divided into three phases. In phases I and II, 25% and 50% lagoon supernatant feed water was prepared by diluting raw lagoon supernatant with deionized (DI) water in 1:3 and 1:1 volume ratio, respectively. Sodium nitrite was supplemented as the NO_2^--N source to bioreactor at a molar ratio of 1.32:1 of NO_2^--N (from $NaNO_2$) to NH_4^+-N (from diluted lagoon supernatant). In phase III, raw lagoon supernatant was used as reactor feed water. The effluent of a continuous operating nitritation reactor treating lagoon supernatant with high ammonium to nitrite conversion rate (> 95%) was used as the NO_2^--N source. The molar ratio of NO_2^--N (effluent from nitritation reactor) to NH_4^+-N (from raw lagoon supernatant) was maintained at 1.32:1.

A sequencing batch reactor (SBR) mode was applied in the anammox reactor. Each SBR cycle was 12 hours, which included 20 min feed, 11 h reaction, 30 min settle, 5 min withdrawal, and 5 min idle. The exchange ratio for each SBR cycle was kept at 20%, which resulted in a hydraulic retention time of 2.5 days. The reactor had a double jacket to maintain constant temperature at 30 °C.

3.2.4. Sample collection and analytical methods

Reactor influent and effluent were sampled every 2-3 days during the operation. Water quality including the concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N were measured with Hach kits (10205, 10206 and 10207, respectively, Loveland, Colorado). Chemical oxygen demand (COD) and 5-day biochemical oxygen demand (BOD_5) were measured according to Standard Methods ((APHA, 2005); 5220D, 5210B, respectively). Collected samples were filtered (0.45 μm pore) and analyzed within 2 h of collection for COD, inorganic nitrogen concentrations (Shao et al., 2017). The biomass in the carriers was measured using methods from Wells et al. (2017). The contribution of the anammox bacteria and heterotrophic denitrification processes on the nitrogen removal was determined according to Ruscalleda et al. (2008).

3.2.5. Specific anammox activity (SAA) tests

The anaerobic ammonia oxidation rates of biofilm and flocs were performed. Biomass samples were obtained at a biofilm to sludge volume ratio of 40 biocarriers: 350 mL sludge under the steady-state conditions in each phase and washed with phosphate buffer to remove residual compounds. The biomass samples were transferred to batch reactors filled with 350 mL substrate solutions containing NH_4Cl and $NaNO_2$ at concentrations of 70 mg NH_4^+-N/L and 95 mg NO_2^--N/L. Three sets of batch experiments were performed for each collection, including flocs only, biofilm only, and abiotic control (no biofilm or flocs). All sets were performed in triplicates. The O_2 was removed by purging nitrogen gas for 20 min, and the batch reactor bottles were immediately sealed with butyl rubber and placed in a shaker at 31 ± 1 °C and 180 rpm. Samples were collected along reaction time. The NH_4^+-

N and NO_2^--N uptake rates were determined using the linear regression of concentration over time divided by the biomass, a method previously demonstrated (Bassin et al., 2012).

3.2.6. q-PCR analysis and 16S rRNA gene sequencing

Biofilm and floc samples were collected for genomic DNA extraction under steady-state conditions of each operation phase, on days 80 (Phase I), 160 (Phase II) and 255 (Phase III). DNA was extracted using MO BIO PowerSoil® DNA Isolation Kits (MoBio Laboratories Inc, Carlsbad, California), according to the manufacturer's instructions.

Population of anammox bacteria and denitrification bacteria were determined by qPCR targeting AMX *nirS* gene for anammox (Li et al., 2011), and *nosZ* (Kim et al., 2011) and *narG* (Kandeler et al., 2006) genes for denitrification using qPCR kits (Bio-Rad Laboratories, Mississauga, Ontario). Information about the primers and qPCR reaction conditions is shown in Table A-1 (Appendix A). The qPCR amplification program for each gene is summarized in Table A-2 (Appendix A).

Extracted DNA samples were sent to a commercial lab for 16S rRNA gene sequencing on an Illumine MiSeq platform at the Research and Testing Laboratory (Lubbock, TX, USA) to determine bacterial community diversity and composition. The V3-V4 regions of 16S rRNA genes were amplified using primer set 357wF (CCT ACG GGN GGC WGC AG) and 785R (GAC TAC HVG GGT ATC TAA TCC). Sequencing data processing and taxonomic classification were analyzed using QIIME pipelines (Caporaso et al., 2010) with references in the Silva database. The alpha-diversity, beta-diversity, and principal coordinates analysis (PCoA) were performed using the "vegan" package in RStudio (Jari Oksanen et al., 2017).

3.2.7. Statistical analysis

T-test and single factor analysis of variance (ANOVA) at a 5% probability level were performed, and were reported as p-values.

3.3. Results and discussion

3.3.1 Reactor performance

Figure 3.1A shows the concentrations of NH_4^+, NO_2^- and NO_3^- in the influent and effluent over the period of 259 days. During phase I (day 0 - day 83), the total NH_4^+-N removal efficiency of over 94% was consistently recorded (Figure 3.1B). The NO_2^--N removal efficiency varied between 93% and 98%. The NO_3^--N concentration was 48.9 ± 4.5 mgNO_3^--N/L, which was produced as part of the NO_2^- was oxidised to NO_3^- to provide electrons for carbon fixation during anammox reaction. The ratio of NO_3^--N generated to NH_4^+-N oxidized was 0.21 ± 0.04 during phase I (Figure 3.1B), which is lower than the theoretical ratio of 0.26 based on the anammox kinetics (Strous et al., 1998); indicating the existence of nitrate reduction through denitrification. Under the phase II conditions (day 84 - day 162), the NH_4^+-N removal efficiency at the steady state slightly decreased to 89 ± 3.2% ($P > 0.05$).

In phase III, effluent from a partial nitrification reactor treating lagoon supernatant was used as the nitrite source. As shown in Figure 3.1B, the total NH_4^+-N nitrogen removal efficiency (87 ± 2.9%) was not significantly affected ($P > 0.05$). The effluent contained 36.2 ± 17, 30.8 ± 12, and 68.4 ± 12 mg/L as NH_4^+-N, NO_2^--N and NO_3^--N, respectively. The ratio of NO_3^--N generated to NH_4^+-N oxidized was 0.17 ± 0.02 (Figure 3.1B). This is slightly lower than in phase II, however, it was not significantly different ($P > 0.05$) even though the COD increased from 305 ± 55 to 583 ± 75 mg/L.

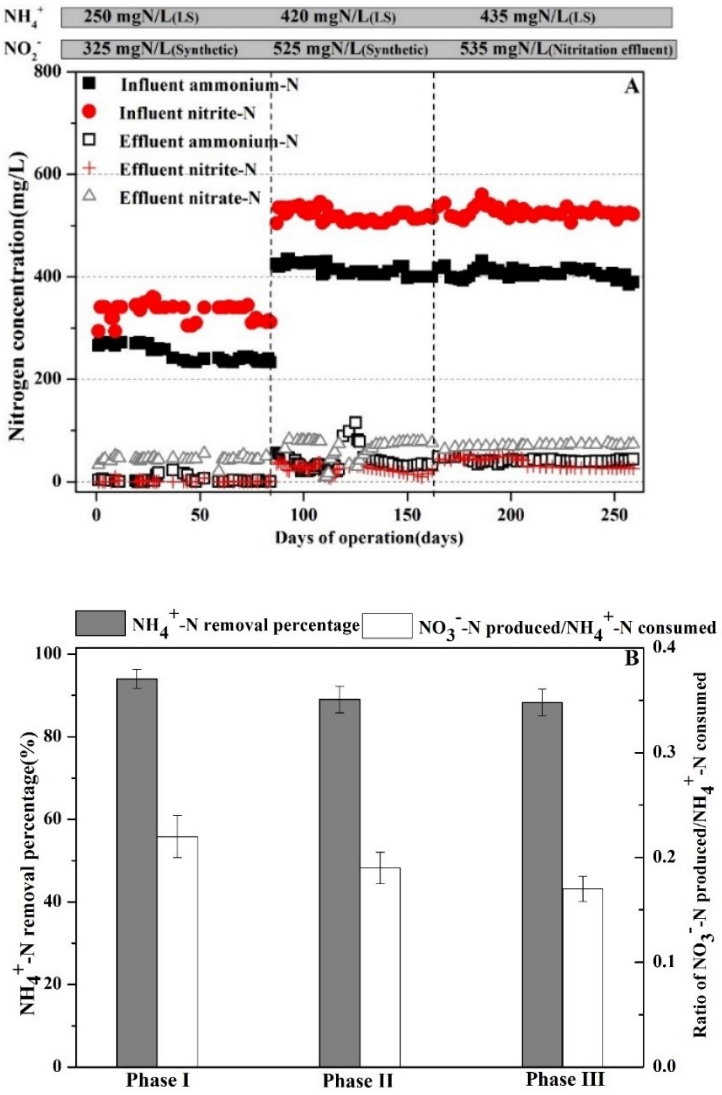

Figure 3.1. IFAS reactor performance during three reactor operation phases treating lagoon supernatant (LS). (A) The influent and effluent water quality along time. Ammonium nitrogen concentrations in the reactor feed in different phases are listed on top of the figure and each phase is divided by dashed lines. (B) The average ammonium removal efficiencies at steady state (last 10 days of each phase) and ratios of NO_3^--N produced per NH_4^+-N removed.

The NO_3^- consumption by denitrification was gradually increased through all phases. The COD removal efficiency was 12 ± 2.4% in phase I and increased to 20 ± 2.1 % ($P < 0.05$) in phase II. The COD removal efficiency in Phase III was 22 ± 2.7%, which was comparable with that in phase II ($P > 0.05$). Although the presence of heterotrophic denitrifiers helps to control effluent NO_3^- concentration and COD impacts, extensive growth of denitrifiers can lead to the reduced anammox activities through competition of the common nitrogen source (NO_2^-). In an IFAS configuration, controlling flocs concentration helps to maintain anammox activities and eliminate competition between denitrifiers and anammox bacteria. In our study, the flocs concentration was maintained at 428 mg/L (Phase I), 645 mg/L (Phase II) and 805 mg/L (Phase III), through controlling the settling time and volume exchange ratio during SBR operation.

Table 3.1 shows the relative contribution of anammox bacteria and heterotrophic denitrification on the nitrogen removal. The average inorganic nitrogen removal percentage in phase I, phase II and phase III by the anammox route were 97.2 ± 2.4%, 94.8 ± 1.6% and 91.4 ± 2.8%, respectively, while heterotrophic denitrification contributions in Phase I, phase II and phase III were 2.8 ± 2.3%, 5.2 ± 1.9% and 8.6 ± 2.7%, respectively.

Table 3.1. The relative contribution of different routes for nitrogen removal.

Consumption	Removal route	Phase I	Phase II	Phase III
NH_4^+-N removed (mg/L)	Anammox	232 ± 12	365 ± 17	345 ± 19
NO_2^--N removed (mg/L)	Anammox	306.2 ± 18	481.8 ± 22	455.4 ± 24
	Denitrification	2.76 ± 1.8	13.2 ± 4.5	39.6 ± 5.2
NO_3^--N removed (mg/L)	Denitrification	5.32 ± 2.1	15.1 ± 3.2	19.7 ± 4.1

The calculated COD removal based on denitrification stoichiometry (Supplementary information) were similar to the measured COD removal (Appendix B Figure B-3.2), indicating that COD consumption contributed to the heterotrophic nitrogen (e.g. NO_3^--N and NO_2^--N) removal, which verified the calculations of inorganic nitrogen removal contribution by anammox bacteria and heterotrophic denitrifiers.

The IFAS system seemed to have established higher resistance to organic substrates in phase III, which could be linked to the acclimatization of anammox bacteria, the higher biomass content in the reactor, and impact of co-existence of biofilm and flocs. These factors are discussed in the following sections.

3.3.2 Biomass content and specific anammox activity (SAA)

Figure 3.2A shows the total biomass content in biofilms and flocs. Biofilm biomass increased from 3.7 g in phase I to 5.1 g and 5.2 g in phases II and III, respectively. The flocs biomass in the reactor increased from 2.4 g in phase I to 2.9 g in phase II, and further increased to 3.8 g in phase III. Overall, biofilm biomass was higher than the flocs biomass, which may be attributed to the longer solid retention time of biofilm microorganisms which supported the growth of slow growing bacteria and retaining more biomass. Figure 3.2B shows the specific anammox activities (SAA) of biomass in biofilm and flocs during different operation phases. Under all conditions, the SAA of biofilm biomass was higher than that of the flocs biomass, indicating that the anammox activity mainly took place in the biofilm. Anammox bacteria are slow growing microbes, which are better retained in the biofilm with higher cell retention time (Fernández et al., 2008).

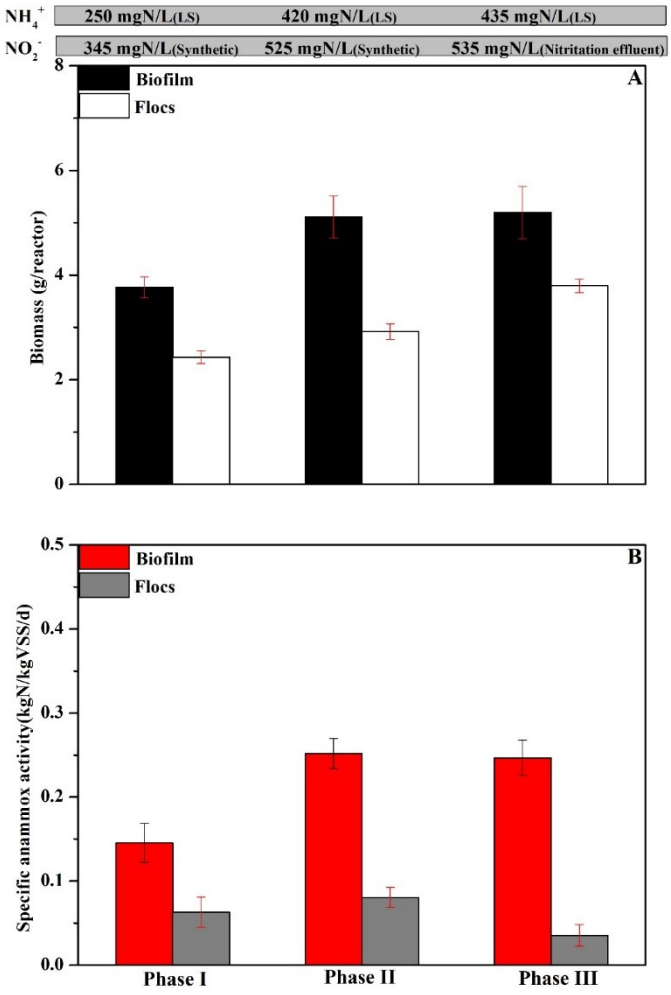

Figure 3.2. (A) Total biomass contents in biofilm and flocs; (B) specific anammox activity in biofilm and flocs.

From phase I to II, the SAA increased significantly in biofilm from 0.16 to 0.25 kg N/kg VSS/d ($P < 0.05$, Figure 3.2B). The increase in biomass and SAA was associated with the increase of NH_4^+-N and NO_2^--N in the feed, indicating active growth of anammox bacteria in the biofilm. In flocs, the slight increase of biomass content and SAA (0.06 to 0.08 kg N/kg VSS/d) could have resulted from detachment of biofilm. The COD increase from

phase I to II did not inhibit microbial growth and anammox activities, but led to a decrease in the ratio of NO_3^--N generated to NH_4^+-N oxidized, indicating denitrification activities. In phase III, the SAA of biofilm (0.24 kg N/kg VSS/d) was comparable with phase II, while the SAA in the flocs decreased to 0.03 kg N/kg VSS/d. Since NH_4^+-N and NO_2^--N concentrations in the feed only changed slightly while COD concentration increased significantly, the SAA decrease in flocs was probably due to the high COD loading. In phase III, it is possible that the COD promoted denitrifier growth in flocs, which competed with anammox bacteria. As a result, anammox activity in flocs was more easily affected than in biofilm. Accordingly, the flocs biomass content was higher in phase III than in phase II due to growth of denitrifiers. The total specific COD reduction rate increased gradually from phase I to phase III (Appendix B Figure B-3.1), confirming that heterotrophic activity was enhanced.

Previous studies showed that both of the anammox activity and population were significantly decreased when COD increased. For instance, an anammox lab-scale up-flow anaerobic sludge blanket (UASB) reactor fed with either UASB-post-digested pig manure or the effluent after partial oxidation showed enhanced denitrification and reduced anammox activities under higher COD loadings(Molinuevo et al., 2009). Tang et al. (2010) reported the activities of anammox bacterial were largely suppressed by denitrifiers under high organic loading conditions (400-600 mg/L of COD concentration in the influent) in high-rate anammox UASB reactor. In the present study, the average COD removals in Phases II (74 mg/L) and III (128 mg/L) were lower than the BOD5 concentrations (103 mg/L and 165 mg/L, respectively), indicating heterotrophic denitrification was not limited by the available BOD_5 (Ruscalleda et al., 2008). Results in our study showed that the

anammox bacteria activity in the biofilm was not affected either in phase II (305 mg/L COD, 103 mg/L BOD_5) and phase III (583 mg/L COD, 165 mg/L BOD_5). . This observation may be explained by the presence of flocs in the system which consumed organic substrates more efficiently and protected the biofilm from high organic substrate loading.

3.3.3. of functional genes by qPCR

The Quantification quantitative real-time PCR analysis was performed to quantify the abundances of anammox bacteria and heterotrophic denitrifiers in the biofilm and flocs. As shown in Figure 3.3A, the amount of anammox functional genes (AMX *nirS*) increased in both biofilm and flocs ($P < 0.05$) as the NH_4^+-N and NO_2^--N feeding concentration increased from phase I to phase II. The abundance of anammox AMX *nirS* in the biofilm increased from 1.15×10^7 to 3.76×10^7 copies/mgVSS in phase II (Figure 3.3A). This confirmed that good anammox activity was established at this high ammonium condition. The flocs exhibited a lower abundance of AMX *nirS* than within biofilm ($P < 0.05$), but a similar increasing trend from phase I (2.12×10^6 copies/mgVSS) to phase II (4.52×10^6 copies/mgVSS). The absolute quantity of anammox bacteria in biofilm was much higher than in flocs, which is in agreement with the anammox activity test results (Figure 3.2B); this is supported by both the higher abundance of biofilm AMX *nirS*, and a higher biofilm biomass concentration (Figure 3.2A) when compared to that of flocs.

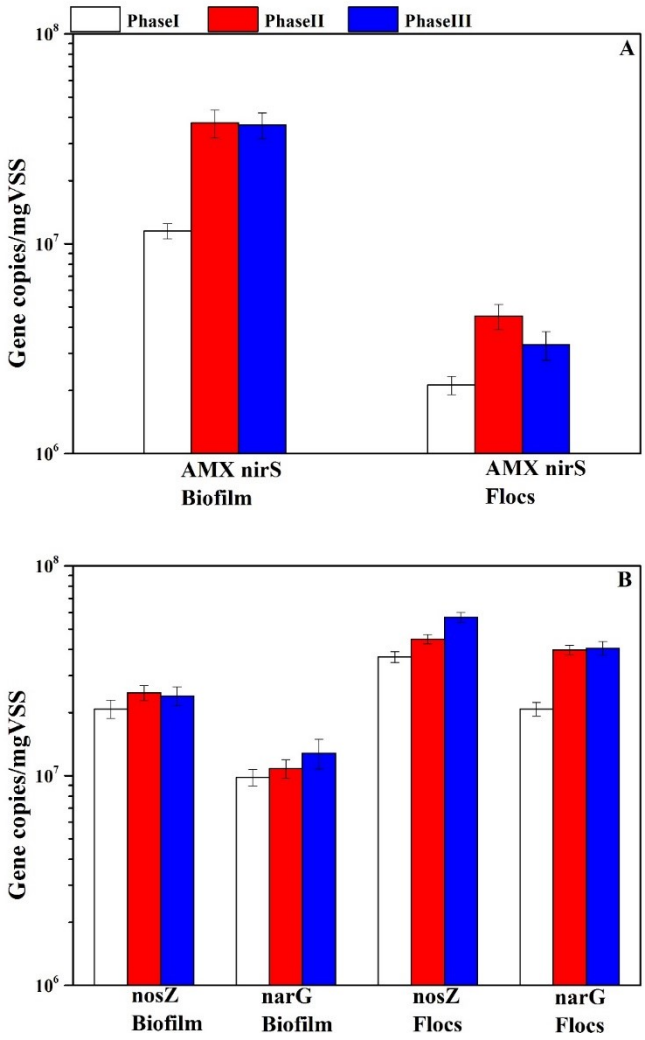

Figure 3.3. qPCR results of (A) functional anammox gene AMX *nirS*; (B) functional denitrification gens *nosZ* and *narG* in different phases in biofilm and flocs.

In phase III, the AMX *nirS* in biofilm remained at a similar level (3.68×10^7 copies/mgVSS) as in phase II. However, AMX *nirS* in flocs reduced to 3.30×10^6 copies/mgVSS. These results confirmed that raw nitritation reactor effluent had a greater affect than synthetic nitrite on anammox bacteria in flocs.

Figure 3.3B shows the gene copy numbers of heterotrophic denitrifiers in biofilm and flocs, based on functional genes *nosZ* and *narG*. In the biofilm, the gene copy numbers of *nosZ* and *narG* did not change significantly ($P > 0.05$); rather, from phase I to phase III they slightly increased from 2.08×10^7 to 2.40×10^7 copies/mgVSS biomass and from 9.80×10^6 to 1.28×10^7 copies/mgVSS biomass, respectively.

In the flocs, the abundances of the denitrification genes *nosZ* and *narG* both increased significantly ($P < 0.05$), from 3.68×10^7 to 4.48×10^7 copies/mgVSS biomass and 2.08×10^7 to 3.98×10^7 copies/mgVSS biomass, respectively. The abundance of *nosZ* in the flocs further increased significantly to 5.68×10^7 copies/mgVSS biomass ($P < 0.05$) in phase III while the *narG* in the flocs remained relatively stabilized with no significant difference ($P > 0.05$). Comparison between biofilm and flocs revealed that anammox bacteria resided mostly in the biofilm, while more denitrifiers resided in the flocs. The changes through different operation phases confirmed that higher organic substrates resulted in higher abundances of heterotrophic denitrifiers and lower anammox bacteria in the flocs.

Other studies have shown that high concentrations of organic matters inhibited anammox activity. For example, COD concentrations higher than 300 mg/L or COD/N ratios higher than two suppressed anammox activity in low-N-strength wastewater (Chamchoi et al., 2008). In high-N-strength wastewater similar with phase I in our study, high COD showed an instant loss in anammox activity; at COD/NO_2^--N ratio of 2.92, the anammox activity nearly ceased (Tang et al., 2010). Increasing the COD/N ratio from 0.6 to 1.4 led to a decreased anammox activity in a flocs reactor (Jenni et al., 2014); whereas in granular sludge system the COD/N ratio had less impact on the anammox activity at values below 3.1 (Ni et al., 2012). In the present study, the COD/N ratio (ranging from 0.58 to 1.34) was

lower than the inhibitory levels reported in literature. Our study showed an increase in COD/N ratio only caused marginal reduction in anammox activity in the biofilm, but significantly reduced anammox activity of flocs. This observation may be attributed to the presence of active denitrifiers in flocs and thus mitigated the organic carbon impacts on anammox activities in biofilm biomass. In addition, as compared to the flocs, biofilm is often reported to be more resistant towards environmental stress due to its unique architectural features (Sheng and Liu, 2011; Sheng et al., 2015). Overall, it appeared that anammox bacteria in biofilm may benefit from heterotrophic bacteria in flocs under conditions of high COD loading.

3.3.4 Microbial community diversity

The microbial community during each operation phase was investigated via 16S rRNA gene sequencing analysis. The alpha-diversity indices (Shannon diversity, observed genera, Chao 1, ACE and Pielou's evenness) varied between different operation phases and between the biofilm and flocs (Table 3.2). The alpha-diversity of biofilm community increased after raising the proportion of lagoon supernatant in the feeding water from 25% (phase I) to 50% (phase II), indicating that the microbial community has developed with higher richness and evenness. In flocs, the Shannon diversity and Pielou's evenness slightly decreased from phase II to III, while other indices slightly increased. Comparing biofilm and flocs, the latter appeared to have higher alpha-diversity, though it was not significant ($P>0.05$). The increased concentration of lagoon supernatant increased the complexity of the microbial community structure, probably due to the complex composition of the feeding water containing various organic carbon substrates, nutrients, and rare microorganisms (Shao et al., 2019). Other studies also showed that microbial communities

in anammox reactor are more diverse when fed with real ammonium-rich wastewater than synthetic ammonium-rich wastewater (Wagner and Loy, 2002).

Table 3.2. Alpha-diversity indices in different operation phases of microbial communities in the biofilm and flocs.

	Shannon Diversity	Observed Genera	Chao 1	ACE	Pielou's Evenness
Biofilm-Phase I	2.36	183	190	189	0.45
Biofilm-Phase II	3.16	244	248	252	0.57
Biofilm-Phase III	3.11	226	230	236	0.57
Flocs -Phase II	3.28	262	265	270	0.59
Flocs -Phase III	3.13	271	273	278	0.56

The beta-diversity among samples was analyzed using Bray-Curtis distance and presented in principal coordinates analysis (PCoA) plot (Figure 3.4). The biofilm and flocs communities were clearly differentiated along the PCoA1 axis (80.7% of total variance), indicating segregated microbial communities. In biofilm, a shift was shown from phase I to II, then remained unchanged in phase III indicating that a stable community had been developed in phase II and was not affected by the changes in feed water in phase III. In flocs, the large distance between phase II and III suggests that its microbial community was changed more dramatically, probably due to the feeding composition changes.

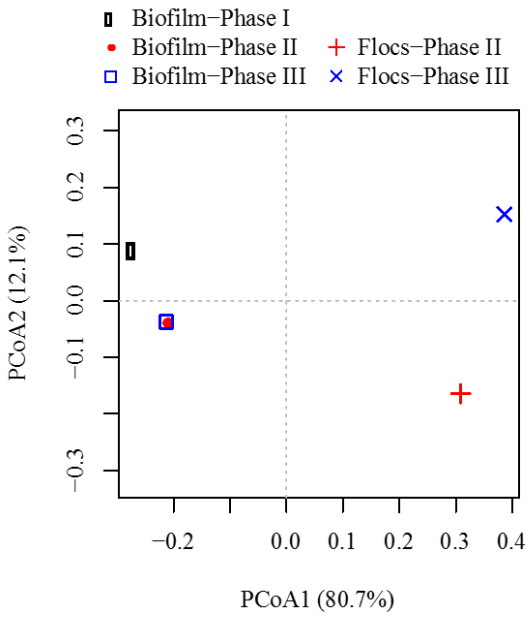

Figure 3.4. Principal coordinates analysis (PCoA) plot of Bray-Curtis distance among biofilm phases I, II, and III, and flocs phases II and III.

3.3.5 Taxonomic analysis

The relative abundances of microbial taxa were demonstrated at the phylum (Figure 3.5A) and genus (Figure 3.5B) levels. In the biofilm samples, the phylum *Ignavibacteriae* (24.6-47.3%) was most predominant, followed by *Planctomycetes* (17.6-22.2%), *Chloroflexi* (10.0–10.5%), *Bacterioidetes* (6.0–9.4%), *Proteobacteria* (5.7–9.3%), and *Chlorobi* (3.6-8.1%). The most abundant phylum in flocs samples was *Bacteroidetes* (41.6%-44.9%). *Ignavibacteriae* (12.6-16.3%) was the second most abundant phylum. Similar to the biofilm communities, *Proteobacteria* (11.8-13.4%), *Chloroflexi* (2.9-3.6%), *Chlorobi* (0.7-3.1%), and *Planctomycetes* (0.7-0.8%) were also abundant. The relative abundance of *Planctomycetes*, which was related to anammox bacteria, was notably enriched from phase I (17.6%) to phase II (22.2%) in the biofilm, and remained at high level (21.2%) in phase

III. In flocs, *Planctomycetes* only took up 0.8% and 0.7% in phase II and phase III, respectively. Other studies also showed that members of phyla other than *Planctomycetes*, namely *Proteobacteria, Bacterioidetes, Chlorobi*, and *Chloroflexi* were present in anammox bioreactors (Gonzalez-Gil et al., 2015; Gonzalez-Martinez et al., 2015).

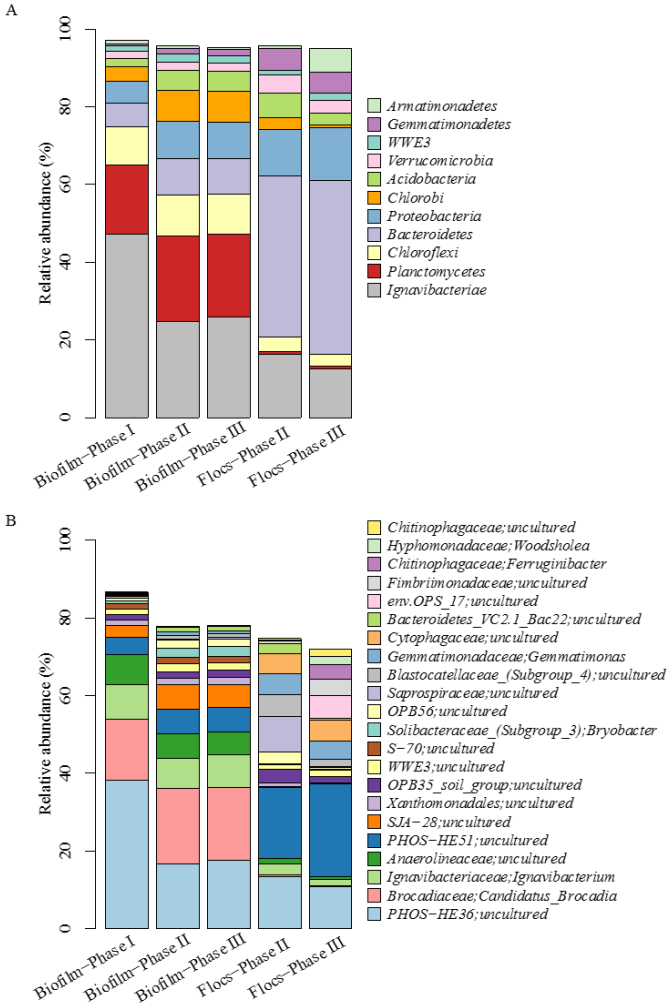

Figure 3.5. (A) Relative taxonomic abundances of microbial community at phylum level with average relative abundance >1%; (B) assemblage of highest 10 genera from each sample.

At the genus level, *Candidatus* Brocadia in the phylum *Planctomycetes* was the predominant identified anammox bacteria (Figure 3.5B), taking up 15.7%, 19.5%, and 18.9% of biofilm communities in Phases I, II, and III, respectively, and only 0.3% of flocs in Phases II and III. Research showed that anammox bacteria tend to live in biofilm in nitritation-anammox bioreactors (Vlaeminck et al., 2010; Speth et al., 2016).

Uncultured bacterium in PHOS-HE36 (phylum *Ignavibacteriae*) were the highest abundant genus in biofilm phase I and second highest abundant in the other samples, which was previously reported in denitrifying communities (Dabert et al., 2001; Koenig et al., 2005). PHOS-HE36 decreased from phase I (38.2%) to phase II (16.6%), indicating that the biofilm community favored the growth of anammox bacteria than heterotrophic denitrifers. Uncultured bacterium in PHOS-HE51 (phylum *Bacterioidetes*) was the most abundant genus in the flocs communities, which was also related to denitrifying populations (Dabert et al., 2001). PHOS-HE51 increased from 18.2% in phase II to 23.7% in phase III in flocs, indicating its enrichment under high COD loadings. Other increased genera include uncultured env.OPS_17, uncultured *Fimbriimonadaceae*, *Ferruginibacter*, *Woodsholea*, and uncultured *Chitinophagaceae*. Decreased genera include uncultured PHOS-HE36, *Ignavibacterium*, uncultured OPB35_soil_group, uncultured *Blastocatellaceae* (Subgroup 4), uncultured *Saprospiraceae*, and *Gemmatimonas.*

The differences between biofilm and flocs community compositions demonstrated biomass segregation between biofilm and flocs: anammox bacteria residing in the biofilm while heterotrophic denitrifiers predominating the flocs. This segregation was related with different solid retention times, substrate accessibility, and interaction between the two matrices (Winkler et al., 2011; Hubaux et al., 2015). Flocs have more efficient access to

substrates whereas biofilm allows sufficient retention time for the slow-growing bacteria. Previous studies of one-stage PN/A systems have shown that IFAS systems enriched fast-growing microorganisms in the flocs and anammox bacteria in the biofilm (Laureni et al., 2019); Our results also showed higher anammox bacteria in the biofilm than in flocs, consistent with these IFAS studies. Moreover, the flocs in our reactor showed beneficial impacts on biofilm when dealing with complex feedings containing high nitrogen and high COD. It is suggested that the flocs play important roles in maintaining stability and resistance of anammox systems treating high-strength real wastewater and should not be neglected in operational and modeling practices (Volcke et al., 2012; Park et al., 2015).

3.4. Conclusions

This study demonstrated that anammox IFAS reactor was successfully treated ammonia-rich digester sludge thickening lagoon supernatant, reaching ammonia removal efficiencies higher than 88% at steady state. Switching from synthetic nitrite feeding to real nitritation effluent feeding led to a significant COD increase without reducing the anammox removal efficiency, reaching a 87% removal under the steady state condition. This suggests that the anammox IFAS reactor is a feasible system for treating ammonia-rich digester effluent.

Microbial activities and community compositions revealed that anammox bacteria (*Candidatus* Brocadia) mainly resided in the biofilm while flocs harbored mainly heterotrophic denitrifiers. The microbial temporal changes during different operation phases and responses to high organic content loading in Phase III showed that the anammox activity was only suppressed in flocs but not in biofilm. The flocs community functioned as a major denitrification player and a protection barrier against high COD inputs to maintain anammox activities in the biofilm. The biofilm- flocs hybrid system benefits from

the segregation of microbial communities and is advantageous in treating high organic content ammonia-rich wastewaters.

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CHAPTER 4 ANAMMOX REACTOR OPTIMIZATION FOR THE TREATMENT OF AMMONIUM RICH DIGESTATE LAGOON SUPERNATANT-STEP FEEDING MITIGATES NITRITE INHIBITION[2]

4.1. Introduction

The presence of high ammonia nitrogen (NH_4^+-N) concentration (*i.e.*, 800–1200 mg L^{-1}) in the digestate lagoon supernatant can cause serious eutrophication problems in receiving waters (Zhang et al., 2013) and thus requires efficient nitrogen reduction treatment before discharge (Dapena-Mora et al., 2004; Shao et al., 2018). However, conventional nitrification/ heterotrophic denitrification process is energy intensive, and the low organic carbon in the lagoon supernatant cannot support denitrification treatment (Shao et al., 2018). Anaerobic ammonia oxidation (anammox) coupled with partial nitritation (nitritation/anammox) has emerged as an efficient alternative to treat ammonia rich wastewater streams. In the first of a two-stage process, ammonia oxidizing bacteria (AOB) convert ammonium (NH_4^+) to nitrite (NO_2^-) by consuming oxygen (O_2) from the surrounding environment. Following the first stage, anammox bacteria anaerobically oxidize NH_4^+ using NO_2^- as the electron acceptor, generating nitrogen gas (N_2) (Strous et al., 1997; Zhang et al., 2015a). The oxygen requirement for the nitritation/anammox process is 60 % less than for conventional nitrification/denitrification (Lackner et al., 2014), as only 50% of the NH_4^+ is oxidized to NO_2^- ions aerobically, while the rest is converted to nitrogen gas via anammox. Therefore, the process can consequently contribute to the

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economical and effective operation of wastewater treatment plants (Mulder, 2003; van Loosdrecht and Salem, 2006; Siegrist et al., 2008; Eskicioglu et al., 2018).

Although nitritation/anammox processes have been applied at the pilot- and full-scale level treating ammonia-rich anaerobic digestate liquor, limited studies have reported on the treatment of digestate lagoon supernatant using this process. Compared to the digestate, which has a long storage time (4-6 months) in the lagoon for thickening, lagoon supernatant has a lower biodegradable chemical oxygen demand (COD), suspended solids and temperature, a higher pH, and a more diverse slow-growing microbial community (Shao et al., 2019).

Considering the long doubling time of anammox bacteria, most frequently studied anammox reactor configurations for this process are biofilm-based (granules or carrier biofilms), including up-flow granular anammox sludge bed reactors (Abma et al., 2010), moving bed biofilm reactors (MBBR) (Rosenwinkel and Cornelius, 2005), fluidized bed biofilm reactors (Ma et al., 2017), membrane biological nutrient removal reactor (Wang et al., 2019) and rotating biological contactors (Egli et al., 2001). Lately, the integrated fixed-biofilm activated sludge reactor (IFAS) has been studied as a promising option to implement the nitritation/anammox process for high ammonia strength wastewater treatment. By introducing flocs to biofilm-based reactors, IFAS may offer various engineering advantages, such as extended biomass retention times from the biofilm configuration (Zhang et al., 2015a; Huang et al., 2016; Arias et al., 2018), and improved substrate transfer from the suspended sludge configuration (Ding et al., 2018). Importantly for nitrogen-reduction, repression of nitrite oxidizing bacteria (NOB) in the flocs and a segregation of anammox bacteria and AOB provided by IFAS could also facilitate the

nitritation/anammox process (Zhang et al., 2015a). However, other than the comparison with biofilm-only systems (*i.e.*, MBBR), limited information is available regarding the process stability evaluation and IFAS operation optimization for high ammonia strength wastewater treatment (a total of 3 publications based on the search from "web of science" database), with a research focus on the COD/N ratio (Wang et al., 2018a), residual ammonium (Yang et al., 2017) or temperature (Dong et al., 2016). High concentration of nitrite can lead to irreversible inhibition (*i.e.*, toxicity) of the anammox bacteria by causing imbalance in cell mass transport and proton gradient (Li et al., 2017), or reversible inhibition on certain metabolic activities (Qiao et al., 2017). The mechanisms of nitrite inhibition was also regarded to be via the form of free nitrous acid (FNA, HNO_2) (Fernández et al. (2012), which can diffuse across the cell membrane. Under high concentration of FNA conditions, FNA can change the pH in the cell and neutralize the transmembrane potential which causes cell death (Kadam et al., 1994).

Our current research was undertaken to investigate the impact of reactor operation on the IFAS anammox process, treating digestate lagoon supernatant. Sequencing batch reactor (SBR) operation strategies were adopted. Nitrite inhibition was monitored when changing the operational hydraulic retention time (HRT) and step feeding was applied after inhibition occurred. Bioreactor treatment performance, microbial population dynamics, and anammox activities under different HRTs and feeding strategies were evaluated and compared.

4.2. Materials and methods

4.2.1. Reactor setup and operation

A laboratory scale integrated fixed-biofilm activated sludge sequencing batch reactor (IFAS-SBR) reactor (6 L working volume) was operated for 339 days. The reactor was initially seeded with Anox^{TM} K5 carriers, kindly provided by Veolia, with a 55% volume fill ratio (*i.e.*, volume ratio of biocarriers and total reactor volume). The reactor influent was a mixture of (i) raw lagoon supernatant from a biosolids digestate thickening lagoon in the City of Edmonton and (ii) the effluent of a continuously operating nitritation reactor treating raw lagoon supernatant. Raw lagoon supernatant was sampled once a month from the lagoon and stored in a 4 °C cold room (Shao et al., 2018). The molar ratio of NO_2^--N (from nitritation reactor effluent) to NH_4^+-N (from raw lagoon supernatant) in the reactor feed water was maintained at 1.32:1 based on the anammox biochemistry indicated in equation (1) (Strous et al., 1997). The water characteristics of raw lagoon supernatant, nitritation reactor effluent, and anammox reactor influent are summarized in Table 4.1.

A peristaltic pump (masterflex, Cole-Parmer, Illinois, USA) automated with an electronic timer was used for influent filling. A thermostatic jacket (Xuanyuan, Yancheng, China) was used to keep the reactor inside temperature at 30 ± 1 °C. A mechanical stirrer (Grainger, USA) was employed in the reactor reaction stage to blend the flocs, carriers and liquid thoroughly. The operation of reactor can be divided into four phases with their HRTs and feeding mode. As shown in Table 4.2, in Phase I (HRT = 2.5 d, days 1-90) and Phase II (HRT = 1.7 d, days 91-190), the reactor feed volume exchange ratio (VEX, the ratio of feed volume added to the reactor each cycle and the reactor working volume) was fixed at 20%. In Phase I, each SBR cycle was 12 hours, which included 15 min filling, 11 h anoxic reaction, 30 min settling, 5 min decant, and 10 min idle. Thus the reactor HRT was 2.5 d in Phase I. The operating cycle in Phase II was 8 hours which included 15 min filling, 7 h

anoxic reaction, 30 min settling, 5 min decant and 10 min idle and thus the HRT was reduced to 1.7 days by running three cycles each day. Starting from day 191 (Phase III), the HRT was further reduced to 1.2 days by increasing VEX to 28% also with an operating cycle length of 8 hours. In Phase IV (days 280-339), a step-feed approach was applied for the SBR operation with the HRT maintained at 1.2 days, where an 8 h operating cycle included a 7.5 min filling followed by a 4 h anoxic reaction, another 7.5 min filling followed by 3 h anoxic reaction, 30 min settling, 5 min decant and 10 min idle. Thus the total amount of influent that used in Phase III was distributed equally to be fed at the beginning and after 4 h anoxic reaction of the operating cycle in Phase IV respectively. The reactor pH was controlled at the anammox bacteria favored range of 7.5-7.8 by periodical addition of diluted hydrochloric acid. The feed was flushed with nitrogen gas at least 1 h per day. Anaerobic conditions were maintained by keeping the reactor sealed and gas outlet tubing submerged.

Table 4.1. Water characteristics of raw lagoon supernatant, nitritation reactor effluent and anammox reactor influent.

Parameters	Raw lagoon supernatant	Nitritation reactor effluent	Anammox reactor influent
TSS ($g\ m^{-3}$)	284 ± 142	325 ± 48	304 ± 78
NH_4^+-N ($mg\ L^{-1}$)	891.9 ± 79	25 ± 15	420 ± 12
NO_2^--N ($mg\ L^{-1}$)	0.4 ± 0.7	825 ± 45	543 ± 20
NO_3^--N ($mg\ L^{-1}$)	0.2 ± 0.7	22 ± 3.5	13.2 ± 2.6
COD ($mg\ L^{-1}$)	644 ± 14.42	450 ± 58	543 ± 65
pH	7.98 ± 0.07	7.38 ± 0.05	7.98 ± 0.05
Alkalinity ($mg\ CaCO_3\ L^{-1}$)	3180 ± 51.9	158 ± 32	1890 ± 75

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

4.2.2. Sample collection and analytical methods

Reactor water samples were collected 2-3 times per week for water quality including COD, NO_3^--N, NO_2^--N and NH_4^+-N concentration measurement with Hach reagent kits (methods 8000, 10205, 10206, and 10207, respectively, Hach Company, USA) (Nze et al., 2018). The inorganic nitrogen was calculated as the summation of NO_3^--N, NO_2^--N and NH_4^+-N (Vazquez-Padin et al., 2010).

Table 4.2. The test conditions of the anammox reactor during different operational phases.

Phase	Operation period (d)	Nitrogen rate (kg m^{-3} d^{-1})		Stoichiometric molar ratio	
		NLR	NRR	R_1*	R_2**
I	1-90	0.38 ± 0.00	0.31 ± 0.01	1.33 ± 0.05	0.22 ± 0.01
II	91-190	0.61 ± 0.02	0.32 ± 0.00	1.32 ± 0.04	0.15 ± 0.01
III	191-279	0.80 ± 0.01	0.49 ± 0.04	1.47 ± 0.05	0.16 ± 0.02
IV	280-339	0.84 ± 0.01	0.59 ± 0.01	1.35 ± 0.06	0.15 ± 0.01

*R1=NO_2^--N conversion/NH_4^+-N oxidation

**R2=NO_3^--N accumulation/NH_4^+-N oxidation

The nitrogen species mass balance is calculated based on the influent and effluent inorganic nitrogen concentrations. The average of nitrogen removal routes by anammox bacteria and heterotrophic denitrification was determined according to equations 1 (Chamchoi et al., 2008; Ruscalleda et al., 2008). FNA concentrations were determined based on the equation from Anthonisen et al. (1976).

Further water quality tests within one SBR cycle (*i.e.*, cycle test) were performed to evaluate the kinetics of NO_2^--N and NH_4^+-N depletion or NO_3^--N accumulation in different

phases. Water samples were subjected to measurement after being filtrated by 0.45 μm pore filters within 2 h of sample collection. Biomass of flocs and biofilm was sampled for measurement weekly following the volatile suspended solids (VSS) analysis method (Eaton et al., 2011).

4.2.3. Specific anammox activity (SAA) analysis

The specific anammox activity (SAA) of flocs and biofilm on the carriers under steady state conditions were analyzed with the procedure described by Jin et al (2012). 400 mL serum bottles with a liquid volume of 350 mL were adopted for the batch study. Biofilm and flocs samples were obtained from the continuously operating reactor according to the biocarrier volume fill ratio, which was 40 biocarriers every 350 mL mixed liquor in each bottle. The collected biofilm and floc samples were washed separately with phosphate buffer for three times before resuspended in 350 mL mineral medium containing 95 mg L^{-1} of NO_2^--N and 70 mg L^{-1} of NH_4^+-N. Hydrochloric acid was used to adjust the medium pH to about 7.5. Anoxic conditions were established by purging nitrogen (99.9% purity) in the serum bottles for 20 min before sealing the bottles with butyl rubbers and incubating them in a thermostatic shaker (180 rpm) at 31 ± 1 °C. Samples were taken for N species analyses periodically without disturbing the anoxic condition. The SAA was calculated as the ratio of maximum substrate consumption rate and biomass concentration. All batch studies were performed in triplicate.

4.2.4. Quantitative polymerase chain reaction (qPCR) analysis

The biomass samples of flocs and biofilm were collected from the reactor under the steady-state condition in each operating phase for DNA extraction using MO BIO PowerSoil® DNA Isolation Kits. The extracted DNA was subjected to the qPCR analyses using a

CFX96^{TM} real-time PCR detection system (Bio-RAD, USA) with the target genes of anammox bacteria and denitrification bacteria (Kim et al., 2011; Huang et al., 2016). Information on qPCR primers for target genes, and qPCR reaction details is provided in Table A-1 and A-2 (Appendix A).

Gene copy numbers were calculated as the relative values of those known plasmid DNA standard concentrations compared to the threshold cycle values obtained in every single qPCR run (Lü et al., 2013). The qPCR reaction without DNA template served as the control and was conducted for every target gene. All qPCR reactions were performed in triplicate.

4.2.5. Statistical significance

Statistical analyses were conducted using Microsoft Excel® single factor analysis of variance (ANOVA) function, and were recorded as p values, which indicate statistical significance under a 5% probability.

4.3. Results and discussion

4.3.1. Performance of the anammox reactor

Throughout the IFAS-SBR's 339 day operation, the influent NH_4^+-N concentration (420 ± 12 mg L^{-1}), NO_2^--N concentration (543 ± 20 mg L^{-1}), and NO_3^--N concentration (13.2 ± 2.6 mg L^{-1}) were maintained (Figure 4.1) whereby the inorganic N loading rate (NLR) experienced a proportional increase as the HRT decreased, which was 0.38 ± 0.00 kg m^{-3} d^{-1} in Phase I (90 days, HRT = 2.5 d), 0.61 ± 0.02 kg m^{-3} d^{-1} in Phase II (100 days, HRT = 1.7 d), 0.80 ± 0.01 kg m^{-3} d^{-1} in Phase III (89 days, HRT = 1.2 d), and 0.84 ± 0.01 kg m^{-3} d^{-1} in Phase IV (60 days, HRT = 1.2 d) (Table 4.2).

The change of HRT significantly affected NH_4^+-N and NO_2^--N removal efficiency and NO_3^--N accumulation (Figure 4.1 and Appendix B Figure B-4.1).

Table 4.3. Average consumption and percentages of different routes for nitrogen removal at different phases.

Consumption	Removal route	Phase I	Phase II	Phase III	Phase IV
NH_4^+-N removed (mg/L)	Anammox	380	383	287	389
NO_2^--N removed (mg/L)	Anammox	501.6	505.6	378.8	513.5
	Denitrification	18.4	16.4	71.2	14.5
NO_3^--N removed (mg/L)	Denitrification	18.8	28.4	18.6	27.1
Total nitrogen removed (mg/L)		908.8	913.4	755.6	924.1
Average nitrogen removal percentage (%)	Anammox	95.5	92.0	86.8	93.6
	Denitrification	4.5	8.0	13.2	6.4

In Phase I, the effluent NH_4^+-N and NO_2^--N concentrations were stabilized at 50.2 ± 5.7 and 30.1 ± 3.4 mg L^{-1} respectively, with NH_4^+-N and NO_2^--N removal efficiencies of 88.0 ± 1.4% and 94.2 ± 0.6% respectively.

Meanwhile, an average of 80.6 ± 4.0 mg L^{-1} of NO_3^--N accumulation occurred as a result of the generation from anammox reaction and the reduction from heterotrophic denitrification (Gilbert et al., 2014). Lagoon supernatant contains low levels of COD, which may facilitate the growth of heterotrophic denitrification activity by providing the external organic carbon source to the denitrifiers (indicated by18.0 ± 5.8% COD reduction in Phase I, as shown in Appendix B Figure B-4.2), consequently leading to a lower observed NO_3^--N accumulation.

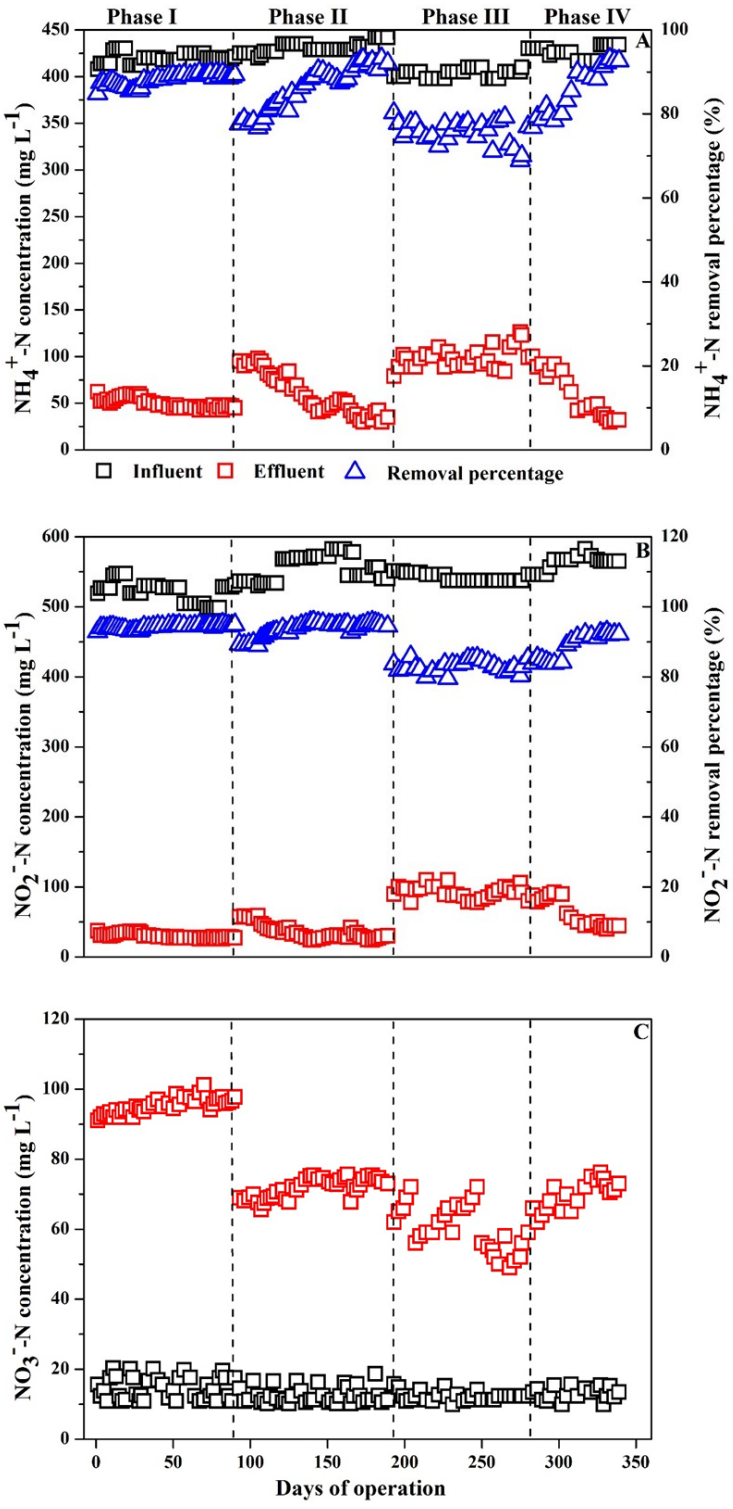

Figure 4.1. The reactor performance of different nitrogen species (A. NH_4^+-N, B. NO_2^--N, C. NO_3^--N) with black square dots representing influent concentration, red square dots

representing effluent concentration and blue triangle dots representing removal efficiency.

However, anammox was still the major route for the observed nitrogen reduction because denitrifiers cannot outcompete anammox bacteria under such low C/N ratio (< 1) conditions (Sobieszuk and Szewczyk, 2006). Further, as a means to measure the extent of anammox process (Trigo et al., 2006), the stoichiometric ratio of NO_2^--N conversion and NH_4^+-N oxidation (R_1) and the stoichiometric ratio of NO_3^--N accumulation and NH_4^+-N oxidation (R_2) were 1.33 ± 0.05 and 0.22 ± 0.01, respectively. This also points to the co-existence of dominant anammox activity and limited heterotrophic denitrification, by comparing with the theoretic values (equation (1)) (Strous et al., 1997) which were 1.32 and 0.26, respectively. Further stoichiometric estimation showed that 95.5 ± 1.2% of inorganic nitrogen removal was through anammox process (Table 4.3). Therefore, the average total inorganic nitrogen (TN) removal efficiency was 81.6 ± 0.7% and the inorganic nitrogen removal rate (NRR) was 0.31 ± 0.01 kg m^{-3} d^{-1} in Phase I (Table 4.2), both of which were in the favorable range for IFAS configuration anammox process performance (Zhang et al., 2015a).

When the HRT was reduced from 2.5 d (Phase I) to 1.7 d (Phase II), an adaptation period (days 91-140) was observed due to the operation condition change (*i.e.*, HRT and NLR), which imposed selective pressure on the microbes (Costa et al., 2014). After that, the effluent NH_4^+-N and NO_2^--N concentrations steadily decreased to 41.2 ± 7.5 mg L^{-1} and 29.1 ± 4.1 mg L^{-1}, respectively, with the NH_4^+-N and NO_2^--N removal efficiencies of 90.5 ± 1.8% and 95.2 ± 0.7%, respectively, which were slightly higher than ($p < 0.01$ for NH_4^+-

N and p = 0.02 for NO_2^--N) that in Phase I, suggesting a slight improvement in anammox activity (R_1 reached 1.32 ± 0.04). Meanwhile, the NO_3^--N accumulation decreased significantly ($p < 0.01$) to 57.6 ± 3.1 mg L^{-1} (R_2 = 0.15 ± 0.01), accompanied with an elevated COD degradation (26.2 ± 2.1%) (Appendix B Figure B-4.2). The stoichiometry analysis also indicated that the anammox process slightly increased the removal efficiencies of NH_4^+-N and NO_2^--N, and the heterotrophic denitrification process contributed to higher removal efficiency of NO_3^--N (Table 4.3). Heterotrophic denitrification contribution to the total inorganic nitrogen removal was increased from 4.5 ± 1.4% (phase I) to 8.0 ± 1.1% (phase II). Our observation indicates heterotrophic denitrification was improved by shortening the HRT, similar to previous reports (Xu et al., 2014). At a shorter HRT of 1.7 d and a subsequently higher NLR of 0.61 ± 0.02 kg m^{-3} d^{-1}, both TN removal efficiency and NRR significantly increased ($p < 0.01$ for both) to 85.7 ± 0.7% and to 0.49 ± 0.04 kg m^{-3} d^{-1}, respectively, which can be attributed to the enhancement of anammox and heterotrophic denitrification activities.

When the HRT was further reduced to 1.2 d (Phase III), significantly increased ($p < 0.01$ for both) NH_4^+-N (98.4 ± 11.8 mg L^{-1}) and NO_2^--N (92.8 ± 9.0 mg L^{-1}) concentrations were observed in the effluent, with a simultaneously decreased NH_4^+-N removal efficiency of 75.6 ± 2.9% and NO_2^--N removal efficiency of 82.8 ± 1.6%, suggesting a diminished anammox activity (R_1 =1.47 ± 0.05). Further, a lower ($p < 0.05$) NO_3^--N accumulation of 48.2 ± 6.8 mg $L^{-1}$was also observed due to enhanced heterotrophic denitrification, as indicated by the improved COD degradation efficiency of 28.7 ± 1.9% (Appendix B Figure B-4.2); the calculated percentage of total inorganic nitrogen removal increased to 13.2 ± 0.9% by heterotrophic denitrification (Table 4.3).

The reported adverse effects of a short HRT that could affect nitrogen removal performance primarily referred to the substantial biomass wash-out or short-cut flow caused by the increased hydraulic pressure (Meng et al., 2018). This could be easily ruled out in our study because heterotrophic denitrification or COD removal performance, occurring mainly in the flocs (to be further discussed in section 3.2), was not compromised. As indicated in the kinetics study (section 3.3), anammox activity was inhibited by the accumulation of NO_2^--N in each operation cycle (Appendix B Figure B-4.3), which was associated with the enhanced inorganic N loading at shorter HRT. Therefore, a deterioration of TN removal efficiency (73.7 ± 0.7%) was still observed regardless of the higher heterotrophic denitrification, which is less efficient than anammox (Zhang et al., 2015b; Qiao et al., 2019). Meanwhile, NRR still increased ($p < 0.01$) to 0.59 ± 0.01 kg m^{-3} d^{-1} with the higher NLR of 0.80 ± 0.01 kg m^{-3} d^{-1}, which was similar to previously reported study (Malovanyy et al., 2015).

Phase IV was considered a restoration stage for the deterioration of nitrogen removal performance in Phase III. Step feed mode was employed in Phase IV. Step feeding strategies have been regarded as an effective way to improve nitrogen removal performance in conventional biological nitrogen removal under higher nitrogen loading conditions through reducing reactor interim substrate concentrations with more uniform substrate inputs (Sahlstedt et al., 2012; Wang et al., 2018b). In Phase IV (HRT of 1.2 d), the inorganic nitrogen loading was distributed equally as a temporal 50%-50% being introduced to each operation cycle. After approximately a 1-month adaptation (days 276-208), the removal efficiencies of NH_4^+-N and NO_2^--N increased significantly ($p < 0.001$ for both) to 90.9 ± 1.8% and 92.1 ± 0.1%, respectively, with the effluent NH_4^+-N and NO_2^--N concentrations

reduced to 38.7 ± 6.9 mg L^{-1} and 45.2 ± 3.2 mg L^{-1}, respectively, while the NO_3^--N accumulation increased ($p < 0.01$) to 59.2 ± 2.9 mg L^{-1}. The stoichiometric ratio R_1 decreased significantly ($p < 0.01$) to 1.35 ± 0.06 while R_2 was relatively stabilized at 0.15 ± 0.01, probably because heterotrophic denitrification was not further improved as evident from the comparable ($p > 0.05$) COD degradation efficiency (28.2 ± 7.8%) with Phase III. The calculated contribution to total inorganic nitrogen removal by the anammox process improved from 86.8 ± 1.8% to 93.6 ± 2.7% compared with that in phase III. Therefore, the restoration was achieved with the enhanced TN removal efficiency of 84.5 ± 0.1% and NRR of 0.71 ± 0.01 kg m^{-3} d^{-1} without compromising the treatment capacity (*i.e.*, without increasing HRT).

Due to the low concentration of biodegradable influent organic matters, the contribution of heterotrophic denitrification was limited under all conditions. Figure B-4.4 (Appendix B) showed the evolution of the nitrogen removal percentages by both processes. The average inorganic nitrogen removal percentage by the anammox route was 93.2 ± 3.8%, while heterotrophic denitrification contribution was 6.8 ± 2.1%. Results indicated that the anammox reaction was the main nitrogen removal route despite the co-existence of heterotrophic denitrifiers. The COD removal percentages calculated based on heterotrophic denitrification stoichiometry (Supplementary information) were similar to the measured values (Appendix B Figure B-4.2), indicating that most COD reduction contributed to the heterotrophic NO_3-N and NO_2-N removal, which verified the calculations of inorganic nitrogen removal contribution by anammox and heterotrophic denitrifiers.

4.3.2. Biomass and specific anammox activities (SAA)

The performance of anammox was determined by evaluating the biomass concentration and SAA (Jin et al., 2013). Figure 4.2A shows that the total biofilm biomass concentration was higher than the floc biomass concentration, which is predictable in the anammox bacteria-dominant reactor where anammox bacteria were retained extremely long in the biofilm configuration. Meanwhile, the presence of the influent exogenous organic carbon prompted heterotrophic denitrifiers to dominate the flocs in the bulk solution (Hubaux et al., 2015).

Both the total biofilm biomass and floc biomass in the reactor increased significantly ($p = 0.022$ for biofilm fraction and $p = 0.001$ for flocs fraction) from 6.5 ± 0.2 g and 3.7 ± 0.1 g to 7.0 ± 0.2 g and to 4.5 ± 0.1 g, respectively from Phase I to Phase II due to the higher substrate (*i.e.*, nitrogen and organic carbon) loading, which was similar to the previous research (Liao et al., 2007; Nze et al., 2018).

However, further reduction of the HRT led to a decrease ($p = 0.02$) in floc biomass (3.5 ± 0.2 g) in Phase III which was possibly caused by biomass wash-out under high hydraulic pressure, while biofilm biomass continued increasing to 7.6 ± 0.2 g in Phase III ($p = 0.03$); this demonstrates the superior biomass retention ability of the biofilm configuration. With more uniform distribution of substrate (Phase IV), the biomass further increased to 4.7 ± 0.1 g in flocs and to 8.1 ± 0.2 g in biofilm ($p = 0.001$).

The specific activities of anammox (SAA) in the four operation phases are shown in Figure 4.2B, which were much higher than the reported value in the one-stage nitritation/anammox reactors (Malovanyy et al., 2015; Wang et al., 2018a). The biofilm SAA increased significantly ($p < 0.05$) from 0.25 ± 0.09 kg N kg^{-1} VSS d^{-1} to 0.41 ± 0.07 kg N kg^{-1} VSS d^{-1} when the HRT was shortened from 2.5 d (Phase I) to 1.7 d (Phase II).

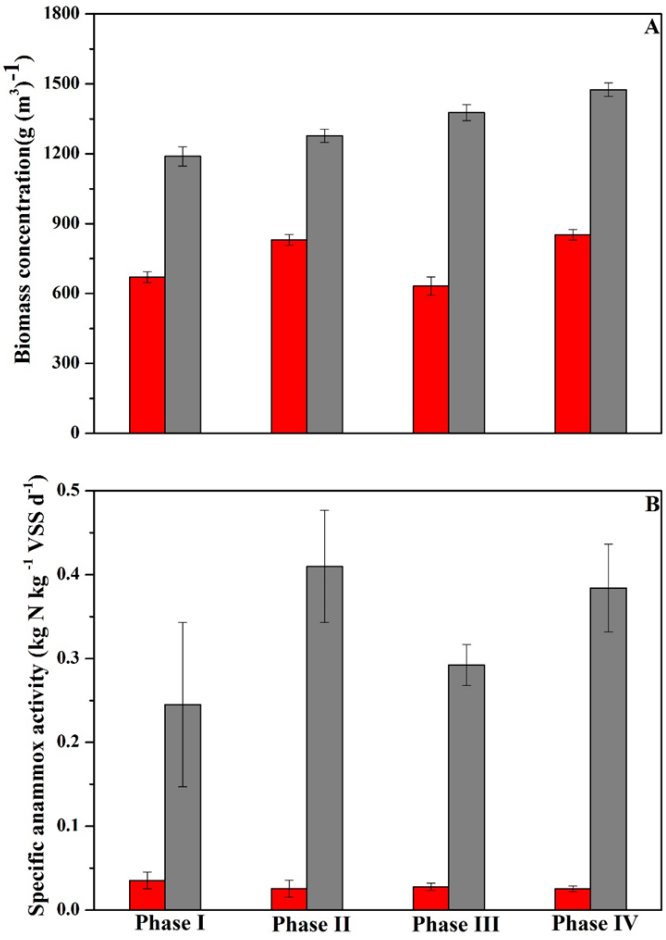

Figure 4.2. Biomass concentration (A) and specific anammox activity (SAA) (B) of flocs (red bars) and biofilm (gray bars) in the reactor under different operation phases.

The biofilm SAA decreased ($p < 0.05$) to 0.29 ± 0.02 kg N kg^{-1} VSS d^{-1} when the HRT was further reduced to 1.2 d (Phase III), and was quickly recovered to 0.38 ± 0.05 kg N kg^{-1} VSS d^{-1} in Phase IV, which were higher than the reported values (0.12 - 0.32 kg N kg^{-1} VSS d^{-1}) in various studies (Vázquez-Padín et al., 2009; Kotay et al., 2013), especially when treating real lagoon supernatant. This change in biofilm SAA correlated well with the nitrogen removal performance as the anammox activity dominated in the nitrogen removal process. On the other hand, the floc SAA contributed to only 6-13% of the total

SAA in the reactor which was reduced significantly ($p < 0.05$) from 0.035 ± 0.010 kg N kg^{-1} VSS d^{-1} at HRT of 2.5 d (Phase I) to 0.025 ± 0.000~0.028 ± 0.004 kg N kg^{-1} VSS d^{-1} at the shorter HRTs of 1.2-1.7 d (Phase II-IV).

As discussed above, different from the biofilm only configuration, where heterotrophs exclusively grow in the outer layer of the biofilms, a better segregation of microbial species and activity can be provided in the IFAS configuration where the majority of the heterotrophic activities were shifted to the bulk (*i.e.*, flocs), whereas the anammox activity dominated the biofilm (Hubaux et al., 2015). The elevated nitrogen and organic loading with the shorter HRT facilitated the shift in spatial partitioning of anammox bacteria and heterotrophic denitrifiers, and subsequently led to a lower SAA in the flocs fraction. This observation was also directly reflected by our microbial analysis (Figure 4.4). From a positive side, the anammox bacteria (in a small amount) which were detached from the biofilm were entrapped in the flocs and therefore were "protected" to the inhibitors, resulting in no further decrease of SAA in the flocs under the nitrite inhibition in Phase III.

4.3.3. Degradation kinetics studies and inhibition mechanism analysis

The change of NH_4^+-N oxidation, NO_2^--N consumption, and NO_3^--N accumulation in typical SBR cycles under each operation conditions are shown in Figure 4.3 and the calculated change rates of each nitrogen species were summarized in Figure B-4.3 (Appendix B). Under a higher NLR (*i.e.*, shorter HRT) condition in Phase II, NH_4^+-N oxidation, NO_2^--N consumption, and NO_3^--N accumulation rates enhanced due to the improved anammox activity. These rates then decreased simultaneously in Phase III because of the nitrite inhibition. The further increased NLR in Phase III exceeded the anammox capacity in the reactor, leading to the NO_2^--N accumulation and its resultant

inhibition of the anammox bacteria. Therefore, the interim NO_2^--N concentration in the operation cycle of Phase III (141.2-219.2 g L^{-1}) was much higher than that in other phases (*i.e.*, 4-132.5 g L^{-1} in Phase I, 25-145 g L^{-1} in Phase II and 40-129 g L^{-1} in Phase IV, as shown in Figure 4.3), which could reflect the inhibition on anammox activity in this phase. As reported previously, nitrite inhibition is a rate limiting criterion for many anammox reactor configurations. High concentration of nitrite can lead to irreversible inhibition (*i.e.*, toxicity) of the anammox bacteria by causing imbalance in cell mass transport and proton gradient (Li et al., 2017), or reversible inhibition on certain metabolic activities (Qiao et al., 2017). In the meantime, the concentration of FNA which was recognized as the actual inhibitor of anammox under high nitrite concentration (Fernández et al. (2012)), was also determined in our study. The FNA concentrations were 1.7 ± 0.8 μg L^{-1} in Phase I, 2.1 ± 1.3 μg L^{-1} in Phase II and 2.8 ± 1.2 μg L^{-1} in Phase IV, which was below the commonly reported FNA inhibition concentration (5.9 - 19.8 μg L^{-1}) for anammox bacteria (Oshiki et al., 2011). However in Phase III, FNA concentration increased to 18.9 ± 8.9 μg L^{-1}. Therefore, both of the concentrations of FNA and NO_2^--N indicate the inhibition of anammox activity occurring in Phase III. Both inhibition mechanisms result in mitigation or even cessation of the reaction as reflected by the lowest nitrogen conversion kinetics in Phase III in our study. Further, the highest NH_4^+-N oxidation rate with step feeding in Phase IV may suggest the reversible nitrite inhibition is the major inhibitory mechanism, as step feeding greatly reduced ($p < 0.05$) the interim NO_2^--N concentration in the operation cycle of Phase IV.

4.3.4. Quantification of functional genes by qPCR

Figure 4.4 shows the gene copy numbers of anammox and heterotrophic denitrifiers in both biofilm and flocs, based on functional gene analysis. AMX *nirS* encoding gene have been widely applied for targeting genus of *Candidatus Brocadia*, *Candidatus Jettenia*, *Candidatus Kuenenia* and *Candidatus Anammoxoglobus* (Li et al., 2011; Yang et al., 2019). Among those four genus, *Candidatus Kuenenia* and *Candidatus Brocadia* are the most commonly found organisms in enrichments from WWTPs (Jetten et al., 2009). Similarly, *nosZ* and *narG* were two most widely applied primer sets for targeting heterotrophic denitrifiers (Henry et al., 2006; Segawa et al., 2014). Overall, the abundance of AMX *nirS* gene for anammox bacteria in biofilm was significantly ($p < 0.01$) higher than that in flocs (*i.e.*, > 90% AMX *nirS* gene detected in the biofilm biomass), suggesting a more important role of the biofilm configuration in ammonium removal, which agrees with the SAA results (Figure 4.2B).

The decrease in HRT from 2.5 d (Phase I) to 1.7 d (Phase II) led to a significant increase in the AMX *nirS* gene copy number from $4.1\times10^6 \pm 4.6\times10^5$ and $3.7\times10^7 \pm 2.1\times10^6$ copies mg^{-1} VSS biomass to $5.6\times10^6 \pm 6.2\times10^5$ and $6.9\times10^7 \pm 5.2\times10^6$ copies mg^{-1} VSS biomass in flocs and in the biofilm respectively, displaying a respective increase of 37.9% and 88.6% of AMX *nirS* gene copy numbers in flocs and biofilm.

Generally, higher nitrogen loading accompanied with shorter HRT in Phase II could encourage the growth of anammox bacteria in both flocs and biofilm. In addition, when biofilm reached a certain thickness, biomass could be detached from biocarriers and further increase the AMX *nir*S gene abundance in the bulk flocs (Qiao et al., 2019).

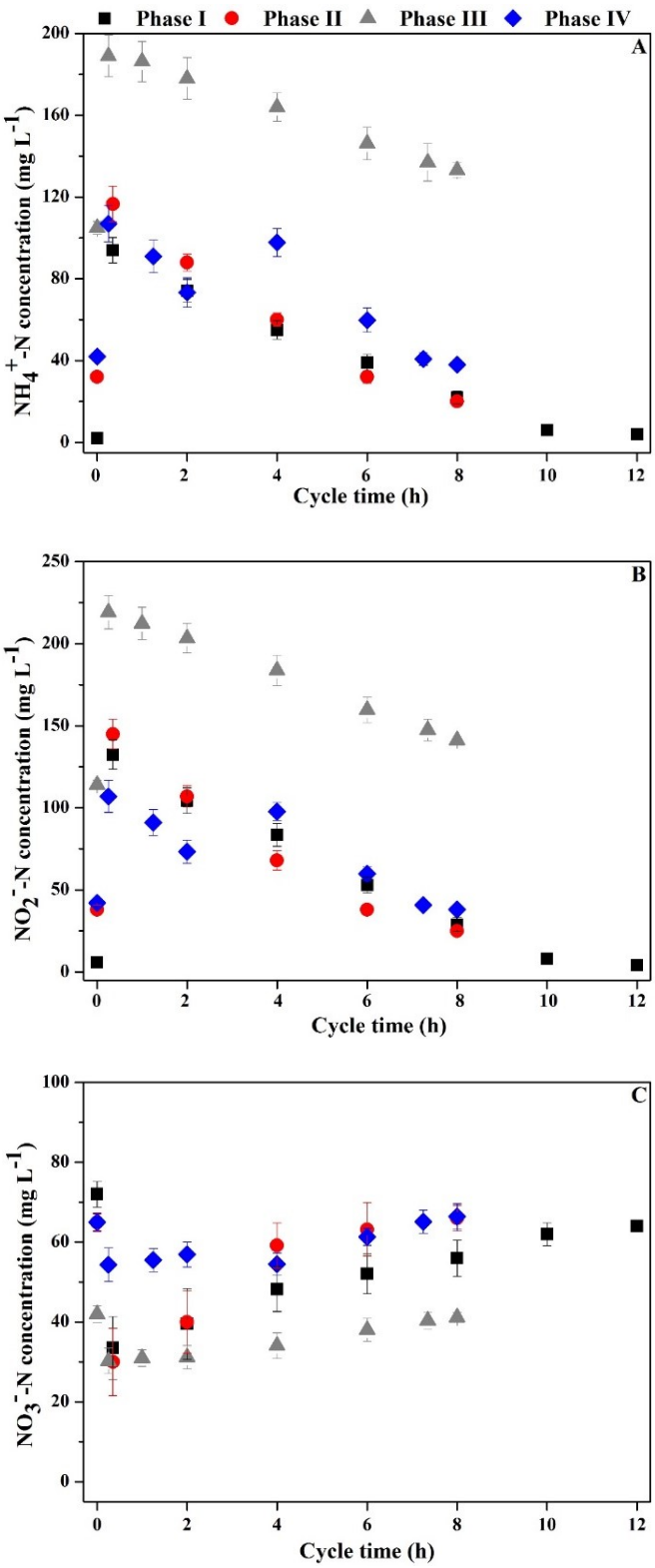
Phase I
Phase II
Phase III
Phase IV
A
NH_4^+-N concentration (mg L^{-1})
Cycle time (h)
B
NO_2^--N concentration (mg L^{-1})
Cycle time (h)
C
NO_3^--N concentration (mg L^{-1})
Cycle time (h)

Figure 4.3. The change of NH_4^+-N, NO_2^--N and NO_3^--N concentrations in each sequencing batch reactor cycles of Phase I (black dots), Phase II (red dots), Phase III (grey dots) and Phase IV (blue dots). Error bar represent duplicate measurements.

In contrast, the lower increase percentage of anammox bacteria in flocs may result in a less susceptible anammox bacteria community to the change of HRT because of the floc structure. Under the nitrite inhibition condition (Phase III), the AMX *nirS* gene copy numbers decreased by 38.0% (from $5.6\times10^6 \pm 6.2\times10^5$ to $3.5\times10^6 \pm 5.9\times10^5$ copies mg^{-1} VSS biomass) and 29.1% (from $6.9\times10^7 \pm 5.2\times10^6$ to $4.9\times10^7 \pm 5.2\times10^6$ copies mg^{-1} VSS biomass) in flocs and biofilm respectively, which correspond to SAA results and nitrogen removal performance as well. After switching the feeding mode to step feeding in Phase IV, our results show that the AMX *nirS* gene copy numbers increased by 24.6% (from $4.9\times10^7 \pm 5.2\times10^6$ to $6.1\times10^7 \pm 5.2\times10^6$ copies mg^{-1} VSS biomass) in biofilm and decreased slightly (from $3.5\times10^6 \pm 5.9\times10^5$ to $3.1\times10^6 \pm 6.3\times10^5$ copies mg^{-1} VSS biomass) in flocs. The recovery in anammox bacteria population abundance was expected after the nitrite inhibition was mitigated in Phase IV. From our previous microbial results, anammox bacteria were mostly affiliated with the genus *Candidatus* Brocadia, which dominated the bacterial biofilm community (Yang et al., 2019).

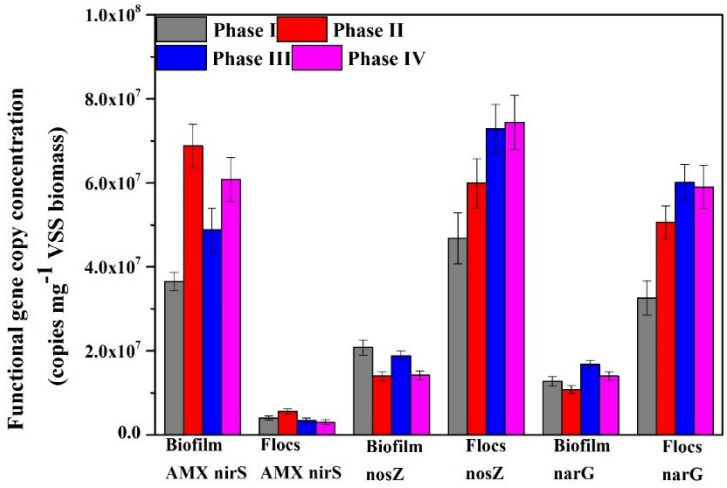

Figure 4.4. 16S rRNA gene copy abundance of anammox bacteria and heterotrophic denitrifiers in biofilm and flocs, respectively in different operation phases (Phase I (gray bars), Phase II (red bars), Phase III (blue bars) and Phase IV (pink bars)).

Meanwhile, the denitrifier gene (primarily targeted *nosZ* and *narG*) abundance in flocs was significantly higher than that in biofilm ($p < 0.01$). Denitrifiers can be more easily detached from the biocarrier as they grow mostly in the outer layer of biofilm. Therefore, a relative stable abundance was observed for the denitrifiers in the biofilm ($1.4\times10^7 \pm 1.1\times10^6$ to $2.1\times10^7 \pm 1.8\times10^6$ copies mg^{-1} VSS biomass for *nosZ* and $1.1\times10^7 \pm 1.0\times10^6$ to $1.7\times10^7 \pm 1.0\times10^6$ copies mg^{-1} VSS biomass for *narG* respectively). In the flocs where denitrifers dominated (> 70% of total denitrifier genes, for both *nosZ* and *narG*), the abundance of the denitrifier genes *nosZ* and *narG* increased significantly ($p < 0.05$) from $4.7\times10^7 \pm 6.1\times10^6$ to $7.3\times10^7 \pm 5.8\times10^6$ copies mg^{-1} VSS biomass and from $3.3\times10^7 \pm 4.1\times10^6$ to $6.0\times10^7 \pm 4.3\times10^6$ copies mg^{-1} VSS biomass, respectively, as the HRT decreased from Phase I to Phase III, while a further increase in denitrifier genes was not observed with the step feeding operation in Phase IV. This is plausible and was similar to the previous

observations which suggests the lower HRT consistently leading to a higher population of heterotrophic bacteria (Xu et al., 2014). Moreover, the limited increase in the abundance of *narG* in Phase III was probably due to the biomass washout at the shorter HRT which correlated well with floc biomass change in Figure 4.2A. The amoA gene copy numbers (representing the AOB bacteria abundance) were about five log units lower than the anammox bacteria copy numbers, indicating their low abundance in the reactor.

4.4. Conclusion

For the first time, the impacts of operating strategies on the IFAS-SBR anammox reactor treating ammonium rich digestate lagoon supernatants was demonstrated. Anammox activity was slightly improved by shortening HRT from 2.5 d to 1.7 d which may reach the maximum anammox capacity of the reactor. The anammox activity was inhibited with the further reduced HRT of 1.2 d due to the interim accumulation of nitrite in the reactor. The strategy of applying step feed at HRT of 1.2 d mitigated the nitrite accumulation and restored the anammox activity. Heterotrophic denitrification co-existed with anammox in the reactor for the nitrogen removal activity, however, with minor contribution and less affected by nitrite accumulation. Our study concluded that optimization of IFAS anammox reactor can be achieved by shortening HRT, but the increased risk of nitrite inhibition of anammox activity should be considered with the increased nitrogen load. Changing the feeding strategy (i.e., applying step-feeding) can effectively mitigate nitrite inhibition. The co-existence of heterotrophic denitrifies contributed to less than 10% of the nitrogen removal under all conditions, without affecting the anammox activity in the IFAS configuration.

It should be noted that our study focused on applying the step feeding strategy to mitigate the nitrite inhibition on the anammox activity. However, the 2nd stage of anammox bioreactor may be optimized through further operation condition optimization (e.g. through reducing cycle duration time, changing VEX percentages) to reduce the potential FNA inhibition of the reactor, which was not studied in the present paper but may be of interest of further studies.

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CHAPTER 5 IMPORTANCE OF CONTROLLING PHOSPHATE CONCENTRATION IN NITRITATION-ANAMMOX REACTOR OPERATION[3]

5.1. Introduction

Anaerobic digester sludge liquor in municipal sewage treatment plants contains high concentrations of ammonia nitrogen (i.e., 800–1200 mg/L NH_4^+-N) and phosphorus (i.e., 180–250 mg PO_4^{3-}-P/L), and has a low biodegradable chemical oxygen demand (COD) (i.e., < 280 mg/L in BOD) (Wang et al., 2014; Shao et al., 2018). Conventional nitrification/denitrification treatment of sludge liquor is energy intensive due to the extensive aeration required for high ammonia removal. Additional organic carbon is required for complete denitrification, which imposes a further cost increase. On the other hand, the application of anaerobic ammonium oxidation (anammox), utilizing bacteria capable of oxidizing NH_4^+ with NO_2^- as the electron acceptor at mesophilic temperatures (30–40 °C) is a cost effective option to treat high ammonia (NH_4^+) strength stream (Rikmann et al., 2018; Zekker et al., 2018). Nitritation-anammox, which depends on ammonia oxidizing bacteria (AOB, e.g., *nitrosomonas*) to partially oxidize NH_4^+ to NO_2^-, and anammox bacteria (e.g., *Candidatus Brocadia*) to convert the remaining NH_4^+ and the produced NO_2^- to N_2 gas, has become an attractive process for enhanced nitrogen removal from ammonia rich wastewater (Rikmann et al., 2018; Zekker et al., 2018).

Full scale application of nitritation-anammox reactors for ammonia-rich sludge supernatant treatment is increasing, but still facing the challenges such as unexplained anammox

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instabilities during the long-term operation (Gilbert et al., 2014; Tenno et al., 2018a; Tenno et al., 2018b). Anammox bacteria are difficult to cultivate because of their low growth rate, low cell yield (0.11 g volatile suspended solids (VSS)/gNH_4^+-N), and high sensitivity to environmental conditions (Gilbert et al., 2014). Various common wastewater constituents have been reported to influence nitritation-anammox stability. In particular, a high free ammonium concentration (> 25 mg NH_3-N/L) has been shown to inhibit anammox under mesophilic conditions (Carvajal-Arroyo et al., 2013). Salinity (dissolved solids concentration > 20 g/L), heavy metals, NO_2^- (> 120 mg N/L), O_2 (DO > 2.5 mg/L), low temperature (< 25 ºC) and H_2S (> 64 mg H_2S-S/L) also inhibit anammox activities, especially over long-term wastewater treatment (Carvajal-Arroyo et al., 2013). A high concentration of phosphate may also impact anammox activities, although only limited reports are available. Van de Graaf et al. (1996) first reported a 100% inhibition on the growth of anammox bacteria in the suspended culture exposed to 160 mg PO_4^{3-}-P/L phosphate in batch experiments. Pynaert et al. (2003) observed an 80% inhibition of anammox bacteria cultivated using a rotating biological contactor (dominated by *Ca. Kuenenia stuttgartiensis*) when the phosphate concentration was above 100 mg PO_4^{3-}-P/L. More recently, Carvajal-Arroyo et al. (2013) found that the specific anammox activity (SAA) of a *Ca*. *Brocadia* dominant anammox culture was reduced by 60%, when phosphate concentrations were elevated from 170 to 1000 mg PO_4^{3-}-P/L. All the reported studies were performed in the anammox only system with synthetic wastewater feeding. The impact of a high phosphate concentration on the performance and stability of microbes in continuously operating nitritation-anammox bioreactors treating real ammonium rich wastewater (e.g., digester thickening lagoon supernatant) has not been reported.

Considering the high phosphorous concentration in anaerobic sludge digester sludge liquor (235 ± 15.4 mg PO_4^{3-}-P/L or 8.0 ± 0.5 mM), it is important to explore the impact of phosphorous on the activity of anammox bacteria in continuous operating bioreactors for nitrogen reduction. If phosphorous inhibition exists, the phosphorous concentration in digester sludge liquor can be reduced by first precipitating phosphorous as struvite ($NH_4MgPO_4 \cdot 6H_2O$, a spin-off product that can be applied as a slow release fertilizer). In full-scale side-stream treatment plants, phosphorus recovery processes, e.g., Ostara® processes, are applied after nitritation-anammox treatment with the goal of preserving sufficient phosphorous for microbial growth in bioreactors (Sharp et al., 2013).

Our current study was undertaken to determine and compare the treatment performance, and dynamic of microbial community and anammox activities in, a nitritation-anammox reactor treating digester sludge thickening lagoon supernatants before (pre-Ostara®) and after (post-Ostara®) struvite recovery (phosphorous reduction). A laboratory integrated fixed-film activated sludge sequencing batch reactor (IFAS-SBR) that carries out one stage nitritation-anammox was run for 575 days to evaluate phosphate impacts. The present study provides much needed information on the impact of phosphorous concentration on nitritation-anammox reactor performance.

5.2. Methods and materials

5.2.1 Reactor feed

Digested sludge liquor thickening lagoon supernatant was collected from a biosolids digester sludge thickening lagoon in the City of Edmonton once a month. To minimize microbial activity, the lagoon supernatant was stored at 4 °C prior to use. An Ostara® facility in Edmonton recovers phosphorus from the lagoon supernatant. Lagoon

supernatant before and after Ostara® treatment was collected and fed into a continuous operating reactor sequentially. 50% pre-Ostara® and post-Ostara® feed water was prepared by diluting raw pre-Ostara® and raw post-Ostara® lagoon supernatant with deionized (DI) water in 1:1 volume ratio, respectively.

5.2.2 Reactor operation

The lab-scale integrated fixed film activated sludge (IFAS) reactor with a working volume of 6 L was run in a sequencing batch mode. The reactor was seeded with colonized AnoxKaldnes K5 biofilm carriers (800 m^2/m^3 protected surface area) with a 38% volume fill ratio, kindly provided by Veolia.

The IFAS-SBR reactor was operated following four operation phases (Table 5.2) for a total of 575 days. In the first two phases, the reactor was fed with 50% and 100% pre-Ostara® lagoon supernatant, respectively; in the last two phases, the reactor was fed with 50% and 100% post-Ostara® lagoon supernatant, respectively. As a result, the respective influent PO_4^{3-}-P concentration was 120 mg/L, 235 mg/L, 15 mg/L and 32 mg/L from Phase I to Phase IV, either through the dilution of lagoon supernatant (to achieve 50% of the lagoon supernatant) or applying Ostara® pretreatment.

The hydraulic retention time (HRT) of the IFAS reactor was maintained at 2.4 days. The IFAS reactor was operated following a sequencing batch model; each sequencing batch reactor (SBR) cycle lasted for 12 hours, which included a 5 min feeding, a 10 hour reaction, a 110 min settling, and a 5 min effluent discharge. The reaction phase consisted of five alternating aeration (80-105 min) and non-aeration (15-40 min) periods (with a total of 2 hours in each period). 0.16–0.24 mg/L of dissolved oxygen was maintained in the aeration periods. The duration of aeration and non-aeration periods was based on the anammox

activity. At a lower anammox activity, a longer non-aeration period was provided to prevent rapid nitrite accumulation produced by AOB bacteria during the aeration period. The sludge retention time (SRT) of the suspension was maintained at 24 ± 3.2 days, considering the mixed liquor suspended solids, and the influent and effluent suspended solid concentrations, while the SRT of attached growth biomass was considered as "infinite" (Veuillet et al., 2014). Reactor pH of 7.2-7.8 was maintained naturally as a result from the diverse metabolic activities of microbial community.

The reactor feed volume exchange ratio (i.e., the ratio of feed volume added to the reactor at the beginning of each cycle to the reactor working volume) was 21%. A thermostatic water jacket was used to keep the temperature of the liquid at 31 ± 2 °C. A mechanical stirrer was used to keep the reactor completely mixed during reaction period.

5.2.3 Sample collection and analytical methods

Reactor influent and effluent were sampled daily or every two days during the operation. Water quality including the concentrations of COD, NH_4^+-N, NO_3^--N, and NO_2^--N and PO_4^{3-}-P were measured with Hach kits (methods 8000, 10205, 10206, 10207 and 10209, respectively, Loveland, Colorado).

Water quality (NH_4^+-N, NO_3^--N, and NO_2^--N) tests were also performed during SBR cycles with proper intervals to determine the nitrogen species transformation and to evaluate the nitrogen reduction kinetics in the reactor. After collection, water samples were filtrated with 0.45 μm filters and measured within 2 h.

5.2.4 Biomass analysis

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured every week according to Standard Methods (APHA, 2011).

Table 5.1. Composition of the pre-Ostara® and post-Ostara® supernatants

Parameters	Unit	Pre-Ostara supernatant	Post-Ostara supernatant
		Mean ± S.D.	Mean ± S.D.
TSS	g TSS/m^3	344±120	305±107
NH_4^+	mg N/L	891.9±79	835±35
NO_2^-	mg N/L	0.4±0.7	0.6±0.5
NO_3^-	mg N/L	0.2±0.7	0.2±0.2
FA[1]	mg NH_3-N/L	13.2 ± 1.2	12.7 ± 1.4
FNA[2]	mg HNO_2-N/L	0.0015±0.0006	0.0010±0.0006
PO_4^{3-}	mg PO_4-P/L	235±15.4	32±5.4
Alkalinity	mg $CaCO_3$/L	3180±51.9	3056±45.6
pH	-	7.98±0.07	7.87±0.15
Alkalinity/NH_4	mg $CaCO_3$/mg N	3.59±1.23	3.43±1.04
COD	mg/L	644±14.42	568±54.42
rbCOD[3]	mg/L	205±25	189±28

[1]FA: free ammonia

[2]FNA: free nitrous acid

[3]rbCOD: readily biodegrable COD

The biofilm biomass was determined using methods reported previously (Shao et al., 2018). The specific anammox activity (SAA) of biomass under steady state conditions was determined with the method described in Jin et al. (2013). The serum bottles were de-oxygenized by purging N_2 gas for 25 min, and were immediately sealed with butyl rubber before being placed in a thermostatic shaker operated at 31 ± 1 °C and 180 rpm. Biomass concentration and pH were measured at the end of each test. SAA was calculated as the maximum substrate consumption rate divided by VSS.

5.2.5 Short-term batch experiments to detect phosphate impacts

Batch experiments were conducted using serum bottles to determine the short-term (4-8 hours) impact of phosphate stress on bacterial activities. Biofilm and flocs samples obtained from the continuous operating reactor (at a biofilm to sludge volume ratio of 40 biocarriers: 280 mL sludge) on days 80, 120, and 150 were inoculated in batch reactors to treat pre-Ostara® and post-Ostara® lagoon supernatant containing various phosphorous concentrations.

Two trials were performed (Appendix A Table A-3). In Trial 1, 12%, 25%, 50%, and 100% pre-Ostara® lagoon supernatant solutions were used to provide phosphate for the batch tests, and the targeted final phosphate concentrations were 30, 60, 120, and 240 mg PO_4^{3-}-P/L, respectively. Two additional higher phosphate concentrations were tested by directly adding inorganic NaH_2PO_4 into 100% pre-Ostara® supernatant samples to reach 350 and 450 mg PO_4^{3-}-P/L. In Trial 2, synthetic NaH_2PO_4 was directly added into the post-Ostara® lagoon supernatant solutions (~ 30 mg PO_4^{3-}-P/L) to obtain targeted phosphate concentrations similar to Trial 1 (30, 60, 120, 240, 350, and 450 mg PO_4^{3-}-P/L). The difference between the first two trials is that Trial 1 examines the impacts of all lagoon supernatant pollutant concentrations on the specific anammox activity (SAA), in addition to the phosphate concentrations examined in Trial 2. With such design, Trial 2 evaluates if an elevated phosphate concentration in the lagoon supernatant could impact the anammox reaction; and the comparison of Trials 1 and 2 examines whether phosphate is the main inhibiting factor in lagoon supernatant.

For both trials, biofilm and flocs were separated to evaluate SAA variations under different phosphate concentrations. Table A-3 (Appendix A) shows the detailed experimental conditions for the short-term phosphate impact on the activity of anammox bacteria.

5.2.6 q-PCR analysis and MiSeq sequencing

The biomass samples of biofilm and sludge were collected during the steady-state conditions of four phases for both denitrification and anammox bacteria. DNA of sludge and biofilm biomass was extracted with MO BIO PowerSoil® DNA Isolation Kits (MoBio Laboratories Inc., Carlsbad, California). qPCR kits were purchased from Bio-Rad Laboratories, Mississauga, Ontario. Table A-1 (Appendix A) shows the detailed information of qPCR primers and target genes.

The extracted DNA samples were analyzed for 16S rRNA gene sequencing on an Illumine MiSeq platform at the Research and Testing Laboratory (Lubbock, TX, USA) to investigate bacteria community diversity and composition. The V3-V4 regions of 16S rRNA genes were amplified using primer set 357wF (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC) (Loh et al., 2018). Sequencing data processing and taxonomic classification were completed using QIIME pipelines with references in the Silva database (Caporaso et al., 2011).

5.2.7 Statistical analysis

T-test and single factor analysis of variance at a 5% probability level was determined to perform the statistical analysis, and were reported as P-values. A statistically significant difference is evident from a p-value smaller than 0.05.

5.3. Results and discussion

5.3.1 Lagoon supernatant characteristics

The water chemistry of digester sludge thickening lagoon supernatant before and after Ostara® treatment is shown in Table 5.1. In the pre-Ostara® lagoon supernatant, the COD concentration was 644 ± 14.42 mg/L with the readily biodegradable COD (rbCOD) concentration of 205 ± 25 mg/L, the ammonium nitrogen concentration was 891.9 ± 79 mg/L, and the phosphate concentration was 235 ± 15.4 mg PO_4^{3-}-P/L. The post-Ostara® stream had relatively lower COD values of 568 ± 54 mg/L with the rbCOD concentration of 189 ± 28 mg/L, a significantly lower phosphate concentration (32 ± 5.4 mg PO_4^{3-}-P/L), and a comparable ammonia concentration (835 ± 35 mg N/L) ($P < 0.01$) compared to the pre-Ostara® lagoon supernatant. Free ammonia concentration was low (< 12.8 mg/L) in the reactor (Table 5.1), as the pH in the reactor was in the range of 7.2-7.8, which was below the commonly reported free ammonia inhibition concentration (25-157 mg NH_3-N/L)(Jin et al., 2012) for both NOB and anammox bacteria. FNA (free nitrous acid) concentrations were negligible in the reactor, which were 0.0015 ± 0.0006 mg NO_2^--N /L and 0.0010 ± 0.0006 mg NO_2^--N/L in the pre-Ostara® lagoon supernatant and post-Ostara® lagoon supernatant, respectively.

5.3.2 Impacts of Ostara® pretreatment on nitritation-anammox reactor performance

The IFAS bioreactor was operated in a sequencing batch mode for 575 days.

As shown in Figure 5.1, when the reactor feed was switched from 50% pre-Ostara® lagoon supernatant (400 ± 32 mg NH_4^+-N/L, 120 ± 12 mg PO_4^{3-}-P/L) to 100% pre-Ostara® lagoon supernatant (891 ± 79 mg NH_4^+-N/L, 235 ± 15.4 mg PO_4^{3-}-P/L), the NH_4^+-N removal percentage was reduced from 88.2 ± 2.5% to 55% ± 9.8%. Previous studies demonstrated that under certain inhibition conditions, microorganisms may adapt to the stress condition over time (Nautiyal et al., 2000; Romano et al., 2015). However, this adaption was not

observed in our study, and instead we observed continuous deterioration of the reactor performance over the 94 days of operation in Phase II. A significant reduction in N removal efficiency was also accompanied by a high NO_2^- accumulation (120-189 mg NO_2^--N/L), indicating an anammox activity inhibition when the raw pre-Ostara® lagoon supernatant was directly used as reactor feed.

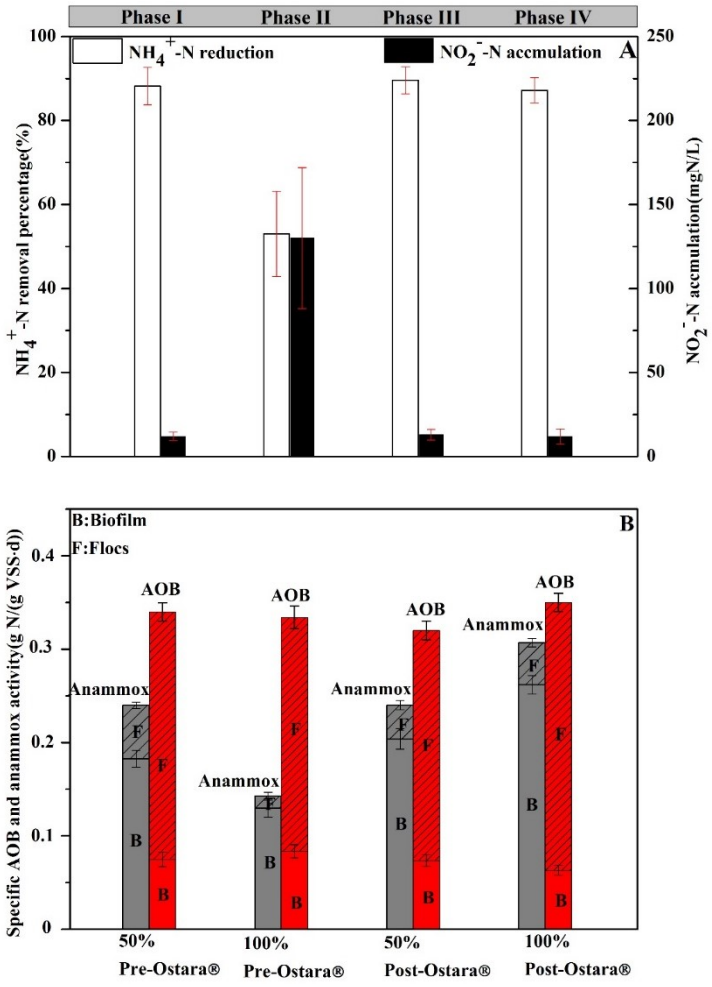

Figure 5.1. Ammonia removal percentage and nitrite accumulation in the IFAS-SBR reactor (A) and relative specific AOB and anammox activity of biofilm and flocs (B) during four different phases: 50% Pre-Ostara® supernatant; 100% Pre-Ostara® supernatant; 50% Post-Ostara® supernatant and 100% Post-Ostara® supernatant.

Treatment of 50% post-Ostara® lagoon supernatant (420 ± 35 mg NH_4^+-N/L, 16 ± 5.4 mg PO_4^{3-}-P/L) achieved an NH_4^+-N removal percentage of 89.6 ± 3.2%. An increase of post-Ostara® lagoon supernatant fraction to 100% did not impact the NH_4^+-N reduction percentage, and led to an NH_4^+-N removal of 88.2 ± 3.6% ($P < 0.01$). Figure 5.1A shows that no significant NO_2^- accumulation was observed when post-Ostara® lagoon supernatant was utilized as reactor feed. As shown in Appendix B Figure B-5.1, although it took 117 days (from day 403 to day 520) for the bioreactor to stabilize when the reactor feed was switched from 50% to 100% post-Ostara® lagoon supernatant (corresponding to a NH_4^+-N loading rate change of 0.17 to 0.34 kg N (m^3·d)), the reactor performance was stable once sufficient biomass was established (from day 520 and from day 575, as discussed below). The volumetric nitrogen removal rates (VNRR), biomass specific nitrogen removal rates (BNRR) and total inorganic nitrogen (TIN) efficiencies calculated for each operation phase are shown in Table 5.2. The highest VNRR, BNRR and TIN achieved were 0.30 ± 0.01 kg N/(m^3·d), 0.13 ± 0.01 g N/(g VSS·d), and 80.2 ± 2.8%, respectively, under 100% post-Ostara® feed condition. The VNRR in the reactor with 100% post-Ostara lagoon supernatant feed was higher than the reported highest value of 0.28 kg N/(m^3·d) treating real anaerobic digester supernatant (Vázquez-Padín et al., 2009). Meanwhile, the stationary phase or stable operation phase in the reactor operation can be defined as the phase where organics and nutrient removal efficiency became stable which happened because the mature microbial community was well developed or acclimated to the operation conditions. The stable operation time in the experimental study can be expected to be at least 2-3 times of the start-up period, which was achieved in our study.

Table 5.2. The test conditions of the nitritation-anammox reactor during different operational phases

Phase	Operation period (d)	Phosphate concentration (mg PO_4-P/L)	TIN[1] removal efficiency (%)	Nitrogen rate		
				VNLR[2] (kg N/ (m^3·d))	VNRR[3] (kg N/(m^3· d))	BNRR[4] (kg N/(kg VSS·d))
I	1-120	120±12	73.6±5.8	0.17±0.02	0.13±0.02	0.065±0.01
II	121-214	235±15.4	34.8±8.9	0.31±0.02	0.12±0.03	0.048±0.01
III	215-403	15±4.2	68.5±3.2	0.16±0.01	0.15±0.02	0.085±0.01
IV	404-575	32±5.4	80.2±2.8	0.33±0.02	0.30±0.01	0.13±0.01

[1]TIN: total inorganic nitrogen

[2]VNLR: volumetric nitrogen loading rate

[3]VNRR: volumetric nitrogen removal rate

[4]BNRR: biomass specific nitrogen removal rate

5.3.3. Anammox bacteria and AOB activities during reactor operation

The specific activities of AOB and anammox bacteria measured in the four operation phases are shown in Figure 5.1B. SAA were 0.24 ± 0.012 g N/(g VSS·d), 0.14 ± 0.014 g N/(g VSS·d), 0.27 ± 0.016 g N/(g VSS·d) and 0.31 ± 0.014 g N/(g VSS·d) from Phase I to Phase IV, respectively; the lowest SAA value occurred when the reactor was fed 100% pre-Ostara® lagoon supernatant, which corresponds to a low ammonia reduction under such feeding condition in the nitritation-anammox reactor. AOB activities were stable during all four phases, indicating that the phosphate concentrations did not have a significant impact on AOB bacteria activities ($P > 0.05$). This result helps to explain the NO_2^--N accumulation observed under 100% pre-Ostara® lagoon supernatant feeding conditions.

It was observed that the phosphate tolerance ability of anammox biomass was dependent on aggregation type. Figure 5.1B shows that the specific anammox activity (SAA) in biofilm was significantly ($P < 0.01$) higher than the SAA in flocs under all treatment conditions. When reactor operation was switched from 50% to 100% pre-Ostara® lagoon supernatant, the SAA in suspended growth biomass and the SAA in biofilm decreased by 78% and 28%, respectively, indicating that flocs was less resistant than biofilm to the reactor feed change. The SAA in both biofilm and flocs increased after switching the reactor feed from pre-Ostara® to post-Ostara® lagoon supernatant. It was also noted that the activity of AOB bacteria was higher in the flocs than in the biofilm. It has been reported that faster growing microorganisms (i.e., the AOB in our study) prefer to inhabit the suspended growth phase, and slower growing microorganisms (i.e., the anammox bacteria in our study) proliferate in the attached growth phase (Veuillet et al., 2014). The enhanced segregation of anammox bacteria and AOB in IFAS system can help mitigate the substrate diffusion limitation which was observed in biofilm alone system (Laureni et al., 2019).

5.3.4 Degradation kinetics

Figure B-5.3 (Appendix B) shows the nitrogen concentration changes in typical SBR cycles under each operation condition. Based on the results shown in Figure B-5.3, ammonium consumption and nitrite accumulation rates in SBR cycles were calculated; the process efficiencies and nitrogen removal rates are shown in Figure 5.2. Figure 5.2 shows that ammonium can be consumed in both aeration and non-aeration phases under all conditions. Under the aeration phase, ammonium was oxidized and nitrite was produced. The consumption of ammonium can be attributed to nitritation (production of NO_2^-), nitrification (production of NO_3^-) and anammox reactions (consumption of NH_4^+ and NO_2^-,

and production of NO_3^-). In the non-aeration (anoxic) stage, concentrations of both NO_2^- and NH_4^+ decreased due to anammox and/or denitrification activities. As shown in Figure 5.2A, ammonium consumption rates in aeration and non-aeration phases were similar for all feeding conditions except for the 100% pre-Ostara® lagoon supernatant feeding condition where the NH_4^+-N consumption rate in the aeration phase was significantly ($P < 0.01$) higher than that in the non-aeration phase. Again, the nitrite accumulation rate in the aeration phase was low for all conditions, except for the 100% pre-Ostara® lagoon supernatant feeding condition; the high nitrite accumulation rate (0.19 ± 0.01 mg/L/min) observed under 100% pre-Ostara® feeding condition can be attributed to the low anammox activities in the aeration phase. Ammonium reduction rates in both aeration and non-aeration periods reached the highest levels under the 100% post-Ostara lagoon supernatant feeding condition.

Nitrate production occurred in both aeration and non-aeration periods. Both NO_2^- oxidation and anammox can produce NO_3^-, while heterotrophic denitrification reduces nitrate concentrations. Figure 5.2B shows a COD reduction of 28%-32% (12-15% in the aeration phase and 14-17% in the non-aeration phase), indicating that denitrification occurred. However, due to a low biodegradable COD in the lagoon supernatant (< 280 mg/L), heterotrophic denitrification was limited (< 36.8 mg N/L, based on a theoretical COD:N ratio of 7.6)(Sobieszuk and Szewczyk, 2006), making anammox the major route of observed nitrogen reduction. Our results showed that the ratio of NO_2^--N consumption to NH_4^+-N consumption in anammox process was not significantly different before (1.38 ± 0.04) and after (1.36 ± 0.03) switching the feed, and indicated the anammox pathway was

the major route in the reactor under both conditions as compared to the theoretical ratio in anammox stoichiometry (i.e., 1 NH_4^+ requires 1.32 NO_2^-) (Gilbert et al., 2014).

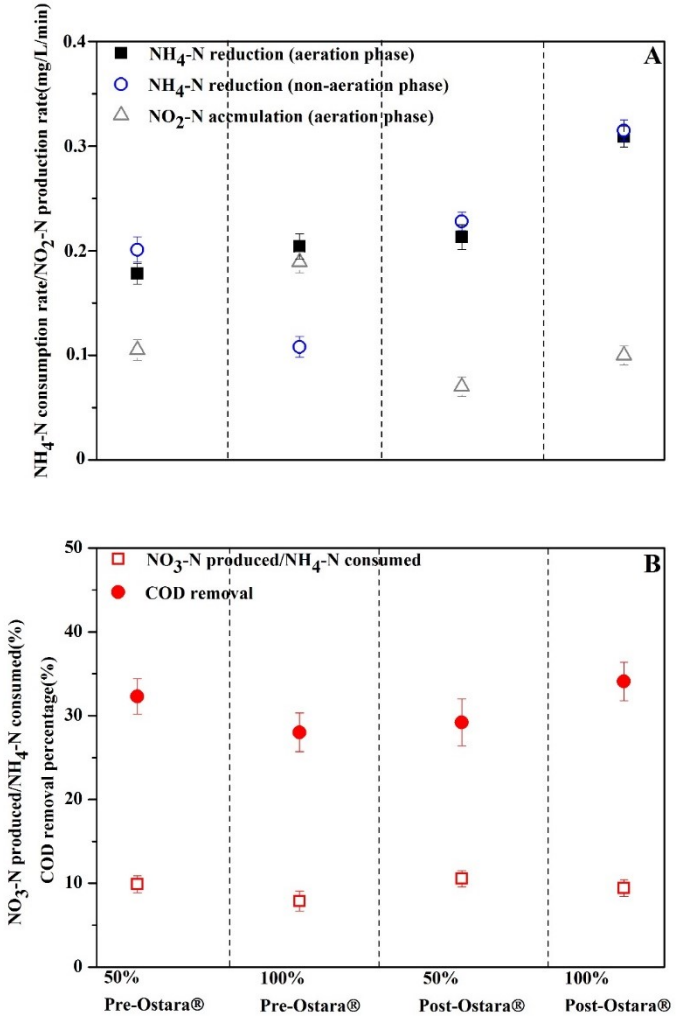

Figure 5.2. Ammonium reduction and nitrite accumulation rates (mg/L/min) in the bioreactor under four operation phases (A), and COD removal percentage and nitrate produced/ammonium consumed during each operation cycle (B).

Meanwhile, according to the anammox stoichiometry, 11% of nitrogen as substrate was converted to NO_3^--N. Thus, a value of NO_3^--N /NH_4^+-N (the fraction of NH_4^+-N converted

to NO_3^--N) greater than 11% suggests that NO_3^--N was produced by nitrite oxidizing bacteria (NOB) oxidation process. A value of NO_3^--N /NH_4^+-N smaller than 11% suggests that NO_3^--N was generated by anammox and/or that nitrification was reduced by heterotrophic denitrification. Our calculations showed that in both aeration and non-aeration phases, NO_3^--N /NH_4^+-N ratios were below 11%, indicating that nitrite oxidation was not significant in our experiments; and that denitrification contributed to nitrogen reduction in the reactor.

It should be noted that the non-aeration phase was slightly adjusted based on reactor performance, as a longer non-aeration phase is needed when anammox activity is low and nitrite accumulation is observed. In the present study, the non-aeration period was maintained at 20 min and 40 min in 50% and 100% pre-Ostara® conditions, respectively, and 15 min in 50% and 100% post-Ostara conditions. Only a short non-aeration phase was required in post-Ostara® conditions due to the high anammox activities in these phases (as shown in Appendix B Figure B-5.3).

5.3.5 Impact of phosphate concentration on specific anammox activity (SAA)

As shown in Figure 5.3, under both Trial 1 (direct dilution of pre-Ostara® lagoon supernatant) and Trial 2 (P addition to post-Ostara® lagoon supernatant) conditions, SAA declined when the phosphate concentration was greater than 120 mg PO_4^{3-}-P/L. In particular, when the phosphate concentration increased from 30 mg PO_4^{3-}-P/L (100% post-Ostara® feed) to 240 mg PO_4^{3-}-P/L (100% pre-Ostara® feed), the SAA declined from 0.30 to 0.17 kg N/(kg VSS·d). SAA inhibition in Trial 1 (when pre-Ostara® feed received different water volumes in order to estimate phosphate concentrations based on dilution) and SAA inhibition in Trial 2 (post-Ostara® feed with direct addition of NaH_2PO_4) were

not statistically different in terms of the relative reduction in SAA (P = 0.84). A comparison of the two trials illustrated that phosphate inhibition was the major factor in the observed anammox activity reduction when pre-Ostara® supernatant was used as reactor feed regardless of the P source.

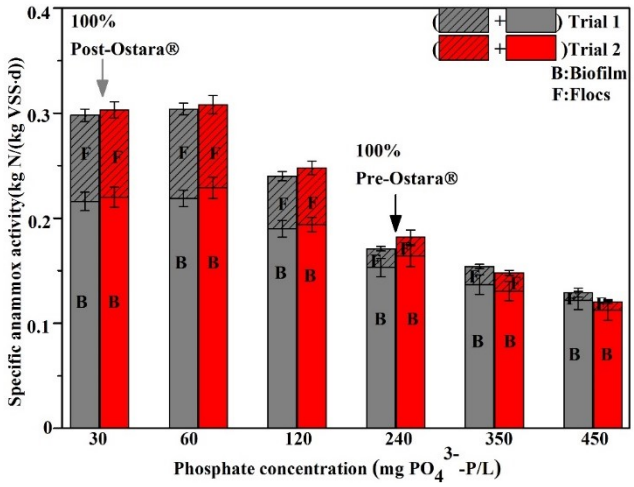

Figure 5.3. Impact of phosphate concentrations (30-450 mg PO_4-P/L) on specific anammox biomass activities (kg N/(kg VSS·d)). Trial 1 examines the impact of phosphate concentrations by diluting pre-Ostara® lagoon supernatant with deionized water. Trial 2 evaluates the impact of phosphate concentrations by adding NaH_2PO_4 directly to post-Ostara® lagoon supernatant.

Further, SAA in biofilm was significantly ($P < 0.05$) higher than SAA in flocs under all P concentration conditions, indicating the significance of biofilms in anammox based nitrogen reduction. Both biofilms and flocs were significantly inhibited when the concentrations of phosphate were above 120 mg PO_4^{3-}-P/L, and biofilms showed greater tolerance than flocs toward the observed phosphorous inhibition ($P < 0.01$).

5.3.6 Microbial community analysis

The microbial community variation under different reactor feeding conditions is shown in Figure 5.4. Figure 5.4A indicates a high prevalence of *Bacteroidetes*, *Proteobacteria*, *Chlorobi*, and *Chloroflexi*, which are commonly reported to be prevalent heterotrophic phyla in anammox bioreactors.(Lawson et al., 2017) *Ignavibacteriae*, previously characterized as cellulolytic bacteria(Podosokorskaya et al., 2013), were abundant in both biofilm and flocs.

The relative abundance of *Planctomycetes* (the *Planctomycetes* phylum covers all five anammox genera discovered to date) (Pereira et al., 2017) was much higher in biofilm than in flocs under all conditions (Figure 5.4A). This observation is supported by results shown in Figure 5.3, where the specific anammox activity is much higher in biofilm than in flocs. Our results underline the importance of biofilms in the control of anammox activities in the reactor. As shown in Figure 5.4, when reactor feed was switched from 50% pre-Ostara® to 100% pre-Ostara® lagoon supernatant, a decrease in the relative abundance of *Planctomycetes* from 8.0% to 5.5% in biofilms was observed. The relative abundance of *Planctomycetes* increased to 8.4% in the 50% post-Ostara® lagoon supernatant feeding condition, and to 17.5% in 100% post-Ostara® lagoon supernatant feeding.

Within the phylum *Planctomycetes*, the dominant anammox bacterial genus was *Candidatus Brocadia*. The abundance of *Ca. Brocadia* decreased from 3.6% to 2.5% in biofilm, and from 3.1% to 0.1% in flocs, when the reactor feed changed from 50% pre-Ostara® supernatant to 100% pre-Ostara® supernatant.

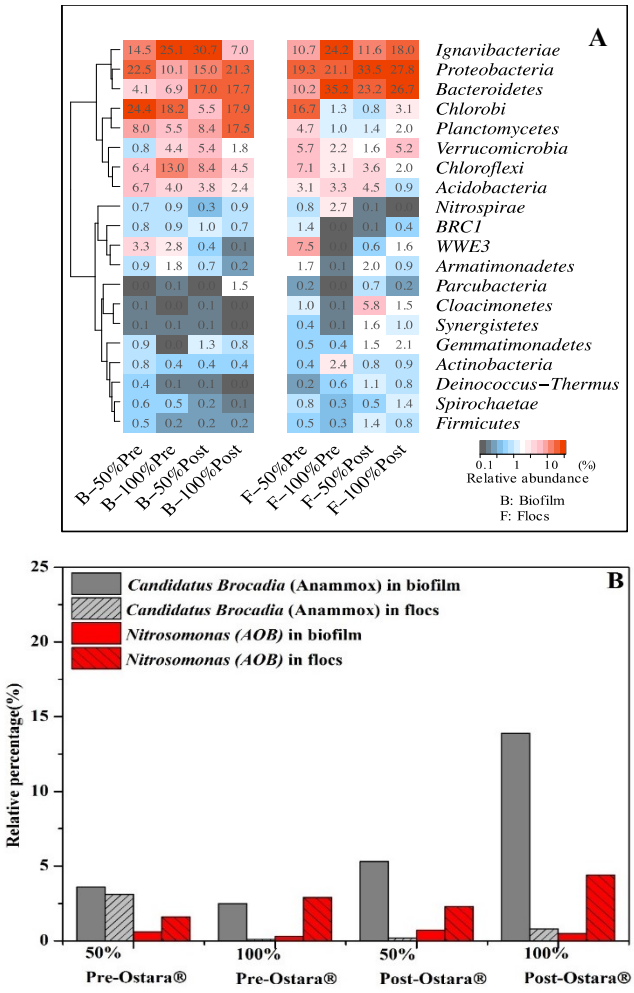

Figure 5.4. Relative read abundance (%) of 16S V4 rRNA gene sequences out of 10 000 reads per sample. Abundance of the major groups (>0.1% in any sample) in the biofilm and flocs at the phylum level (A) during four different phases. Abundance of *Candidatus Brocadia* (anammox) and *Nitrosomonas* (AOB) in biofilm and flocs at the genus level (B) during four different phases.

Thus, 100% pre-Ostara® supernatant negatively affected *Ca. Brocadia* in both biofilm and suspended biomass. The relative abundance of *Ca. Brocadia* increased to 5.3% and 0.2% in biofilm and flocs, respectively, when the reactor was fed with 50% post-Ostara®

supernatant, and the relative abundance of *Ca. Brocadia* further increased to 13.9% and 0.8% in biofilm and flocs, respectively, when the reactor feed was switched to 100% post-Ostara® supernatant. Thus post-Ostara® lagoon supernatant feeding promoted the growth of anammox bacteria better than pre-Ostara® lagoon supernatant.

Nitrosomonas was the most abundant AOB genus in the present study, and was detected in both biofilm and flocs (Figure 5.4B). The relative abundance of AOB was greater in flocs than in biofilm, in agreement with AOB activity results and qPCR studies. *Nitrospira*, known as a nitrite oxidizer, was detected with an abundance of less than 0.2%. The key microorganism responsible for COD removal was identified as *Thauera* (5.1%), which was previously reported in denitrifying communities as a phylum of *Proteobacteria* (Pereira et al., 2017). Denitrifying phosphorous accumulating organisms (PAO) which was identified in the enhanced phosphorus removal flocs system (Mandel et al., 2019), however, was not dominant in our nitritation-anammox system.

5.3.7 Quantitative PCR assay

The key microbes involved in the N removal process was identified as anammox bacteria (dominant by *Candidatus Brocadia*) and AOB (dominant by *Nitrosomonas*). Anammox bacteria and AOB were quantified by real-time qPCR performed on the AMX nirS and amoA genes, respectively. Figure 5.5 shows gene copy numbers of anammox bacteria, *Nitrosomonas* (AOB) and *Nitrospira* (NOB), based on functional gene analysis. The increase in phosphate concentration when reactor feed was switched from 50% pre-Ostara® lagoon supernatant (120 mg PO_4^{3-}-P/L) to 100% pre-Ostara® lagoon supernatant (235 mg PO_4^{3-}-P/L) led to a significant decrease in AMX nirS gene copy numbers (for anammox bacteria) from 9.6×10^6 and 2.6×10^7 copies/mg VSS biomass to 3.0×10^6 and 1.68×10^7

copies/mg VSS biomass in suspension and in biofilm, respectively; representing a decrease of 69% and 35% of AMX nirS gene copy numbers in suspended biomass and biofilms, respectively ($P < 0.05$).

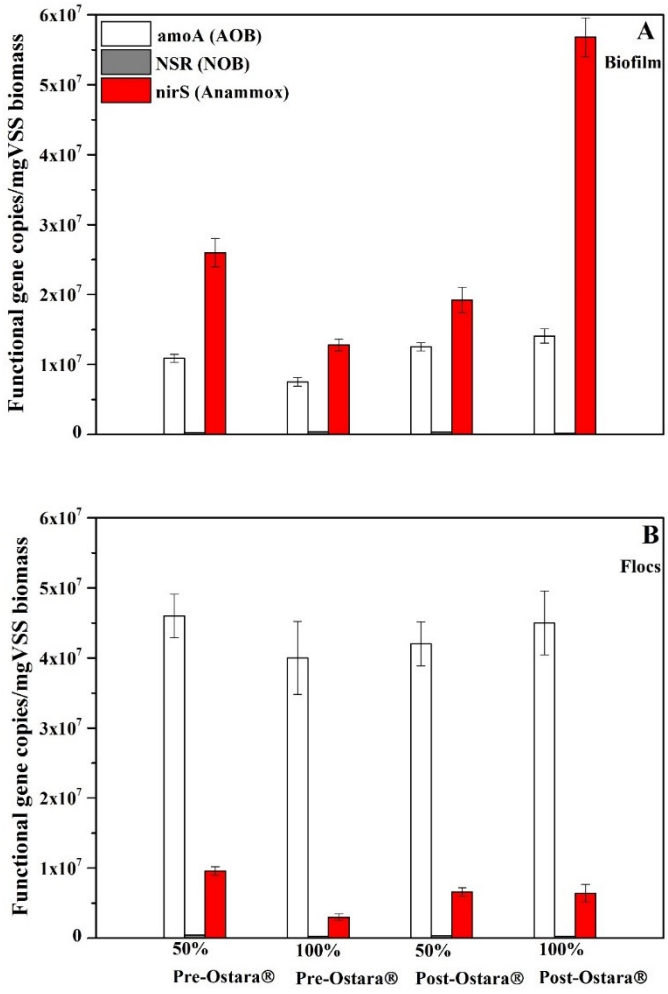

Figure 5.5. qPCR results of biofilm (A) and flocs (B) during four operation conditions. The AMX nirS gene is the functional nirS gene of anammox bacteria. The amoA gene is the functional gene of AOB (*Nitrosomonas*). And the NSR gene is the functional gene of NOB (*Nitrospira*).

After switching the feeding type to post-Ostara® lagoon supernatant, AMX nirS gene copy numbers increased in both biofilm and flocs. AMX nirS gene copy numbers increased by 2.38 times (from 1.68×10^7 to 5.68×10^7 copies/mg VSS biomass) in biofilm and 1.13 times (from 3.0×10^6 to 6.4×10^6 copies/mg VSS biomass) in sludge when the reactor feed changed from 100% pre-Ostara® lagoon supernatant feeding to 100% post-Ostara® lagoon supernatant feeding ($P < 0.05$).

AOB abundance was relatively stable under all reactor feeding conditions, which were 4.6×10^7 copies/mg VSS biomass, 4.0×10^7 copies/mg VSS biomass, 4.2×10^7 copies/mg VSS biomass and 4.5×10^7 copies/mg VSS biomass from Phase I to Phase IV, respectively. The only exception was in the 100% pre-Ostara® lagoon supernatant condition in which amoA gene copy numbers were slightly reduced. In flocs, the abundance of the amoA genes per mg VSS biomass (representing AOB abundance) was significantly higher in flocs than in biofilms ($P < 0.01$). These results agree with the AOB activity results discussed above (Figure 5.1B).

NSR gene copy numbers (representing nitrite oxidizer abundance) were about two log units lower than AOB copy numbers under all feeding conditions, indicating the successful suppression of nitrite oxidizing bacteria. No significant difference in NSR gene copy numbers was observed in flocs and biofilm ($P > 0.01$).

5.4. Conclusion

For the first time, the effects of phosphate concentration in a continuous operating nitritation-anammox reactor treating ammonium rich digester sludge thickening lagoon supernatant were explored. Ammonium reduction efficiency significantly improved when a phosphorus recovery treatment (the Ostara® process) was implemented before lagoon

supernatant was introduced to the bioreactor. Our studies concluded that phosphate concentration was the major inhibitor of anammox bacteria in the reactor; and anammox bacteria in biofilm showed greater tolerance than anammox bacteria in flocs to phosphate stress. Further studies on the impacts of environmental and operation conditions on the phosphorous inhibition should be carried out to elucidate the overall impact of phosphorus on such processes. Results demonstrated that the by placing Ostara® process as the pretreatment for phosphorus reduction, the nitrogen removal efficiency was improved in IFAS-SBR nitritation-anammox process treating raw sludge thickening lagoon supernatant. It should be noted that our study focused on elucidating the impact of phosphorus concentrations on anammox activities. However, anammox bioreactor treating Pre-Ostara® supernatant may be optimized through further operation condition optimization (e.g., through reducing nitrogen loading rate, adjusting aeration strategies, changing the biofilm media filling ratio, and/or feeding strategies), which was not studied in the present paper but may be of interest for future studies.

5.5. References

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CHAPTER 6 IMPROVING NITROGEN REMOVAL IN AN IFAS NITRITATION-ANAMMOX REACTOR TREATING LAGOON SUPERNATANT BY MANIPULATING BIOCARRIER FILLING RATIO AND HYDRAULIC RETENTION TIME[4]

6.1. Introduction

Biological nutrient removal (BNR) is gaining prevalence in wastewater treatment plants (WWTP), especially as city water demand increases and wastewater treatment regulations become more stringent (Xu et al., 2014; Nze et al., 2018; Zhang et al., 2019). Lagoon supernatant is a side stream in WWTP that is rich in ammonium (NH_4^+) when compared with the frequently studied anaerobic digestate liquor (Shao et al., 2018). Rather than the immediate mechanical separation (*e.g.*, centrifugation) of the digestate, lagoon supernatant offers a natural settling process by storing the digestate for approximately five months, thus generating the supernatant feathering with lower temperature and alkalinity and less biodegradable organic matter and suspended solids. Approximately 800-1200 mg L^{-1} NH_4^+ in lagoon supernatant is recirculated to the mainstream and contributes over 1/3 of the total nitrogen loading in WWTP (Shao et al., 2018). Therefore, removing the nitrogen from lagoon supernatant is an urgent need and straightforward option to reduce the nitrogen level in wastewater discharge.

Nitritation-anammox process is an emerging BNR technology used for WWTP NH_4^+ removal, especially in wastewater stream that contain high NH_4^+ concentration and low

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biodegradable organic matters (*i.e.*, low C/N ratio) (Boiocchi et al., 2015; Gilbert et al., 2015; Persson et al., 2017). This process is more energy/cost efficient (less oxygen input and no extra carbon supplementation) and process effective (in a shortcut of NH_4^+—NO_2^-—N_2 pathway) as compared to the traditional nitrification and denitrification pathways (Rezania et al., 2015; Ramalingam et al., 2017). Nitritation-anammox process can be achieved in one- or two-stage reactors where NH_4^+ is partially oxidized to NO_2^- by ammonium oxidizing bacteria (AOB) (nitritation step, equation [1]) and the spare NH_4^+ react with the AOB produced NO_2^- with the assistance of anammox bacteria to produce N_2 gas, achieving N removal (anammox step, equation [2]). The activity of AOB contributes to dissolved oxygen consumption which positively influence the maintenance of anoxic environment which anammox bacteria prefer. NO_3^- is a by-product that can be generated in the entire process either via the anammox reaction itself (equation [2]) or through the further oxidation of NO_2^- (Strous et al., 1997; Zhang et al., 2015b). Major technical challenges exist in both nitritation and anammox steps, including the continuous repression of NO_2^- oxidation and the difficult cultivation of anammox bacteria in the reactor due to their long generation time and low tolerance to environmental condition changes (Strous et al., 1999; Thamdrup, 2012; Ding et al., 2018).

The one-stage nitritation-anammox reactor is of utmost engineering importance in full-scale application due to its process compactness (Lackner et al., 2014; Gilbert et al., 2015). Achieving synchronous aerobic and anaerobic ammonium oxidation and heterotrophic denitrification (if present) in one reactor requires a precise process control to attain an effective balance of microbial metabolic activities in the competition for available resources, such as substrates (*e.g.*, NH_4^+, NO_2^- or organic carbons), dissolved oxygen (DO),

and inhabiting spaces (Agrawal et al., 2018). For example, sufficient DO should be provided for the growth and activities of AOB. However, too much DO can lead to overgrowth of nitrite oxidizing bacteria (NOB), which hamper the nitritation-anammox kinetics (Shao et al., 2018; Wang et al., 2019). The presence of organic matter in wastewater not only increases the abundance and diversity of heterotrophic bacteria, but also proliferates denitrifiers, especially at high ratios of chemical oxygen demand (COD) to nitrogen (N) (*e.g.*, COD/N ≥ 2) (Wang et al., 2018). Heterotrophic bacteria affect the nitritation-anammox process via competing for the available DO with AOB or competing for NO_2^- with anammox bacteria (*i.e.*, heterotrophic denitrification) (Wang et al., 2018). In addition, in the biofilm or flocs system, fast growing bacteria (*e.g.*, AOB and heterotrophic bacteria) tend to grow on the outer layer of biofilms or flocs, which may limit the accessibility of nutrients to anammox bacteria (Tsushima et al., 2007; Costa et al., 2014), while protecting anammox bacteria from the hostile environment (*i.e.*, oxygen or organic matter) (Agrawal et al., 2018).

The integrated fixed-film activated sludge (IFAS) reactor acts as a unique implementation for the nitritation-anammox process by combining both flocs and biofilm configurations. The one-stage IFAS nitritation-anammox process offers a better segregation of anammox bacteria and other microbes (*e.g.*, AOB and heterotrophic denitrifiers, if present) within one reactor due to the respective favorable inhabiting environment (*i.e.*, biofilm or flocs) (Zhang et al., 2019). Based on the physical structure and mass transferring feathers of biofilm and flocs, the majority of anammox bacteria are retained in the biofilm and AOB aggregate within flocs, providing more flexibility on controlling the individual nitritation and anammox steps as compared to other one-stage nitritation-anammox reactors (Zhang

et al., 2015b). Additional advantages from this process come in the form of a lower optimum DO range and easier suppression of NOB growth (Hubaux et al., 2015). Therefore, up to 200% higher nitrogen removal efficiency can be achieved in the IFAS type one-stage nitritation-anammox reactor when compared to the parallel operated moving bed biofilm reactor (MBBR) reactor (Malovanyy et al., 2015; Trojanowicz et al., 2016). The highest reported nitrogen removal efficiency of 84% is reported in a study by Zhang et al (2015b), where the synthetic digestate liquor was fed at ammonium loading rates of 0.7-1.3 Kg N m^{-3} d^{-1}. However, other than the explication on reactor feathers, the optimization on the IFAS nitritation-anammox reactor operation is still largely unknown, especially for low C/N wastewater such as lagoon supernatant.

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad [1]$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad [2]$$

This study established a lab scale one-stage IFAS sequencing batch reactor (IFAS-SBR) for lagoon supernatant nitritation-anammox treatment. The objectives of this study were to investigate the reactor nitrogen removal performance at (1) different biocarrier filling ratios and (2) different hydraulic retention times (HRTs). The key factors that affect the nitrogen removal rates or nitrogen removal efficiency of the process are discussed. Kinetic study and microbial analysis were also performed to assess the respective response of flocs and biofilm biomass under different operation conditions.

6.2. Materials and Methods

6.2.1. Reactor operation

Nitritation-anammox process was operated in a lab-scale IFAS reactor with a diameter of 17.8 cm, height of 35.6 cm, and effective volume of 6 L (Appendix B Figure B-5.4). The reactor was seeded with Veolia Anox K™5 carriers (Veolia water technologies, Canada) at a biocarrier filling ratio of 35% or 55%. The IFAS reactor was operated in sequencing batch mode (so-called "IFAS-SBR") which comprised of two daily cycles. Each SBR cycle lasted for 12 h, including a 5 min feeding, 10 h reaction with intermittent aeration (*i.e.*, in the alternating 105 min aerobic and 15 min anaerobic conditions), 110 min settling, and 5 min effluent withdrawing. The reactor feed volume exchange ratio (VEX), which represents the ratio of influent volume added at the beginning of each cycle and the reactor effective volume, varied from 20% to 42%, leading to the operating HRTs of 1.2 d to 2.5 d. The selection of operating parameters was mainly based on our previous reactor operation and available operating knowledge from the similar research (Li et al., 2016; Zhang et al., 2016; Yang et al., 2019b).

Table 6.1. The test conditions of the one-stage nitritation-anammox reactor during different operational phases.

Phase	HRT (d)	Biocarrier filling ratio	VEX[1] (%)	Nitrogen rate (kg m^{-3} d^{-1})	
				NLR[2]	NRR[3]
I	2.5	35%	20	0.32 ± 0.00	0.25 ± 0.00
II	2.5	55%	20	0.33 ± 0.00	0.29 ± 0.00
III	1.5	55%	33	0.56 ± 0.01	0.46 ± 0.01
IV	1.2	55%	42	0.70 ± 0.01	0.54 ± 0.00

[1]VEX: Volume Exchange Ratio

[2]NLR: Nitrogen Loading Rates

[3]NRR: Nitrogen Removal Rates

The 500 d operation included four different operation stages, distinguished by different biocarrier filling ratios (the ratio of total volume of biocarriers to the reactor working volume) or different HRTs. The details of the operation conditions are shown in Table 6.1. Similar to our previous research (Yang et al., 2019a), the reactor was fed with lagoon supernatant with the water chemistry parameter showing in Table 6.2. The resultant volumetric ammonium loading rates (ALR), or nitrogen loading rates (NLR, as the influent NO_2^- and NO_3^- were negligible), for Stages I, II, III, and IV were 0.32 ± 0.00 kg N m^{-3} d^{-1}, 0.33 ± 0.00 kg N m^{-3} d^{-1}, 0.56 ± 0.01 kg N m^{-3} d^{-1}, and 0.70 ± 0.01 kg N m^{-3} d^{-1}, respectively. A mechanical stirrer (Grainger, Illinois, USA) was used to ensure the reactor was well mixed in the reaction phase. An aeration pump (Active Aqua, California, USA) was used to ensure the reactor maintained a constant DO between 0.24-0.26 mg L^{-1} in the aerobic phase. A thermostatic water jacket was used to ensure the liquid temperature in the reactor was stabilized at 31±2 °C. There was no sludge discharge during the 500 d operation. Due to the metabolic activities of different microbial species (Poot et al., 2016), pH was measured in the range of 7.8 - 8.1 at the beginning of each SBR cycle and in the range of 7.2-7.4 at the end of each cycle.

6.2.2. Specific activity analysis of anammox bacteria and ammonium oxidizing bacteria

The specific activity of anammox bacteria (SAA) and specific activity of ammonium oxidizing bacteria (SAOB) of flocs in the bulk solution and biofilm on the carriers was measured at each stage by the method described previously (Jubany et al., 2009; 2012; Jin et al., 2013; Gilbert et al., 2014). Floc and biofilm biomass pellets (after centrifugation) were washed with phosphate-buffered saline (PBS) at least three times before being re-

suspended in mineral medium containing target substrates (*i.e.*, NH_4^+ and NO_2^- for SAA and NH_4^+ for SAOB).

Table 6.2. Water characteristics of raw lagoon supernatant.

Parameters	Unit	Lagoon supernatant
		Mean ± S.D.
TSS	g TSS/m^3	305±107
NH_4^+	mg N/L	855±45
NO_2^-	mg N/L	0.6±0.5
NO_3^-	mg N/L	0.2±0.2
PO_4^{3-}	mg PO_4-P/L	32±5.4
Alkalinity	mg $CaCO_3$/L	3056±45.6
pH	-	7.87±0.15
COD	mg/L	568±54.42

The anaerobic and aerobic condition was maintained separately for SAA and SAOB tests. The SAA and SAOB was calculated as the ratio of maximum substrate consumption rate and biomass concentration. All batch studies were performed in triplicate.

6.2.3. Microbial analysis

Quantitative polymerase chain reaction (qPCR) experiments were performed for microbial analysis of biofilm and floc biomass at each stage. DNA isolation kits (MO BIO Laboratories Inc., Carlsbad, California) were used to extract DNA from biomass samples from flocs and biofilm after biomass samples collected, which were collected from the steady-state of each reactor stage. The extracted DNA was quantified by a DNA concentration measurement instrument (NanoDrop One, Thermo Scientific, Walthan, MA) before being subjected to qPCR experimentation (performed by CFX96TM Real-Time Detection System, Bio-Rad Laboratories Ltd., Mississauga, Ontario). The genes targeted

by qPCR and corresponding primers used for anammox bacteria, ammonia oxidizing bacteria, and denitrification bacteria can be found in Table A-1 (Appendix A). The qPCR reaction conditions are summarized in Table A-2 (Appendix A). Each qPCR experiment was performed in triplicate for individual sample with target genes.

6.2.4. Chemical and statistical analysis

Water quality metrics, including COD, NH_4^+-N, NO_2^--N, and NO_3^--N concentrations, were measured with Hach reagent kits (Hach Company, Loveland, Colorado) after the liquid samples were filtered by 0.45 μm filters (fisherSci, Canada) (Xu et al., 2018). The total nitrogen concentration was determined by the addition of NH_4^+-N, NO_2^--N, and NO_3^--N concentrations. Volatile suspended solids (VSS) was measured for the solid samples to evaluate the biomass concentration of flocs and biofilm (Eaton et al., 2011).

The widely used statistical analysis method "analysis of variance (ANOVA)" in single factor model was performed for the statistical comparison of different samples with p-values smaller than 0.05 indicating the existence of statistical difference (Xu et al., 2019; Yang et al., 2019a; Zhang et al., 2019).

6.3. Results and Discussion

6.3.1. Reactor performance

6.3.1.1. Nitrogen removal performance

The influent NH_4^+-N concentrations and effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations from the IFAS-SBR reactor were monitored during the 500 d operation (Figure 6.1). In the initial 129-d operation (Stage I), at the volumetric ammonium loading rate (ALR) of 0.32 ± 0.00 kg N m^{-3} d^{-1}, the effluent NH_4^+-N concentration decreased consistently from approximately 260 mg L^{-1} to below 160 mg L^{-1} (*i.e.*, 150.1 ± 6.1 mg L^{-1},

days 107-129). The effluent NO_2^--N concentration fluctuated in the range of 17-32 mg L^{-1} during the first 2 months and started to stabilize at 10.2 ± 1.6 mg L^{-1} during the middle of Stage I. The effluent NO_3^--N concentration fluctuated before stabilizing at 44.5 ± 4.1 mg L^{-1}. At the end of Stage I, the removal efficiencies of NH_4^+-N and total nitrogen (TN) were 81.8 ± 0.6% and 74.8 ± 1.1%, respectively (Appendix B Figure B-6.2); the nitrogen removal rate (NRR) reached 0.25 ± 0.00 kg N m^{-3} d^{-1} (Table 6.1).

When the biocarrier filling ratio was increased from 35% to 55%, the reactor was operated at HRT of 2.5 d for another 134 days (Stage II; days 130-265). With a constant volumetric ALR of 0.33 ± 0.00 kg N m^{-3} d^{-1}, as in Stage I, the effluent NH_4^+-N concentration decreased from 138.8 ± 11.5 mg L^{-1} (days 130-159) to 39.1 ± 3.5 mg L^{-1} after day 238. The effluent NO_2^--N concentration remained relatively stable at 10.6 ± 2.4 mg L^{-1} from the beginning of Stage II, with the exception of a few small fluctuations. In contrast, there were larger fluctuations of the effluent NO_3^--N concentration through the entire stage with the peak value of 70 mg L^{-1} on day 209 and lowest value of 42 mg L^{-1} on day 160. At the end of Stage II (days 244-265), the removal efficiencies of NH_4^+-N and TN reached 95.2 ± 0.4% and 87.4 ± 0.8%, respectively (Appendix B Figure B-6.2), with NRR being 0.29 ± 0.00 kg N m^{-3} d^{-1} (Table 6.1). Both nitrogen removal efficiency and removal rates were significantly higher ($p < 0.001$ and $p < 0.05$, respectively) than those in Stage I, indicating a nitrogen removal performance improvement at the higher biocarrier filling ratio.

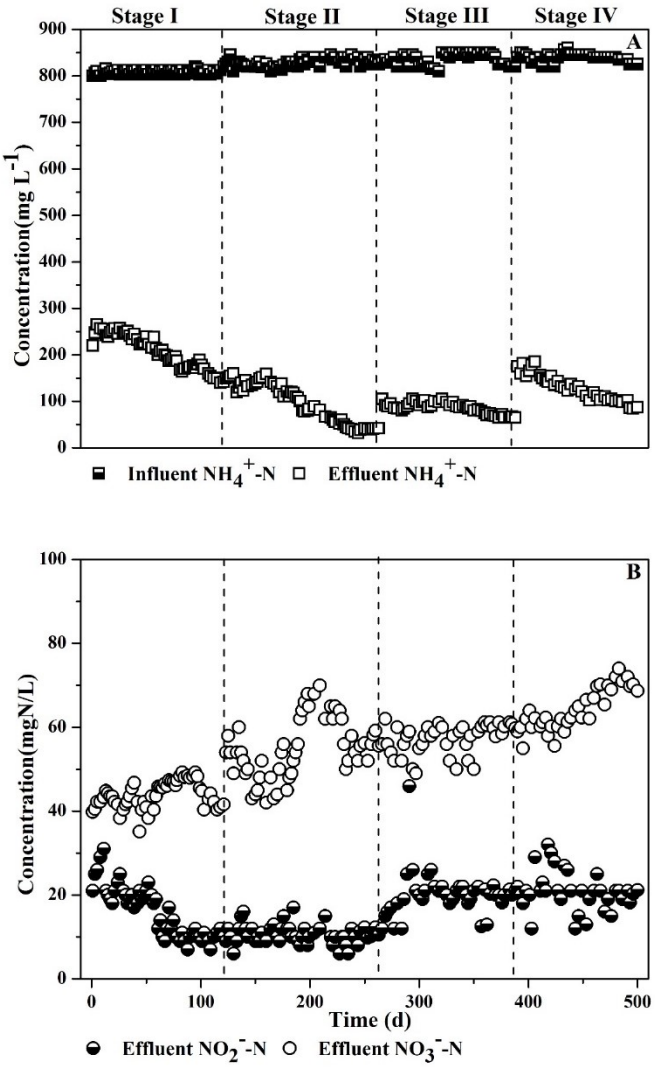

Figure 6.1. Changes in reactor influent and effluent NH_4^+-N concentrations (A), effluent NO_2^--N and NO_3^--N concentrations (B) under different operation stages.

At the transition from Stage II to Stage III (*i.e.*, day 266), the effluent NH_4^+-N concentration immediately increased to 93.5 ± 7.5 mg L^{-1}, which is likely explained by the sudden increase in volumetric ALR (0.56 ± 0.01 kg N m^{-3} d^{-1}) or decrease in HRT (1.5 d). After approximately 90 days of continuous operation, the effluent NH_4^+-N concentration became stable at 70.0 ± 4.6 mg L^{-1} until the last day of Stage III (day 389), which was still higher

than the lowest level in Stage II (*i.e.*, 39.9 ± 3.5 mg L^{-1}). Meanwhile, the effluent NO_2^--N concentration increased from 10.6 ± 2.4 mg L^{-1} to 21.3 ± 5.1 mg L^{-1} within the first 20 d operation in Stage III and was stabilized at the latter level until the end of stage. On the contrary, the effluent NO_3^--N concentration remained relatively constant at 57.3 ± 3.7 mg L^{-1} during the entirety of Stage III. Therefore, in the end of operation in Stage III (days 365-389), although NRR increased significantly ($p < 0.001$) to 0.46 ± 0.01 kg N m^{-3} d^{-1} when compared with Stage II, the removal efficiencies of NH_4^+-N and TN reduced ($p < 0.05$) to 91.8 ± 0.3% and 82.1 ± 0.3%, respectively (Appendix B Figure B-6.2); this is discussed further in section 3.1.2.

The further increase of volumetric ALR (*i.e.* 0.70 ± 0.8 mg L^{-1} as HRT reduced to 1.2 d) in Stage IV (days 390-500) initially led to a quick accumulation of NH_4^+-N (171.2 ± 11.0 mg L^{-1}) in the reactor effluent (days 390-406), which gradually declined to 85.8 ± 1.2 mg L^{-1} after continuously operating for 2 months. The effluent NO_2^--N concentration remained stable at 19.9 ± 1.5 mg L^{-1} after some peak values occurring at the beginning, which did not appear to be affected by the elevated ALR. Meanwhile, the effluent NO_3^--N concentration expressed an ascending trend in this stage (IV), increasing gradually from the 60.2 ± 2.5 mg L^{-1} (days 390-436) to 69.6 ± 0.8 mg L^{-1} (days 491-500). This may be due to the higher anammox activities at the enhanced ALR (Yang et al., 2019b). Finally, by comparison to Stage III, the removal efficiencies of NH_4^+-N and TN in Stage IV dropped further ($p < 0.05$) to 89.6 ± 0.1% and 78.8 ± 0.2% (Appendix B Figure B-6.2), respectively, while NRR further increased to 0.54 ± 0.00 kg N m^{-3} d^{-1} (Table 6.1) at the end of Stage IV (days 491-500).

6.3.1.2. Biomass concentration and specific biomass ammonium loading rates

The biomass concentration was determined in both biofilm and flocs configurations (Figure 6.2). In principal, the flocs are mainly formed via the proliferation of fast-growing organisms feeding on the organics or nutrients of lagoon supernatant and the biomass detachment from the inoculated biocarriers. From Stage I to Stage II, with the increase of biocarrier filling ratio from 35% to 55%, the biofilm biomass increased significantly ($p < 0.01$) from 5.2 ± 0.1 g to 7.4 ± 0.2 g while the flocs biomass remained relatively similar 9.9 ± 0.1 g and 9.5 ± 0.2 g ($p = 0.2$), leading to a slight increase of total biomass in the reactor from 15.1 g to 16.9 g. In Stage III, both biofilm and flocs biomass increased to 8.6 ± 0.3 g ($p < 0.01$) and 11.3±0.4 g ($p = 0.03$), respectively, as more organics and nutrients were available for bacteria growth at the higher influent loading rate (Xu et al., 2014; Nze et al., 2018). However, only biofilm biomass further increased to 10.1 ± 0.6 g ($p < 0.01$) when the influent loading rate continued increasing, while the floc biomass remained similar at 12.9 ± 0.4 g ($p = 0.06$) which was likely a compromised result under both biomass growth and loss via "wash-out". This observation underlines the advantage of biofilm configuration compared to flocs as the flocs were likely more sensitive to the increased influent loading rate and were "washed out" with the concurrently increased hydraulic pressure (van der Star et al., 2007; Zhang et al., 2015b).

Other than the operating parameter "volumetric ALR", the specific biomass ALR (ALR in unit biomass) is another critical parameter that could affect the nitrogen removal performance (Appendix B Figure B-6.3). The reactor biomass enhancement under the increased volumetric ALR was primarily associated with the stimulated growth of the nitrifying bacteria and heterotrophic bacteria, as observed in flocs systems (Xu et al., 2014), which in turn contributed to the nitrogen removal in the reactor. Particularly with lagoon

supernatant nitritation-anammox treatment, the population of heterotrophic bacteria in biomass is minor due to the low C/N ratio (*i.e.*, < 0.6), resulting in anammox bacteria and nitrifying bacteria (*i.e.*, AOB) in the dominant position of the biomass components (see section 3.2.).

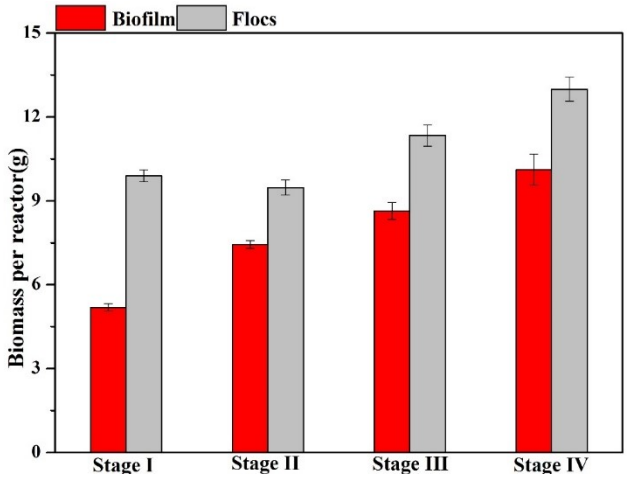

Figure 6.2. Biomass concentration in both biofilm and flocs configurations under different stage operations. Error bars represent standard deviations from different samples (the same for the following figures).

However, the growth of biomass associated with the reduced HRT was probably not sufficient to withstand the elevated volumetric ALR, which can be attributed to the slow growth rate of anammox bacteria and the substrate transfer restriction in biofilm configuration (van der Star et al., 2007). Therefore, the declined NH_4^+-N and TN removal efficiencies in Stages III and IV may result from the higher ($p < 0.01$) specific biomass ALRs in these stages (0.17 ± 0.00 kg N kg^{-1}VSS d^{-1} and 0.18 ± 0.00 kg N kg^{-1}VSS d^{-1}, respectively) (Appendix B Figure B-6.3).

6.3.2. Specific activities of anammox bacteria and AOB bacteria

The specific anammox activity (SAA) under the steady-state of different stages were shown in Figure 6.3A. The SAA of biofilm biomass was significantly higher than that in flocs ($p < 0.001$), as anammox bacteria prefer to aggregate in biofilm to obtain the long retention time. This has been directly indicated by our microbial community analysis results, which showed a significantly higher (*i.e.*, 1~2 log higher) abundance of AMX *nirS* gene (representing anammox bacteria) in biofilm than that in flocs. Increasing biocarrier filling ratio did not affect ($p = 0.09$) the SAA in the biofilm, leading to the SAA in Stage I and Stage II of 0.26 ± 0.00 kg N kg^{-1} VSS d^{-1} and 0.27 ± 0.00 kg N kg^{-1} VSS d^{-1}, respectively. However, the biofilm SAA significantly increased to 0.28 ± 0.00 kg N kg^{-1} VSS d^{-1} in Stage III and to 0.33 ± 0.02 kg N kg^{-1} VSS d^{-1} in Stage IV ($p = 0.02$ and $p < 0.001$, respectively) as ALRs increased. Meanwhile, the anammox bacteria gene abundance in biofilm of each operation stages were (5.25 ± 0.15)×10^7, (5.38 ± 0.27)×10^7, (5.78 ± 0.30)×10^7, and (6.71 ± 0.21)×10^7 copies mg^{-1} VSS biomass respectively, which showed an increasing trend ($p < 0.05$) from Stage II to Stage IV and provides an explanation of the SAA changes (Figure 6.3A); this is plausible since simply adding more biocarriers to the reactor does not improve the anammox bacteria density in biofilm, while increasing the ALR does. On the other hand, the floc SAAs were 0.09 ± 0.01, 0.09 ± 0.01, 0.07 ± 0.01 and 0.04 ± 0.01 kg N kg^{-1} VSS d^{-1} in Stages I, II, III, and IV, respectively (Figure 6.3A), which was not affected by the increased biocarrier filling ratio either ($p > 0.05$); however, it started to decrease when the HRT declined from Stage II to Stage IV ($p = 0.015$ and $p = 0.003$, respectively). Meanwhile, a slight decrease of the anammox bacteria abundance in flocs was observed from (6.20 ± 0.21)×10^6 copies mg^{-1} VSS biomass in Stage II to (4.31 ± 0.43)×10^6 copies mg^{-1} VSS biomass in Stage IV ($p < 0.05$). The slowly growing bacteria, such as anammox bacteria,

in our study are less resilient to increased hydraulic pressure when the HRT reduced, and therefore are more easily flushed out as compared to the microbes which grow faster (van der Star et al., 2007).

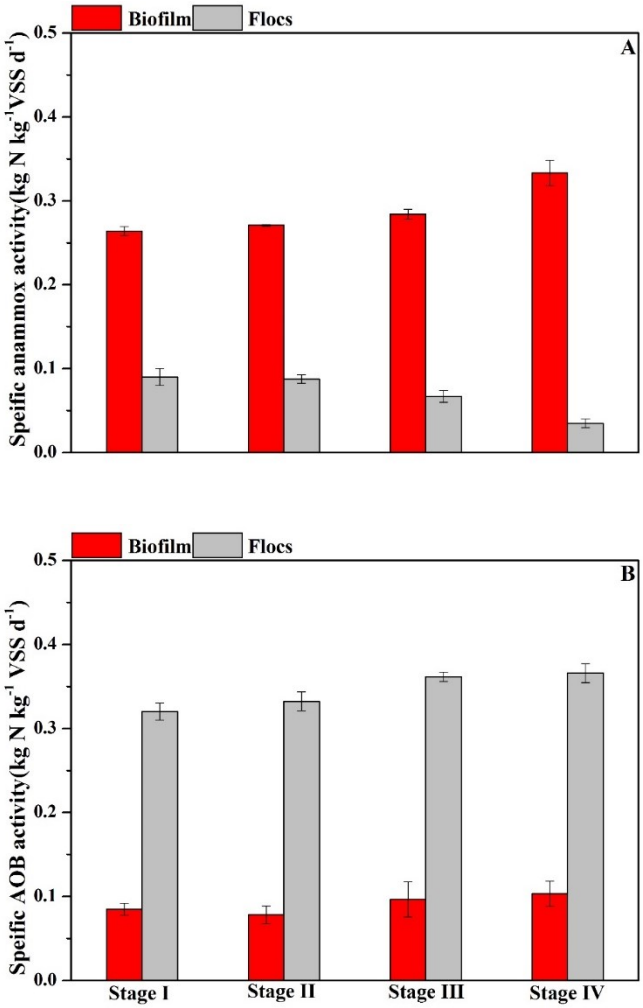

Figure 6.3. Specific activities of anammox bacteria (A) and ammonium oxidizing bacteria (B) in both biofilm and flocs configuration in different stages

On the contrary, the specific activity of another important bacteria in reactor — AOB — was significantly higher in flocs than in biofilm (Figure 6.3B), due to the fact that the permeable and loose structures of flocs provide better access of NH_4^+-N and O_2 to AOB.

SAOB were 0.32 ± 0.01 and 0.33 ± 0.01 kg N kg^{-1} VSS d^{-1} in the suspended flocs of Stages I and II, respectively, which was not influenced ($p = 0.23$) by the increased biocarrier filling ratio. Flocs SAOB increased to 0.36 ± 0.01 kg N kg^{-1} VSS d^{-1} ($p = 0.015$) in Stage III and further increased to 0.38 ± 0.00 kg N kg^{-1} VSS d^{-1} ($p = 0.027$) in Stage IV, which was associated with the increased volumetric ALR. Likewise, SAOB was related to the density of AOB in biomass (Figure 6.3B). During the operation condition changes, only the increase of volumetric ALR could encourage the growth of AOB, leading to a higher density of AOB in floc biomass. This is indicated by the abundance dynamics of amoA gene (as AOB representative gene) in flocs , which showed a similar abundance ($p > 0.05$) in Stage I ([3.51 ± 0.25]×10^7 copies mg^{-1} VSS biomass) and Stage II ([3.6 ± 0.08]×10^7 copies mg^{-1} VSS biomass), and higher abundances in Stage III ([4.59 ± 0.20]×10^7 copies mg^{-1} VSS biomass) and Stage IV ([4.99 ± 0.20]×10^7 copies mg^{-1} VSS biomass) ($p = 0.046$). In comparison, SAOB in biofilm were in the significantly lower range of 0.08 ± 0.01~0.10 ± 0.02 kg N kg^{-1} VSS d^{-1} during the entire operation, showing no statistical difference between stages ($p = 0.08$, $p = 0.4$, and $p = 0.2$, respectively). As is evident from the amoA gene abundance in biofilm, only a few AOB grew in the biofilm (*i.e.*, 2~3 fold lower than that in flocs), with a relatively stable ($p > 0.9$) abundance under all operation conditions, which were (1.66 ± 0.12)×10^7, (1.64 ± 0.32)×10^7 (1.19 ± 0.15)×10^7, and (1.18 ± 0.15)×10^7 copies mg^{-1} VSS biomass, in Stages I, II, III, and IV, respectively. Based on the multi-layer structure of biofilm, most of biofilm AOB aggregated on the biofilm surface and protected AOB from the O_2 inhibition (Zhang et al., 2015a).

Overall, a better segregation of anammox bacteria and AOB was observed in such IFAS configurations, which is highlighted with the decreased HRTs. The AOB dominant flocs

can supply an enhanced NO_2^- flow towards the anammox bacteria dominant biofilm biomass to support anammox bacteria growth and metabolic activities (Hubaux et al., 2015; Zhang et al., 2015b).

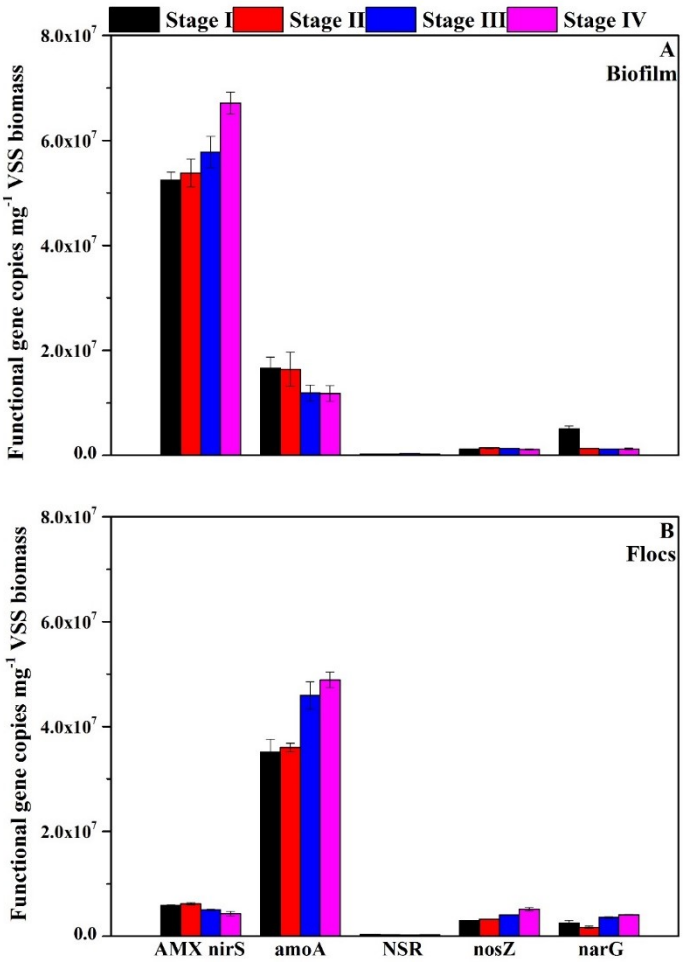

Figure 6.4. Gene abundance of anammox bacteria (AMX *nirS* gene), ammonium oxidizing bacteria (*amoA* gene), nitrite oxidizing bacteria (*Nitrospira* (*NSR)* gene) and heterotrophic denitrifiers (*nosZ* and *narG* genes) in both biofilm and flocs configurations in different stages.

NOB (Figure 6.4) was highly suppressed in this reactor under all operation conditions, as indicated by the negligible gene abundance of NOB (*i.e.*, *Nitrospira* [*NSR*] gene), which was $(1.95 \pm 0.10)\times10^5 \sim (3.57 \pm 0.30)\times10^5$ copies mg^{-1} VSS biomass in biofilm phase and $(2.43 \pm 0.30)\times10^5 \sim (3.34 \pm 0.40)\times10^5$ copies mg^{-1} VSS biomass in flocs phase, which was lower than the other selected genes (*i.e.*, AOB, AMX *nirS*, *narS* and *nosZ*).

The low biodegradable organic carbon in the lagoon supernatant limited the growth of heterotrophic bacteria in the nitritation-anammox process. The population of heterotrophic denitrifers (represented by *nosZ* and *narS* genes in our study), which contributed for the NO_3^- /NO_2^- reduction and COD removal under anoxic condition, were in scarcity compared to the anammox bacteria and AOB (Figure 6.4). This is similar to other research in the anammox process for wastewater with a low C/N ratio (Malovanyy et al., 2015).

6.3.3. Nitrogen removal kinetics

Nitrogen removal kinetics can be reflected by NH_4^+-N oxidation rate, NO_2^--N accumulation or consumption rates, and NO_3^--N accumulation rates in an SBR operating cycle (Figure 6.5), which were calculated from the changes of intermediate NH_4^+-N, NO_2^--N and NO_3^--N concentrations in typical SBR cycles under different operation stages (Appendix B Figure B-6.4). In particular, NH_4^+-N can be depleted by both aerobic oxidation (nitritation) and anaerobic oxidation (anammox). In the aerobic phase (105 min out of the 120-min cycle), NH_4^+ was mainly oxidized to NO_2^- by AOB, with a very limited amount of NO_2^- further oxidized by NOB to NO_3 (nitrification), with both NO_2^- and NO_{3-} accumulating under aerobic condition. The aerobic oxidation rates of NH_4^+-N were 0.20 ± 0.02, 0.26 ± 0.01, 0.36 ± 0.01, and 0.42 ± 0.01 g m^{-3} min^{-1} for Stages I, II, III, and IV, respectively, while the accumulation rates of NO_2^--N were 0.05 ± 0.01, 0.10 ± 0.01, 0.13 ± 0.01, and

0.20 ± 0.06 g m^{-3} min^{-1}, respectively. Regardless of the operation condition difference, the oxidation rates of NH_4^+-N were 2.1~3.8 times higher than the accumulation rates of NO_2–, which may be attributed to the further oxidation of NO_2^-. The presence of anammox activities under such conditions as anoxic zones can be developed inside the flocs and biofilm layers (Joss et al., 2009). However, with the repression of NOB, the further oxidation of NO_2^- to NO_3^- was at a low rate (*i.e.*, $0.01 \pm 0.01 \sim 0.03 \pm 0.01$ g m^{-3} min^{-1}) and significantly ($p < 0.01$) lower than the accumulation rate of NO_2^-. Further, both NH_4^+-N oxidation rates and NO_2^--N / NO_3^--N accumulation rates increased from Stage I to Stage IV, perhaps due to the increased biomass concentration.

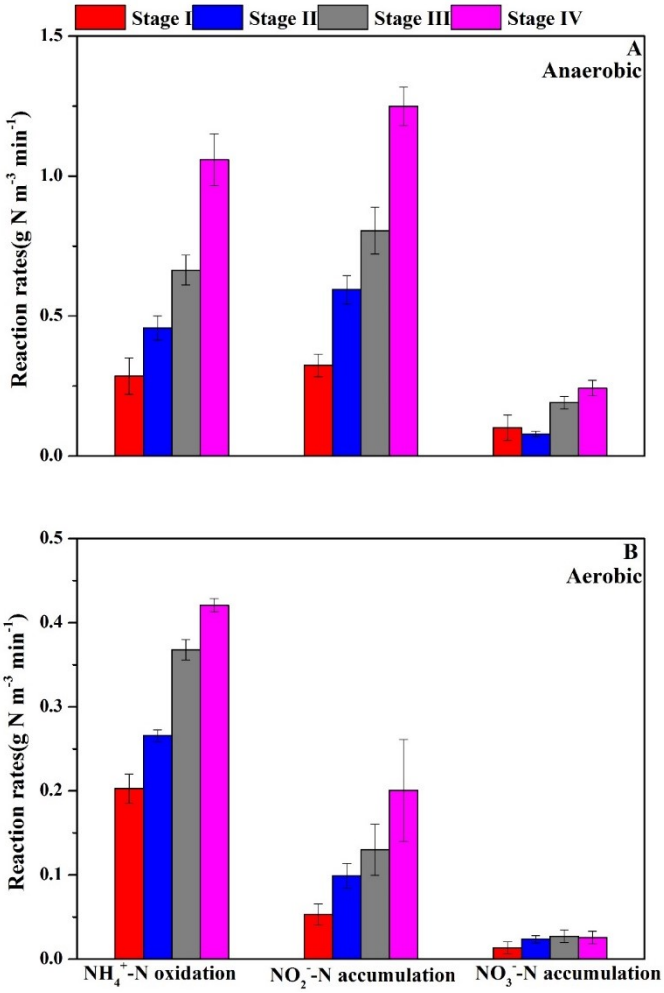

Figure 6.5. The rates of NH_4^+-N oxidation, NO_2^--N consumption and NO_3^--N accumulation in anaerobic phase of each SBR cycle in different stages (A); the rates of NH_4^+-N oxidation, NO_2^--N accumulation and NO_3^--N accumulation in aerobic phase of each SBR cycle in different stages (B).

During the anoxic phase (15 min out of the 120 min cycle) which was shorter than the aerobic phase, both NH_4^+ and NO_2^- were further utilized by anammox bacteria to produce N_2 gas with a few NO_3^- generated as a "by-product" in high efficiency. Meanwhile, a certain portion of NO_3^- was reduced by heterotrophic denitrifiers to nitrogenous gas (N_2 or N_2O) through heterotrophic denitrification. The anoxic oxidation rates of NH_4^+-N were 0.29 ± 0.07, 0.46 ± 0.04, 0.67 ± 0.04, and 1.06 ± 0.05 g m^{-3} min^{-1} for Stages I, II, III, and IV, respectively. These values are much higher than aerobic oxidation rates of NH_4^+-N in the according stages; the consumption of NO_2^--N was 0.32 ± 0.04, 0.59 ± 0.05, 0.81 ± 0.05, and 1.25 ± 0.08 g m^{-3} min^{-1}, for Stages I, II, III, and IV, respectively. Both NH_4^+-N and NO_2^--N consumption rates constantly increased from stage I to Stage IV, providing kinetic evidence for the improved nitrogen removal rates.

With regards to the anammox reaction stoichiometry (Equation [2]), the ratios of NO_2^--$^-$N consumption to NH_4^+-N consumption were 1.14, 1.30, 1.21, and 1.18 for Stages I, II, III, and IV respectively, of which the ratio in Stage II was closest to the theoretical value (1.32), indicating anammox activity contributed to most N reduction in Stage II. Moreover, the ratio (0.17) of NO_3-$^-$N accumulation to NH_4^+-N oxidation in Stage II was lower than the theoretical value of 0.26, which suggests a possible co-existence of heterotrophic denitrification, however, this did not significantly contribute to the nitrogen removal.

6.3.4. Environmental application and implication

In the conventional BNR process, IFAS is well-known as a practical technology to upgrade or retrofit the existing flocs process in municipal WWTP. The treatment capacity and nitrogen removal performance of the IFAS BNR process can be further improved by increasing the biocarriers filling ratios, which increased biomass density, or by increasing the ALR, which encouraged the growth of nitrifying and denitrifying bacteria (Nze et al., 2018). After optimization, the highest nitrogen removal rates (0.56 kg N m^{-3} d^{-1}) and removal efficiencies (87.4%) were achieved in our IFAS nitrtitation-anammox reactor. The operation cost linked to the optimization strategy of the IFAS-SBR is likely be impacted when the reactor filling ratio increases, or the HRT decreases, which affects energy consumption associated with liquid mixing, reactor heating, aeration and pumping. On the other hand, enhanced treatment efficiency reduces the footprint of the treatment system, and improves effluent quality (Piemonte et al., 2014; Boiocchi et al., 2017; Giwa et al., 2018). Details of techno-economic analysis should be conducted when designing nitritation-anammox processes. Our research provides important insights on nitritation-anammox process treating ammonium rich wastewater for the future scale-up.

6.4. Conclusions

A lab-scale one-stage IFAS nitritation-anammox reactor was established for lagoon supernatant treatment and optimized by altering biocarrier filling ratio and HRTs during 500 day operation. The nitrogen removal performance was evaluated and compared during different reactor operation stages. Nitrogen removal rates were improved from 0.25 kg m^{-3} d^{-1} to 0.29 kg m^{-3} d^{-1} by both increasing the biocarrier filling ratio from 35% to 55%, and further improved from 0.29 kg m^{-3} d^{-1} to 0.54 kg m^{-3} d^{-1} by decreasing HRTs from 2.5 d to

1.2 d, due to the increased reactor biomass, while nitrogen (NH_4^+-N and TN) removal efficiencies were only increased with the operation under higher biocarrier filling ratio instead of shortening HRTs with the highest NH_4^+-N and TN removal efficiency of 95.2% and 87.4%, respectively. A distinct segregation of anammox bacteria and AOB was achieved in the IFAS system and was highlighted by the decreased HRTs, which also contributed to the excellent nitrogen removal; this was also evident from the SAA and SAOB analysis and microbial community analysis. The investigation and comparison of nitrogen conversion kinetics supported that the nitritation-anammox process feathers under different operation conditions. The specific anammox activity was higher in biofilm than in flocs, and vice versa for the specific AOB activity. The present study provides critical knowledge for optimizing the one-stage IFAS nitritation-anammox reactor treating supernatant of anaerobically digested sludge thicken lagoon.

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CHAPTER 7 THE IMPORTANCE OF INTEGRATED FIXED FILM ACTIVIATED SLUDGE REACTOR AND INTERMITTENT AERATION IN NITRITATION-ANAMMOX SYSTEMS: UNDERSTANDING REACTOR OPTIMIZATION FOR LAGOON SUPERNATANT TREATMENT[5]

7.1. Introduction

Supernatant of anaerobically digested sludge thickening lagoon contains high NH_4^+ concentration (Shao et al., 2019). One stage nitritation-anammox process has been proven to be an efficient mean of nitrogen removal in lagoon supernatant treatment due to its low aeration and C/N ratio requirements, and high ammonium removal efficiencies (Lotti et al., 2019; Shao et al., 2019; Yang et al., 2019a).

The dominant microorganisms in the nitritation-anammox system are anammox bacteria (*i.e.*, *Candidatus Brocadia*; *Candidatus Kuenenia; Candidatus Anammoxoglobus, Candidatus Jettenia* and *Candidatus Scalindua*) (Lotti et al., 2014; Rodriguez-Sanchez et al., 2016) and AOB (*i.e.*, *Nitrosomonas*; *Nitrosospira; Nitrosolobus; Nitrosovibrio* and *Nitrosococcusare*) (Pynaert et al., 2003; Blackburne et al., 2007). Nitrite oxidizing bacteria (NOB) are a group of nitrifier bacteria easily accreting with AOB in the aerobic environment, which compete for oxygen and nitrite sources with AOB and anammox bacteria, respectively (Yang et al., 2015). Therefore, as widely reported, suppressing the

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activity and population of NOB (*i.e.*, termination of nitrification) while maintaining the activity of both AOB and anammox bacteria is critical to maintain the nitritation-anammox process (Li et al., 2011; Malovanyy et al., 2015b; Laureni et al., 2016; Qiu et al., 2019).

Operation under DO limited conditions can effectively inhibit NOB growth and activity. Both continuous aeration and intermittent aeration have been applied in nitritation-anammox systems with low DO conditions (*i.e.*, < 0.5 mg L^{-1}) (Molinuevo et al., 2009; Yang et al., 2015). For instance, Malovanyy et al. (2015a) found that the activity of NOB and associated nitrate production was strongly limited at a DO concentration of 0.5 mg L^{-1}. By applying a sufficient duration of non-aeration phase between aeration periods, the activity of NOB is highly limited, which contribute to the suppression of NO_3^- production by NOB (Corbalá-Robles et al., 2016). However, the impact of intermittent aeration on the growth of AOB is still not well understood.

Systems with biofilm only (*i.e.* moving bed biofilm reactor [MBBR]) and hybrid systems of flocs and biofilm (*i.e.* integrated fixed film activated sludge reactor [IFAS]) have been explored for the implementation of the nitritation-anammox process (Wells et al., 2017). In Veuillet *et al* (2014)'s study, IFAS showed a 3 - 4 times higher nitrogen removal capability than MBBR treating anaerobic digestate. The objective of this study was to identify options to improve the treatment efficiency of nitritation-anammox processes when treating ammonium rich lagoon supernatant. During the 438-day reactor operation, this study compared (i) MBBR and IFAS configurations, and (ii) continuous and intermittent aeration strategies, and evaluated nitrogen removal efficiencies and microbial community changes.

7.2. Methods and materials

7.2.1 Reactor set up

A bench scale reactor (working volume of 6 L) was operated in a continuous flow mode. The reactor was seeded with Veolia Anox K™5 carriers (Veolia Water Technologies, Canada) with 55% volume fill ratio. Digested sludge liquor thickening lagoon supernatant (Table 7.1) to be used as reactor feed was collected from a biosolids digester sludge thickening lagoon in the City of Edmonton once a month. To minimize microbial activity, the collected lagoon supernatant was stored at 4 °C prior to use.

Table 7.1. Composition of the lagoon supernatant in the feed

Parameters	Unit	Lagoon supernatant
		Mean ± S.D.
TSS	g TSS $(m^3)^{-1}$	305 ± 107
NH_4^+	mg N L^{-1}	835 ± 45
NO_2^-	mg N L^{-1}	0.6 ± 0.5
NO_3^-	mg N L^{-1}	0.2 ± 0.2
PO_4^{3-}	mg PO_4-P L^{-1}	32 ± 5.4
Alkalinity	mg $CaCO_3$ L^{-1}	3056 ± 45.6
pH	-	7.87 ± 0.15
Alkalinity/NH_4	mg $CaCO_3$ mg N^{-1}	3.43 ± 1.04
COD	mg L^{-1}	568 ± 54.42
rbCOD[1]	mg L^{-1}	189 ± 28

[1]rbCOD: readily biodegrable COD

The 438 days operational period for nitritation-anammox reactors was divided into five periods (Table 7.2). In Periods I and II, MBBR operation mode was applied. In Periods III to V, IFAS operation mode was applied and sludge from the treated effluent was collected

and returned back to the bioreactor (Appendix B Figure B-7.1). The reactor was fed with 75 % lagoon supernatants in Period I and 100 % during Periods II to V. 75% feed water was prepared by diluting the raw lagoon supernatants with deionized (DI) water in a 3:1 (feed water: DI water) volume ratio. Hydraulic retention time (HRT) of 2.5 d were applied in both Periods I and II, then increased to 3.2 d in Periods III and IV by decreasing the influent flowrate from 1.6 mL min^{-1} to 1.25 mL min^{-1} (Peristaltic pump model: 77200-60, Masterflex, Cole-Parmer, Illinois, USA), then reduced to 2.5 d in Phase V. Continuous aeration was applied in Periods I to III which was replaced by an intermittent aeration regime (i.e., alternating 105 min aeration and 15 non-aeration) in Periods IV to V with DO maintained between 0.18 - 0.19 mg L^{-1} (Table 7.2). The reactor was equipped with a heat jacket (Xuanyuan, Yancheng, China) to maintain a constant liquid temperature of 28.5± 1.4 °C.

7.2.2 Sample collection and analytical methods

Reactor influent and effluent samples were collected every day for COD, NH_4^+, NO_3^-, and NO_2^- analysis using reagent kits (methods 8000, 10205, 10206, and 10207, respectively, Hach Company, Loveland, Colorado). The system was considered stable if effluent characteristics became stable for at least one week. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured weekly using Standard Methods 2540B (Eaton et al. 2011).

7.2.3 Activity measurement

The specific activity of anammox (SAA) and ammonium oxidizing bacteria (SAOB) under steady state conditions were determined following methods described by Jin et al. (2013).

Table 7.2. Summary of performance and efficiency of the reactor in Periods I-V.

Period	I	II	III	IV	V
Days of operation	1 - 72	73 - 144	145 - 230	231 - 370	371- 438
HRT(d)	2.5	2.5	3.2	3.2	2.5
DO (mg L^{-1})[a]	0.18 ± 0.01	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.01
Aeration	Continuous	Continuous	Continuous	Intermittent[b]	Intermittent[b]
Sludge recirculation	No	No	Yes	Yes	Yes
Raw supernatant (%)	75	100	100	100	100
NH_4^+-N influent (mg N L^{-1})	620 ± 38	830 ± 25	825 ± 45	832 ± 38	840 ± 32

[a]Dissolved oxygen concentration during aerated phase.

[b]Intermittent aeration with 15 min non-aerated phase/105 min aerated phase per 120 minutes

[c]NRR: Nitrogen removal rates

Serum bottles were deoxygenized by purging N_2 gas for 25 min, and then immediately sealed with butyl rubber before being placed in a thermostatic shaker operated at 31 ± 1 °C and 180 rpm. The biomass concentration and pH were measured at the end of each test. SAA was calculated as the maximum substrate consumption rate divided by VSS. Activity of heterotrophic denitrifiers was determined by the same methodology. Sodium nitrate and sodium acetate were used as the substrates with concentrations of 50 and 100 mg COD L^{-1}, respectively. The oxygen uptake rate (OUR) was measured indirectly by tracking the pressure decrease in the headspace, which was used to determine the aerobic bacteria (AOB

and NOB) activity (Gilbert et al., 2014b). Sodium azide (NaN_3) is a commonly used inhibitor to supress NOB activity and was used for testing specific AOB activity. Sludge samples were suspended in mineral medium containing substrates (i.e., NH_4^+ and NO_2^- for SAA and NH_4^+ for SAOB, NO_2^- for SNOB and sodium nitrate and sodium acetate for specific activity of denitrifier).

7.2.4. q-PCR analysis and MiSeq sequencing

Sludge and biofilm samples were collected from the reactor during different periods under stable conditions. DNA was extracted from sludge and biofilm samples with MO BIO PowerSoil® DNA Isolation Kits (MoBio Laboratories Inc., Carlsbad, California). Anammox bacteria, AOB bacteria, NOB bacteria, and denitrification bacteria were analyzed by qPCR (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). Information about qPCR primers and target genes is provided in Table A-1 (Appendix A). Extracted DNA samples were sequenced on an Illumina MiSeq platform at the Research and Testing Laboratory (Lubbock, TX, USA) to investigate diversity and community composition. The detailed analyze information is described in our previous study (Yang et al., 2019b).

7.2.5. Statistical analysis

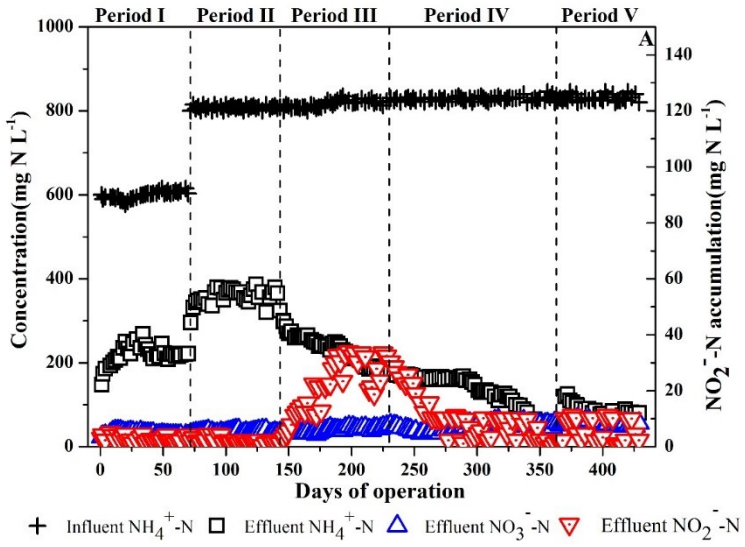
Statistical analysis was performed using the t-test at 5 % probability level and reported as p-values. A statistically significant difference is evident from a p-value smaller than 0.05.

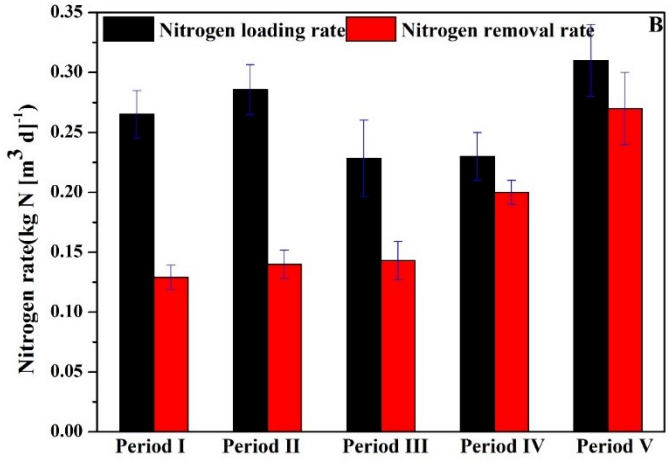
7.3. Results

7.3.1. Reactor operation

Figure 7.1 shows the bioreactor performance in terms of the influent and effluent concentrations of NH_4^+, NO_2^- and NO_3^-. Figure 7.1B shows the nitrogen loading rate (NLR) and nitrogen removal rate (NRR) at different periods. The NH_4^+ removal percentage decreased from 65 ± 2.3 % in Period I (days 1 - 72) to 56 ± 5.5 % in Period II (days 73 – 144) ($P < 0.05$), likely due to change in reactor feed, which was increased from 75 % lagoon supernatant (*i.e.* , 620 ± 27 mg NH_4^+-N L^{-1}) to 100 % lagoon supernatant (*i.e.*, 835 ± 45 mg NH_4^+-N L^{-1}). The NO_2^- concentration in the reactor effluent was as low as 1.5 ± 1.3 mg NO_2^--N L^{-1} in Period I and 2.0 ± 1.1 mg NO_2^--N L^{-1} in Period II, which is below the reported NO_2^--N accumulation range causing inhibition of anammox activity (Wells et al., 2017).

The AOB-produced NO_2^- was consumed immediately by anammox bacteria. A low aerobic ammonium oxidation rate in Periods I and II could lead to a relatively low anammox oxidation rate, which was likely the reason for the low NH_4^+ removal percentage. Although a higher influent NH_4^+ concentration in Period II may have stimulated the growth of AOB, it did not improve the NH_4^+ removal rate by a significant amount. In order to examine the impact of IFAS operation on reactor performance, IFAS mode (days 145 - 230) was applied in Period III. In the meantime, HRT was increased from 2.5 to 3.2 d to maintain the reactor stability due to the change of reactor operation strategy. An enhanced NH_4^+ removal percentage (74 ± 4.8 %) with a significant accumulation of NO_2^- accumulation (23.5 ± 5.6 mg NO_2^--N L^{-1}) was observed. This was significantly higher when compared to previous periods ($P < 0.05$). Our batch studies demonstrated that AOB activity was higher in the sludge (0.318 ± 0.017 kg N [kg VSS $d]^{-1}$), as compared to the biofilm (0.085 ± 0.010 kg N [kg VSS $d]^{-1}$). By returning the settled flocs from the clarifier into the bioreactor, more AOB can be retained in the reactor.


Period I
Period II
Period III
Period IV
Period V
A
Concentration(mg N L-1)
NO2--N accumulation(mg N L-1)
Days of operation
Influent NH4+-N
Effluent NH4+-N
Effluent NO3--N
Effluent NO2--N


Nitrogen loading rate
Nitrogen removal rate
B
Nitrogen rate(kg N [m3 d]-1)
Period I
Period II
Period III
Period IV
Period V

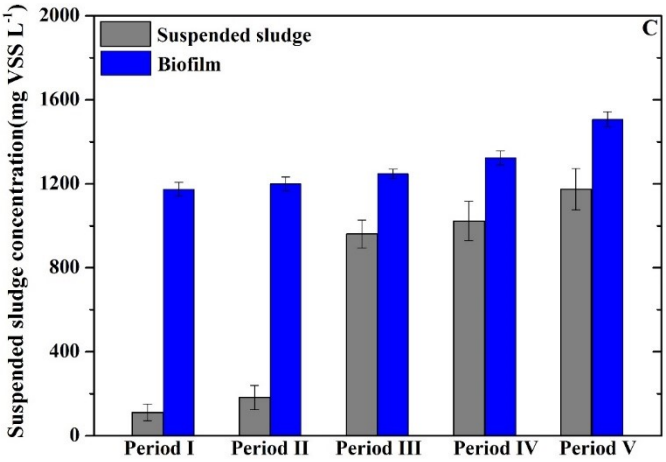

Suspended sludge
Biofilm
C
Suspended sludge concentration(mg VSS L-1)
Period I
Period II
Period III
Period IV
Period V

Figure 7.1. A: the bioreactor performance in terms of the influent and effluent concentrations of NH_4^+-N, NO_2^--N and NO_3^--N; 1B: the nitrogen loading rate (NLR) and nitrogen removal rate (NRR) at different phases; 1C: Flocs /biofilm concentration in the reactor.

However, the decreased NLR along with the increased HRT may reduce the anammox activity in biofilm, leading to an accumulation of NO_2^- (Yang et al., 2019b). This is a clear evidence of the suppression of NOB, which could otherwise oxidize the accumulated NO_2^- in the system. With the application of intermittent aeration in Period IV (days 231 - 370), the effluent concentration of NO_2^- decreased to 12.3 ± 2.3 mg NO_2^--N L^{-1}) ($P < 0.05$) with the NH_4^+ removal percentage significantly increasing to 91.8 ± 1.2 %. Results indicated that the application of intermittent aeration favored the growth of anammox bacteria, which may optimize the living environment for anammox bacteria, as shown by Miao et al. (2017). The NH_4^+ removal percentage in Period IV (92.2 ± 2.1%) was not significantly affected ($P > 0.05$) by a reduction of HRT from 3.2 d to 2.5 d when operation switched to Period V (days 371 - 438), indicating the nitrogen removal rates can be improved without compromising nitrogen removal efficiency in the IFAS system.

From Period I to II, biofilm biomass remained relatively stable between 1174 ± 34 mg VSS L^{-1} to 1200 ± 32 mg VSS L^{-1} ($P > 0.05$) while the flocs concentration was measured as low as 110 ± 38 mg VSS L^{-1} and 182 ± 36 mg VSS L^{-1} , attributed largely from the detachment of biofilm biomass and suspended solids in influent with an actual SRT of 2.5 d. Starting from Period III, the discharged flocs was recirculated to the reactor where a SRT of 14 ± 2.1 d was maintained. This led to a significant increase of flocs concentration to 961 ± 67

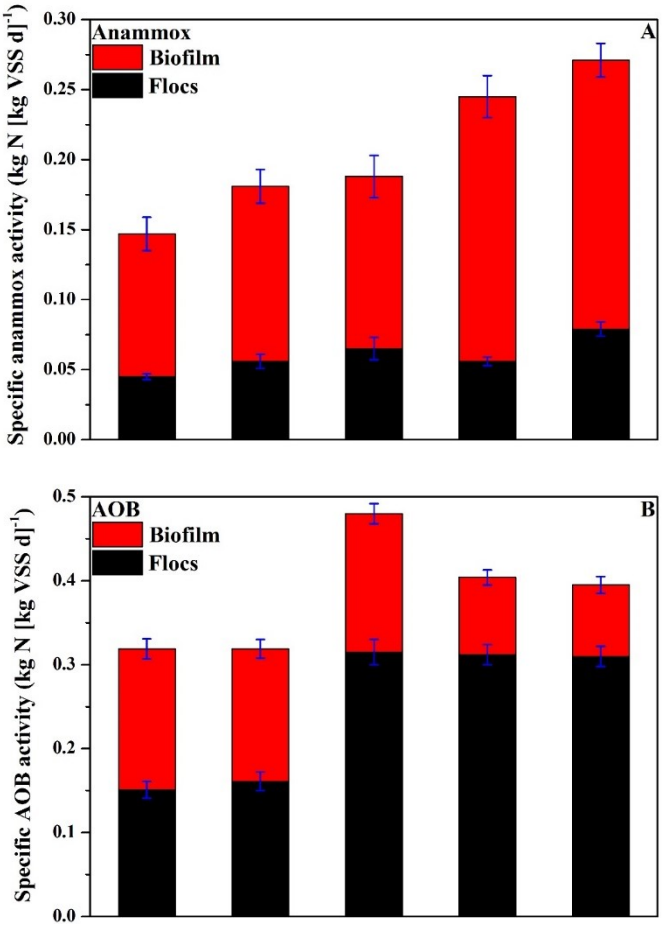
mg VSS L^{-1} ($P < 0.05$). In Period IV, with the application of intermittent aeration, the flocs concentration reached a comparable concentration of 1023 ± 93 mg VSS L^{-1} ($P > 0.05$) while the biofilm biomass further increased from 1247 ± 23 mg VSS L^{-1} to 1323 ± 34 mg VSS L^{-1} ($P < 0.05$). This observation was likely linked to the application of non-aeration phase resulting in more favorable conditions for anammox bacteria growth; which is discussed further in 3.3. In Period V, both of the biofilm and flocs concentrations significantly increased to 1507 ± 35 mg VSS L^{-1} and 1174 ± 98 mg VSS L^{-1} ($P < 0.05$), respectively. This can be explained by the fact that a higher NLR (*i.e.*, from 0.23 kg N [m^3 d]$^{-1}$ in period IV to 0.31 kg N [m^3 d]$^{-1}$ in period V) provided more available nutrients (*i.e.,* NH_4^+ and NO_2^-) for the growth and activity of autotrophic bacteria (*i.e.* AOB and anammox bacteria) (Yang et al., 2019b), which improved N removal efficiency.

7.3.2. Activities of different groups of microorganisms

The activities of AOB, anammox bacteria, NOB, and heterotrophic denitrifiers were determined in both biofilm and flocs to further compare the operation at different periods (Figure 7.2). Specific AOB activity (SAOB) in the flocs significantly increased from 0.151 ± 0.015 kg N (kg VSS d)$^{-1}$ in Period I to 0.161 ± 0.012 kg N (kg VSS d)$^{-1}$ in Period II, then to 0.315 ± 0.015 kg N (kg VSS d)$^{-1}$ in Period III, then to 0.312 ± 0.012 kg N (kg VSS d)$^{-1}$ in Period IV, and finally to 0.318 ± 0.017 kg N (kg VSS d)$^{-1}$ in Period V ($P < 0.05$ for all increases). This was due to the change in operation mode from MBBR to IFAS. The resulting SRT for flocs increase from 2.5 d to 14 d retained a significantly higher population of AOB in the suspended phase. Meanwhile, the application of intermittent aeration in Periods IV to V led to a decrease of SAOB in the biofilm configuration from 0.168 ± 0.012, 0.158 ± 0.011, and 0.165 ± 0.012 kg N (kg VSS d)$^{-1}$ in Periods I to III, respectively, to

0.092 ± 0.009 kg N (kg VSS d)$^{-1}$ in period IV and 0.085 ± 0.010 kg N (kg VSS d)$^{-1}$ in period V in the biofilm phase ($P < 0.05$). Therefore, the application of intermittent aeration led to the different responses of SAOB in biofilm and flocs based on our observation.

The specific anammox activity (SAA) of the biofilm biomass was significantly higher ($P < 0.01$) than that of the flocs in all the operation periods, which was similar to many previous studies (Zhang et al., 2015; Yang et al., 2019a; Yang et al., 2019b). Anammox bacteria prefer to proliferate in the biofilm phase where an extremely long SRT can be reached (Yang et al., 2019b).



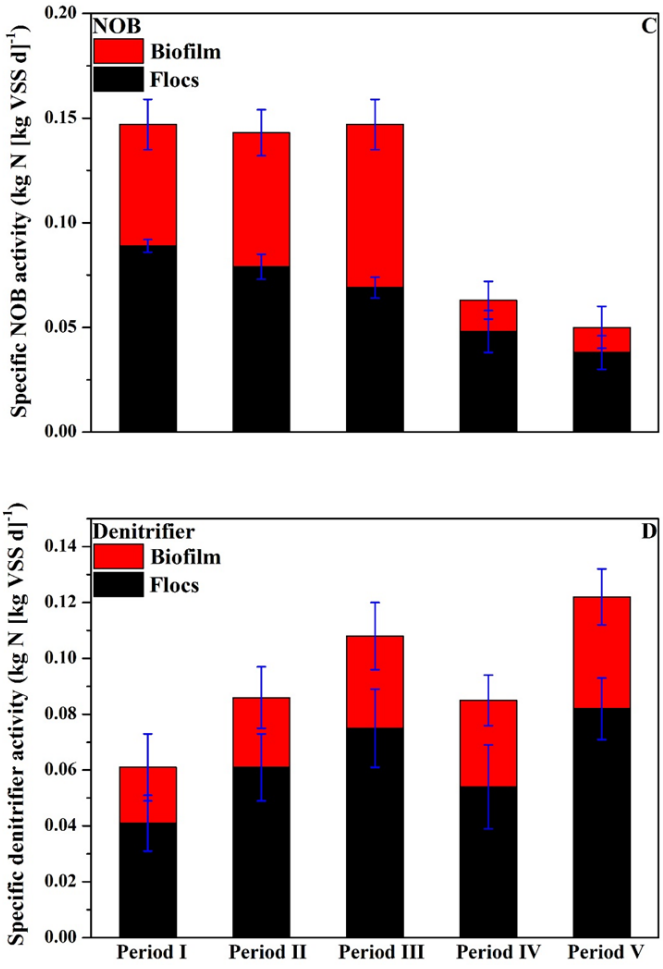


Figure 7.2. Specific activity of different groups of bacteria (anammox bacteria (A), AOB (B), NOB bacteria (C) and denitrifies (D)) in biofilm and flocs

In Periods I to III, the SAA of biofilm remained between 0.102 - 0.123 kg N (kg VSS d)$^{-1}$ where continuous aeration strategy was applied. With the application of intermittent aeration in Periods IV to V, the SAA increased significantly to 0.189 ± 0.015 kg N (kg VSS d)$^{-1}$ and 0.192 ± 0.012 kg N (kg VSS d)$^{-1}$, respectively. While in the flocs configuration, the SAA was relative stable during Periods I to V, which were at 0.045 ± 0.002, 0.056 ± 0.005, 0.065 ± 0.008, 0.056 ± 0.003, and 0.079 ± 0.005 kg N (kg VSS d)$^{-1}$,

respectively, indicating an equal distribution of anammox bacteria in the flocs irrespective of the operation status change.

Specific NOB activities were at a comparably low level in both the biofilm and flocs phase. The application of intermittent aeration in Periods IV to V significantly reduced the specific NOB activity from 0.078 ± 0.012 kg N (kg VSS d)$^{-1}$ (Period III, continuous aeration phase) to 0.012 ± 0.01 kg N (kg VSS d)$^{-1}$ (Period V, intermittent aeration phase) in biofilm biomass ($P < 0.01$) and from 0.069 ± 0.005 kg N (kg VSS d)$^{-1}$ (Period III, continuous aeration phase) to 0.038 ± 0.008 kg N (kg VSS d)$^{-1}$ (Period V, intermittent aeration phase) in floc biomass ($P < 0.01$). Meanwhile, it has been noticed that the NOB activity in biofilm became significantly lower than that of flocs after applying intermittent aeration. The ratio of AOB activity and NOB activity was 2.2 times higher in flocs than that in biofilm biomass. NOB growth has previously been reported to be successfully suppressed by applying intermittent aeration accompanied by low DO condition with an low NOB abundance of 2.0 - 2.6 % (Ma et al., 2015), which helps explain the similar observation in our study.

Due to the low C/N ratio in the feed, the activity of heterotrophic denitrifiers was limited. The activity of heterotrophic denitrifies in biofilm phase (0.02 to 0.05 kg N [kg VSS d]$^{-1}$) was significantly lower ($P < 0.01$) than that in the flocs phase (0.13 ± 0.02 kg N [kg VSS d]$^{-1}$), which may due to the better accessibility of nutrients in the flocs (Hubaux et al., 2015).

7.3.3 Microbial community dynamics

The microbial community structure in biofilm biomass and flocs biomass at the end of period V were investigated. The relative abundances of microbial taxa were demonstrated at the phylum (Appendix B Figure B-7.2A) and genus (Appendix B Figure B-7.2B) levels.

In the biofilm biomass samples, the phylum *Bacterioidetes* (55.2 %) was most prominent, followed by *Chloroflexi* (13.0 %), *Planctomycetes* (12.7 %), *Proteobacteria* (9.9 %), *Acidobacteria* (2.0 %), and *Gemmatimonadetes* (1.8%). The most abundant phylum in flocs samples was *Bacteroidetes* (41.3 %), followed by *Proteobacteria* (28.3 %), *Chloroflexi* (12.2 %), *Gemmatimonadetes* (5.9 %), *Acidobacteria* (5.24 %), and *Planctomycetes* (2.18 %). At the genus level, *Candidatus Brocadia* in the phylum *Planctomycetes* was most prominent (Appendix B Figure B-7.2), accounting for 11.63 % of biofilm communities, but only 1.04 % of flocs in period V. The most abundant AOB genus was *Nitrosomonas*, which was detected in both biofilm (0.13 %) and flocs (0.57 %). Only one genus of NOB, *Nitrospira* was detected with a relative abundance below 0.10 %. *Thauera* (2.12 % in flocs phase and 0.65 % in biofilm phase) was the dominant heterotrophic denitrifiers.

The quantitative real-time PCR analysis was performed to quantify the abundances of anammox bacteria, AOB, NOB, and heterotrophic denitrifies in the biofilm and flocs (Figure 7.3). The abundance of AMX *nir*S in both the biofilm biomass and floc biomass increased significantly ($P < 0.05$) from 1.02×10^7 copies mg^{-1} VSS and 1.12×10^6 copies mg^{-1} VSS respectively in period I to 4.17×10^7 copies mg^{-1} VSS and 4.30×10^6 copies mg^{-1} VSS respectively in period V (Figure 7.3A), although relatively low abundance was observed in the flocs. This correlated well with the maximum nitrogen removal efficiency observed in Period V.

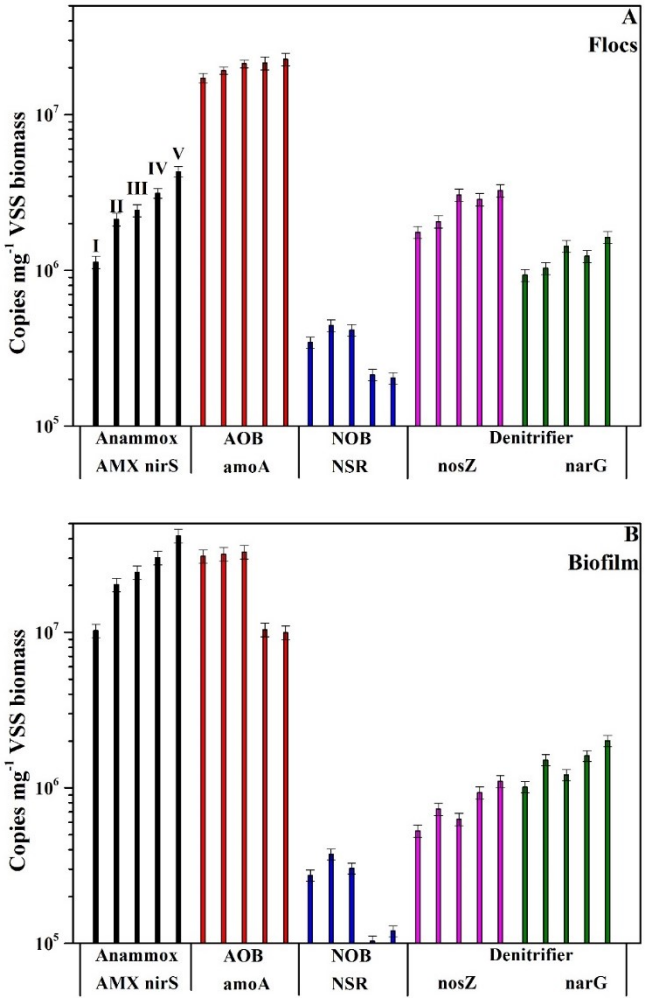


Figure 7.3. A: Abundance of nitrogen transforming groups of bacteria (anammox bacteria, AOB, NOB and denitrifiers) in the flocs; 3B: Abundance of nitrogen transforming groups of bacteria in the biofilm on the carriers. Note: The units for all numbers are gene copies/mg VSS biomass. amoA is the targeted functional gene for AOB; NSR is for Nitrospira; nosZ, and narG are for denitrifiers' functional genes; AMX nirS is for the Anammox.

The amoA gene copy concentration represents the AOB abundance in the reactor. In the biofilm phase, the AOB abundance remained constant in Periods I to III ($P > 0.05$) and

then decreased significantly ($P < 0.02$) from 3.28×10^7 copies mg^{-1} VSS in period III to 1.04×10^7 copies mg^{-1} VSS in Period IV, then further to 9.98×10^6 copies mg^{-1} VSS in Period V when operation switched from continuous aeration condition to intermittent aeration conditions. In the flocs phase, the abundance of AOB increased significantly ($P < 0.01$) to 2.12×10^7 copies mg^{-1} VSS after altering operation mode from MBBR to IFAS in Period III. After that, the intermittent aeration application did not further lead to a significant change in AOB abundance in flocs, ($P > 0.05$) which was 2.14×10^7 copies mg^{-1} VSS in Period IV and 2.26×10^7 copies mg^{-1} VSS in Period V .The abundance analysis of AOB correlated well with the results of SAOB.

Figure 7.3C shows the gene copy numbers of heterotrophic denitrifiers in biofilm and flocs, based on functional genes nosZ and narG. Both, nosZ and narG remained relatively stabilized in the biofilm biomass with no significant difference ($P > 0.05$). In the flocs, the abundance of nosZ and narG increased from 2.06×10^6 copies mg^{-1} VSS and 1.03×10^6 copies mg^{-1} VSS respectively in Period II to 3.06×10^6 copies mg^{-1} VSS and 1.43×10^6 copies mg^{-1} VSS in Period III respectively with no further significant changes in the remaining operation periods. This indicates that the heterotrophic denitrifiers have a better growth in IFAS systems (*i.e.*, mainly in the flocs) as compared to MBBR, which is plausible since the flocculent structure provides ease of accessibility of substrates for heterotrophic growth (Zubrowska-Sudol and Walczak, 2014). However, under organic-limiting conditions, the growth of heterotrophic bacteria was not competitive with anammox bacteria overall, as can be seen in Figure B-7.3 (Appendix B).

NOB abundance remained low during all five periods. After applying intermittent aeration, the abundance of NOB became significantly lower ($P < 0.01$) due to the influence of the

non-aeration period, indicating an improved suppression of NOB occurring under intermittent aeration operation (Sobotka et al., 2015).

7.4. Discussion

7.4.1 MBBR VS IFAS operation

It has been demonstrated in previous research that IFAS has a 3 - 4 times higher nitrogen removal than MBBR systems operating in parallel (Veuillet et al., 2014). Our results agree with these findings and have built upon this knowledge with the application for the treatment of lagoon supernatant. Given both biofilm and suspended floc configurations, IFAS provided better segregation of AOB and anammox bacteria, leading to an optimized inhabiting environment for each microorganisms (Malovanyy et al., 2015a). For example, the diffusion limitation of dissolved oxygen and NH_4^+-N in biofilm configuration suppresses the growth of AOB and subsequently affects anammox activity (Miao et al., 2017). As a relatively fast growing bacteria, AOB tends to grow in the outer layer of biofilm, which likely leads to a denser and thicker biofilm, thus decreasing the accessibility of substrates for other microorganisms (*e.g.*, anammox bacteria) (Verhamme et al., 2011). This likely limited the growth of AOB in turn due to the biofilm thickness limitation. Therefore, a larger population of AOB can be retained in the IFAS system (*i.e.*, in the flocs), which optimizes the NO_2^- flow for anammox bacteria in the biofilm (Veuillet et al., 2014). When the suspended biomass concentration reached its highest at 1320 ± 125 mg L^{-1} in Period V, the NRR increased by 2 - fold from 0.14 ± 0.012 to 0.28 ± 0.03 kg N $(m^3 \cdot d)^{-1}$, and the NH_4^+-N removal efficiency significantly increased ($P < 0.01$) from 56 ± 5.5 % to 92.2 ± 2.1 %, as compared to Period I. Both the IFAS and application of intermittent aeration contributed to the performance improvements. As the limiting step of nitritation-

anammox process (Szatkowska et al., 2007), NO_2^- production via the activity of AOB is critical and can be better conducted in IFAS systems; this was reflected by the higher N removal efficiency in Period III as compared to Periods I and II. This finding was also confirmed by the significantly higher AOB activity of the flocs (Figure 7.3) accompanied with the higher flocs concentration (Figure 7.2) in Period III, leading to an improved contribution of NO_2^- production from the flocs configuration. Although some research (Gatti et al., 2015) indicated the longer SRT (*i.e.*, 14 d in the IFAS) may improve the retention of NOB in the reactor, the NOB population did not further increase in Period III (Figure 7.3).

The reduced NLR in Period III compromised the activity improvement of anammox bacteria in the biofilm (Figure 7.2). Thus, the imbalance of SAA and SAOB at this period caused the NO_2^- accumulation (Figure 7.1) in the reactor and increased the risk of enhancing NOB activity (Figure 7.2C). Although the activity of heterotrophic denitrifiers improved in the IFAS during Period III, further enhancement of heterotrophic denitrification may have been limited by the lack of available biodegradable COD (Appendix B Figure B-7.3).

7.4.2 Intermittent aeration impact on nitrogen removal

Low DO concentration (0.18 - 0.19 mg L^{-1}) in both continuous and intermittent mode successfully suppressed the NOB growth (Figure 7.3), which gave anammox bacteria an advantage in competing for the NO_2^--N with NOB bacteria, as comparable with previous studies (Wang et al., 2018; Yang et al., 2019b), regardless of reactor configuration. However, as reported in Zekker et al. (2017)'s study, the continuous aeration may increase the chance of NOB acclimation to the low DO environment, which could lead to a failure

of suppression of NOB in the long-term operation. In our study, the continuous aeration led to an accumulation of NO_2^- (Period III), which disturbed the subsequent anammox activity. Therefore, with the introduction of non-aeration in intermittent aeration regime, the activity of AOB in biofilm configuration was reduced while the anammox activity in biofilm was enhanced (Figure 7.2), which led to an increase in the overall nitrogen removal performance. The decrease of AOB activity in the biofilm (Periods III to V) may be attributed to (1) the low amount of oxygen supplied during non-aeration phases and (2) substrate diffusion limitation for the biofilm biomass. However, non-aeration phase provided favorable conditions for the growth of anammox bacteria through reducing oxygen inhibition (Ma et al., 2015; Qiu et al., 2019). The growth of NOB (Figure 7.3) was also significantly reduced under intermittent aeration and led to a lower specific activity of NOB (Figure 7.2), which is in agreement with previous studies (Gilbert et al., 2014a; Zhang et al., 2017).

According to the nitritation-anammox stoichiometry reaction (Equation S2), 11% of NH_4^+ is converted to NO_3^- by anammox bacteria (Yang et al., 2019b). In theory, effluent NO_3^- concentration would be 33.5 ± 2.2, 45.6 ± 4.1, 54.2 ± 5.9, 66.7 ± 2.8, and 84.2 ±7.1 mg NO_3^--N L^{-1} Periods I through V, respectively. In our study, the practical effluent NO_3^- concentration was 23.1 ± 2.2, 22.1 ± 3.1, 38.9.1 ± 5.8, 52.1 ± 6.2, and 62.1 ± 3.6 mg NO_3^--N L^{-1} in Periods I through V, which is much lower than the theoretical effluent NO_3^--N concentrations. Heterotrophic denitrification may have occurred, which reduced NO_3^- concentration through COD oxidation. As shown in Figure B-7.3 (Appendix B), COD removal percentages were in the range of 22% to 35% in the present study.

7.5. Conclusion

Optimized nitrogen removal efficiency and nitrogen removal rates in a single-stage nitritation-anammox CSTR treating lagoon supernatant were achieved by both recirculating flocs and applying intermittent aeration. The highest NH_4^+ removal efficiency of 92.2 ± 2.1 % was achieved under a NLR of 0.34 ± 0.03 kg N $(m^3 \cdot d)^{-1}$ in the IFAS-mode operated CSTR with intermittent aeration. The successful suppression of NOB was observed under all operation periods and the growth of NOB bacteria was significantly reduced by applying intermittent aeration. Heterotrophic denitrification was also improved under the optimized operation condition.

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CHAPTER 8 LONG-TERM CONTINUOUS NITRITATION-ANAMMOX REACTOR AERATION OPTIMIZATION AT DIFFERENT NITROGEN LOADING RATES FOR THE TREATMENT OF AMMONIUM RICH DIGESTATE LAGOON SUPERNATANT

8.1. Introduction

Excessive nitrogen in wastewater effluents threatens ecological systems and public health by causing eutrophication in natural water bodies (Xu et al., 2014; O'Brien et al., 2019). Nitrogen reduction from wastewater is mandatory in many countries, which is typically achieved through nitrification-denitrification under aerobic then anoxic conditions, respectively (Xu et al., 2013). Though effective, it is costly, energy-demanding and less efficient when treating high-strength ammonium wastewaters. Further, such biological nitrogen reduction often required the addition of organic carbon (e.g. methanol) to optimize the carbon-to-nitrogen ratio (C/N) (Hvala et al., 2018), adding to overall treatment costs.

Since the 1990s, along with the research of novel nitrogen reduction strategy known as "anammox" (i.e., anaerobic ammonium oxidation) (van der Star et al., 2010), the rapid development of partial nitritation and anammox (or nitritation-anammox) processes was identified for high ammonium wastewater treatment. Unlike the conventional nitrification-denitrification process, where influent ammonium (NH_4^+) is completely converted into nitrate (NO_3^-), which is then reduced to nitrogen gas through heterotrophic denitrification, nitritation process only partially oxidizes NH_4^+ to nitrite (NO_2^-), while anammox bacteria denitrify NO_2^- using NH_4^+ as the electron donor. The nitritation-anammox process therefore, results in more energy-efficient and less costly treatment than the conventional nitrification-denitrification process (Fux et al., 2002).

Persistent suppression of nitrite oxidizing bacteria (NOB) converting NO2- to NO3- is imperative for high and stable performance of the nitritation-anammox process to ensure the availability of NO2- for anammox bacteria. The abundance of NOB has been observed to be less than 1% in a successful nitritation-anammox reactor (Wang et al., 2018). Generally, NOB can be eliminated by adjusting operational parameters. For example, maintaining short solids retention time (SRT) (i.e., 1~2 d) has been shown to effectively wash out NOB (Liu and Wang, 2014), while at the same time short SRTs eliminate anammox bacteria in flocs. On the other hand, operating nitritation-anammox reactors under low dissolved oxygen (DO) environments favors AOB due to their relatively higher oxygen affinity when compared to NOB (Guisasola et al., 2005). However NOB may acclimatize to these conditions over the long term (Zekker et al., 2017). In contrast, application of an intermittent aeration regime provides a better solution for the suppression of NOB under low DO conditions (Liu and Wang, 2013). Additionally, the non-aerated phases are beneficial to the anammox bacteria and heterotrophic denitrifiers as they allow the deoxygenation of the outer layer of the biofilm and flocs associated with treatment (Zubrowska-Sudol et al., 2011).

The optimization of operational parameters for intermittent aeration such as the duration and DO level of aeration cycles is well studied. Zubrowska-Sudol et al. (2011) reported that longer aeration periods encouraged NOB suppression while Gilbert et al. (2014) found NOB require a longer resuming time than AOB at the switch from anoxic to aerobic environments. Furthermore, the adjustment of DO concentration for NOB suppression has been already investigated (Wyffels et al., 2004; Zheng et al., 2016) as have strategies to optimize oxygen supply in nitritation/anammox systems (Mauricio-Iglesias et al., 2015;

Boiocchi et al., 2016). However, many of these studies just focus on one average nitrogen loading rate and neglect the variety of operational limitations when different nitrogen loadings are applied. In the current work, we undertook a more comprehensive investigation of the partial nitritation/anammox process where we not only changed the aeration regime but also the nitrogen loading rate to comprehensively assess a single-stage IFAS partial nitritation/anammox system.

8.2. Materials and methods

8.2.1. Feed water quality

As disclosed in the introduction, the influent water used was the anaerobically digested biosolid thickening lagoon supernatant. Lagoon supernatant is an ammonium rich side stream in wastewater treatment plant. As shown in Table 8.1, NH_4^+-N (855 ± 45 mg/L) was the main inorganic nitrogen species in the reactor influent, which can be transformed into NO_2^--N, NO_3^--N, or nitrogen gas by different microbial communities through nitritation-anammox and heterotrophic denitrification processes (Yang et al. 2019a). The soluble COD concentration was 568 ± 54.42 mg/L with the biodegradable fraction of 182 ± 32 mg/L, providing a low C/N ratio. The PO_4^{3-}-P concentration (32 ± 5.4 mg/L), FA concentration (12.7 ± 1.4 mg/L) and FNA concentration (0.0010 ± 0.0006 mg/L) were below the commonly reported inhibition ranges for both NOB and anammox bacteria (Yang et al. 2019a).

8.2.2. Reactor set up and operation

The IFAS system was implemented as a continuous operation with a working volume of 6 Liters. It was operated for 312 days, during which the following phases can be identified according to the accomplished HRT: Phase I with an HRT of 2.5 d (from day 1 to 64), Phase II with an HRT of 2.0 d (from day 65 to 162), Phase III with an HRT of 1.5 d (from day 163 to 246), and Phase IV with an HRT of 1.2 d (from day 247 to 312). The raw lagoon supernatant (see Table 8.1) was supplied continuously to the reactor by a peristaltic pump (Easy - load® II, Model: 77200-60, Masterflex, Cole-Parmer, Illinois, USA) at influent flow rates of 1.61, 1.97, 2.65, and 3.32 mL/min in Phase I to Phase IV, respectively, to accomplish the different HRTs.

Table 8.1. Lagoon water composition

Parameters	Unit	Lagoon supernatant
		Mean ± S.D.
TSS	g TSS/m^3	305±107
NH_4^+	mg N/L	855±45
NO_2^-	mg N/L	0.6±0.5
NO_3^-	mg N/L	0.2±0.2
PO_4^{3-}	mg PO_4-P/L	32±5.4
Alkalinity	mg $CaCO_3$/L	3056±45.6
pH	-	7.87±0.15
Soluble COD	mg/L	568±54.42
Biodegradable COD	mg/L	182±32

The anammox inoculum was seeded with Veolia Anox K™5 carriers (Veolia Water Technologies, Canada) with 55% filling ratio (Yang et al. 2019a). The temperature of the

mixed liquor was controlled at 29.2 ± 1.2 °C using a heat jacket (Xuanyuan, Yancheng, China).

A mechanical stirrer (Grainger, USA) was used to mix the liquid in the reactor thoroughly, thus allowing biomass-substrate contact. The intermittent aeration was applied throughout all the phases with cycles of 105-minute aerated periods followed by 15 minutes of non-aeration. The DO during the aeration period was altered in each phase and sub-phase by adjusting the air pump flow rate (Active Aqua, Model: AAPA45L, Hydrfarm, California, USA) using an air flow meter (Model: MR3A02SVVT, Pennsylvania, USA). The bulk DO concentration, monitored daily using a DO meter (YSI 6050020 Pro20, Cole- Parmer, Illinois, USA), ranged as follows: 0.18-0.20 mg/L in Phase I and in Phase II-1 (days 65-115), 0.22-0.24 mg/L in Phase II-2 (days 116-162) and in Phase III-1 (days 163-203), 0.24-0.28 mg/L in Phase III-2 (days 204-246) and in Phase IV-1 (days 247-286), and 0.28-0.35 mg/L in Phase IV-2 (days 287-312).

Effluent from the biological reactor was carried to a clarifier in order to achieve high retention time of biomass flocs. Specifically, a sludge recycle ratio of 1:1 was maintained for influent wastewater. The SRT was maintained at 12.6 ± 4.2 days throughout the entire operational period. The liquid pH in the reactor was between 7.1 - 7.6 for the duration of operation according to the daily measurements.

8.2.3. Reactor performance analysis

The reactor performance was evaluated by influent and effluent water chemistry determination. The influent and effluent water samples were collected at least twice a week and filtered through 0.45 μm filters (fisherSci, Canada) before intermediate measurement

(Xu et al. 2018b; Yang et al. 2019a). Concentration of soluble chemical oxygen demand (COD), NH_4^+-N, NO_2^--N, and NO_3^--N were chosen as targets and measured using the Hach methods (Hach Company, Colorado, U.S.). Inorganic nitrogen balance was performed with the individual inorganic nitrogen species. Inorganic nitrogen removal efficiency was calculated based on influent and effluent total inorganic nitrogen concentrations. Free ammonia (FA) and free nitrous acid (FNA) concentrations were determined based on Equations 1 and 2.

$$\text{FA as } NH_3\ (\text{mg}/L) = \frac{17}{14} \times \frac{total\ ammonia\ as\ N\ (mg/L) \times 10^{pH}}{K_b/K_w + 10^{pH}} \quad \text{Equation 1}$$

Where $K_b/K_w = e^{(6334/273+℃)}$

$$\text{FNA as } HNO_2\ (\text{mg}/L) = \frac{46}{14} \times \frac{NO_2^- - N\ (mg/L) \times 10^{pH}}{K_a \times 10^{pH}} \quad \text{Equation 2}$$

Where $K_a = e^{(-2300/273+℃)}$

Analysis of variance (ANOVA) was performed for the statistical comparison of different samples with a p-value less than 0.05 indicating a statistically significant difference.

8.2.4. Biomass and microbial analysis

Biomass samples of biofilm and mixed liquor were collected during the optimum steady-state conditions of each phase for both volatile suspended solids (VSS) and microbial analysis. Quantitative polymerase chain reaction (qPCR) experiments were carried out using the exacted DNA of biomass samples following the method described in our previous study (Yang et al. 2019a) (see Appendix A Table A-1). The biofilm thickness was measured with a confocal microscope (Zeiss LSM 710), following the method described in our previous study (Xu et al. 2018a).

8.2.5. Specific biomass activities

Within each stage, the specific activity of anammox bacteria (SAAB) and ammonium oxidizing bacteria (SAOB) of flocs and biofilm on the carriers were measured by a commonly used batch experiment method (Gilbert et al. 2014; Jubany et al. 2009). Floc and biofilm biomass pellets, obtained via centrifugation, were washed with phosphate-buffered saline at least three times before being suspended in mineral medium containing target substrates (*i.e.*, NH_4^+ and NO_2^- for SAAB and NH_4^+ for SAOB). Ideal anaerobic and aerobic conditions were maintained separately for SAAB and SAOB tests. The SAAB and SAOB were calculated as the ratio of maximum substrate consumption rate and biomass concentration. All batch studies were performed in triplicate.

8.3. Results

8.3.1. Nitrogen removal performance

Figure 8.1 shows the average ammonium and total nitrogen removal efficiencies achieved during each operating phase, while Figure B-8.1 (Appendix B) in the Supplementary Information shows detailed dynamic measurements.

As can be seen in Figure 8.1, from Phases I to II at an influent NH_4^+-N of 826.8 ± 6.6 mg/L, the average removal efficiency of NH_4^+-N decreased from 88.6 ± 1.4% to 81.5 ± 1.6% ($p < 0.01$), and the TN removal efficiency decreased from 79.3 ± 1.4% to 70.2 ± 2.1% ($p < 0.01$). This could be attributed to the fact that the elevated nitrogen loading rate (NLR) (*i.e.*, from 0.33 ± 0.003 kg N/m^3/d to 0.41 ± 0.003 kg N/m^3/d) and the associated HRT reduction increased oxygen requirements by AOB.

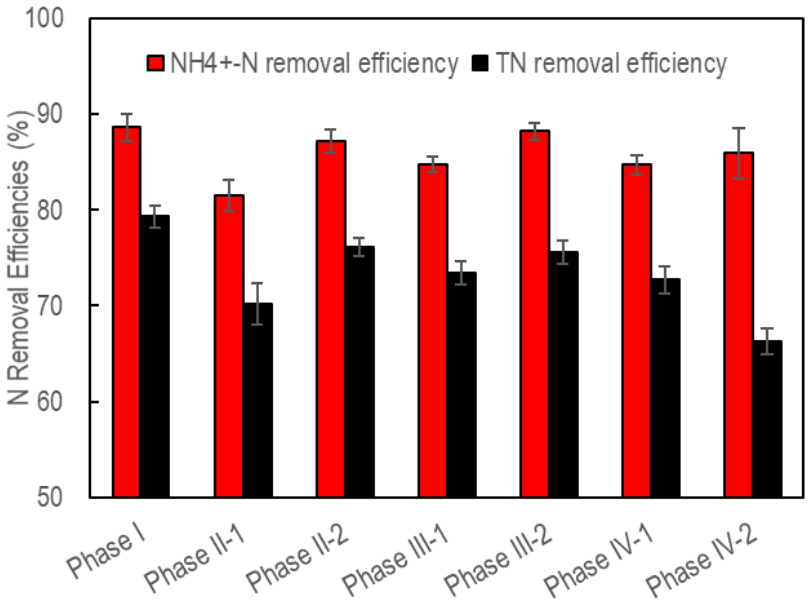


Figure 8.1. Removal efficiency of NH_4^+-N and total nitrogen during different operation phases. Error bars represent the standard deviation of samples.

As a matter of fact, with the increase of bulk DO from 0.18-0.20 to 0.22-0.24 mg/L from Phase II-1 to Phase II-2, the NH_4^+-N removal efficiency recovered from 81.5 ± 1.6% to 87.2 ± 1.2% ($p < 0.01$), and the TN removal efficiency increased from 70.2 ± 2.1% to 76.1 ± 0.9% ($p < 0.01$).

Similarly to the transition from Phase I to Phase II-1, when the HRT was further reduced from 2.0 days (Phase II) to 1.5 d (Phase III), the ammonium removal efficiency dropped from 87.2 ± 1.2% to 84.7 ± 0.8% ($p < 0.01$) in Phase III-1, with the TN removal efficiency decreasing from 76.1 ± 0.9% to 73.4 ± 1.2% ($p < 0.01$). As DO was increased to 0.24-0.28 mg/L in Phase III-2, the ammonium removal efficiency recovered to 88.2 ± 0.9%, and the TN removal efficiency raised to 75.6 ± 1.2% ($p<0.01$).

Finally, when the HRT was further reduced to 1.2 d in Phase IV, the ammonium removal efficiency decreased to 84.7 ± 1.0% ($p<0.01$), while the TN removal efficiency decreased from 75.6 ± 1.2% to 72.7 ± 1.4% ($p<0.01$) in Phase IV-1. Remarkably, when DO was increased to 0.28-0.35 mg/L in Phase IV-2 in an attempt to improve nitrogen removals, a significant accumulation of NO_2^- was observed (see Appendix B Figure B-8.1), which led to a relevant decrease in the TN removal efficiency from 72.7 ± 1.4% to 66.3 ± 1.3% ($p<0.01$), although the NH_4^+-N removal efficiency was recovered to 85.9 ± 2.6% ($p = 0.08$). This decrease in TN removal efficiency can be attributed to the oxygen inhibition on anammox responsible for NO_2^- reduction.

With regards to the effluent nitrogen species apart from NH_4^+-N, the effluent NO_3^--N concentration was less than 11% (*i.e.*, 7.0%-9.6%) of the influent NH_4^+-N concentration, indicating the co-existence of heterotrophic denitrification, similar to reported studies (Yang et al. 2019a). The degradation of soluble COD, which is shown in Figure B-8.2 (Appendix B), increased significantly from 29.7 ± 4.6 % at an HRT of 2.5 d to 38.70 ± 4.8% at HRT of 1.2 d. This could be attributed to the increase in the oxygen concentration which sped up aerobic heterotrophic activity.

Upon HRT change, a constant increase of the biofilm thickness was observed, which was measured as 145.6 ± 10.2 µm in Phase I, 171.6 ± 5.8 µm in Phase II, 184.6 ± 8.1 µm in Phase III and 214.2 ± 9.6 µm in Phase IV). In our study, biofilm thickness could be as well correlated with substrate removal rate, as shown in Figure 8.2. It is however in our case study more difficult to have an unbiased clear correlation between the two, as nitrogen removal rate does not exclusively depend on the biofilm biological activity but also on the

suspended biomass. By allowing the growth of AOB which consumes oxygen diffusing into the biofilm from the bulk, thicker biofilm may help to protect anammox bacteria from higher DO concentrations.

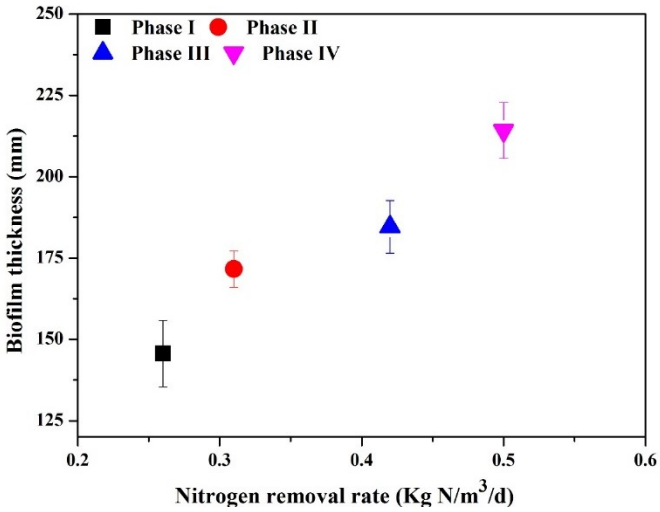
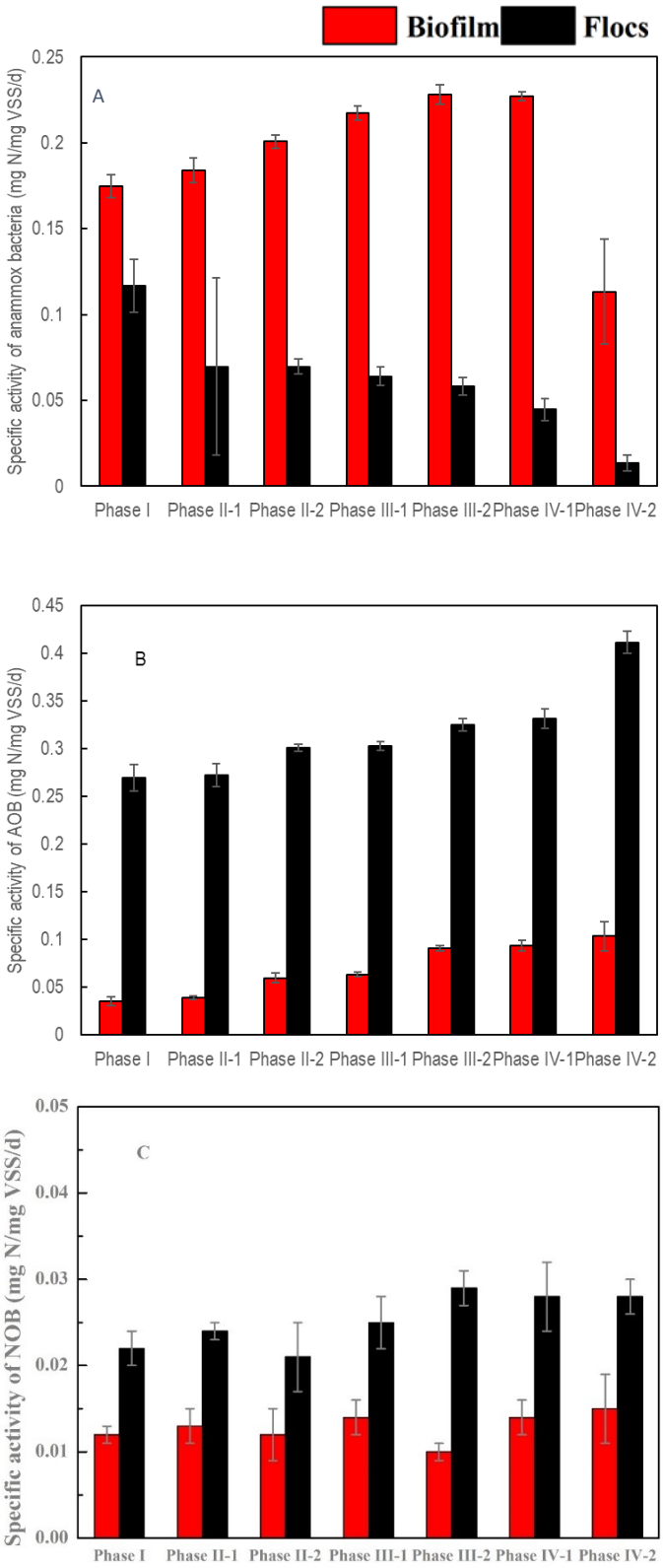


Figure 8.2. The biofilm thickness change with the nitrogen removal rate (NRR) increase from Phase I to Phase IV. The numbers for biofilm thickness and NRR were related to the end of each phase.

8.3.2. The specific activities of AOB and anammox bacteria

Anammox bacteria and AOB dominated the microbial populations in the system, and their specific activities, determined as explained in Section 2.4, are exhibited in Figure 8.3A and 8.3B, respectively.


Biofilm
Flocs
A
Specific activity of anammox bacteria (mg N/mg VSS/d)
0.25
0.2
0.15
0.1
0.05
0
Phase I
Phase II-1
Phase II-2
Phase III-1
Phase III-2
Phase IV-1
Phase IV-2
B
Specific activity of AOB (mg N/mg VSS/d)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
C
Specific activity of NOB (mg N/mg VSS/d)
0.05
0.04
0.03
0.02
0.01
0.00

\

Figure 8.3. The specific activity of anammox bacteria (A), AOB (B) and NOB (C) under different operation phases red bars representing biofilms and black bars representing flocs)

With the extended SRT, anammox bacteria dominated the biological activities in the biofilm. Biofilm structure is known to exhibit a higher mass transfer resistance of substrates and DO, as compared to flocs (Zhang et al. 2015). As can be seen in Figure 8.3A, when the HRT was reduced from 2.5 d (Phase I) to 2.0 d (Phase II), biofilm SAAB changed from 0.17 ± 0.01 mg N/mg VSS/d to 0.18 ± 0.01 mg N/mg VSS/d in Phase II-1 ($p = 0.17$) at DO of 0.18-0.20 mg/L, and increased slightly to 0.20 ± 0.00 mg N/mg VSS/d ($p = 0.025$) when DO in Phase II was increased to 0.22-0.24 mg/L in Phase II-2. When the HRT was further reduced to 1.5 d, biofilm SAAB further increased to 0.22 ± 0.00 mg N/mg VSS/d ($p = 0.007$), but remained stable at 0.23±0.01 mg N/mg VSS/d with the higher DO of 0.24-0.28 mg/L ($p = 0.055$). Adjustments of DO levels enabled maintaining potential high AOB activity which provided anammox bacteria with a larger amount of NO_2^- as nitrogen loading increased. This helped explain the slight increase of anammox activity from Phase I to Phase IV-1. However, the increase of DO levels to 0.28-0.35 mg/L in Phase IV-2 made anammox activity drop significantly to 0.11 ± 0.03 mg N/mg VSS/d ($p = 0.003$), which consequently led to the aforementioned nitrite accumulation.

A small portion of anammox bacteria inhabited the flocs. Floc SAAB consistently dropped as a consequence of HRT reduction from Phase I to Phase IV ($p = 0.12$). Floc SAAB was measured at 0.12 ± 0.02 mg N/mg VSS/d and 0.10 ± 0.00 mg N/mg VSS/d ($p = 0.17$) in

Phases I and II, respectively, and significantly decreased to 0.07 ± 0.00 mg N/mg VSS/d ($p = 0.00033$) with the increased DO at a HRT of 2.0 d. This may be explained by the higher DO inhibition on anammox activity in the floc configuration, where less oxygen transfer limitation occurs due to the looser floc structure compared to that of biofilm. The floc SAAB accounted for 11-40% of the overall anammox activity, with a constant decrease from Phase I to Phase IV-2, as shown in Figure 8.3A. More anammox bacteria are retained in biofilm with the HRT reduction, which is consistent with the conclusion of our previous study (Yang et al. 2019b). The bulk DO raise further increased the retain ability of anammox bacteria within biofilm, where a larger DO transfer resistance applies. As shown in Figure 8.3B, the specific activity of AOB (SAOB) in flocs was significantly higher than that of biofilm, as observed previously (Yang et al. 2019b). This is because, contrarily to anammox bacteria, AOB growth is favored by oxygen. When the HRT was reduced from 2.5 d (Phase I) to 2.0 d (Phase II-1), floc SAOB remained stable at 0.27 ± 0.014 mg N/mg VSS/d and 0.272 ± 0.012 mg N/mg VSS/d ($p = 0.81$), respectively, and increased significantly to 0.301 ± 0.004 mg N/mg VSS/d ($p = 0.019$) with an increase of DO in Phase II-2 (0.22-0.24 mg L^{-1}). The activity of AOB in the flocs increased with higher DO because of low resistance in DO accessibility from bulk liquid to the flocs. Similarly, the reduction of HRT to 1.5 d in Phase III-1 did not affect the flocs SAOB (0.303 ± 0.005 mg N/mg VSS/d [$p = 0.6$]). However, it increased to 0.325 ± 0.007 mg N/mg VSS/d ($p = 0.009$) with an increase of DO in Phase III-2 (0.24-0.28 mg/L). When the HRT was further reduced from 1.5 d to 1.2 d in Phase IV-1, the floc SAOB was stable at 0.331 ± 0.010 mg N/mg VSS/d ($p = 0.29$) and further increased to 0.412 ± 0.012 mg N/mg VSS/d ($p = 0.0008$)

with an increase of DO. AOB within flocs exhibited greater sensitivity to changes in bulk DO concentration than to changes in the NLR.

The change of biofilm SAOB followed the same pattern in the operation as that in the flocs. The contribution of SAOB in flocs account for 78-88% in the overall SAOB of reactor, indicating a successful segregation of AOB and anammox bacteria in the IFAS type reactor.

8.3.3. Biomass

Monitoring reactor biomass is important because longer biomass retention times are necessary for the slow growth of anammox bacteria and monitoring the biomass changes at different operational phases can give us valuable information related with its removal performance. Figure 8.4 shows the biomass mass as biofilm and as flocs during the different operating phases. Under the well-maintained SRT throughout the entire operational period, floc biomass concentrations were impacted only slightly by the change of operating conditions. For instance, floc biomass increased significantly from 4.28 ± 0.26 mg VSS to 5.02 ± 0.33 mg VSS ($p = 0.025$) when the HRT was reduced from 2.5 d in Phase I to 2.0 d in Phase II-1 and became stable in Phase II-2 at 5.17 ± 0.28 mg VSS/L ($p = 0.15$) with the increase of DO. This is consistent with the observation in other flocs systems which indicated the increased influent loading encouraged the biomass growth (Sheng et al. 2016; Xu et al. 2018b). Flocculent biomass overall keeps increasing with higher nitrogen loading rate and higher oxygen concentrations throughout the operational period.

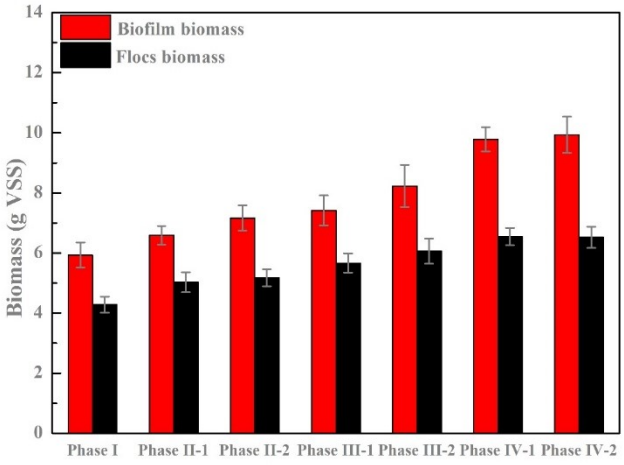


Figure 8.4. The biomass of biofilm (left column) and floc (right column) during different phases.

The biofilm biomass was slightly higher than that of the flocs, due to the superior retention of biomass in the biocarriers. Both the decrease of HRT and increase of DO led to an increase ($p < 0.05$) of biofilm biomass along with the operation (from 5.9 ± 0.4 g VSS in Phase I to 9.9 ± 0.6 g VSS in Phase IV), mainly due to the growth of AOB and anammox bacteria in the biofilm, as observed previously (Yang et al. 2019b). The increased biomass at a lower HRT may explain the higher DO requirement when considering the fixed DO mass transfer rate. As reported in the study by (Wang et al. 2019), DO concentration in the inner biofilm layers is lower than that of the bulk solution because of the accessibility limitation, thus higher DO may be required to maintain the similar DO level in biofilm when the biofilm thickness or density increase.

8.3.4. Microbial analysis

Figure 8.5 shows 16S rRNA gene abundance of anammox bacteria, AOB, NOB, and heterotrophic denitrifiers in biofilm (A) and flocs (B). The qPCR (targeting anammox *nirS* gene) results showed that the biofilm anammox population increased from (4.13 ± 0.09) $\times 10^7$ to (4.46 ± 0.09) $\times$ 10^7 gene copies/mg VSS biomass ($p = 0.02$) from Phase I to II-2, respectively, and further increased to (5.55 ± 0.09) $\times 10^7$ gene copies/mg VSS biomass ($p = 0.0002$) from Phase II-2 to III-2. It then decreased to (4.2 ± 0.06) $\times 10^7$ gene copies/mg VSS biomass ($p < 0.0001$) from Phase III-2 to IV-1, and finally decreased to (1.02 ± 0.1) $\times 10^7$ gene copies/mg VSS biomass ($p < 0.0001$) in Phase IV-2. The floc anammox bacteria population were relatively stable in Phases I-III, measuring (4.14 ± 0.16) $\times 10^6$, (3.98 ± 0.13) $\times 10^6$, and (3.57 ± 0.3) $\times 10^6$ gene copies/mg VSS biomass ($p = 0.26$ and 0.07), respectively. From Phase III to IV, the floc anammox bacteria population decreased to (2.78 ± 0.1) $\times 10^6$ gene copies/mg VSS biomass ($p = 0.009$) in Phase IV-1 and to (1.35 ± 0.4) $\times 10^6$ gene copies/mg VSS biomass ($p = 0.003$) in Phase IV-2. The change of anammox bacteria in both biofilm and floc configuration at each phase correlated well with corresponding SAAB results. From Phase I to Phase III-2 (*i.e.*, from HRT of 2.5 d to 1.5 d) the increase of anammox bacteria population in biofilm was continuously observed, but in flocs anammox population constantly decreased. It is worth to point out that the presence of anammox in the flocs is a result of different contribution such as biofilm detachment, settling and operating conditions such as oxygen. In general, anammox bacteria in flocs are more affected by higher oxygen concentration which, as previously reported, is increased from Phase I to Phase IV. The results suggest that when oxygen increased moderately, namely from Phase I to III, anammox bacteria tend to build up more in the biofilm than in

the bulk. However, when oxygen concentration is increased above the threshold of 0.28 mg/L, also the anammox bacteria in the biofilm get affected.

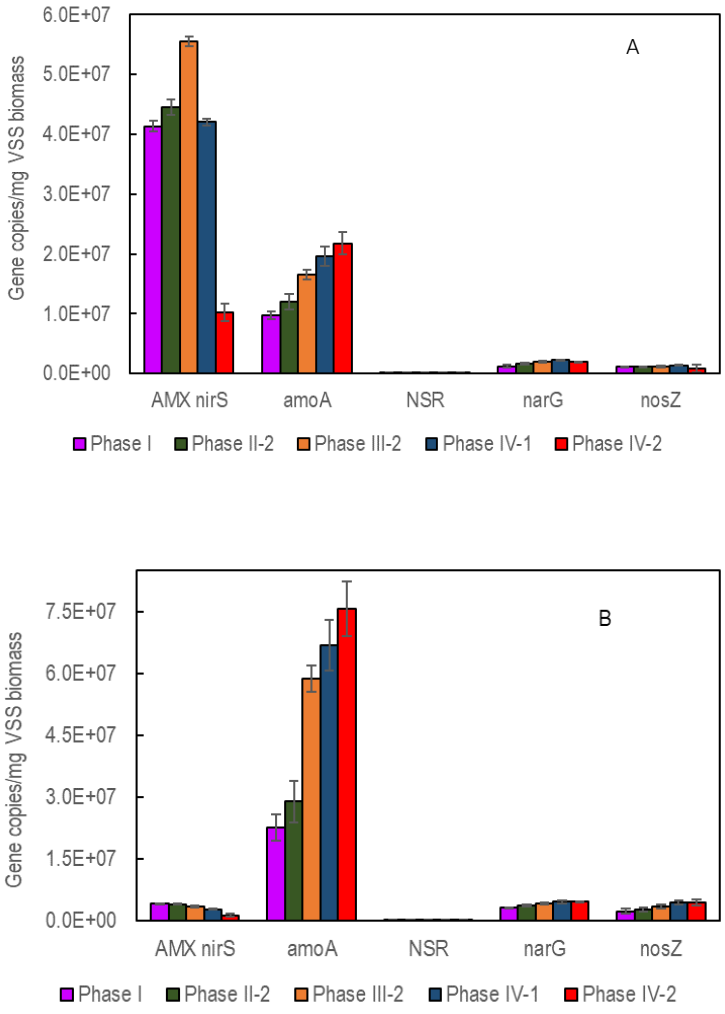


Figure 8.5. The 16S rRNA gene abundance of anammox bacteria (targeting AMX nirS), AOB (targeting amoA gene), NOB (targeting NSR gene), and denitrifiers (targeting narG and nosZ gene) in biofilm (A) and flocs (B).

The qPCR results targeting the amoA gene indicated flocculent AOB population was significantly higher than biofilm AOB. In the bulk, AOB population increased slightly from $(2.26 \pm 0.3) \times 10^7$ gene copies/mg VSS biomass in Phase I to $(2.90 \pm 0.5) \times 10^7$ gene copies/mg VSS biomass in Phases II-2, and then significantly increased to $(5.88 \pm 0.3) \times 10^7$ gene copies/mg VSS biomass ($p = 0.001$) in Phase III-2. In Phase IV-1, the floc AOB population increased further to $(6.69 \pm 0.6) \times 10^7$ gene copies/mg VSS biomass, to $(7.57 \pm 0.7) \times 10^7$ gene copies/mg VSS biomass in Phase IV-2. These results demonstrate that AOB population increases with the increase of their substrate in the feeding, provided oxygen is supplied in a sufficient amount. As for the difference in the AOB qPCR results between Phase IV-1 and Phase IV-2, it could be that the oxygen inhibition on anammox made more ammonium available for AOB, leading to their augmentation.

Biofilm AOB increased continuously from Phase I to Phase IV-1, with populations of $(9.73 \pm 0.7) \times 10^6$, $(1.20 \pm 0.1) \times 10^7$ ($p < 0.05$), $(1.65 \pm 0.08) \times 10^7$ ($p = 0.007$), and $(1.96 \pm 0.2) \times 10^7$ ($p = 0.04$) gene copies/mg VSS biomass in Phases I, II-2, III-2, and IV-1, respectively. The qPCR results (targeting NSR gene) demonstrated that the population of NOB were considerably sparse compared to the population of AOB, anammox bacteria, and the detected heterotrophic denitrifiers. The population of *Nitrospira* were also relatively stable with the change of operation conditions, which were $(1.38 \pm 0.4) \times 10^5$ ~$(2.48 \pm 0.2) \times 10^5$ and $(1.97 \pm 0.4) \times 10^5$ ~$(3.60 \pm 0.17) \times 10^5$ gene copies/mg VSS biomass in biofilm and flocs, respectively. By applying intermittent aeration, NOB are easily outcompeted by AOB as NOB need a longer time to recover from the non-aeration period (Li et al. 2011), or are inhibited by the NH_2OH that is likely produced in the intermittent aeration process (Trojanowicz et al. 2019).

Heterotrophic denitrifiers were measured by qPCR targeting both *nosZ* gene and *narG* gene, which were extensively studied for the heterotrophic denitrifier detection (Yang et al. 2019b). From Figure 8.5 the activity of heterotrophic denitrifiers can be observed to be considerably lower than that of anammox bacteria, indicating heterotrophic denitrifiers were not dominant in the reactor. This can be attributed to the low available biodegradable carbon source, and has been widely observed in the low C/N prevailing reactors (Veuillet et al. 2014; Yang et al. 2019b).

The increase of NO_2^- along with the decrease of NO_3^- in the Phase IV-2 clearly indicate the scarce abundance of NOB in the system, which has been reported previously for such intermittently aerated systems (Li et al. 2011). As a matter of fact, if NOB presence were relevant, the AOB-produced NO_2^- would be converted into NO_3^- instead of accumulating. The presence of NO_2^- was associated to an accumulation of FNA in the system, which were detected in the range of 1.4 ± 1.0 μg/L- 2.4 μg/L in Phase I to IV-1, until increased to 21.7 ± 4.1 μg/L in Phase IV-2. The FNA concentration in Phase IV-2 is beyond the reported FNA inhibition thresholds (*i.e.*, 5.9-19.8 μg/L) on anammox bacteria (Jin et al. 2012).

As can be observed from this experience, being HRT the same (namely, within the same phase), oxygen concentration in the bulk needs to be raised in order to increase ammonium nitrogen removal. Furthermore, as HRT decreases, oxygen set point must be increased. However, it can be noted that when HRT is too small (1.2 days), the required oxygen concentration needed in order to improve ammonium removal efficiency inhibits anammox bacteria and this in turn worsens total nitrogen removal efficiency. Our study shows that 0.28 mg/L is the threshold bulk oxygen concentration above which anammox inhibition starts taking place. Other studies found higher oxygen values at which anammox bacteria

are strongly inhibited. For instance, Kalvelage et al. (2011) reported anammox activity being completely inhibited at DO of 0.64 mg/L. However, the threshold oxygen concentration depends on several other operational features among which biofilm thickness and density, mixing regime, coexistence with AOB in the biofilm and oxygen diffusivity.

In addition to the inhibitory effect of DO, in long term the accumulation of FNA in the system due to the absence of NOB can be considered responsible for further anammox activity drops.

Furthermore, the feasibility of using intermittent aeration to maintain a relatively stabilized anammox activity and suppress NOB activity was demonstrated. This is because the non-aeration period provides favorable conditions for the growth of anammox bacteria in both flocs and biofilm. Although the NH_4^+ and the AOB-produced NO_2^- can be oxidized either during aeration stage or non-aeration stage in this study thanks to the oxygen transfer limitations in the biofilm, it was observed that the NO_2^- utilization rate during non-aeration stage was 2.2 times higher than that during aeration stage ($P < 0.01$) indicating that non-aeration enhanced the activity of anammox bacteria. Furthermore, thanks to the non-aerated periods NOB was more easily outcompeted even under a relatively high DO concentration of 0.35 mg/L indicating. In the study by Brockmann and Morgenroth (2010), the activity of NOB bacteria was recovered slowly after 110 days of stabilized operation in a nitritation system under continuous aeration conditions. On the contrary, in this study the specific activities of NOB bacteria were always kept low (0.012 ± 0.001 - 0.015 ± 0.002 mg N/mg VSS/d in the biofilm 0.022 ± 0.005 - 0.028 ± 0.004 mg N/mg VSS/d in the flocs).

8.4. Conclusions

The present work shows how to regulate oxygenation regime of IFAS-continuous operation nitritation-anammox systems at different nitrogen loading rates. It was found that higher DO concentrations need to be established in order to maintain high total nitrogen removal efficiency at increased nitrogen loading rates. However, this cannot be done endlessly. As a matter of fact, when DO concentration is higher than 0.28 mg/L, inhibition on anammox bacteria starts taking place due to increased oxygen concentrations in the biofilm. In escalation, if no NOB are present in the system, the AOB-produced nitrite starts accumulating and this in turn can further compromise anammox bacteria activity via free nitrous acid inhibition.

From this experience, it can be observed that at nitrogen loading rates equal or higher than 0.69 kg $N/m^3/day$, alternative ways of maintaining high TN removal efficiencies – other than increasing DO concentration - should be identified.

In addition, the present study confirms the use of intermitted aeration as a strategy to effectively avoid NOB growth.

Finally, knowing the maximal nitrogen loading rate at which an IFAS one-stage partial nitritation/anammox system can work is crucial when identifying the ideal reactor volumes for larger scales.

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CHAPTER 9 PERFORMANCE ANALYSIS AND OPTIMIZATION OF NITRITATION-ANAMMOX PROCESS FOR AUTOTROPHIC NITROGEN REMOVAL IN DIFFERENT REACTOR CONFIGURATION: A STUDY FOR THE TREATMENT OF AMMONIA-RICH LAGOON SUPERNATANT

9.1. Introduction

Technologies for treating high-ammonia, dewatered-sludge return-flow separately prior to blending with the main sewage treatment train are urgently needed to meet stringent nitrogen removal effluent standards. Compared with conventional biological nitrogen removal processes, the anammox process has many advantages, *i.e.*, low oxygen demand, low excess sludge, reduced CO_2 emissions, and no requirement for external carbon sources (Van Hulle et al., 2010). Thus, the anammox process is a promising technology for ammonia-rich sludge-liquor treatment.

The nitritation-anammox process can be configured as either a one-stage or two-stage configuration. One-stage configurations include: DEMON (Deammonification) (Wett et al., 2015), OLAND (oxygen limited autotrophic nitrification denitrification) (Schaubroeck et al., 2012), CANON (completely autotrophic nitrogen removal over nitrite) (Vázquez-Padín et al., 2009), and SNAP (single-stage nitrogen removal using anammox and nitritation) (Zhang et al., 2014). Two-stage configurations include Sharon-Anammox (single reactor high activity ammonia removal over nitrite-anaerobic ammonium oxidation) (Hwang et al., 2005).

Various operational conditions may be applied for ammonia reduction. Previous studies have demonstrated that oxidation–reduction potential (ORP) (Lackner et al., 2012), oxygen

transfer rate (OTR) (Akaboci et al., 2018) and online NH_4^+ signal (Joss et al., 2011) are all important process control parameters. Previous studies have demonstrated that changes in reactor configuration and operational conditions can significantly impact reactor performance (Tao et al., 2012). The aim of the present work was to summarize these published studies and compare the nitrogen removal treatment performance and microbial communities between bioreactors operating under various configurations and operational conditions. Operational strategies that can enhance treatment effectively and stability have been identified and discussed.

9.2. Material and methods

9.2.1. Lagoon supernatant characteristics

The details of characteristics details are shown in the study of Yang et al. (2019a).

9.2.2 Reactor set-up

9.2.2.1. Anammox IFAS configuration (2nd stage of two-stage nitritation-anammox)

A bench-scale integrated fixed film activated sludge (IFAS) reactor was operated in a sequencing batch (SBR) mode. The NO_2^--N substrate was from nitritation reactor effluent treating lagoon supernatant.

9.2.2.2. Nitritation-anammox SBR configuration (one-stage SBR nitritation-anammox)

SBR operation mode was applied which was divided into 4 stages for each SBR cycle: influent stage for 5 min, reacting stage for 10 hour, settling stage for 110 minute, discharge stage for 5 minute.

9.2.2.3. Nitritation-anammox continuous operation configuration (one-stage continuous operation nitritation-anammox)

A bench scale was operated in a continuous operation mode. An intermittent aeration regime was applied. In every 2 hours, there had 105 minutes of aeration stage with 15 minutes of anoxic stage.

9.2.3. Sample collection and analytical methods

Measurement methods are described in the study Yang et al. (2019b).

9.2.4. Activity measurement

The specific anammox activity (SAA), AOB activity (SAOB), and NOB activity (SNOB) of biomass were measured with the method described by Yang et al. (2019c).

9.2.5. q-PCR analysis and MiSeq sequencing

Information about qPCR primers and target genes is shown in Appendix A-1 and A-2.

9.3. Discussion

9.3.1 One-stage vs. Two-stage

The highest NRR occurred in the anaerobic-anammox IFAS configuration (0.61 ± 0.02 kg N m^{-3} d^{-1}) while NRR in the one-stage SBR and continuous operation configurations was 0.54 ± 0.01 and 0.46 ± 0.01 kg N m^{-3} d^{-1}, respectively. The advantage of anammox IFAS configuration (2^{nd} stage of two-stage PN/A reactor) is that anammox bacteria activities are most robust due to the favorable conditions provided by the separated reactor system (Yang et al., 2019a) (see Chapter 3). For the two-stage reactor, biodegradable COD in the raw wastewater can be utilized by the 1^{st} stage of the nitritation reactor and thus reduce the

competition with heterotrophs for the NO_2^- substrates. The nitritation and anammox processes are separated and offer a wider range of optimal process conditions than in a one-stage system. However, NO_2^- can easily accumulate, especially at high loading rates (Yang et al., 2019d) (see Chapter 4). In the one-stage systems (*i.e.*, IFAS or continuous operation configurations), NO_2^- was consumed and produced simultaneously and there was no NO_2^- accumulation (Yang et al., 2019c) (see Chapter 6). Under DO conditions, anammox bacteria can be protected by the inner layer of biofilm. Therefore, the process-design considerations should be based on expected operating. For example, if anammox activities are low during the start-up period, a two-stage reactor is recommended. If anammox activities become stable and active, switching to one-stage operation is an appropriate option.

9.3.2. IFAS vs. MBBR

In order to evaluate the impacts of MBBR and IFAS on the nitrogen removal efficiency, MBBR and IFAS modes were applied in Chapter 7 (Yang et al., 2020). Results showed that when the MBBR operation was switched into the IFAS operation, where the flocs biomass increased, the NRR increased 2-times. The activity of AOB in the flocs was much higher than that in the biofilm due to the diffusion limitation (*e.g.* dissolved oxygen and substrates) to the biofilm. Results showed that AOB population was significantly improved because of the returned settling sludge back into the reactor system.

9.3.3. Aeration strategies

Both continuous and intermittent aeration were compared in the one-stage nitritation-anammox process in Chapter 7 (Yang et al., 2020). Study showed that NOB was effectively

inhibited under 0.20 mg L^{-1} of DO condition. However, study indicted that the NOB activity would recover with the long term operation under continuous aeration condition (Jianlong and Ning, 2004). Results showed that by applying intermittent aeration, NOB activity remained low during different stages. Furthermore, the enhancement of anammox bacteria activity was proved when non-aeration stage was applied.

The anammox activity was significantly enhanced.

9.3.4. Continuous operation vs. SBR

SBR operation ensures a long retention time of bacteria effectively growing in the reactor. It was proven that anammox activity was significantly improved at a higher substrate loading rates in Chapter 4 and Chapter 6 (Yang et al., 2019c; Yang et al., 2019d). Continuous flow operation can reduce the high substrate loading shocks. A shorter SRT has the advantage of selecting AOB growth while suppressing NOB growth. Our results indicate that the NRR during SBR and continuous operation operations were stabilized at 0.54 ± 0.01 and 0.26 ± 0.005 kg N m^{-3} d^{-1}, respectively.

9.3.5. Microbial community comparison

Among the three reactor configurations, the amount of AMX *nirS* in biofilm was highest ($P < 0.05$), at 6.85×10^7 copies mg^{-1} VSS biomass in the anammox IFAS configuration. On the other hand, anammox population in the flocs remained low at different stages indicting that the anammox activity was more prevalent in the biofilm versus the flocs, regardless of reactor configuration (*i.e.*, one-stage reactor vs two-stage reactor). The following are our recommended ways to improve the abundance of anammox bacteria, preceded by the rationale. (1) Higher abundances of nutrients improved the growth of bacteria (AOB and

anammox bacteria. In Yang et al. (2019d) of Chapter 4, the decrease in HRT from 2.5 to 1.7 day resulted in 1.9-times increase in the anammox population. (2) In Yang et al. (2019d) of Chapter 4, results showed that after switching to step-feeding mode, the population of anammox bacteria increased from 4.9×10^7 to 6.1×10^7 copies mg^{-1} VSS biomass in biofilm. Thus, it is recommended that step feed mode in SBR operation can be applied for preventing high influent NO_2^- inhibition. (3) In Yang et al. (2020) of Chapter 7, the abundance of AMX *nirS* in biofilm improved effectively after switching to intermittent aeration. Thus, it is recommended that intermittent aeration can be applied for maintaining anammox bacteria activity active. (4) Anammox population can be affected by different DO concentrations. In chapter 8, the qPCR (targeting AMX *nirS* gene) the biofilm anammox population decreased from 4.13×10^7 to 1.02×10^7 gene copies mg^{-1} VSS biomass ($P < 0.01$) when the DO was increased from 0.24 - 0.28 mg L^{-1} to DO of 0.28 - 0.35 mg L^{-1}, respectively. Thus, it is recommended that DO concentration in one-stage should not exceed 0.28 mg L^{-1} to prevent the inhibition impact on the anammox bacteria.

The qPCR results show that the AOB population was much higher in the flocs than in the biofilm. In order to enhance the overall AOB population, it is recommended that the flocs should be kept in one stage operation system. For example, in Chapter 7, the AOB population in the flocs improved significantly by IFAS mode. Further, a higher DO concentration has been shown to improve the growth rate of AOB; in Chapter 8, the AOB population was highest (7.57×10^7 gene copies/mg VSS biomass) at a DO of 0.28 - 0.35 mg/L.

NOB was suppressed under all operation conditions, as shown by the low NOB populations. This indicates that a low NOB population can be ensured by (1) applying intermittent

aeration (Chapter 7), (2) keeping low DO (lower than 0.28 mg L^{-1}) (Chapter 8), and (3) high free ammonia inhibition (Chapter 6).

9.3.6. Operational recommendations

(1) The start-up time for anammox bacteria can be long (1-2 years) due to its extremely low growth rate, therefore, an anammox IFAS configuration is recommended for the start-up phase. After the anammox activity builds up by introducing the nitritation sludge into anammox IFAS configuration, the one-stage nitritation-anammox configuration can be feasible and reliable. (2) Online monitoring of NO_2^- and O_2 concentrations are necessary for achieving a stabilized nitrogen removal efficiency. Since anammox can be inhibited by the high accumulation of NO_2^- (above 125 - 150 mg N L^{-1}) and DO (above 0.35 mg L^{-1}) in the bulk liquid, monitoring can provide reliable information to run nitritation-anammox system, and operational adjustments can be made accordingly when NO_2^- accumulates or high/low DO concentration are present in the bulk liquid. (3) During operations, SAA and SAOB tests are highly recommended since maintaining active anammox activity and AOB activity are crucial. Relying on online probes and MLSS measurements are not usually accurate because such measurements may not provide a good correlation with bacteria activity. The best solution to get accurate information is to measure the activities of AOB and anammox bacteria in batch tests.

9.4. Conclusion

1. The anammox IFAS was successfully established. Anammox activity was improved when HRT was reduced from 2.5 day to 1.7 day. At HRT of 1.2 day, it was found that the anammox activity was inhibited because of high nitrite accumulation especially in the

beginning of feed phase per SBR cycle. The impact of NO_2^- inhibition was reduced by applying step-feed strategy. The anammox IFAS configuration achieved the highest NRR (0.61 ± 0.02 kg N m^{-3} d^{-1}) and anammox activity (0.38 ± 0.05 kg N kg VSS^{-1} d^{-1}). Microbial activities and community compositions showed that anammox bacteria dominated in the biofilm while heterotrophs mainly stayed in the flocs.

2. The anammox activity significantly improved when a phosphorus concentration reduced from 235 ± 15.4 mg PO_4^3-P L^{-1} to 32 ± 5.4 mg PO_4^3-P L^{-1}. Batch study indicated that the anammox bacteria in the biofilm showed greater tolerance when facing higher phosphate concentration conditions than anammox bacteria in the flocs. Result demonstrates that nitrogen removal efficiency can be significantly improved on treating ammonia rich lagoon supernatant once PO_4^{3-} is precipitated during the Ostara® process as pre-treatment.

3. In the nitritation-anammox configuration, by reducing HRTs from 2.5 day to 1.2 day and increasing biocarrier filling ratio from 35% to 55%, the nitrogen removal rates (NRR) was significantly improved. SBR operation have higher NRR (0.54 ± 0.01 kg N m^{-3} d^{-1}) and anammox activity (0.33 ± 0.02 kg N kg VSS^{-1} d^{-1}) than continuous operation operation at an HRT of 1.2 day.

4. By applying intermittent aeration and introduction of returned sludge into system, continuous operation configuration can reach a maximum NRR (0.46 ± 0.01 kg N m^{-3} d^{-1}) and anammox activity (0.23 ± 0.01 kg N m^{-3} d^{-1}), which can make up for the disadvantages of continuous operation configuration operation.

5. The impact of different DO concentrations on the nitrogen removal efficiency in one-stage continuous operation nitritation-anammox was also investigated. Results suggested

that AOB activity was significantly improved when DO increased from 0.18 to 0.35 mg L^{-1}. However, when DO concentration was higher than 0.28 mg/L, NO_2^- concentration began to accumulate and inhibition on anammox bacteria occurred indicting that the optimal DO range in our study should be from 0.30 to 0.35 mg L^{-1}.

9.5. References

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CHAPTER 10. CONCLUSIONS AND RECOMMENDATIONS

10.1. Conclusions

Conclusions derived from research described in chapters 3 - 9 are reported below:

This lab-scale study showed that applications for lagoon supernatant treatment are achievable by applying nitritation-anammox process, either by one-stage or two-stage operation. Under optimized conditions, the anammox IFAS configuration achieved the highest NRR (0.61 ± 0.02 kg N m^{-3} d^{-1}) and anammox activity (0.38 ± 0.05 kg N kg VSS^{-1} d^{-1}) at an HRT of 1.2 day. The anammox activity were 2-times higher in the anammox IFAS configuration than in the one-stage nitritation-anammox operation.

For the nitritation-anammox configuration, SBR operation have higher NRR (0.54 ± 0.01 kg N m^{-3} d^{-1}) and anammox activity (0.33 ± 0.02 kg N kg VSS^{-1} d^{-1}) than CSTR operation at an HRT of 1.2 day. The operation of the SBR configuration achieved 1.4-times higher anammox activity than the CSTR configuration. As compared to the MBBR configuration (with no flocs), the IFAS configuration significantly enhanced reactor performance in one-stage nitritation-anammox operation. Further, intermittent aeration resulted in 1.5-times higher SAA compared with continuous aeration. DO concentration in mixed liquor needs to be continuous monitored and adjusted to maintain high AOB activities, and thus overall NRR. After optimization, CSTR configuration can reach a maximum NRR (0.46 ± 0.01 kg N m^{-3} d^{-1}) and anammox activity (0.23 ± 0.01 kg N m^{-3} d^{-1}) by applying intermittent aeration and introduction of returned sludge into system, which can make up for the disadvantages of CSTR configuration operation.

Among all operation parameters tested, the most significant improvements in NRR were observed when (i) aeration was switched from continuous to intermittent; (ii) continuous DO adjustment as biomass develops, and (iii) maintaining suspended biomass (as in one-stage CSTR reactor).

10.2. Recommendations for further studies

1. Nitrous oxide (N_2O) is a potent greenhouse gas and long-term exposure to N_2O in a work environment may cause long-term complications. N_2O can be formed during biological treatment processes. AOB denitrification and incomplete heterotrophs denitrification are two main pathways. For the side stream treatment, further studies are recommended to identify N_2O produced pathways and emissions. Most importantly, studies should focus on how to mitigate N_2O emissions under various operation conditions.

2. Further studies are recommended to evaluate the operation and maintenance cost of nitritation-anammox process for treating side stream wastewater

3. Feasibility of nitritation-anammox process in a pilot scale is recommended.

4. Further studies are recommended to determine the removal efficiency at lower temperatures for two-stage or one-stage nitritation-anammox process on treating side stream wastewater.

5. Compared with the traditional nitrification-denitrification process, nitritation-anammox process is a cost-efficiency technology. Mainstream wastewater treatment with low ammonia nitrogen loading will be a challenge by applying nitritation-anammox process. The study on the low strength nitrogen wastewater are recommended.

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APPENDIX A: Supporting Tables

Table A-1. Information about qPCR primers and target genes

	Primer	Nucleotides sequence 5'-3'	Target
Anammox	AnnirS379F	TCTATCGTTGCATCGCATTT	AMX *nirS* gene
	AnnirS821R	GGATGGGTCTTGATAAACA	
AOB	amoA-1F	GGGGTTTCTACTGGTGGT	*amoA* gene of betaproteobacteria AOB
	amoA-2R	CCCCTCTGCAAAGCCTTCTTC	
NOB	Nitro 1198f	ACCCCTAGCAAATCTCAAAAAA CCG	Nitrobacter spp. *16S rDNA* gene
	Nitro 1423r	CTTCACCCCAGTCGCTGACC	
Denitrification	nosZ 2F	CGCRACGGCAASAAGGTSMSSGT	*nosZ* gene
	nosZ 2R	CAK RTG CAK SGC RTG GCA GAA	
	narG 1960m2f	TAYGTSGGGCAGGARAAACTG	*narG* gene
	narG 2050m2r	CGTAGAAGAAGCTGGTGCTGTT	

Table A-2. qPCR amplification programs

Target gene	Initial denaturation	Cycles				Final extension (72 °C)
		Cycles	Denaturation	Annealing	Extension (72 °C)	
AMX *nirS*	95 °C, 5 min	35	95 °C, 30 s	54 °C, 45s	1 min	10 min
amoA	95 °C, 15 min	45	95 °C, 60 s	54 °C, 60s	1 min	10 min
Nitrobacter spp. 16S rDNA gene	95 °C, 10 min	40	94 °C, 20 s	54 °C, 60s	1 min	10 min
narG gene	95 °C, 30 s	35	95 °C, 15 s	58 °C, 30 s	31 s	-
nosZ gene	95 °C, 30 s	30	95 °C, 15 s	60 °C, 30 s	31 s	-

Table A-3. Phosphate concentrations in the test of the short-term impacts on one-stage nitritation-anammox SBR process

Conditions						
Trial1	20X dilution Pre-Ostara supernatant	4X dilution Pre-Ostara supernatant	2X dilution Pre-Ostara supernatant	Pre-Ostara supernatant	Pre-Ostara supernatant +110mgP/L of synthetic P	Pre-Ostara supernatant+ 210mg P/L synthetic P
Trial 2	2X dilution Post-Ostara supernatant	Post-Ostara supernatant+30 mgP/L synthetic P	Post-Ostara supernatant+90 mgP/L synthetic P	Post-Ostara supernatant+210 mgP/L synthetic P	Post-Ostara supernatant+320 mgP/L synthetic P	Post-Ostara supernatant+ 420 mgP/L synthetic P
Final Phosphorus concentration (mg/L)	12	60	120	240	350	450

Table A-4. Specific AOB and anammox activities in flocs and biofilm in one-stage nitritation-anammox CSTR process

	Period I	Period II	Period III	Period IV	Period V
Specific AOB activity in flocs in *kg N (kg VSS d)$^{-1}$*	0.151	0.161	0.315	0.312	0.318
Specific anammox activity in flocs in *kg N (kg VSS d)$^{-1}$*	0.0450	0.0560	0.0650	0.0560	0.0790
Flocs biomass in $\times 10^{-4}$ *kg VSS*	6.68	10.9	57.6	61.4	70.5
Specific AOB activity in biofilms in *kg N (kg VSS d)$^{-1}$*	0.168	0.158	0.165	0.092	0.085
Specific anammox activity in biofilms in *kg N (kg VSS d)$^{-1}$*	0.102	0.125	0.123	0.189	0.192
Biofilm biomass in $\times 10^{-4}$ *kg VSS*	70.5	72.1	74.9	79.4	90.4
Total specific AOB activity in $\times 10^{-4}$ *kg N d^{-1}*	12.8	13.1	30.5	26.5	29.5
Total specific anammox activity in $\times 10^{-4}$ *kg N d^{-1}*	7.48	9.61	12.9	18.5	22.9

Denitrification processes were calculated using equation S1 and equation S2. The influent COD was likely to be fermented resulting in biodegradable COD, which is represented by butyric acid in the equations (Ahn et al., 2004).

$$NO_2^- + 0.19CH_3CH_2CH_2COOH + H_2CO_3 \rightarrow 0.037C_5H_7O_2N + HCO_3^- + 1.14H_2O + 0.585CO_2 + 0.481N_2 \quad \text{(S1)}$$

$$NO_3^- + 0.29CH_3CH_2CH_2COOH + H_2CO_3 \rightarrow 0.034C_5H_7O_2N + HCO_3^- + 1.54H_2O + 0.986CO_2 + 0.483N_2 \quad \text{(S2)}$$

The specific activity of AOB was calculated as Equation S3.

$$\text{SAOB} = \frac{\Delta p \cdot V_{headspace}}{R \cdot T \cdot VSS} \cdot \frac{1.5 \cdot 14}{V_{liquid} \cdot \Delta t \cdot 32} \quad \text{(S3)}$$

Where SAOB is the specific AOB activity in kg N (kg VSS d)$^{-1}$, Δp is the pressure change in Pascal (N m^{-2}], R is the gas constant of 8.3145 J mol^{-1} K^{-1}, T is the temperature in K, Δt is the time span in s, and V is the volume in m^3.

$$NH_4^+ + 1.5O_2 + 0.028HCO_3^- \rightarrow 0.43N_2 + 0.11NO_3^- + 0.028CH_2O_{0.5}N_{0.15} + 1.44H_2O + 0.51H^+ \quad \text{(S4)}$$

APPENDIX B: Supporting Figures

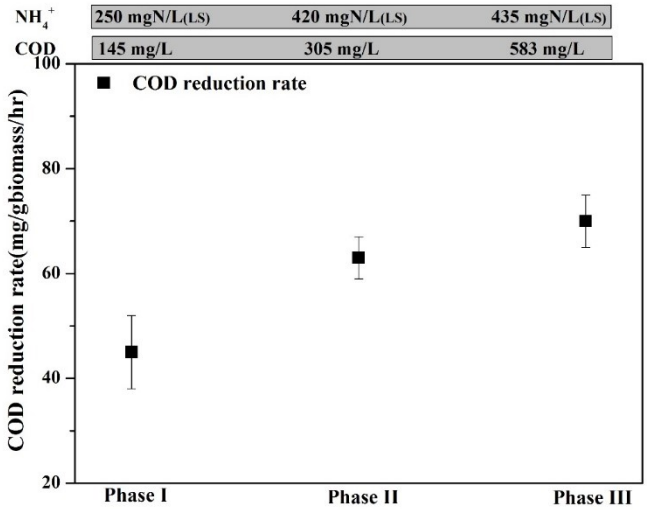

Figure B-3.1. Specific COD reduction rate during three different phases.

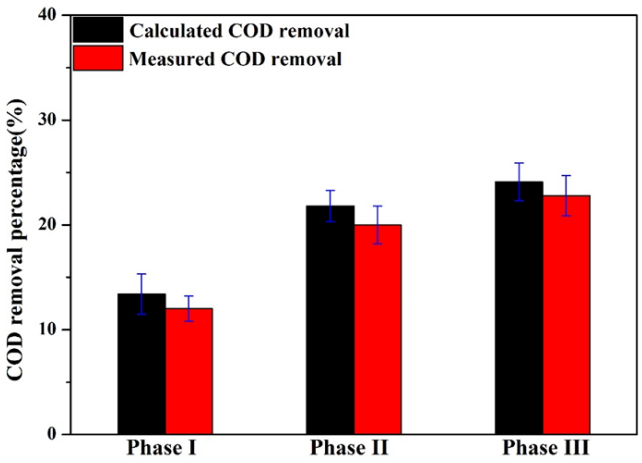

Figure B-3.2. Calculated COD removal percentage (equations S1 and S2) and measured COD removal percentage in the reactor under different operation phases. Error bars represent the standard deviations obtained from different samples.

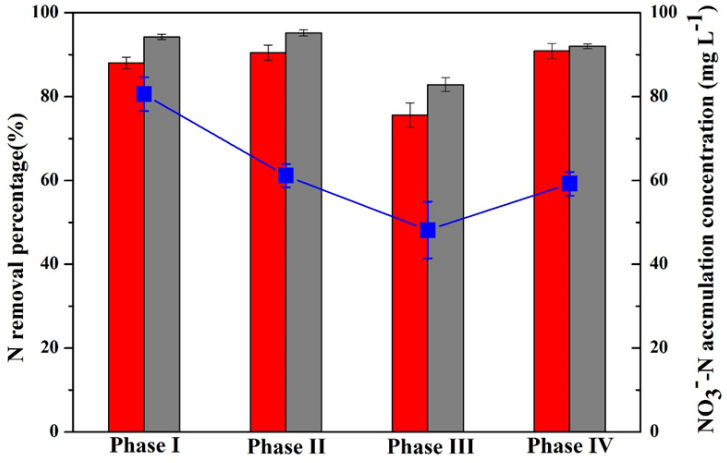

Figure B-4.1. NH_4^+-N (red bars) and NO_2^-N (gray bars) removal percentages and NO_3^- N (blue dots) accumulation concentrations in different operation phases. Error bars represent the standard deviations obtained from different samples (the same for all the following figures).

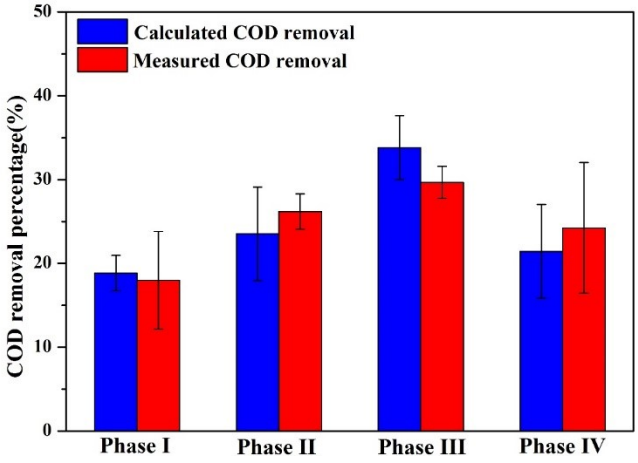

Figure B-4.2. Calculated COD removal percentage (equations S1 and S2) and measured COD removal percentage in the reactor under different operation phases. Error bars represent the standard deviations obtained from different samples.

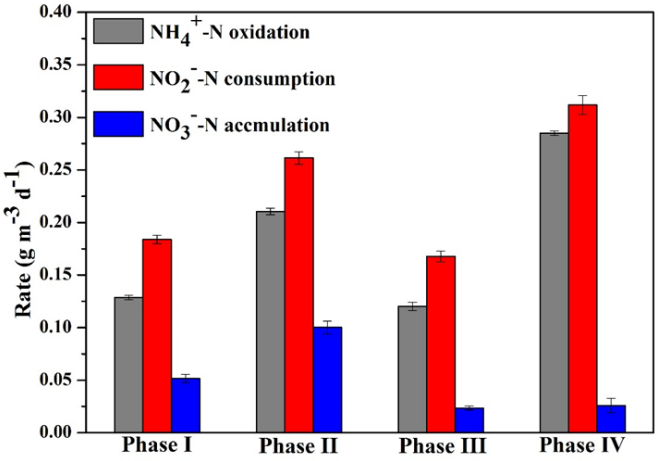

Figure B-4.3. The rates of NH_4^+-N oxidation (gray bars), NO_2^-N consumption (red bars) and NO_3^-N accumulation (blue bars) in each sequencing batch cycle of different operation phases.

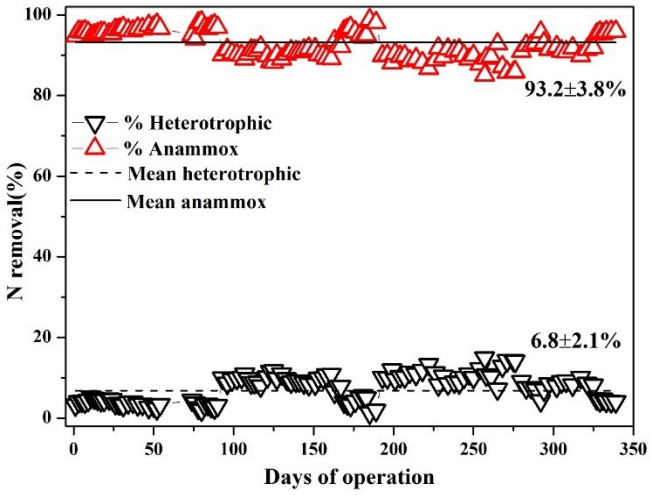

Figure B-4.4. Evolution of the nitrogen removal percentages of both processes in the IFAS anammox SBR.

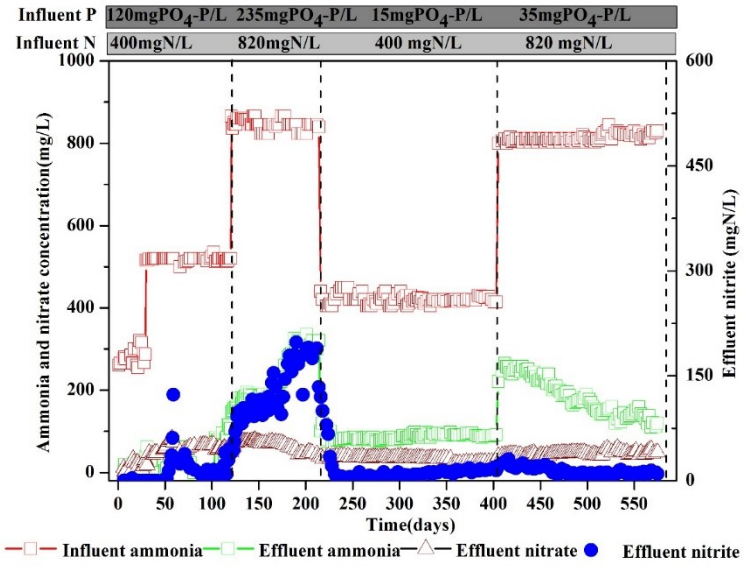

Figure B-5.1. Influent and effluent concentrations of ammonia nitrogen, nitrite nitrogen, nitrate nitrogen during four different operational phases.

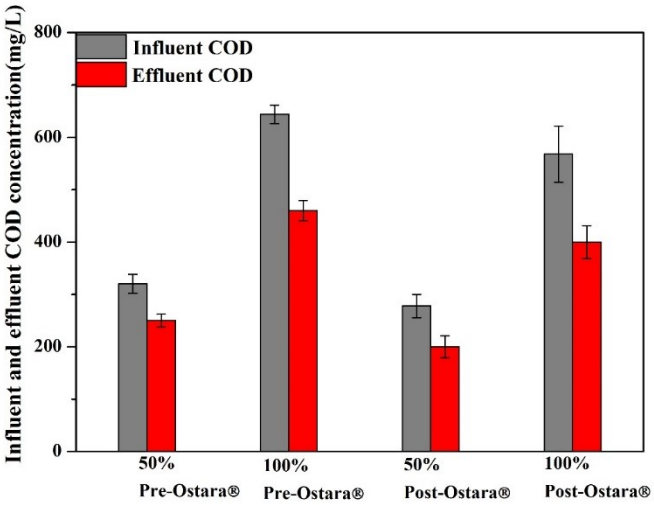

Figure B-5.2. Influent and effluent concentrations of average of COD and COD removal efficiency during four different operational phases.

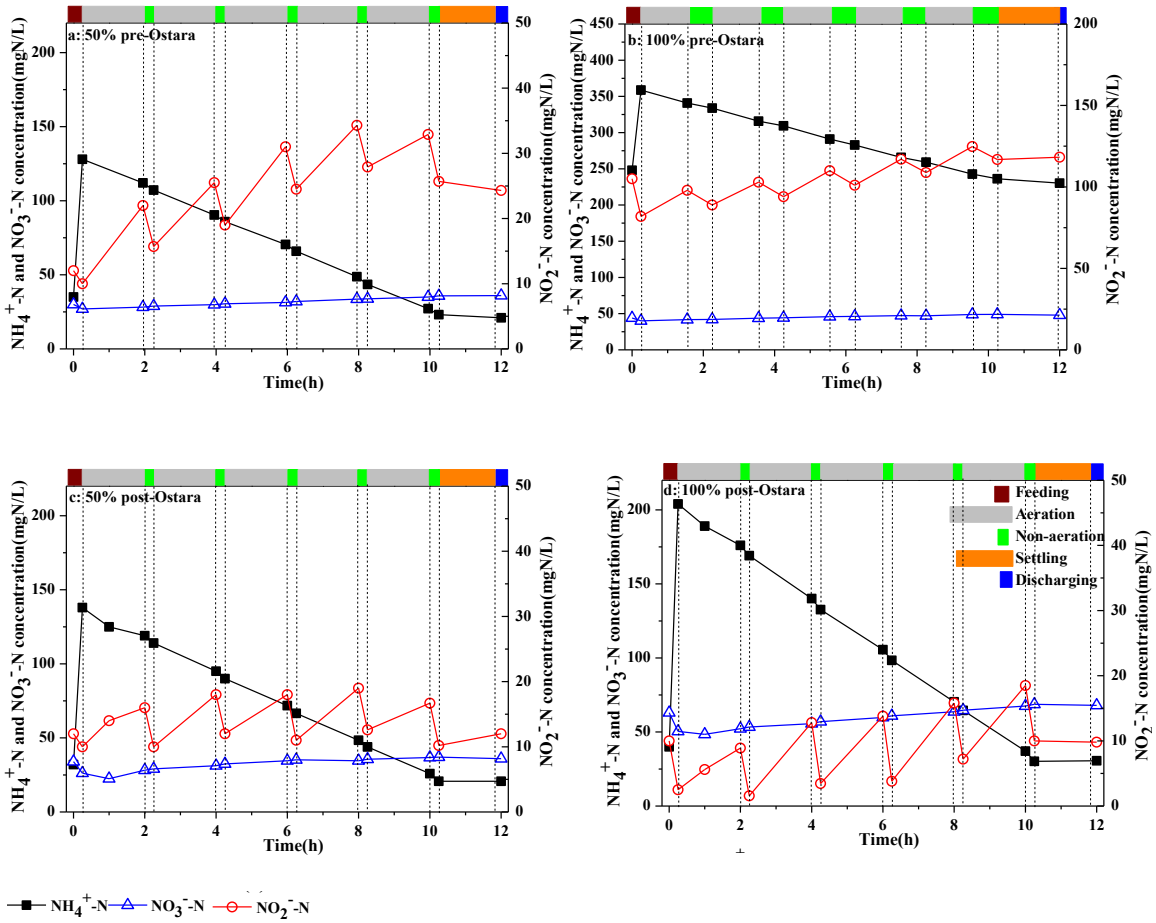

Figure B-5.3. Inorganic nitrogen transformation concentrations in four typical SBR cycles with intermittent aeration applied during four different phases (a: 50% Pre-Ostara®; b: 100% Pre-Ostara®; c: 50% Post-Ostara® and d: 100% Post-Ostara®).

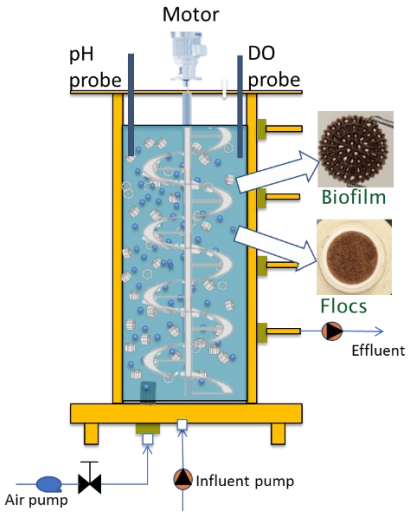

Figure B-5.4. Schematic diagram of the IFAS reactor for one stage nitritation-anammox process

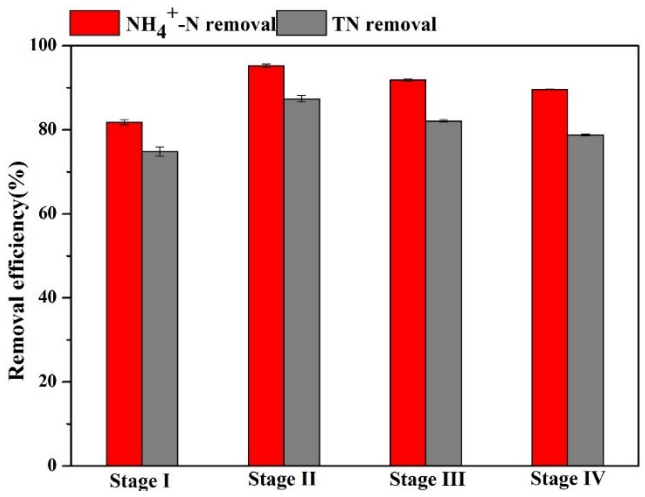

Figure B-6.1. Removal efficiencies of NH_4^+-N and total nitrogen (TN) in different stages, and biomass concentration in both biofilm and flocs configurations under different stage operations.

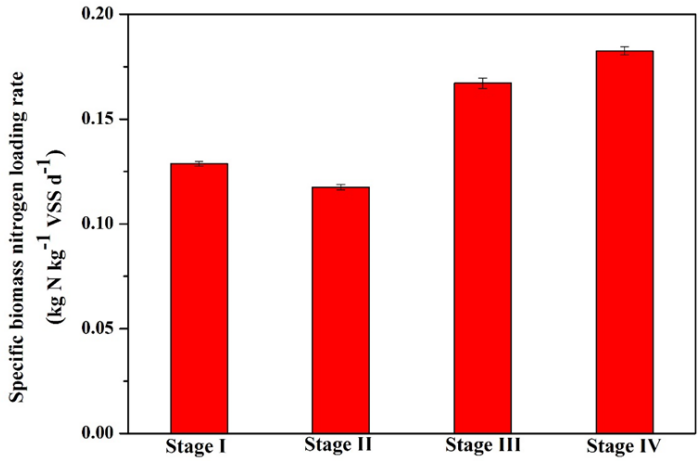

Figure B-6.2. Specific biomass nitrogen loading rates in the reactor operated under different operation stages. Error bars represent standard deviations from different samples

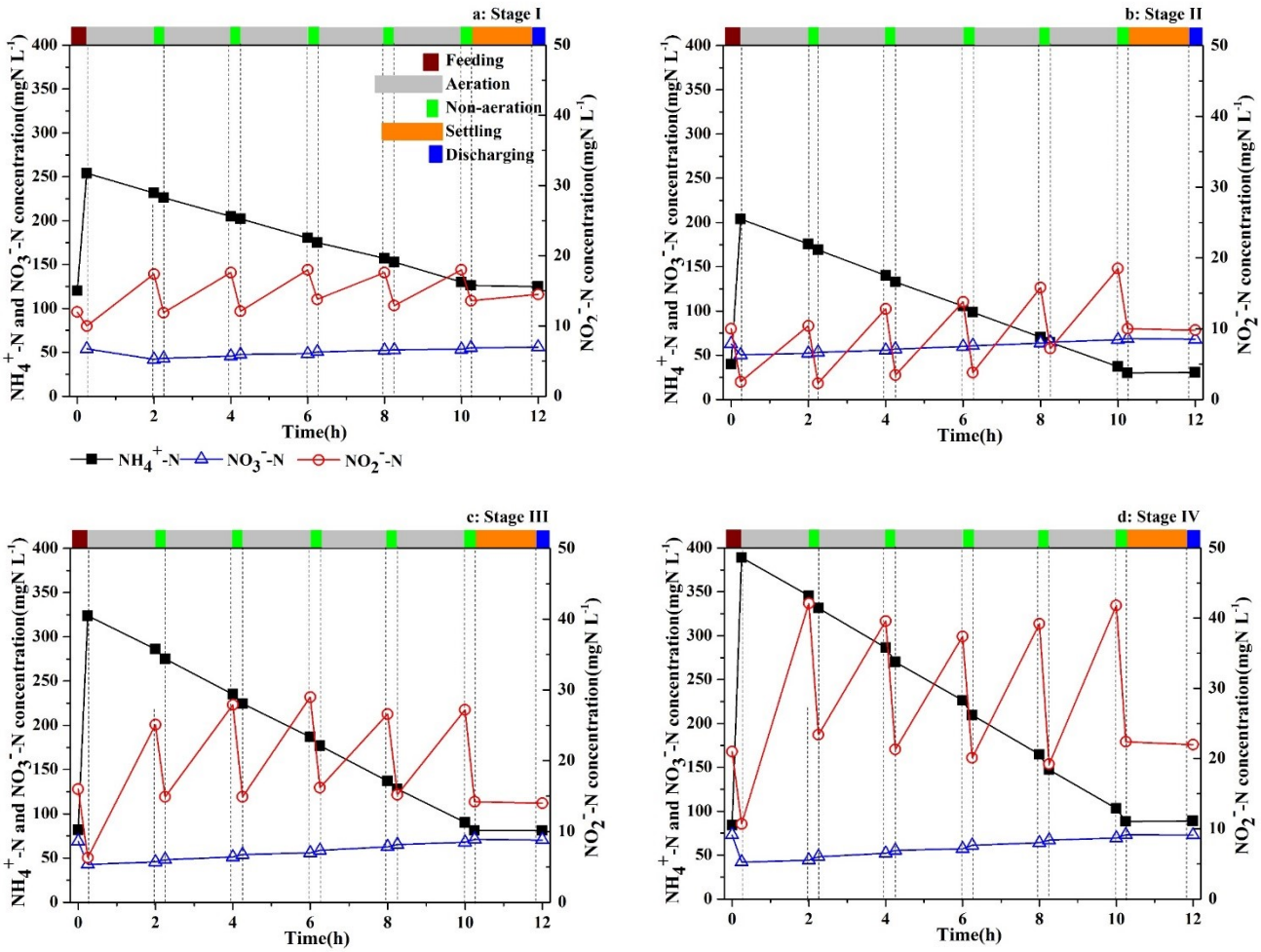

Figure B-6.3. Changes of NH_4^+-N, NO_2^--N and NO_3^--N concentrations in respective each SBR cycle under different operation stages.

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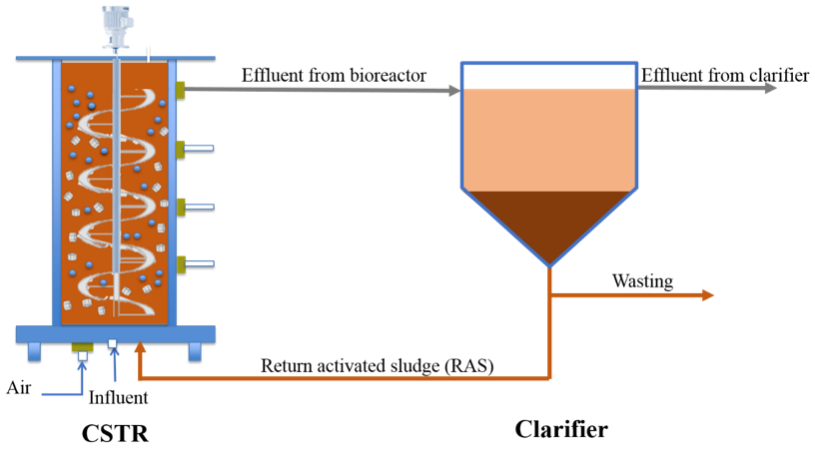


Figure B-7.1. CSTR system with MBBR configuration (without clarifier) and IFAS configuration (with clarifier)

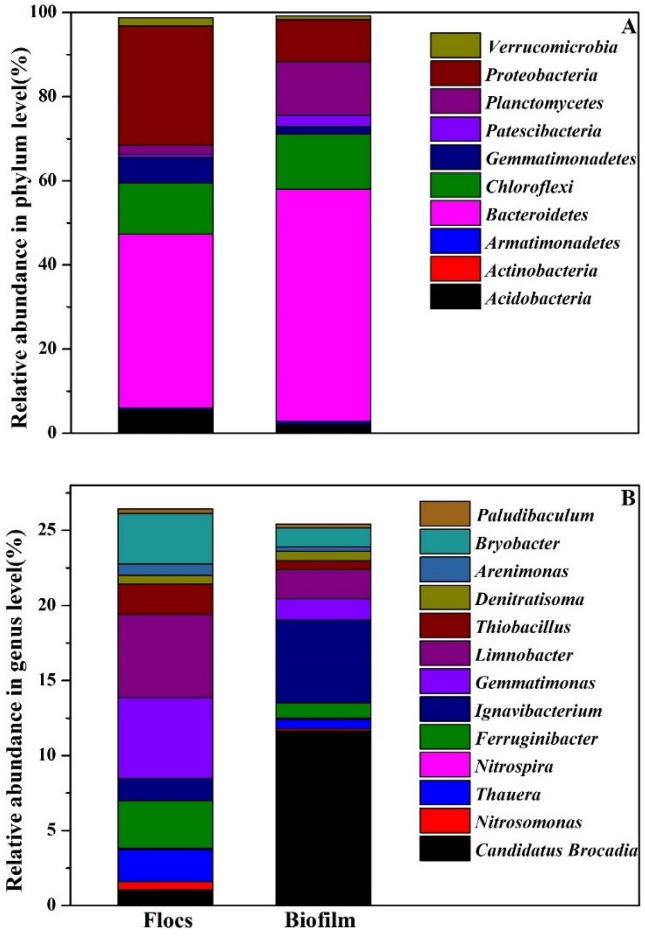


Figure B-7.2. (A) Relative taxonomic abundances of microbial community at phylum level with average relative abundance >1%; (B) assemblage of highest 13 genera from each sample.

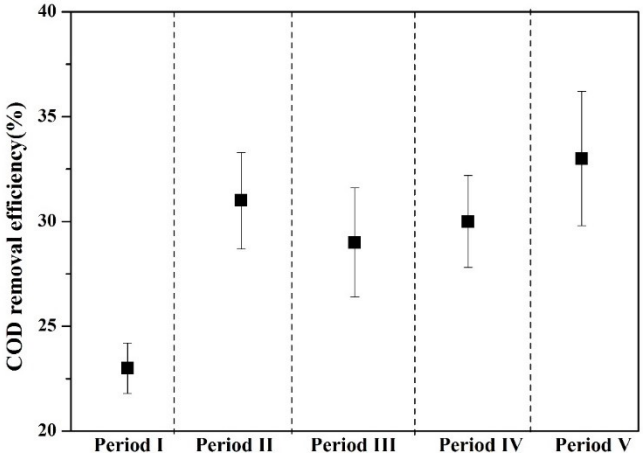
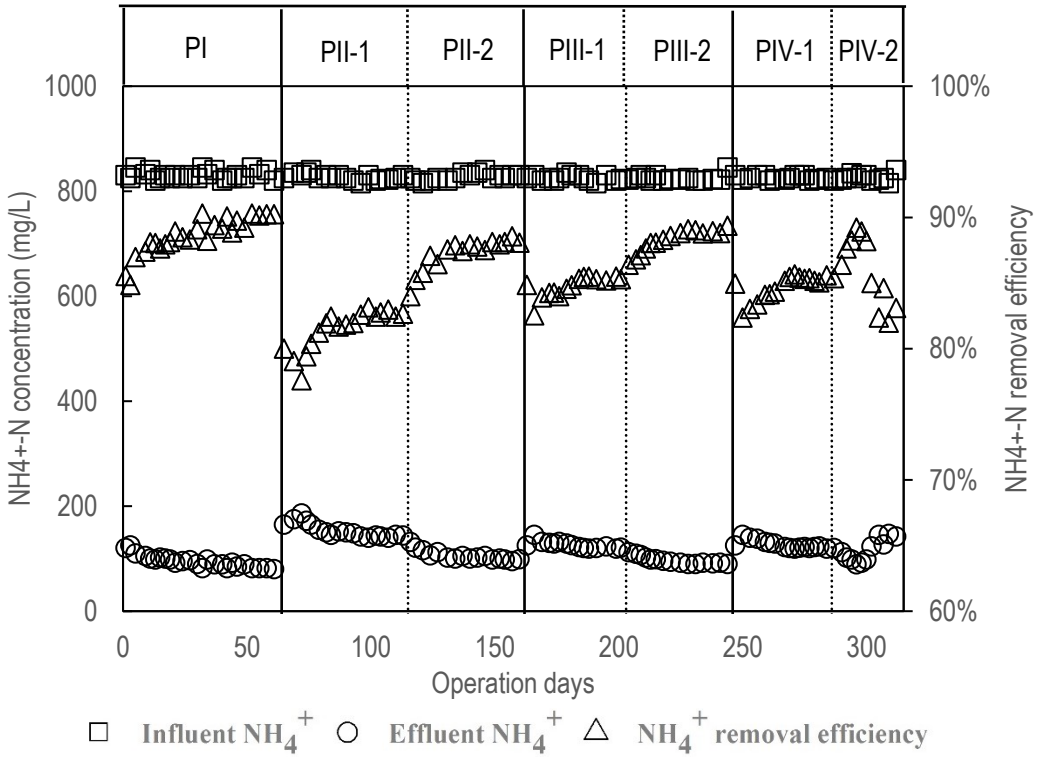


Figure B-7.3. COD reduction efficiency during different periods

(A)


PI
PII-1
PII-2
PIII-1
PIII-2
PIV-1
PIV-2
1000
800
600
400
200
0
100%
90%
80%
70%
60%
NH4+-N concentration (mg/L)
NH4+-N removal efficiency
0
50
100
150
200
250
300
Operation days
Influent NH_4^+
Effluent NH_4^+
NH_4^+ removal efficiency

(B)

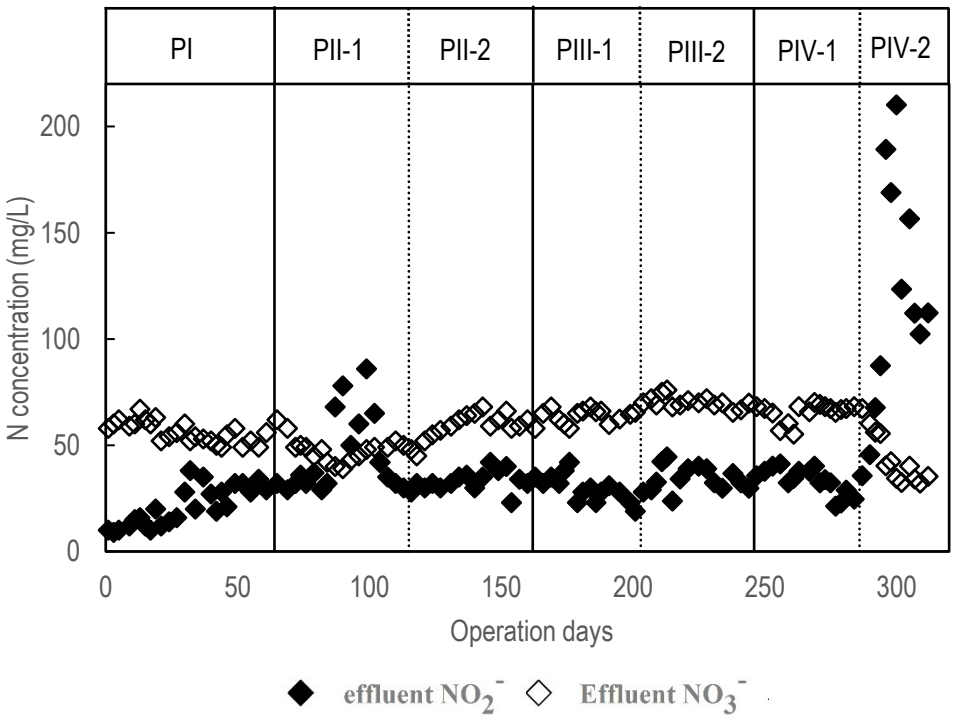

PI
PII-1
PII-2
PIII-1
PIII-2
PIV-1
PIV-2
200
150
100
50
0
N concentration (mg/L)
0
50
100
150
200
250
300
Operation days
effluent NO_2^-
Effluent NO_3^-

Fig. B-8.1. The influent (square symbols) and effluent (circle symbols) NH_4^+-N concentrations, NH_4^+-N removal efficiency (triangle symbols) changes in different operation phases (A), and the effluent NO_2^- -N (filled diamond symbols) and NO_3^--N (non-filled diamond symbols) concentrations in different operation phases (B).

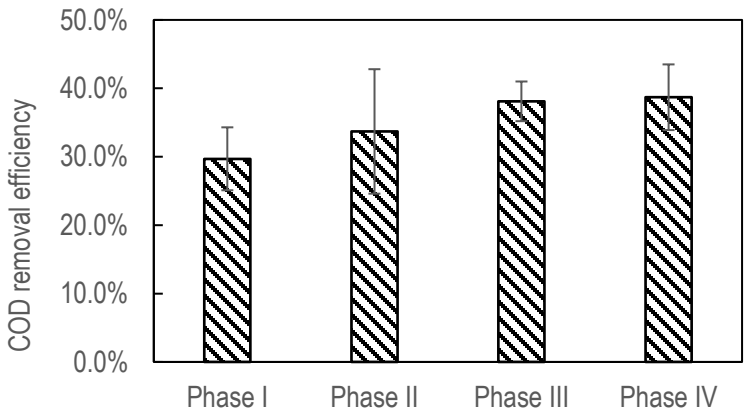


Fig. B-8.2. Soluble COD removal efficiency in different operation phases.