

**Impact of chemical oxygen demand to nitrogen ratio  
on ANAMMOX bacterial growth in an up-flow  
anaerobic sludge blanket reactor**

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Science: Biotechnology in the Department of Biotechnology and  
Food Sciences, Faculty of Applied Sciences at the Durban  
University of Technology, Durban, South Africa**

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**2023**

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## **Approval**

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## **Declaration**

# **Impact of chemical oxygen demand to nitrogen ratio on ANAMMOX bacterial growth in an up-flow anaerobic sludge blanket reactor**

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I, Sandile Simiso Msimango, hereby declare that this dissertation and the content herein is entirely my own work. It has never before been submitted for any diploma, degree or examination to any other University, Technikon or Tertiary Educational Institute.

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06.04.23

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Date

## Reference Declaration in Respect of a Master's Dissertation

I, Sandile Simiso Msimango (full name of student) and Sheena Kumari (full name of supervisor) do hereby declare that in respect of the following dissertation:

The impact of chemical oxygen demand to nitrogen ratio on ANAMMOX bacterial growth in an up-flow anaerobic sludge blanket reactor

- as far as we know and can ascertain:
- no other similar dissertation exists;
- the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

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- All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

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## Abstract

The anaerobic oxidation of ammonium (ANAMMOX) process has been suggested as an economical and innovative means of removing nitrogen from wastewater. Nevertheless, very few studies have evaluated the effect of the chemical oxygen demand (COD) to nitrogen (N) ratio on bacterial communities in an ANAMMOX-mediated system. Heterotrophic bacteria can readily outcompete the slow-growing ANAMMOX bacteria in the presence of organic carbon. This study examined the effect of the organic carbon to nitrogen (C/N) ratio on the performance of ANAMMOX in an upflow sludge blanket reactor using synthetic wastewater as the feedstock. Two UASB reactors (UASB-A and UASB-B) were seeded with biomass from a lab-scale ANAMMOX reactor and operated for a period of 593 days. Both reactors were operated using similar operational conditions during the enrichment phase (0-400 days). Thereafter, the addition of organic carbon in the medium altered the C/N ratio of one of the reactors (UASB-B). During this period, UASB-A served as a control reactor. A CN ratio of 1.0, 1.5, and 2.0 was achieved in the UASB B reactor by increasing the organic carbon concentration every 60 days. The reactors were analyzed at three-day intervals per week for nitrogen and COD removal efficiency. The quantitative PCR method was used to detect the dominant N-removing organisms within both reactors at different phases. In addition, cDNA quantification or reverse transcriptase qPCR (RT-qPCR) was also conducted to determine the dominant and active nitrifying communities.

The results indicated that when the C/N ratio is 1.0, almost complete removal of  $\text{NH}_4^+\text{-N}$  is observed (92%), and nitrogen removal efficiency (NRE) is approximately 82%. The ratios of  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios during this phase (C/N=1) fluctuated from >1.25 to <1.6 and from >0.35 to <0.45 <0.11 to >1.6, respectively, which was within the range of the expected ANAMMOX stoichiometric ratio. In addition, when the C/N ratio was increased from

1 to 1.5, NRE rose from 82 to 88%. However, a decrease of NRE to 83% was observed when the C/N ratio was further increased to 2. The quantitative PCR results showed an increase in total bacteria from  $1.4 \times 10^6$  copies/ $\mu\text{L}$  to  $2.3 \times 10^6$  copies/ $\mu\text{L}$ , and  $2.4 \times 10^6$  copies/ $\mu\text{L}$  as the ratio of C/N increased from 1.0 to 1.5 and thereafter to 2, respectively. ANAMMOX bacteria showed an increase from  $16 \times 10^3$  copies/ $\mu\text{L}$  to  $6.5 \times 10^4$  copies/ $\mu\text{L}$ , and  $2.06 \times 10^5$  copies/ $\mu\text{L}$  when the C/N ratio was increased from 1 to 1.5, and 2, respectively. The cDNA analysis further showed an increase of ANAMMOX bacteria transcript abundance from  $4.6 \times 10^4$  copies/ $\mu\text{L}$  to  $2.52 \times 10^6$  copies/ $\mu\text{L}$  with an increase in C/N ratio to 1.5. Subsequently, a decrease in ANAMMOX bacteria transcript abundance to  $1.09 \times 10^6$  copies/ $\mu\text{L}$  was observed when the C/N ratio was further increased to 2. The expression of the *hzo* gene encoding for hydrazine dehydrogenase (HDH), which catalyses the oxidization of the unique ANAMMOX intermediate hydrazine to  $\text{N}_2$  was 169 folds of expression, which was very high at C/N=1, but showed a decrease to 39 folds expression at C/N=1.5. Almost complete inhibition of *hzo* gene was observed when the C/N ratio was further increased to 2. Based on chemical analysis, it was further confirmed that the decrease of both ANAMMOX and AOB abundance at a higher C/N ratio caused an increase in effluent  $\text{NH}_4^+$ -N concentrations. In conclusion, the study has shown that a higher C/N ratio could significantly affect the overall nitrogen removal rate and the activity of the diverse microbial populations, more specifically the ANAMMOX bacterial activity.

## **Dedication**

*To my late grandmother, Nomusa Mfomude Mhlongo  
who loved us and worked hard to provide for us when we had no parents but could not live to  
witness this achievement. Love you granny.*

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## List of Abbreviations

ANAMMOX	Anaerobic ammonium oxidation
AOB	Ammonia oxidizing bacteria
BNR	Biological nitrogen removal
bp	Nucleic acid base pairs
C	Carbon
CANON	Completely autotrophic nitrogen- removal over nitrite
cDNA	Complementary deoxyribonucleic acid
COD	Carbon oxygen demand
CO <sub>2</sub>	Carbon dioxide
COMAMMOX	Complete ammonia oxidizing
CSTR	Continuous stirred tank reactor
ddH <sub>2</sub> O	Double-distilled deionised water
DEAMOX	Denitrifying Ammonia Oxidation reactor configuration
DEMON	Deammonification reactor configuration
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
FA	Free ammonia
FISH	Fluorescence <i>in situ</i> hybridisation
FNA	Free nitrous acid
GHGs	Greenhouse gases

HRT	Hydraulic retention time
MBR	Membrane bioreactor
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
N	Nitrogen
N <sub>2</sub>	Nitrogen gas
N <sub>2</sub> O	Nitrous oxide
NH <sub>3</sub>	Ammonia
NH <sub>4</sub> <sup>+</sup>	Ammonium ion
NLR	Nitrogen loading rate
NO	Nitric oxide
NO <sub>x</sub>	Generic nitrogen oxides
NO <sub>2</sub> <sup>-</sup>	Nitrite ion
NO <sub>3</sub> <sup>-</sup>	Nitrate ion
NOB	Nitrite oxidizing bacteria
NRR	Nitrogen removal rate
NRE	Nitrogen removal efficiency
O <sub>2</sub>	Oxygen gas
OLAND	Oxygen limited autotrophic nitrification and denitrification
PCR	Polymerase chain reaction
pH	Potential of hydrogen ion
PN	Partial nitrification
PO <sub>4</sub> <sup>-</sup>	Orthophosphate ion
PVP	Polyvinyl pyrrolidone

qPCR	Quantitative polymerase reaction
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
SHARON	Single reactor high activity ammonium removal over nitrite
SNAD	Simultaneous nitrification, ANAMMOX, denitrification
SO <sub>4</sub> <sup>2-</sup>	Sulphates
UASB	Up-flow anaerobic sludge bed
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

## **Thesis structure**

The thesis is divided into five chapters: introduction (Chapter 1), literature review (Chapter 2), technical Chapter 1 (Chapter 3, which includes the first objective of the study), technical Chapter 2 (Chapter 4, which covers objective 2, 3 and 4 of the study). The methodology, results, discussion, and conclusions for each technical chapter are presented in their respective sections. Finally, the last chapter includes a general conclusion and recommendations, followed by a list of references.

## **Preface**

- 1. Msimango, S. S., Nasr, M., Bux, F., Kumari, S. K. S. 2022.** Impact of chemical oxygen demand to nitrogen ratio on bacterial communities in the ANAMMOX-mediated system. Paper presented at South African Society of Microbiology (SASM) Conference, 4th – 6th May 2021 (Oral presentation).
- 2. Msimango, S. S., Nasr, M., Bux, F., Kumari, S. K. S. 2022.** Impact of chemical oxygen demand to nitrogen ratio on bacterial communities in the ANAMMOX-mediated system. (Paper In preparation)

## CHAPTER 1: INTRODUCTION

The elimination of nitrogen compounds from wastewater is essential to prevent the eutrophication of receiving freshwater bodies (Zhu *et al.*, 2008). An activated sludge process is a commonly used wastewater treatment method to remove organic compounds and nutrients from wastewater. Conventional nitrogen removal processes, such as nitrification and denitrification, are employed within wastewater treatment systems as biologically mediated processes for nitrogen removal (Rahimi *et al.*, 2020).

The nitrification process involves the oxidation of ammonium ( $\text{NH}_4^+$ ) to nitrate ( $\text{NO}_3^-$ ) by a series of reactions. This can be either carried out in a single step by complete ammonia oxidizing (COMAMMOX) bacteria (Fowler *et al.*, 2018), or performed in a two-step reaction by the mutualistic activities of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Rahimi *et al.*, 2020). The COMAMMOX bacteria oxidize  $\text{NH}_4^+$  directly to  $\text{NO}_3^-$  in single step reaction using oxygen as an electron acceptor (Annavaajhala *et al.*, 2018). The carbon source consumed through this process is inorganic carbon (Pinto *et al.*, 2016). On the other hand, in a two-step nitrification process, the first step is performed by AOB by oxidizing  $\text{NH}_4^+$  to  $\text{NO}_2^-$  and secondly, NOB oxidize  $\text{NO}_2^-$  to  $\text{NO}_3^-$  under strict aerobic conditions. The oxidized nitrogen species such as  $\text{NO}_2^-$  and  $\text{NO}_3^-$  are then reduced to gaseous nitrogen by a heterotrophic denitrification process under anaerobic conditions, consuming organic matter as an energy source and  $\text{NO}_3^-$  or  $\text{NO}_2^-$  as electron acceptors (Rahimi *et al.*, 2020). However, the conventional nitrogen removal process is often limited by its efficiency, cost of operation and high sludge production (van der Star *et al.*, 2007). This has demanded the invention of novel microbial processes, such as the anaerobic ammonium oxidation (ANAMMOX) process, for cost effective nitrogen removal from wastewater (Zhu *et al.*, 2008).

ANAMMOX was discovered in the 1980s, and it has been regarded as a promising method for autotrophic nitrogen removal. The process involves the oxidation of ammonium to  $N_2$  gas in a single step reaction with nitrite as the electron acceptor. The technology caught the attention of wastewater treatment practitioners worldwide due to its advantages over conventional nitrification processes, such as; 1) no need for external carbon source addition, 2) lower oxygen demand (up to 60% less than conventional process) and 3) decreased sludge production (Hu *et al.*, 2013b). The ANAMMOX bacteria responsible for this process are particularly found in the deep-branching planctomycetes group (Zhu *et al.*, 2008). They are detected from both natural systems and wastewater treatment plants (Zhang *et al.*, 2008, Hu *et al.*, 2013b). Compared to the conventional nitrification-denitrification process for nitrogen removal, ANAMMOX is regarded as a more sustainable and economical alternative, especially for wastewater treatment with a low C/N ratio (He *et al.*, 2018). However, ANAMMOX bacteria with slow growth rate are vulnerable to external environmental factors such as temperature, pH, dissolved oxygen (DO), salinity, substrate concentration, etc. For example, DO concentrations above 0.32 mg/L could inhibit ANAMMOX bacteria. The ANAMMOX bacteria has been reported to be inhibited by the coexistence of organic matter with  $NH_4^+$  in wastewater. This inhibition of ANAMMOX process by the presence of organic matter has been a considerable concern regarding its application at the full-scale level (He *et al.*, 2018). This has caused limitations on ANAMMOX application on high C/N ratio wastewater, such as for mainstream applications where organic matter is the main composition.

It has been reported that the presence of a low concentration of organic matter in ANAMMOX systems could play a vital role in the improvement of nitrogen removal efficiency (NRE), by promoting cooperation between the ANAMMOX and denitrification processes (Sheng *et al.*, 2018, Wang *et al.*, 2019b). However, at a high C/N ratio, ANAMMOX bacteria have been shown to be inhibited by heterotrophic denitrifying bacteria (Sheng *et al.*, 2018). Elevated

organic carbon concentrations in an ANAMMOX system would cause an extreme propagation of heterotrophic bacteria, which would cause a competition for electron acceptors with ANAMMOX bacteria, thus inhibiting the activities of slow growing ANAMMOX (Molinuevo *et al.*, 2009). In a study conducted by Chamchoi *et al.* (2008), “ANAMMOX bacteria were exceeded by heterotrophic denitrification bacteria when the C/N ratio was increased above 2 with a COD value of 400 mg/L”. In the literature, however, there is no general agreement on the exact range of the C/N ratio and the organic matter concentration that inhibits the ANAMMOX process. This might be because of the variation in operating conditions, enrichment of ANAMMOX, combined processes, type of reactors, etc.

The ANAMMOX bacteria, have a long doubling time and slow metabolism, which present a big challenge during ANAMMOX enrichment as it prolongs the start-up of reactors (van der Star *et al.*, 2008). It has been reported that the ANAMMOX bacteria can be successfully cultivated by selective bacterial enrichment strategies using customised bioreactor systems (Banihani *et al.*, 2012b). Similarly, ANAMMOX bacterial activity is influenced by factors such as pH (Anjali and Sabumon, 2014) and temperature (Jin *et al.*, 2012). Therefore, maintaining optimum pH and temperature within the reactor is critical for ANAMMOX activity. Similarly, the co-existence and interactions of ammonium oxidizers, denitrifiers and nitrifiers have been important in wastewater treatment plants for efficient nitrogen removal (Li *et al.*, 2018a).

In addition, the reactor configuration could influence the start-up duration of ANAMMOX process under different C/N ratios through the provision of conducive operating conditions and retention of biomass within the reactors. For this purpose, different types of reactors have been designed to address the challenges associated with ANAMMOX bacterial enrichment and cultivation (Ibrahim *et al.*, 2015). The up-flow anaerobic sludge blanket (UASB) reactor is considered optimum in treating high-strength organic wastewaters because of its characteristic features such as high biomass concentration, rich microbial diversity and simplicity of

operation (Ibrahim *et al.*, 2015). However, not many studies have been conducted to assess the impact of COD/N ratios on ANAMMOX process performance and other associated nitrogen-converting microorganisms and their activity using UASB reactors. Thus, this study aimed to evaluate the effects of the chemical oxygen demand to nitrogen (C/N) ratio on ANAMMOX and associated nitrifying bacterial groups using lab-scale UASB reactors.

## **1.1. Aim and Objectives**

### **1.1.1. Aim**

The aim of this study is to investigate the effects of different chemical oxygen demand to nitrogen ratios on the activities of ANAMMOX and nitrifying bacteria in an upflow sludge blanket reactor.

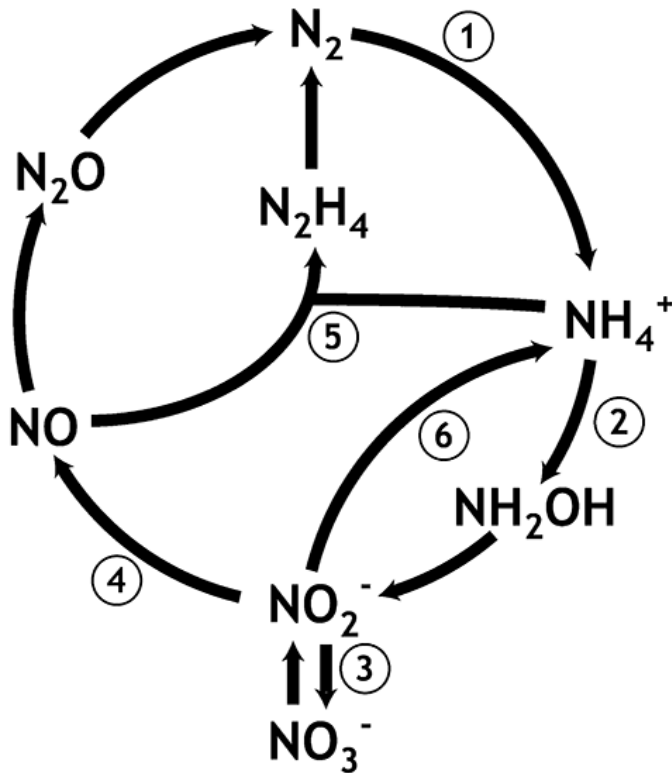
### **1.1.2. Objectives**

- i. To enrich ANAMMOX bacteria using a upflow sludge blanket reactor .
- ii. To determine the effect of different C/N ratio (1.0, 1.5 and 2.0) on the performance of the ANAMMOX-mediated system.
- iii. To evaluate the impact of C/N ratio on the dominance of nitrifying community using quantitative polymerase chain reaction (qPCR).
- iv. To assess the impact of C/N ratio on the activity of ANAMMOX and nitrifying bacteria using reverse transcriptase PCR (RT PCR).

## CHAPTER 2: LITERATURE REVIEW

### 2.1. Nitrogen removal

As a result of industrialization and anthropogenic activities, nitrogenous compounds have become more prevalent in the environment, posing substantial challenges to natural ecosystems. Therefore, the presence and removal of additional ammonia from wastewater have become a worldwide concern (Zhu *et al.*, 2008). Biological wastewater treatment methods have been reported to be more efficient and effective in removing nitrogen compounds than physical and chemical methods (Wiszniewski *et al.*, 2006). As a result, numerous processes and biological technologies have been developed for nitrogen removal from wastewater based on the metabolism of specific microbial populations in the nitrogen cycle. Figure 1 Indicates different biological processes in the nitrogen cycle, including the recently discovered anaerobic oxidation process (Jetten, 2008, Zhu *et al.*, 2008).

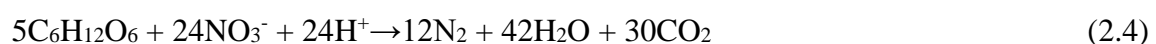


**Figure 1.1:** Microbial nitrogen cycle. (1) DiN<sub>2</sub> fixation; (2) aerobic ammonium oxidation by bacteria and archaea; (3) aerobic nitrite oxidation; (4) denitrification; (5) anaerobic ammonium oxidation; and (6) dissimilatory nitrate and nitrite reduction to ammonium (Jetten, 2008).

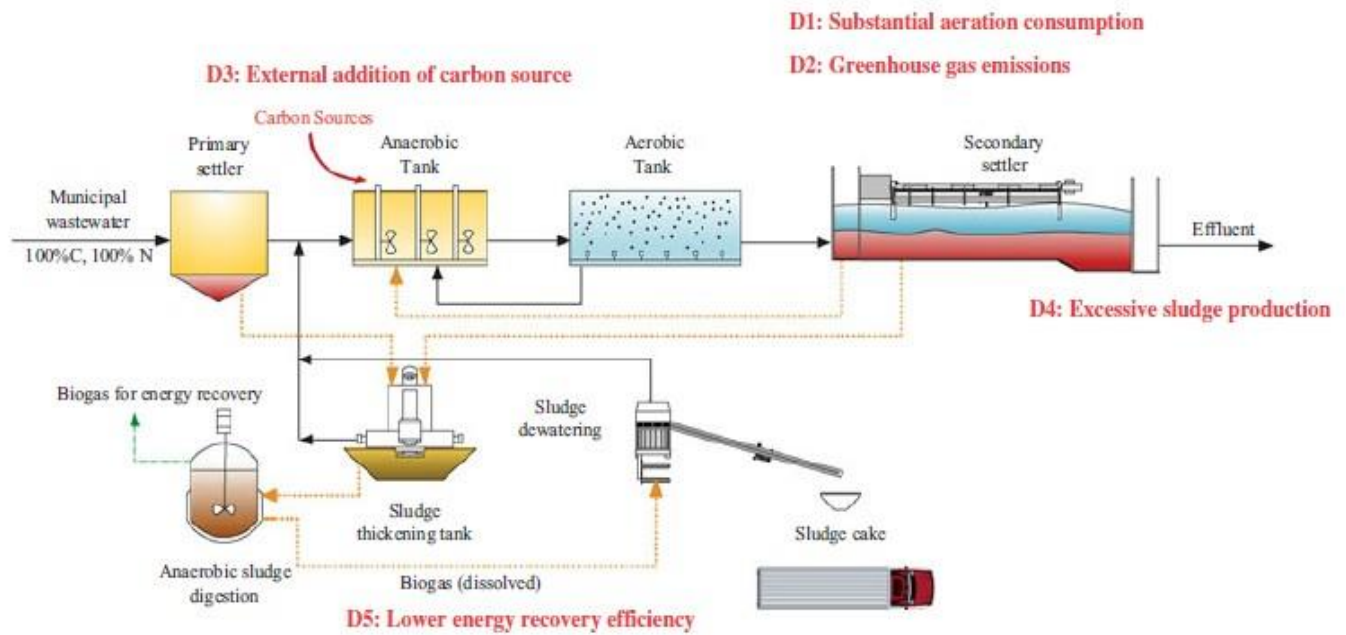
### 2.1.1. Biological nitrogen removal

Conventional nitrogen removal in any biologically mediated system can be attributed to two co-dependant and interlinked processes: nitrification and denitrification (Wang *et al.*, 2016). These processes occur synergistically in natural and engineered ecosystems, each mediated by specific and different microbial populations (Rahimi *et al.*, 2020). The initial step of nitrification involves AOB which is responsible for the oxidation of ammonium (NH<sub>4</sub><sup>+</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>) followed by the NOB which oxidizes nitrite (NO<sub>2</sub><sup>-</sup>) to nitrate (NO<sub>3</sub><sup>-</sup>). The nitrate thus formed is then converted into dinitrogen gas through denitrification by a group of heterotrophic bacteria under anoxic conditions (Mao *et al.*, 2020). The following step is achieved with the addition of an external electron donor or with the residual biological oxygen demand (BOD) as the electron donor (Mao *et al.*, 2020).

*Nitrosomonas*, *Nitrospira*, *Nitrosovibrio*, *Nitrosoglobus* and *Nitrosococcus*, are the known AOBs and *Nitrobacter*, *Nitrospira* and *Nitrococcus* spp are the known NOBs that are commonly reported (Bock *et al.*, 1995, Burrell *et al.*, 1998). The anoxic denitrification process is performed by heterotrophic microorganisms, which include *Pseudomonas*, *Alcaligenes* and *Paracoccus* etc., that use NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> as final electron acceptors (Ciudad *et al.*, 2005).



However, some limitations have been highlighted in these conventional nitrogen removal systems, as shown in Figure 2.2. The first limitation is substantial aeration consumption. Conventional biological technologies are costly and energy-intensive, as extensive aeration is required to oxidize the nitrogenous compounds (Liu *et al.*, 2019). More than 60 % of the power consumption in WWTPs is typically accounted for by aeration alone (Ji, 2022). The emission of greenhouse gas is a second limitation. Notable amounts of N<sub>2</sub>O gas have been reported in wastewater treatment processes (Lu *et al.*, 2018b). It has been reported that the WWTP contribute approximately 3% N<sub>2</sub>O emission worldwide (Law *et al.*, 2012), which can have a global warming potential estimated to 300 times greater than CO<sub>2</sub> over a 100-year horizon (Liu *et al.*, 2019). The third restriction is the requirement for external carbon sources for denitrification. Heterotrophic denitrification is the final step of nitrogen removal which is completed in the anoxic reactor tank and the presence of decomposable organic carbon determines the performance (Liu *et al.*, 2019). An inadequate C/N ratio in municipal wastewater results in unacceptable effluent quality (Yang *et al.*, 2007), and the operation costs increase because the external carbon addition in the tank will be required. The fourth limitation is the excessive sludge production in WWTPs because of the high yields of sludge of heterotrophic processes (Bandosz and Block, 2006).



**Figure 2.2:** Schematic diagram for the conventional wastewater treatment based on nitrification/denitrification processes and five major limitations have been highlighted (Liu *et al.*, 2019)

## 2.1.2. Novel biological processes for nitrogen removal

### 2.1.2.1. Complete ammonia oxidation (COMAMMOX)

Aerobic nitrification was considered to be a two-step process involving nitrification and denitrification until the discovery of the complete ammonia oxidizer (COMAMMOX) process (Daims *et al.*, 2015). The discovery of COMAMMOX organisms, i.e., the bacterial group that can oxidize  $\text{NH}_4^+$  to  $\text{NO}_3^-$  in a single-step reaction has brought a new dimension to the conventional nitrification process. Comammox bacteria has the entire gene sets essential for  $\text{NH}_4^+$  oxidation, i.e., hydroxylamine dehydrogenase (also known as hydroxylamine oxidoreductase) (hao) and ammonia monooxygenase (amoA, amoB, amoC) (Pinto *et al.*, 2016). Although molecular, physiological, and other studies of COMAMMOX bacteria properties are continuing, their affinity for  $\text{NH}_4^+$  has been reported to be greater than that of most  $\text{NH}_4^+$  oxidizers in some other studies (Kits *et al.*, 2017). Moreover, the biomass yield of

COMAMMOX bacteria, such as *Candidatus Nitrospira inopinata* was reported to be higher than that of incomplete ammonia oxidizers (AOB) (Kits *et al.*, 2017). To date, the detection of COMAMMOX bacteria in different biological nitrogen removal (BNR) systems has shown a vital role played by COMAMMOX bacteria in nitrogen conversion (Cotto *et al.*, 2020, Annavajhala *et al.*, 2018).

All COMAMMOX bacteria identified thus far belong to the genus *Nitrospira* of the class *Nitrospira*, which also includes canonical nitrite-oxidizing bacteria and have been discovered in a range of engineered and natural environments (Van Kessel *et al.*, 2015, Daims *et al.*, 2015, Palomo *et al.*, 2016, Pinto *et al.*, 2016), except the marine environments (Fowler *et al.*, 2018). The disadvantage of comammox bacteria is that they have restricted to representatives in lineage II of the *Nitrospira*. Compared to the single step of the canonical nitrifier, the composite reactions of the complete nitrifier could have a competitive advantage to outcompete the canonical “incomplete nitrifiers” such as AOB (Eq. 5) and NOB (Eq. 6) due to the higher energy yield of complete nitrification (Eq. 7) (Daims *et al.*, 2015, Van Kessel *et al.*, 2015).



### **2.1.2.2. Anaerobic ammonia oxidation (ANAMMOX)**

ANAMMOX process involves the oxidation of ammonium to  $\text{N}_2$  by anaerobic ammonium oxidizing (ANAMMOX) bacteria using nitrite as an electron acceptor in anaerobic conditions (Cao *et al.*, 2017, Van de Graaf *et al.*, 1996, Strous *et al.*, 1998). The treatment of domestic wastewater using ANAMMOX process has been reported to have the potential to meet effluent

standards while achieving net energy creation (Maktabifard *et al.*, 2018). The researchers have been focusing on linking ANAMMOX process to other treatment technologies to develop more sustainable domestic wastewater treatment. For ANAMMOX process to occur, a share of ammonium is needed to be oxidized to nitrite, followed by the production of N<sub>2</sub> by ANAMMOX bacteria using the produced nitrite together with the residual ammonium (Zhu *et al.*, 2008). Presently, ANAMMOX process has been reported as a cost-effective alternative to the traditional BNR because there is no requirement for external organic carbon source addition for denitrification; it reduces alkalinity for nitrification and demands oxygen supply reduction by 60% (Rahimi *et al.*, 2020). Furthermore, added advantages are offered, such as the reduction unwanted by-products such as greenhouse gases (GHGs) and decreasing biomass yields, resulting in an important reduction in operational costs (Kartal *et al.*, 2013). Consequently, ANAMMOX process has also been regarded as a sustainable and environmentally friendly process for nitrogen removal from wastewater (Magri *et al.*, 2013).

## **2.2. The history of the anaerobic ammonia oxidation process**

The existence of the ANAMMOX was predicted over four decades ago by Broda (1977) who first postulated the ANAMMOX process as one of the various microbial conversions “missing” in nature (Tal *et al.*, 2006). He assumed that this type of microbial conversion method could exist because the conversion of ammonium and nitrite is a reaction with a large negative free energy. The first experimental observation was made by Mulder (1992) while studying the autotrophic denitrification process whereby sulphide is used as the electron donor (Van Loosdrecht *et al.*, 2004). The initial assumption he made was nitrate as the preferred electron acceptor. The culturing of reproducible ANAMMOX cultures in lab-scale reactors became possible only when it was realised that nitrite is the actual electron acceptor (Van Loosdrecht *et al.*, 2004). From reported estimation, approximately 50% of all N<sub>2</sub> released into the atmosphere is accounted for by the ANAMMOX bacteria (Kartal *et al.*, 2008). The

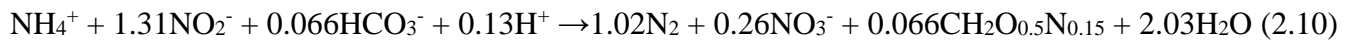
ANAMMOX mechanism involved is well-characterised and the catabolic route has been established (Khin and Annachhatre, 2004). The overall ANAMMOX reaction consists of the production of N<sub>2</sub> from the oxidation of ammonia, using nitrite as the electron acceptor as shown in (Eq. (8)).



Agreeing with the van de Graaf *et al.* (1996) report, the key product during ANAMMOX process was N<sub>2</sub>, but roughly 10% of the N in the influent (NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) was converted to NO<sub>3</sub><sup>-</sup>. The ratio of NH<sub>4</sub><sup>+</sup> conversion to NO<sub>2</sub><sup>-</sup> conversion of 1:1.31 ± 0.06, and the ratio of NO<sub>2</sub><sup>-</sup> conversion to NO<sub>3</sub><sup>-</sup> production were 1:0.22 ± 0.02 was observed (Van de Graaf *et al.*, 1996), and the complete nitrogen balance is shown in Eq. 9.



According to the estimations by Strous *et al.* (1998), ANAMMOX stoichiometry based on mass balance over ANAMMOX enrichment cultures is presented in Eq. (10)

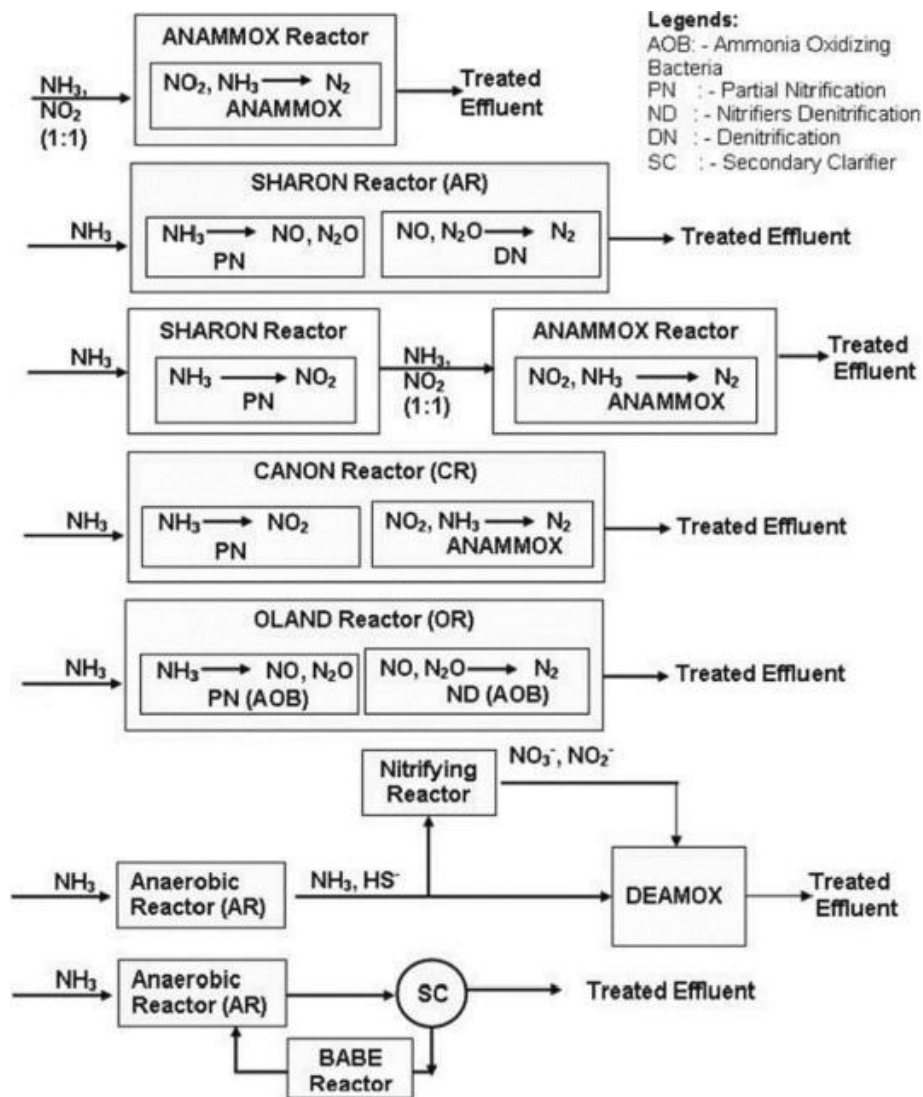


However, the ANAMMOX process poses some limitations, such as the slow growth rate and sensitivity of the ANAMMOX bacteria that mediate this process. This characterizes a major blockage to be overcome before successful widespread full-scale application of this process (Zhang *et al.*, 2022). Also, there are nitrite limitations in the influent wastewater when applying the ANAMMOX process for domestic wastewater as well as high COD-containing wastewaters that necessitate combining ANAMMOX with other technologies (Li *et al.*, 2021b).

### 2.2.1. Combined processes

Previous studies have reported that the ANAMMOX process can be combined with other processes depending on the specific case. The first successful combined processes developed

for commercial applications were the two-stage SHARON process as well as the single-stage CANON process (Figure 2.3). In SHARON process, partial nitrification (PN) can be used to provide  $\text{NH}_4^+/\text{NO}_2^-$  with a ratio near 1 for the ANAMMOX (Ren *et al.*, 2022). CANON process is a second combination which refers to the association of ANAMMOX processes and PN (CANON-ANAMMOX) in one reactor (Wang *et al.*, 2019a). Compared to SHARON-ANAMMOX, a savings of 63 % oxygen has been reported in the CANON process and 100 % organic carbon consumption and investment costs (Ren *et al.*, 2022). There is no need to regulate pH and COD in SHARON-ANAMMOX and CANON compared with traditional nitrification and denitrification processes; they both have low sludge yield features. Third combination is the DEAMOX (DENitrifying Ammonium OXidation) process that requires sulfide as an electron donor for the transformation of nitrate to nitrogen gas (Dehestaniathar *et al.*, 2021). DEAMOX was proposed by Mulder (2007), which combines the autotrophic denitrification process with ANAMMOX process using sulphide as an electron donor for converting nitrate to nitrite within an anaerobic biofilm (Kalyuzhnyi *et al.*, 2007). This process has been reported to be suitable for sulfurization of wastewater containing nitrogen pollutants (Ren *et al.*, 2022).



**Figure 2.3:** Schematic Figure displaying microbial principles in some of the new biological nitrogen removal processes that can be combined with ANAMMOX process (Ren *et al.*, 2022).

More effectiveness, flexibility, and stability were reported in the two-stage system than in the one-stage process. This is because nitrification and ANAMMOX processes can be optimised and controlled separately (Hu *et al.*, 2013b). For example, the inhibitory consequence of O<sub>2</sub> on the performance of ANAMMOX bacteria can be relieved (Khin and Annachhatre, 2004). The development of novel and innovative processes has sustained non-stop, besides the systems that have been discussed previously. For example, the reported possible co-existence of nitrite-dependent anaerobic methane oxidation bacteria (n-damo) and ANAMMOX may enable the

use of these two communities to wastewater containing considerable quantities of both ammonium and dissolved methane (van Kessel *et al.*, 2018). It has been reported as an important pathways in the microbial nitrogen cycle (Nie *et al.*, 2020). Table 2.1 presents the reaction equations involved in these advanced BNR processes.

**Table 2.1:** Summary of innovative BNR processes.

<i>Process</i>	<i>Reaction equation</i>
<i>SHARON-ANAMMOX</i>	Nitritation: $2\text{NH}_4^+ + 1.5\text{O}_2 + 2\text{HCO}_3^- \rightarrow \text{NH}_4^+ + \text{NO}_2^- + 2\text{CO}_2 + 3\text{H}_2\text{O}$ ANAMMOX: $\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$ Total: $2\text{NH}_4^+ + 1.5\text{O}_2 + 2\text{HCO}_3^- \rightarrow \text{N}_2 + 2\text{CO}_2 + 5\text{H}_2\text{O}$
<i>CANON</i>	$\text{NH}_4^+ + 0.85\text{O}_2 \rightarrow 0.13\text{NO}_3^- + 0.435\text{N}_2 + 1.4\text{H}^+ + 1.3\text{H}_2\text{O}$
<i>OLAND</i>	$2\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{N}_2 + 2\text{H}^+ + 3\text{H}_2\text{O}$
<i>n-damo</i>	$3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}$
<i>DEAMOX</i>	$\text{NO}_3^- + 0.25\text{HS}^- \rightarrow \text{NO}_2^- + 0.25\text{SO}_4^{2-} + 0.25\text{H}^+$

### 2.2.2. ANAMMOX bacteria

ANAMMOX bacteria belong to the Phylum Planctomycetes, order “*Candidatus Brocadiales*”, and family “*Candidatus Brocadiaceae*” (Manasa and Mehta, 2021, Ibrahim *et al.*, 2015). Sequence analysis and phenotypic studies were used to separate the ANAMMOX bacteria from other prokaryotes (Fuerst, 2013). ANAMMOX bacteria resembles a less than 1  $\mu\text{m}$  diameter coccus shape (An *et al.*, 2013). They have a doubling time of 10 to 30 days and are physiologically different from the other known Planctomycetes in that they are anaerobic chemolithoautotrophs capable of transforming  $\text{NH}_4^+$  to  $\text{N}_2$ , using  $\text{NO}_2^-$  as the electron acceptor (Khin and Annachhatre, 2004).

**Table 2.2:** The taxonomy of ANAMMOX bacteria and its sources (Ibrahim *et al.*, 2015, Manasa and Mehta, 2021)

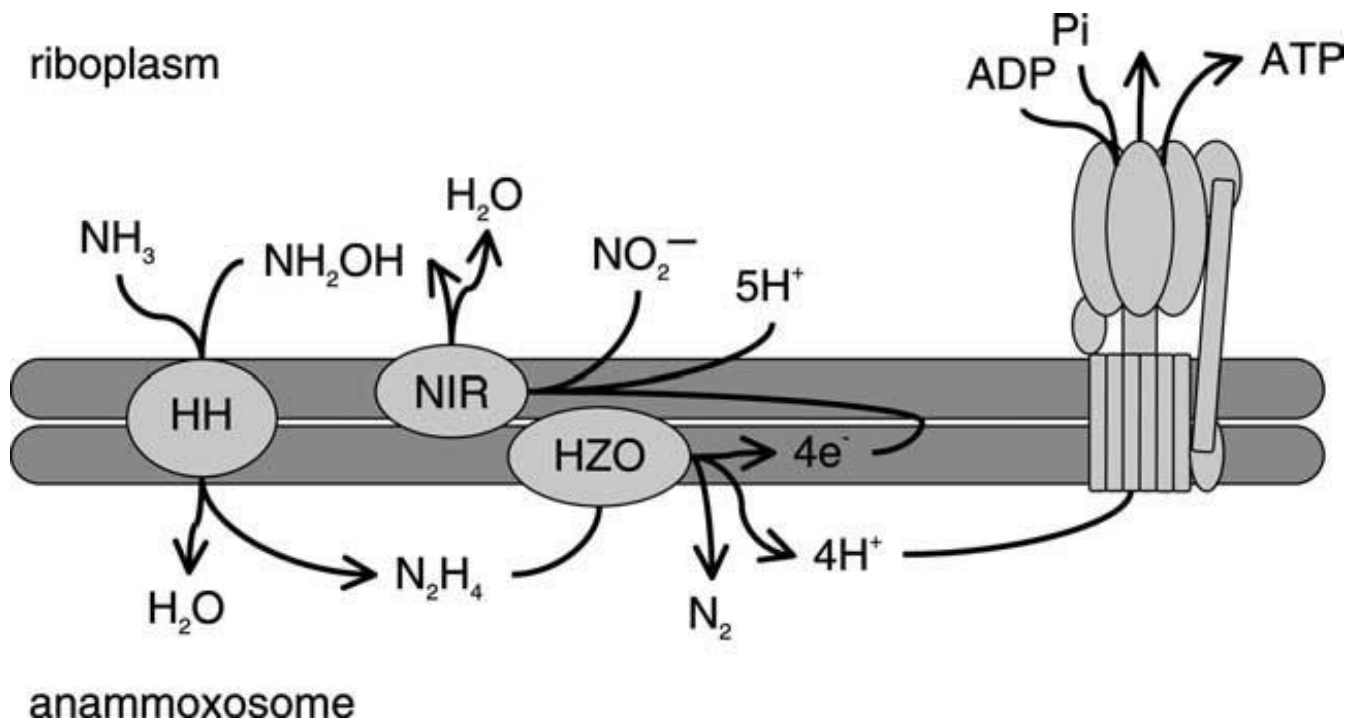
<i>GENUS</i>	<i>SPECIES</i>	<i>SOURCE</i>
<i>Brocadia</i>	' <i>Candidatus</i> Brocadia ANAMMOXidans'	Wastewater
	' <i>Candidatus</i> Brocadia sinica'	Wastewater
	' <i>Candidatus</i> Brocadia fulgida'	Wastewater
<i>Kuenenia</i>	' <i>Candidatus</i> Kuenenia stuttgartiensis'	Wastewater
	' <i>Candidatus</i> Jettenia asiatica'	Wastewater
<i>Jettenia</i>	' <i>Candidatus</i> Jettenia caeni'	Wastewater
	' <i>Candidatus</i> ANAMMOXoglobus propionicus'	Wastewater
<i>Scalindua</i>	' <i>Candidatus</i> Scalindua brodae'	Wastewater and marine
	' <i>Candidatus</i> Scalindua wagneri'	Wastewater and marine
	' <i>Candidatus</i> Scalindua sorokinii'	Sea water
	' <i>Candidatus</i> Scalindua marina'	Marine sediments
	' <i>Candidatus</i> Scalindua profunda'	Marine
	' <i>Candidatus</i> Scalindua arabica'	Marine (Arabian sea)
	' <i>Candidatus</i> ANAMMOXomicrobium moscowii'	Wastewater sludge

Until now, there are 14 different ANAMMOX species divided over six genera that have been enriched and identified (Table 2.2) (Manasa and Mehta, 2021). It has been very difficult to perform isolation of these genera as pure cultures due to their slow specific growth rates; therefore, they all have been given the taxonomical status of ‘Candidatus’ (Kartal *et al.*, 2012). The presence of a specialised organelle called ANAMMOXosome is a common and unique characteristic of ANAMMOX bacteria. This organelle is enclosed by a specific lipid that contains a remarkable enzyme system (Peng *et al.*, 2022a, Kartal *et al.*, 2013).

#### **2.2.2.1. The unique cell structure of ANAMMOX bacteria**

There is no peptidoglycan in the cell wall of ANAMMOX bacteria, like other members of the Phylum Planctomycetes (van Teeseling *et al.*, 2015). According to recent studies, the ANAMMOX bacteria, *Kuenenia Stutgartiensis* has been redefined as a Gram-negative bacterium based on the discovery and characterisation of a thin peptidoglycan layer contained within the cell envelope (Fuerst, 2013). There are three cytoplasmic compartments dividing the cytoplasm separated by single bilayer membranes. “Unique lipids are contained in the ANAMMOXosome membrane with sequential structures of four-membered aliphatic cyclobutane rings arranged like a ‘staircase’ at the end of the hydrocarbon chains. These unique lipids are called ‘ladderane’ lipids. They form an exceptionally dense membrane that likely offers a tight wall against diffusion (Ratray *et al.*, 2008). A toxic and mutagenic compound, hydrazine, is made by the enzyme hydrazine hydrolase as an intermediate product in the ANAMMOX reaction., The ladderane lipid membrane would shelter the genetic material and the remainder of the ANAMMOX cell from exposure by trapping the hydrazine inside the ANAMMOXosome, because the reaction is suggested to be confined to the ANAMMOXosome (Ratray *et al.*, 2008, van Niftrik *et al.*, 2008). The ANAMMOXosome is a big intracytoplasmic compartment bounded by a single bilayer ladderane membrane containing lipid present in the ANAMMOX bacteria that perform anaerobic ammonium

oxidation (ANAMMOX)” (Kuenen, 2008, van Niftrik *et al.*, 2008, Rattray *et al.*, 2008). This specific organelle is the site of ANAMMOX process (Figure 2.4).



**Figure 2.4:** Postulated anaerobic ammonium oxidation coupled to the ANAMMOXosome membrane in ANAMMOX bacteria resulting in a proton motive force and subsequent ATP synthesis via membrane bound ATPases (van Niftrik *et al.*, 2004).

### 2.2.3. ANAMMOX bacterial cultivation

ANAMMOX bacteria can be successfully cultivated by selective bacterial enrichment strategies using modified bioreactor systems (Banihani *et al.*, 2012b). Since the first report, ANAMMOX-based processes for wastewater treatment have changed, and the first full-scale ANAMMOX reactor was started in Rotterdam by scaling-up directly from laboratory-scale. Partially nitrated sludge liquor from an adjusted nitrification process was fed into the ANAMMOX reactor (van der Star *et al.*, 2007). According to the previous studies, the ANAMMOX bacteria has been reported to have a long doubling time and slow metabolism which present a big challenge during ANAMMOX enrichment as it prolongs the start-up of

reactors (van der Star *et al.*, 2007, Banihani *et al.*, 2012b, Kartal *et al.*, 2012, Hu *et al.*, 2013a, Hu *et al.*, 2013b). The doubling time of these microorganisms is approximately 10–30 days under optimal conditions (Kartal *et al.*, 2012), although there are strategies that can be performed to enhance a better start-up time of the reactor. For example, modifying the cultivation conditions to promote biofilms or the formation of aggregates such as flocs and granules can promote fast ANAMMOX enrichment (van der Star *et al.*, 2007). Although granular biomass is often accompanied by flocculent biomass, ANAMMOX bacteria are dominant in the granules, while the AOB and NOB populations are significantly higher in flocs than in the granules (Wang *et al.*, 2017, Adams *et al.*, 2020). Therefore, ANAMMOX bacteria demand a well-designed bioreactor with a highly effective biomass-retaining ability (Rahimi *et al.*, 2020). Additionally, a high specific surface area in ANAMMOX reactors is necessary for a good ANAMMOX reaction to occur. Previously, ANAMMOX bacteria have been successfully cultivated in both suspended and attached growth reactors (Oshiki *et al.*, 2013).

#### **2.2.4. Co-existence and interactions of nitrifying, denitrifying and ammonium oxidizing bacteria in wastewater**

ANAMMOX reactors may contain a range of other microbial communities besides having ANAMMOX bacteria, making them biologically complex habitats (Pereira *et al.*, 2017). The investigation of different microbial populations in ANAMMOX reactors has been done in lab-scale reactors, and there are inadequate studies of microbial communities in full-scale reactors (Pereira *et al.*, 2017).

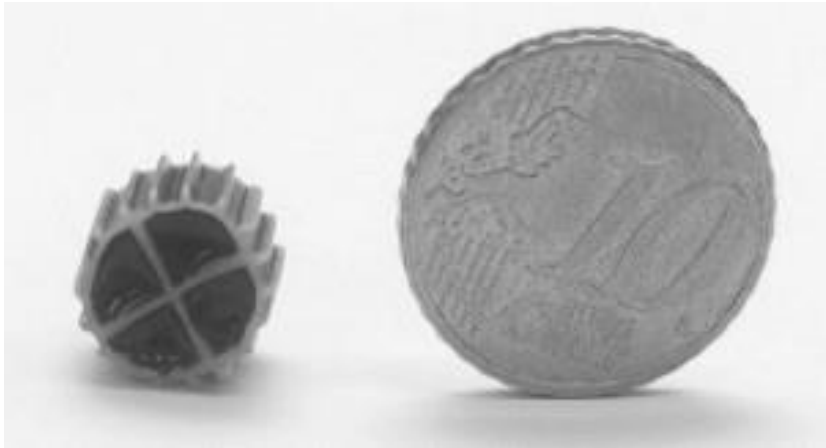
##### **2.2.4.1. Interactions of ANAMMOX and nitrifying bacteria in suspended-growth ANAMMOX-mediated systems**

The niche separation within the ANAMMOX reactors could be driven by the affinity for substrate between different bacterial groups, with r-strategists (bacteria with high maximum

specific growth rates) being outcompeted by K-strategists (bacteria with high affinity for substrate) at low-substrate concentration systems, while the r-strategists outcompete the K-strategists in high-substrate concentration systems. *Nitrospira* spp., *Nitrosospira* spp. and Candidatus *Kuenenia* spp. are regarded as K-strategists while *Nitrosomonas* spp., *Nitrobacter* spp. and Candidatus *Brocadia* spp. are regarded as r-strategists (Park *et al.*, 2015). The key factors that have been demonstrated to influence the diversity of different microbial communities in ANAMMOX-mediated systems are operating conditions (Park *et al.*, 2015, Gonzalez-Martinez *et al.*, 2016). The inoculum was reported to have an influence on the diversity of microbial communities in the ANAMMOX-mediated system (Agrawal *et al.*, 2017). Candidatus *Brocadia* spp. have been reported to thrive easily in ANAMMOX-mediated systems treating wastewaters containing organic carbon, as they had been linked with COD consumption, giving them an additional advantage over other species (Winkler *et al.*, 2012). The high ammonia concentrations in side stream ANAMMOX-mediated systems could result in the dominance of *Nitrosomonas* spp. Though the dominance of *Nitrosococcus*-like bacteria in ANAMMOX-mediated systems has not been reported in previous studies, their growth was reported at pH below 6 (Fumasoli *et al.*, 2017). The prevailing DO concentrations could drive niche segregation between *Nitrospira* and *Nitrobacter* related species, with *Nitrobacter* expected to dominate at DO concentrations above one mg/L whilst *Nitrospira* could dominate at DO concentrations below 0.5 mg/L as *Nitrobacter* have lower affinity for oxygen than *Nitrospira*. The regulation of the duration of the settling phases to maintain an optimal fraction of flocculent biomass in the SBRs is extensively believed as a regulation of the growth of NOB in the reactors through washout” (Bowden *et al.*, 2015, Val del Río *et al.*, 2016).

#### **2.2.4.2. Interactions of ANAMMOX and nitrifying bacteria in attached-growth ANAMMOX-mediated systems**

Similarities in bacterial communities have been reported in comparative studies of attached-growth and granular (Guo *et al.*, 2016, Bhattacharjee *et al.*, 2017). However, *Nitrospira* spp., can adapt well to biofilm conditions (Park *et al.*, 2015). In the study conducted by Park *et al.* (2015), the ratio of *Nitrospira* spp. in biofilms to suspension increased with the reduction in HRT, whereas that of *Nitrobacter* spp. remained largely unaffected. This occurred because of high affinities for oxygen (Park *et al.*, 2015). *Nitrospira* spp. and ANAMMOX bacteria have been reported to have an evolutionary connection to and share similar forms of the enzyme nitrite oxidoreductase (*nxr*) and other proteins involved in metabolic activities (Lucker *et al.*, 2010). On the other hand, *Nitrobacter* spp. has a lower biomass yield than *Nitrospira* spp., not only has a lower affinity for both oxygen and nitrite (Park *et al.*, 2017). In natural ecosystems, a common form of existence of microorganisms in biofilms. These biofilms provide a shield from external stress factors to microorganisms in the communities (Jin *et al.*, 2012). In multi-species biofilms, the results of the interactions between microbial populations show up more than the phenotypic features of each population in the biofilm. These interactions include competition, commensalism and proto-cooperation (Nozhevnikova *et al.*, 2015, Wang *et al.*, 2022c). By enhancing biofilm formation, biomass can be preserved, promoting good process stability. Several biofilm strategies exist for bioreactors, among them granules, rotating biological contactors, trickling filters, and moving bed biofilm bioreactors (MBBRs) (Bertino, 2011, Zhang *et al.*, 2016). In MBBRs, small plastic carriers are applied in the bioreactor, which could provide a sheltered area where the biofilm can propagate (Figure 2.5) (Suarez, 2017, Adams *et al.*, 2020).



**Figure 2.5:** K1 carrier (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden) with a PNA biofilm, and a 10 euro cent coin is shown for size comparison (Suarez, 2017).

Biofilms formed in MBBR reactors are formed on carrier particles that provide protected surfaces where ammonia oxidizing bacteria (AOB) and ANAMMOX bacteria coexist (Xiao *et al.*, 2009). On the biofilms, AOB occupies the layers closer to the biofilm-water interface, whereas ANAMMOX bacteria occupy deeper layers where they are protected from inhibition (Nozhevnikova *et al.*, 2015). AOB utilize oxygen under aerobic conditions for the oxidation of ammonium to nitrite (Xiao *et al.*, 2009). This generates nitrite for the ANAMMOX bacteria and protects the ANAMMOX bacteria in the process from the inhibitory effects of the oxygen, while AOB benefits from nitrite removal as it could inhibit their activities (Nozhevnikova *et al.*, 2015). Competitive and synergistic interactions between ANAMMOX bacteria and their companion bacteria are thus controlled by ecological factors such as dissolved oxygen (DO), substrate ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) and organic matter (Talan *et al.*, 2021). AOB activity is influenced by the corresponding free ammonia (FA) concentration (Jin *et al.*, 2012), because FA, rather than  $\text{NH}_4^+$ , acts both as substrate and inhibitor for AOB. NOB and ANAMMOX bacteria are inhibited by free  $\text{NH}_3$  at different concentrations. The co-existence and interactions of ammonium oxidizers, denitrifiers and nitrifiers has been important in wastewater treatment plants for nitrogen removal. Understanding the underlying microbial mechanisms and

interactions of these microbial populations is important in overcoming the challenges faced during application in wastewater treatment plants by coordinating the activities of the different organisms involved (Hubaux *et al.*, 2015). For instance, while on the one hand, sufficient oxygen needs to be supplied for aerobic ammonium oxidizing bacteria (AOB), on the other hand, excess oxygen supply promotes the growth of nitrite oxidizing bacteria (NOB) and inhibits the growth of the ANAMMOX bacteria (Xie *et al.*, 2017).

#### **2.2.4.3. Co-existence of ANAMMOX and denitrifying bacteria**

In the environments with high  $\text{NO}_3^-$ , ANAMMOX process depends on other processes for  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  and the matter in the existence of ANAMMOX bacteria with denitrifiers is the availability of  $\text{NO}_2^-$  (Kumar and Lin, 2010). Denitrifiers can reduce  $\text{NO}_3^-$  or it can be performed by other species encouraging the dissimilatory reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  (DNRA). This occurs under anoxic conditions where  $\text{NO}_2^-$  is released as a free intermediate (Kumar and Lin, 2010). Then,  $\text{NO}_2^-$  can be taken up by ANAMMOX bacteria as an electron acceptor during the oxidation of  $\text{NH}_4^+$  (Eq. 8). The most serious factor that will determine the direction of a dissimilative pathway to either DNRA or denitrification in wastewater is the C/N ratio (Krishna Mohan *et al.*, 2016). At high organic matter concentration (high C/N ratio), there will be a reduction of nitrate to ammonia because of excessive reducing power (Wang *et al.*, 2020b). The connection of denitrification and ANAMMOX will be positive only when there is no competition for  $\text{NO}_2^-$  between these two processes. The preferable way is partial denitrification (Cho *et al.*, 2019). There will be a potential for  $\text{NO}_2^-$  accumulation because the first two steps in the denitrification pathway are uncoupled. Therefore, the optimum additions of inhibitors such as  $\text{N}_2\text{H}_4$ , allylthiourea and acetylene could be beneficial to control ANAMMOX and denitrification at lab-scale investigations but are not possible to be applied at a field-scale (Jensen *et al.*, 2007).

### **2.3. Bioreactors for ANAMMOX enrichment**

Reactor configuration influences the start-up duration of ANAMMOX reactors through the provision of conducive operating conditions and retention of biomass within the reactors. For this purpose, different types of reactors have been designed to address the challenges associated with ANAMMOX bacterial cultivation (Ibrahim *et al.*, 2015)

#### **2.3.1. Suspended-type sequencing batch reactor (SBR)**

The SBRs are the most preferred reactors for ANAMMOX bacterial cultivation due to their simplicity and flexibility. The motion of agitators in SBR promotes the homogenous distribution of biomass and substrate within the reactor, thus avoiding the negative effect of high nitrite concentrations. However, since ANAMMOX bacteria can be inhibited by their own substrates, batch mode operation may not be able to provide the appropriate conditions for long-term cultivation of the ANAMMOX bacteria (Gilbert *et al.*, 2013).

#### **2.3.2. Continuously stirred tank reactor (CSTR)**

The CSTR is equipped with a stirrer that promotes continuous, homogenous distribution of biomass and substrate, with the only difference with SBR being the operations, are continuous. The CSTR offers a much longer hydraulic retention time (HRT), a continuous source of the fresh substrate, preventing starvation of bacteria, and toxic metabolic by-products are simultaneously removed. However, Biomass retention is a challenge with CSTR, especially since ANAMMOX bacteria grow slowly (Ding *et al.*, 2018).

#### **2.3.3. Up-flow anaerobic sludge blanket (UASB) reactor**

The UASB is a system used to treat wastewater by application of an anaerobic microorganism to the suspended growth-type system in which feed is supplied from the bottom of the reactor and the effluent is removed at the top. UASB is designed with suitable separation of liquid, solids and gas to retain granular sludge (Ibrahim *et al.*, 2015). Ideally, the UASB reactor uses

shear forces and flow rates to encourage the formation of compact granules (van der Star *et al.*, 2007). It has been reported as a reliable system and is among the most stable and effective setups for culturing ANAMMOX. UASB provides high NRE and outstanding biomass retention (Peng *et al.*, 2022b, Ni *et al.*, 2011, Ibrahim *et al.*, 2015). The upflow reactor configuration is the most reliable system that is capable of good sludge settling and high NRE. According to Ibrahim *et al.* (2015), “the range of most usually applied temperature for the UASB functioning system is ranging from 32 to 36°C”. Though, a slightly lower temperature of 25°C was used during the successful enrichment of ANAMMOX bacteria within 4.5 months with in a UASB (Banihani *et al.*, 2012a).

#### **2.3.4. Membrane bioreactor (MBR)**

Another type of suspended bioreactor configuration used for wastewater treatment is an MBR, predominantly when it is combined with a membrane process (Ibrahim *et al.*, 2015). Microfiltration is performed using a membrane material resistant to microbial cells during the membrane process to efficiently retain biomass (Ibrahim *et al.*, 2015). Biomass retention in a membrane bioreactor (MBR) is not based on settling biomass like in SBR but on the permeability of membrane modules to bacterial cells. The effluent is withdrawn through a membrane module which is impermeable for microbial cells, thereby effectively retaining all the bacterial biomass in the reactor. The MBR, therefore, allows the cultivation of slow-growing bacteria with full biomass retention but without a selection on settling ability (Mao *et al.*, 2020). The application of MBRs has been regarded as an alternative technique to get full biomass holding during ANAMMOX enhancement (Trigo *et al.*, 2006).

#### **2.3.5. Moving bed biofilm reactor (MBBR)**

The MBBR is a system that uses low-density (about 950 kg/m<sup>3</sup>) carrier particles on which bacterial biofilms form (Mao *et al.*, 2020), that can be moved around using agitator(s).

Although the system promotes excellent biomass retention, the lack of optimal mixing and limited mass transfer to biofilms present unprecedented challenges. Nevertheless, many full-scale MBBR systems have been implemented to date (di Biase *et al.*, 2019).

### **2.3.6. Gas-lift bioreactor**

A gas-lift bioreactor is a type of suspended biofilm system that applies an artificial airlift approach such as gas bubbles or compressed air for either liquid elevating, biomass blending, maintaining the biomass fluidisation state or anaerobic condition at some point during the reactor operation. This reactor can uphold a very decent transfer of gas to liquid capability as well as suitable working surroundings for the conservation and culture of ANAMMOX bacteria (Ibrahim *et al.*, 2015, Kilonzo and Margaritis, 2004).

### **2.3.7. Fluidised bed reactor (FBR)**

The fluidised bed reactor is an attached growth-type bioreactor that uses a fluidisation procedure during which the biomass is passed through a granular material supported by a porous plate (distributor). The fluidisation process can be achieved by using enough velocity to suspend the bed, allowing it to act as a fluid. Biomass particles can be maintained in the fluidised state by controlling the velocity and this, therefore, promotes better mass transfer characteristics. FBR enhances the formation of ANAMMOX granules, thereby reducing the washout of suspended biomass during the operation (Ibrahim *et al.*, 2015).

## **2.4. The factors influencing ANAMMOX enrichment in bioreactors**

### **2.4.1. Temperature**

Optimum ANAMMOX activity has been reported at temperatures ranging from 35 to 40°C (Ibrahim *et al.*, 2015). Low temperatures ( $\leq 10^\circ\text{C}$ ) reportedly to reduce its growth rates (Ibrahim *et al.*, 2015), and high temperatures above 40 °C cause cell lysis (Tomaszewski *et*

*al.*, 2017). The temperature could affect the normal function of ANAMMOX bacteria, in turn influencing the reaction rate kinetics (Talan *et al.*, 2021). Therefore, strategies such as heating coils or water jackets have been used to maintain temperatures within the optimal range in ANAMMOX systems. The temperature has been reported to influence the detachment of ammonium and nitrite to free ammonia (FA) and free nitrous acid (FNA), respectively, which are toxic to ANAMMOX bacteria (Dosta *et al.*, 2008). The tolerance, however, depends on the species of ANAMMOX bacteria (Zhang and Okabe, 2020). The influence of temperature as a critical parameter in wastewater treatment has been extensively studied and recorded (Dosta *et al.*, 2008). Insufficient research has been done to determine the reasons for the high low-temperature activity of ANAMMOX bacteria.

#### **2.4.2. pH**

High ANAMMOX activity occurs at pH values between 6.5 and 8.8 (Ibrahim *et al.*, 2015). Therefore, if the pH is too high, the metabolic activity of ANAMMOX bacteria is suppressed. It is thus desirable to maintain the pH within this range for stable performance of the ANAMMOX process (Anjali and Sabumon, 2014). To achieve this, automatic pH controllers are normally incorporated into ANAMMOX systems. Similar to temperature, pH influences the dissociation of ammonium and nitrite to free ammonia and free nitrous acid, respectively, which are toxic to ANAMMOX bacteria (Dosta *et al.*, 2008). Since pH is also an important control parameter during the operation of the ANAMMOX bioreactor, extensive research has been conducted on the effect of pH on treatment performance. In an ANAMMOX reactor, the pH of the effluent increases significantly when the reactor has a high nitrogen loading rate (NLR) and nitrogen removal rate (NRR) (Hendrickx *et al.*, 2012). In the ANAMMOX reaction process,  $H^+$  is consumed when ANAMMOX bacteria utilise nitrite as the electronic acceptor to oxidize ammonia. This consumption of acidity results in an increase in the pH and is

considered the main reason for the variations in the pH in the ANAMMOX process (Tomaszewski *et al.*, 2017).

#### **2.4.3. Dissolved Oxygen (DO)**

ANAMMOX bacteria are obligate anaerobes therefore, they are very sensitive to oxygen. If oxygen is not completely removed from the reactor, the growth of ANAMMOX bacteria will be inhibited. In anoxic reactors, ANAMMOX bacteria dominate since the anaerobic conditions inhibit the oxygen-dependent AOB and other aerobic nitrogen-consuming bacteria (Ibrahim *et al.*, 2015).

#### **2.4.4. Substrate concentration**

The primary substrate of ANAMMOX bacteria is ammonium ( $\text{NH}_4^+$ ) which is oxidized in the presence of nitrite ( $\text{NO}_2^-$ ) to  $\text{N}_2$  (Peng *et al.*, 2022b). The reported ANAMMOX stoichiometric ratio of nitrite to ammonium is 1: 1,32 (Pereira *et al.*, 2021). The nitrogen in the influent to ANAMMOX reactors should thus be supplied at 1-part  $\text{NH}_4^+$ : 1.32 parts  $\text{NO}_2^-$ . However, high concentrations of ammonium and nitrite could lead to the inhibition of ANAMMOX bacterial activities (Lotti *et al.*, 2012). According to previous studies, the treatment performance of ANAMMOX is enhanced by increasing the substrate concentration of ammonia, nitrite, and  $\text{HCO}_3^-$  (Wang *et al.*, 2022b). Nevertheless, there is a potential serious inhibition of the ANAMMOX process at an overload of substrates. Free ammonia (FA) has been shown to be the factual inhibition factor in the ANAMMOX process (Jin *et al.*, 2012).

#### **2.4.5. Salinity**

The effect of high salinity is a result of high osmotic pressure, and the microorganism is reported to die or cause plasmolysis and dormancy at high salinity concentrations (Cho *et al.*, 2019). Consequently, osmotic pressure in concentrated wastewater could strictly cause inhibition of bacterial growth. The ANAMMOX process has been reported to be promising in

the treatment of wastewater with elevated salinity concentrations, even though anaerobic biological treatment processes are recognised to be inhibited by brininess (Zhang and Okabe, 2020). The reason for this is that the ANAMMOX pathway was generally found in anoxic marine environments (Engström *et al.*, 2005). Therefore, it is essential to consider the impact of salinity as a potential inhibitory factor when performing the ANAMMOX process in wastewater that contains high salts. Salinity has been reported to promote ANAMMOX granulation and increase bacteria retention in the reactor at certain concentrations, but still, the ANAMMOX is inhibited at high salinity concentrations (Cho *et al.*, 2019).

#### **2.4.6. Heavy metals**

Some types of nitrogen-rich wastewater, such as landfill leachate (Kimura and Isaka, 2014), have been reported to often contain high levels of heavy metal ions. The accumulation of heavy metals in organisms can cause biological accumulation toxicity because heavy metals are not easily biodegradable (Gutwiński *et al.*, 2021). Not much research has been conducted to understand the ANAMMOX inhibition by heavy metals in the removal of nitrogen from heavy metals-containing wastewater. Some studies reported that the ANAMMOX activity was fully inhibited by one mmol/L HgCl<sub>2</sub> (Jin *et al.*, 2012). Although there are limited studies on heavy-metal ANAMMOX inhibition, further studies should be conducted.

#### **2.4.7. Organic matter, C/N ratio**

Municipal and domestic wastewater has been reported to be a very complex mixture of dissolved and suspended materials. Most municipal and domestic wastewater contains large amounts of dissolved organic matter after secondary microbial treatment (Perez-Garcia *et al.*, 2011). The organic matter (OM) dissolved in municipal sewage contains a mixture of recalcitrant compounds such as pharmaceutical compounds, endocrine disrupting compounds, personal care products, disinfection by-products, and metabolites which can be released into the water bodies through discharge (Vimala *et al.*, 2020). ANAMMOX bacteria are

chemoautotrophic microorganisms that have been reported to use  $\text{CO}_2$  or  $\text{HCO}_3^-$  as the only carbon source (He *et al.*, 2015). Therefore, the concentration of bicarbonate has been reported to be essential in the influent of the ANAMMOX process for the cultivation of ANAMMOX bacteria. The addition of inorganic carbon has been reported to promote the growth or enhance the activity of ANAMMOX bacteria (Jin *et al.*, 2012). ANAMMOX bacteria do not require organic carbon to carry out their metabolic activities, and the presence of organic carbon could affect their growth. Although there have been demonstrations that anaerobic ammonia removal is possible in the presence of organic matter on account of the versatility of the ANAMMOX bacteria metabolism, by an enzymatic anoxic oxidation mechanism (Ma and Wang, 2018, Sabumon, 2007).

It is generally believed that organic matter is causing some adverse effects on ANAMMOX bacteria (Ni *et al.*, 2012). Some of the organic carbon compounds, such as alcohols, are toxic to ANAMMOX bacteria even at low concentrations (Jin *et al.*, 2012). Organic carbon could also lead to the proliferation of heterotrophic bacteria, resulting in competition with ANAMMOX bacteria for nitrite and afterwards out-compete the ANAMMOX bacteria (Jin *et al.*, 2012). Heterotrophic bacteria compete with ANAMMOX and AOB for  $\text{NO}_2^-$  and oxygen in the reactors (Li *et al.*, 2018a).

It could be possible that ANAMMOX bacteria first reduce  $\text{NO}_2^-$  to  $\text{NH}_4^+$  and then subsequently oxidize  $\text{NH}_4^+$  to  $\text{N}_2$  through the normal pathway or use the denitrification pathway (Kumar and Lin, 2010). Güven *et al.* (2005) reported that approximately 50% of the organic carbon is oxidized to  $\text{CO}_2$  while less than 10% is incorporated into the cell biomass. In a different study, it was reported that an enhancement in  $\text{N}_2$  production in an ANAMMOX-mediated reactor at 10 mM acetate concentration while at 25 mM and 50 mM acetate concentrations, there was 22% and 70% inhibition of ANAMMOX activities, respectively (Chamchoi *et al.*, 2008).

Denitrification is possible through autotrophic and/or heterotrophic routes, and that organic matter may result in inhibition of the ANAMMOX process (Xie *et al.*, 2017).

Most of the wastewater contain both organic carbon and nitrogen (Vimala *et al.*, 2020); therefore, a better understanding of the inhibition mechanism in ANAMMOX due to the organic matter would benefit the applications of the ANAMMOX process in nitrogen removal from different wastewater type.

# **CHAPTER 3:**

## **ENRICHMENT OF THE ANAMMOX USING AN UP-FLOW SLUDGE BLANKET REACTOR**

### **3.1. Introduction**

ANAMMOX normally takes months to years to start up because ANAMMOX bacteria grow slowly with doubling times reported as 10 to 12 days (Ibrahim *et al.*, 2015, Adams *et al.*, 2020). The slow growth rate of ANAMMOX has been associated with lower production of sludge, but it also causes complications during start-up and contributes to the longer enrichment period. Other main difficulties contributing to the extended start-up of the ANAMMOX process are (i) unsuitable seed sludge selection (Qian *et al.*, 2021), (ii) substrate inhibition and toxicity (You *et al.*, 2020) and (iii) biomass washout during reactor operation (Peng *et al.*, 2022b). Hence, many studies have concentrated on developing methods for ANAMMOX bacteria enrichment with a shorter start-up period (Ibrahim *et al.*, 2015, Peng *et al.*, 2022b).

The reactor configuration affects the start-up time of ANAMMOX reactors by providing conducive operating conditions and retaining biomass within the reactor (Adams *et al.*, 2020). For this purpose, different types of biological reactors have been designed to address the challenges associated with ANAMMOX bacterial cultivation (Ibrahim *et al.*, 2015). This includes both biofilm-based reactors as well as suspended growth reactors (Vandekerckhove *et al.*, 2020). In the biofilm-based reactors, carrier particles are added to the moving liquid medium to which the different groups of bacteria attach (Tian *et al.*, 2020), whereas the suspended particles largely rely on granular sludge formation (Hubaux *et al.*, 2015). Both these growth modes are imperative to overcome the challenges of biomass wash out from the

reactors. A diversity of different configurations of reactors have been established for the ANAMMOX process start-up. This includes types such as an up-flow anaerobic sludge blanket (UASB) (Izadi *et al.*, 2022), continuous stirred tank reactor (CSTR) (Díaz *et al.*, 2020), sequencing batch reactor (SBR) (Zhang *et al.*, 2021), and membrane bioreactor (MBR) (Singh *et al.*, 2022), all show advantages and disadvantages. The UASB reactor has been reported to be among the most effective and stable setups for culturing ANAMMOX (Ibrahim *et al.*, 2015). The UASB has also been reported as a reliable system that provides good sludge settling competence and a high NRE production. Therefore, the up-flow configuration is considered in treating high strength organic wastewaters because of its high biomass concentration, rich microbial diversity and simplicity of operation. It is possible to promote the growth of a desirable microorganism in the enrichment bioreactor while inhibiting the proliferation of competitors by strictly controlling the bioreactor conditions.

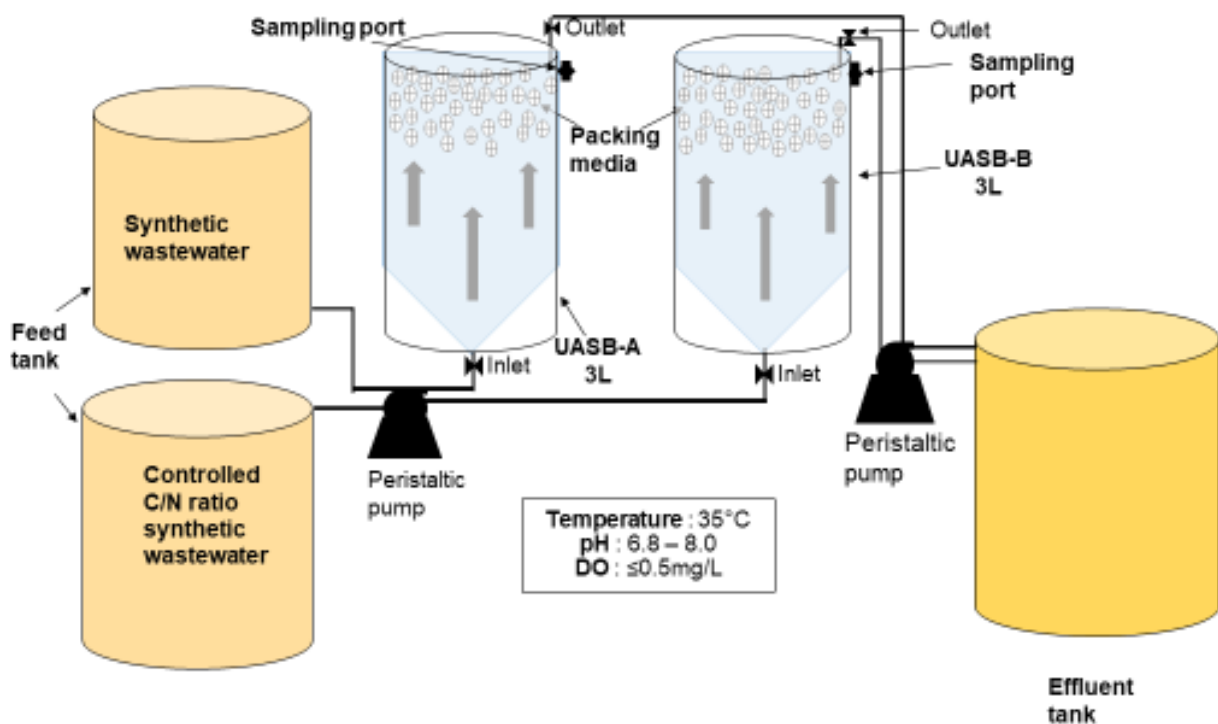
Operational conditions such as temperatures, pH, dissolved oxygen (DO) and other influent compositions have shown to influence ANAMMOX enrichment process(Cho *et al.*, 2019). It is therefore essential to optimize the reactor configuration, seed inoculum and operating conditions during the start-up of the ANAMMOX reactors (Choi *et al.*, 2019). Thus, it could be essential to characterize the bacterial communities present in biological systems in order to describe the process performance in ANAMMOX-mediated systems. This chapter focused on start-up and operation of a laboratory scale UASB reactor for the enrichment of ANAMMOX bacteria using synthetic wastewater.

## **3.2. Materials and method**

### **3.2.1. Reactor construction and operational conditions**

Two 5 L UASB reactors (UASB-A and UASB-B) were constructed using Perspex cylinders and Perspex sheets as shown in the schematic diagram (Figure 6). Both the reactors were

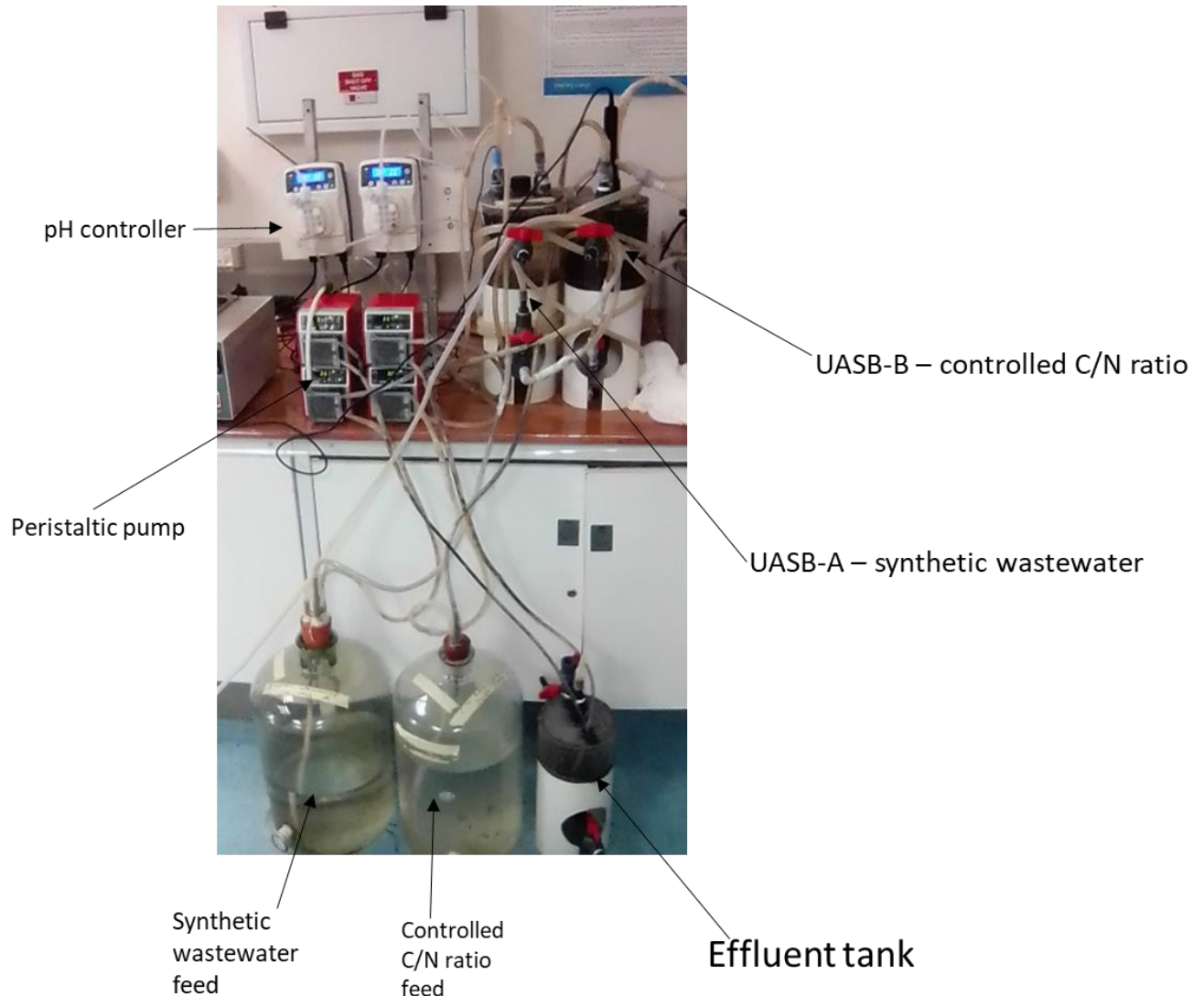
operated at an operating capacity of 3 L (Diameter 10 cm and height 40 cm). Polyvinyl chloride (PVC) pipes and connectors were used to join different pieces of the reactors together. PVC ball valves were used in all the effluent lines and influent lines. Silicone tubes (4 mm internal diameter) were used in the effluent lines to interconnect the reactors to the effluent tank as well as in the feed lines to interconnect the feed tank and the reactors.



**Figure 3.1:** Schematic diagram of UASB-A and UASB-B

Each reactor consisted of a port situated at the bottom to feed wastewater from a holding tank via a peristaltic pump (Watson-Marlow, UK). This feeding method is used to avoid dead zone formation in the reactors. Another port is located at the top of the reactor to discharge the treated effluent using a PVC tube. Reactors were equipped with an external water bath to maintain the temperature at  $35 \pm 1$  °C. Polyethylene K1-type carrier materials (Azacore, South Africa) were added, 100 particles in each reactor during inoculation as a packing media (Figure

3.1). Each of the carrier materials contained 25 equally spaced fins that were 1.5 mm long and 0.1 mm thick. The carrier materials were 14 mm long and 20 mm in diameter. The inner sections of the carrier materials were subdivided into 8 chambers by 0.2 mm thick crosses.



**Figure 3.2:** Photographic image of UASB-A and UASB-B

### 3.2.2. Synthetic feed and composition

Both reactors (UASB-A and UASB-B) were enriched using synthetic ANAMMOX media (Vander Graaf, 1997). During the enrichment period, the media composition and operational conditions was kept constant in both reactors. The composition of the ANAMMOX growth media and trace elements solution is outlined in Table 3.1 and Table 3.2, respectively, as originally described by van de Graaf *et al.* (1997). The  $\text{NH}_4^+\text{-N}$  (added as  $(\text{NH}_4)_2\text{SO}_4$ ) and the

$\text{NO}_2^-$ -N (added as  $\text{NaNO}_2$ ) concentrations were fixed throughout the course of the enrichment, keeping to a relative  $\text{NH}_4/\text{NO}_2$  ratio of ~1:1.32

**Table 3.1:** Media composition for ANAMMOX bacteria

<b>Compound</b>	<b>Concentration (g/L)</b>
$(\text{NH}_4)_2\text{SO}_4$	0.060
$\text{NaNO}_2$	0.0792
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.300
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.180
$\text{NaHCO}_3$	1
$\text{K}_2\text{HPO}_4$	0.0136
$\text{KH}_2\text{PO}_4$	0.0136

A mixture of trace elements was further added to the medium to promote the growth of the ANAMMOX bacteria. Trace Element Solution 1 comprised an iron solution, while Trace Element Solution 2 contained a mixture of other physiologically relevant metals. Both solutions were made bioavailable through chelation with EDTA, and were each added to the media at a concentration of 5 mg/L

**Table 3.2:** Trace elements for ANAMMOX media

<b>Compound</b>	<b>Concentration (g/L)</b>
<b>TRACE I</b>	
<b>EDTA</b>	0.005
<b>FeSO<sub>4</sub></b>	0.005
<b>TRACE II</b>	

<b>EDTA</b>	0.015
<b>ZnSO<sub>4</sub>.7H<sub>2</sub>O</b>	0.00043
<b>CoCl<sub>2</sub>.H<sub>2</sub>O</b>	0.00024
<b>MnCl<sub>2</sub>.4H<sub>2</sub>O</b>	0.00099
<b>H<sub>3</sub>BO<sub>4</sub></b>	0.000014
<b>CuSO<sub>4</sub>.5H<sub>2</sub>O</b>	0.00025
<b>NaMoO<sub>4</sub>.2H<sub>2</sub>O</b>	0.00022
<b>NiCl<sub>2</sub>.6H<sub>2</sub>O</b>	0.00019
<b>NaSeO<sub>4</sub>.10H<sub>2</sub>O</b>	0.00021
<b>NaWO<sub>4</sub>.2H<sub>2</sub>O</b>	0.00005

The pH of the reactors was maintained between 6.8 and 7.5 and the DO was kept below 0.5 mg-O<sub>2</sub>/L by sparging argon/CO<sub>2</sub> mixture for 10 to 15 minutes during the feeding stage. Hydraulic retention time (HRT) was maintained at 0.42 throughout the study and was calculated using equation 3.1.

$$\text{HRT} = \frac{\text{Reactor Volume (L)}}{(\text{Volume Decanted per Cycle (L)}) \times (\text{Cycles per day})} \quad (3.1)$$

### 3.2.3. Inoculum preparation

The seed inoculum was obtained from an ongoing lab scale ANAMMOX reactor with approximately 2.5 g/L mixed liquor-suspended solids (MLSS) and 1.8 g/L mixed liquid volatile solids (MLVSS). A total biomass volume of approximately 50 mL was used to inoculate each of the two 3 L UASB-A and UASB-B reactors. The biomass was treated with a phosphate buffer (0.14 g/L KH<sub>2</sub>PO<sub>4</sub> and 0.75 g/L K<sub>2</sub>HPO<sub>4</sub>) before inoculation (Gasa *et al.*, 2019).

### 3.2.4. Biomass determination

MLSS and MLVSS were measured in duplicate according to the standard methods with slight modifications (Azimi *et al.*, 2019). For the MLSS measurement, 50 mL of the stock sample was added to a cleaned, pre-weighed ceramic crucible. These crucibles were heated at 120 °C for 5 h, after which they were placed in a desiccator containing silica gel to cool. The crucibles were then weighed by an analytical balance (Mettler-Toledo International Inc., USA) to determine the dry biomass. To calculate the MLVSS, these crucibles were subsequently incinerated at 550 °C for 15 min (equation 3.2). The crucibles were cooled in a desiccator containing silica gel and weighed on an analytical balance. The difference in grams between the empty crucible and post heating at 120 °C was recorded as MLSS (equation 3.3), while the difference between the crucible and post heating at 550 °C was recorded as MLVSS.

$$\text{MLVSS} = \text{Mass (Crucible+Biomass)} - \text{Mass (Crucible after incineration at 550}^\circ\text{C)} \quad (3.2)$$

$$\text{MLSS} = \text{Mass (Crucible+Biomass)} - \text{Mass (Crucible)} \quad (3.3)$$

### 3.2.5. Analytical procedure

All samples were consistently obtained directly from the reactor and effluent every 3 days a week using a 60 mL syringe, filtered through a 0.45 µm cellulose acetate syringe filter (Merck Millipore, USA), and immediately analysed.

#### 3.2.5.1. Nitrogen

The transformation of Nitrogen species is an indirect indicator of the metabolism of ANAMMOX bacteria, nitrifying bacteria and denitrifying bacteria. Nitrogen in the forms of Ammonia ( $\text{NH}_4^+\text{-N}$ ), Nitrate ( $\text{NO}_3^-\text{-N}$ ), and Nitrite ( $\text{NO}_2^-\text{-N}$ ) were measured spectrophotometrically using the Gallery Autoanalyser (ThermoScientific, USA), according to the protocols outlined in Standard Methods (Azimi *et al.*, 2019). The Gallery was calibrated

with a linear calibration curve for the  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$ , with an  $R^2$  value of  $0.998 \pm 0.003$  and  $0.999 \pm 0.002$ , respectively. Bacterial activities were monitored in the three reactors based on the stoichiometric ratios of  $\text{NO}_2^-\text{-N}$  consumed to  $\text{NH}_4^+\text{-N}$  consumed ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) (equation 3.4) and  $\text{NO}_3^-\text{-N}$  produced to  $\text{NH}_4^+\text{-N}$  consumed ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) (equation 3.5). The NRE was calculated according to equations 3.6

$$\Delta\text{NO}_2^-/\Delta\text{NH}_4^+ = \frac{((\text{Influent NO}_2^-) - (\text{Effluent NO}_2^-))}{((\text{Influent NH}_4^+) - (\text{Effluent NH}_4^+))} \quad (3.4)$$

$$\Delta\text{NO}_3^-/\Delta\text{NH}_4^+ = \frac{(\text{Effluent NO}_3^-)}{((\text{Influent NH}_4^+) - (\text{Effluent NH}_4^+))} \quad (3.5)$$

$$\text{NRE (\%)} = \frac{(\text{Total influent N} - \text{Total effluent N})}{\text{Total influent N}} * 100 \quad (3.6)$$

### 3.2.5.2. Chemical oxygen demand (COD)

COD was analysed using open reflux method according to the standard methods for the examination of water and wastewater (Azimi *et al.*, 2019).

### 3.2.5.3. pH

The influent media pH was measured using the Orion Dual Star Benchtop pH meter (ThermoScientific, USA). The pH meter was calibrated using different buffer solutions.

The pH in the influent was maintained at  $6.5 \pm 0.2$  to  $7.5 \pm 0.2$  using  $\text{CO}_2$  gas and  $\text{NaHCO}_3$

### 3.2.5.4. Dissolved Oxygen (DO)

DO was measured using Vernier LabQuest® detector. The concentration of DO in the feed was maintained below  $0.5 \text{ mg-O}_2/\text{L}$  by purging with argon-carbon dioxide ( $\text{CO}_2$ ) mixture (95% argon, 5%  $\text{CO}_2$ ) at  $5 \text{ L/min}$  throughout the study.

### **3.2.6. Genomic DNA extraction**

Samples were collected from the inoculum and during different experimental runs on a monthly basis. The obtained samples were centrifuged at 10,000 g for 5 min, washed with distilled water and the genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. Genomic DNA was extracted from inoculum and reactor samples to confirm the presence of ANAMMOX and other nitrifying bacterial groups within the samples. The concentration and purity of the extracted DNA were determined using Implen NanoPhotometer NP 80 (Implen GmbH, Munich, Germany) and the quality of the extracted DNA was evaluated on 1% agarose gel. The extracted DNA was stored at -20 °C for further analysis.

### **3.2.7. PCR amplification**

The genomic DNA was amplified using the ThermoScientific PCR Master Mix in a T100 Thermal Cycler (Bio-Rad PCR system), according to optimized reaction conditions outlined (Table 3.3). The PCR reactions were run in a 25 µL volume containing 12.5 µL of the DreamTaq PCR Master Mix (2X) (ThermoFisher Scientific, USA), 1 µL of each primer, 2 µL of diluted DNA template and 8.5 µL of sterile water. One percent agarose gel electrophoresis stained with ethidium bromide was used to assess the size and quality of PCR amplicons by visualizing under UV light using a ChemiDoc™ XRS+Systems with Image Lab™Software (BIO-RAD, USA).

### **3.2.8. Quantitative polymerase chain reaction (qPCR)**

Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) was used to carry out the qPCR reactions using the specific primer sets as listed in Table 3.3. The qPCR reactions were performed in a total volume of 10 µL containing 4 µL of Sso Advanced™ Universal SYBR® Green Supermix (BIO-RAD, USA), 0.4 µL of forward primer, 0.4 µL of reverse primer, 2 µL of template DNA, and 3.2 µL sterile distilled water. The qPCR conditions

were optimized for each set of primers accordingly. For each experimental set up, appropriate negative controls and reference standard DNA were subjected to the same amplification condition. The specificity of each qPCR assay was confirmed by using both melting curve analysis and agarose gel electrophoresis.

**Table 3.3:** Summary of primers that were used in this study

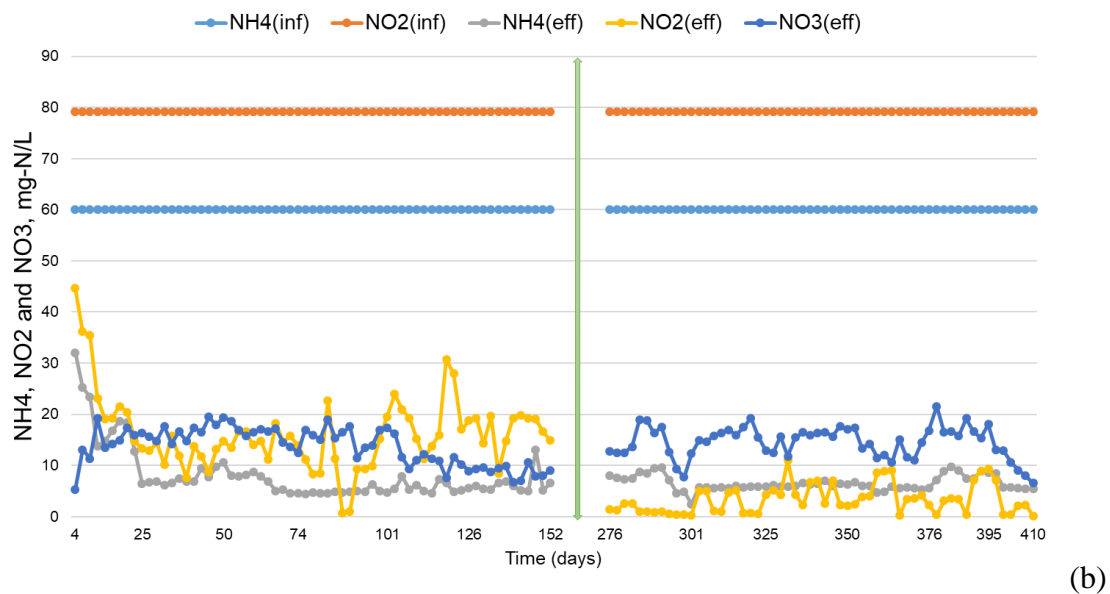
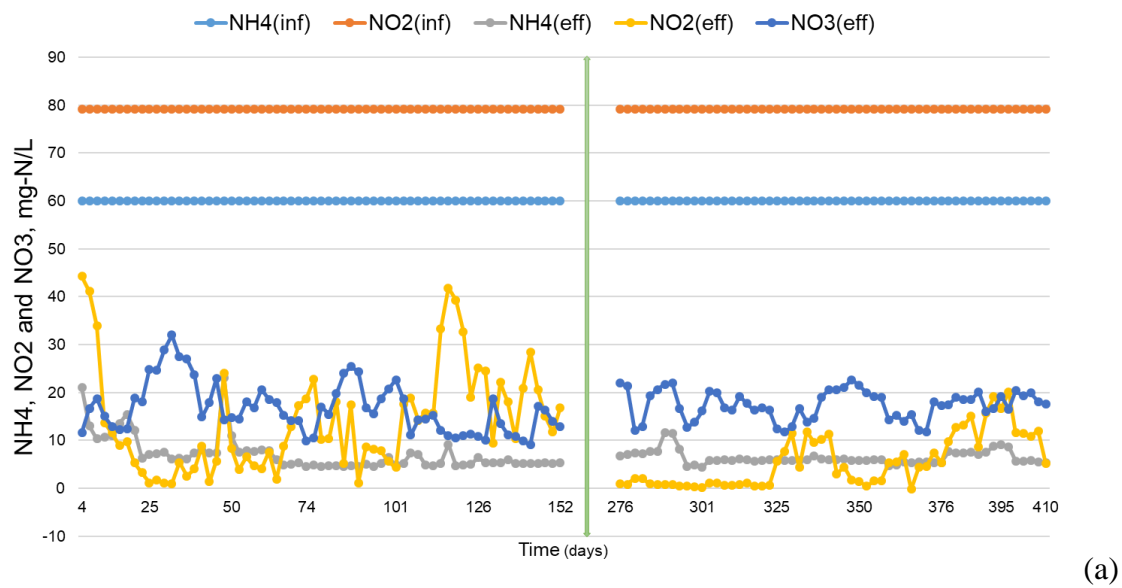
<b>Primer</b>	<b>Sequence 5'-3'</b>	<b>Specificity</b>	<b>Base pair</b>	<b>Reference</b>
<i>PLA46 F</i>	GGATTAGGCATGCAAGTC	ANAMMOX	691	(van der Star <i>et al.</i> , 2007)
<i>Amx667 R</i>	ACCAGAAGTTCCACTCTC			
<i>Hzocl1F1</i>	CACTCCAGATRTGCTGACC	Hzo gene	470	(Schmid <i>et al.</i> , 2008)
<i>Hzocl1R1</i>	TGYAAGACYTGYCAYTGG	(ANAMMOX)		
<i>CTO 189fA/B</i>	GGAGRAAAGCAGGGGATGG	AOB 16S rRNA	467	(Wang <i>et al.</i> , 2018)
<i>CTO 189fC</i>	GGAGGAAAGTAGGGGATCG			
<i>RT1r</i>	CGTCCTCTCAGACCARCTACTG			
<i>amoA-1f</i>	GGGGTTTCTACTGGTGGT	(amo-A gene)	491	(Lee <i>et al.</i> , 2014)
<i>amoA-2r</i>	CCCCTCKGSAAAGCCTTCTTC	AOB		
<i>Nitro1198f</i>	ACCCCTAGCAAATCTCAAAAAACCG	<i>Nitrobacter spp.</i>	229	(Park <i>et al.</i> , 2010)
<i>Nitro1423r</i>	CTTCACCCCAGTCGCTGACC			
<i>nxA F1</i>	CAGACCGACGTGTGCGAAAG	<i>nxA gene</i>	322	(Saijai <i>et al.</i> , 2016)
<i>nxA r2</i>	TCCACAAGGAACGGAAGGTC	( <i>Nitrobacter spp.</i> )		
<i>NSR 1113f</i>	CCTGCTTTCAGTTGCTACCG	<i>Nitrospira spp.</i>	151	(Dionisi <i>et al.</i> , 2002)
<i>NSR 1264r</i>	GTTTGCAGCGCTTTGTACCG			
<i>NitrospiraG2-a-F</i>	ACGTCAAATCACGCAGCTG	<i>Nitrospira nxB</i>	123	(Sun <i>et al.</i> , 2021)
<i>NitrospiraG2-a-R</i>	CGGCATCGAAAATGGTCATCC	<i>uwn-2</i>		
<i>1055f</i>	ATGGCTGTCGTCAGCT	EUB	337	(Orschler <i>et al.</i> , 2019)
<i>1392r</i>	ACGGGCGGTGTGTAC			

### 3.3. Results

During the enrichment period, the slow growing characteristics and sensitive nature of this bacteria presented a major challenge, as it required a long start-up time (an average of 60 days reported in the literature) and constant monitoring. Additionally, the national lockdown following the outbreak of Covid-19 in 2020 impacted reactor operations for 124 days. The reactors were monitored minimally during this period, as indicated by the green line in the Figures (Figure 3.3(a), 3.3(b), 3.4, 3.5 and 3.6).

#### 3.3.1.1. Effluent nitrogen concentrations

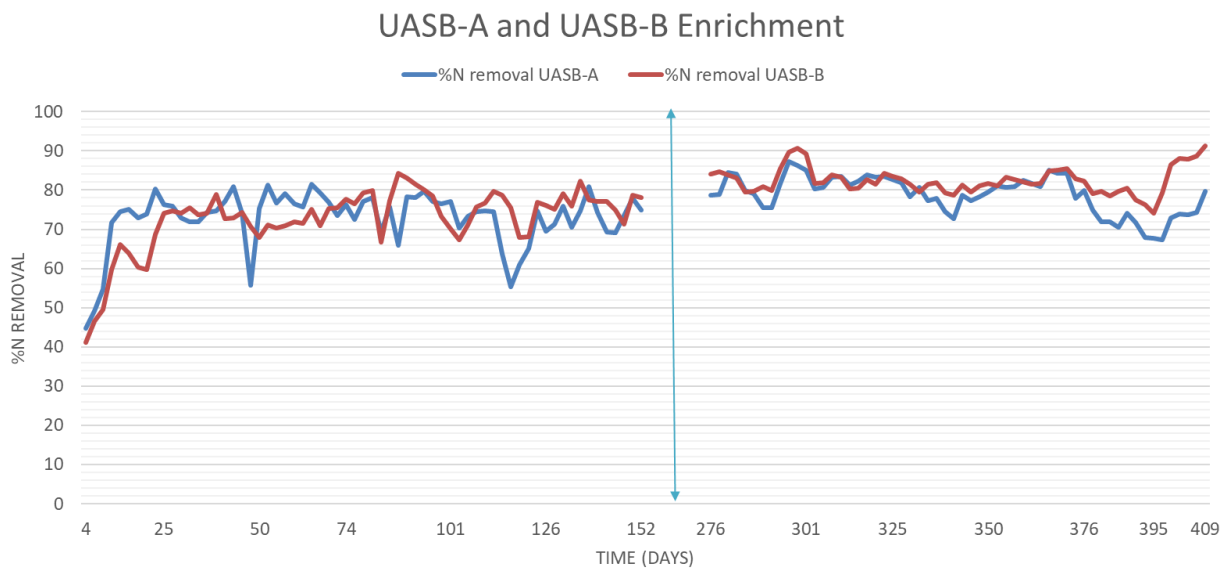
The effluent  $\text{NH}_4^+$  concentration was high during the initial stage of the reactor operation. Between day 1 and day 6 of enrichment, the average  $\text{NH}_4^+$  concentration in UASB-A was 21.01 mg/L (65%  $\text{NH}_4^+$  removal), while for UASB-B it was 32.02 mg-N/L (46.6%  $\text{NH}_4^+$  removal). However, after 25 days of operation, a drastic decrease in average effluent  $\text{NH}_4^+$  concentrations were observed in both reactors indicating an efficient  $\text{NH}_4^+$  removal (Figure 3.3(a) and 3.3(b)). From day 25 to 152 days, the average effluent  $\text{NH}_4^+$  concentrations in UASB-A decreased to 6.28 mg-N/L (89.5%  $\text{NH}_4^+$  removal). Higher concentrations of  $\text{NO}_2^-$  in the effluent were also observed in the beginning of the operation (Day 1 to 6) in both reactors, similar to the concentrations of  $\text{NH}_4^+$  (Figure 3.3(a) and 3.3(b)). Initially, from day 1 to 4, the average effluent  $\text{NO}_2^-$  concentrations were 44.3 mg-N/L and 44.6 mg-N/L in UASB-A and UASB-B, respectively. However, a decrease in the average effluent  $\text{NO}_2^-$  concentrations to 3.29 mg-N/L and 12.12 mg-N/L was observed in UASB-A and UASB-B, respectively, between days 25 to day 49. A sharp increase in effluent  $\text{NO}_2^-$  concentrations to 41.82 mg-N/L and 30.76 mg-N/L was observed in UASB-A and UASB-B, respectively, between days 120 to 128. Both UASB reactors were halted from day 152 to day 276. During this period, the reactors were maintained by increasing substrate concentrations ( $\text{NH}_4^+\text{-N}$  (mg/L) from 60 to 90 and  $\text{NO}_2^-\text{-N}$  (mg/L) from 79.2 to 118.8 )and reducing the influent flow rate from 5 mL/min to 0.5 mL/min.



**Figure 3.3:** Influent and effluent nitrogen concentrations in UASB-A (a) and UASB-B(b). The green line between days 152 – 276 indicates the lockdown period.

On day 276, the reactor operations were resumed after the national lockdown, and the enrichment experiment continued until a steady state was reached. Between days 276 and 410, the average  $\text{NO}_3^-$  concentrations for UASB-A and UASB-B were 17.46 mg-N/L and 14.54

mg-N/L, respectively. Between day 395 and 410, the effluent  $\text{NO}_3^-$  concentrations in UASB-B decreased from 18.05 to 6.55 mg-N/L, while  $\text{NH}_4^+$  concentrations decreased from 8.54 to 5.36 mg-N/L and remained close to these concentrations until the end of enrichment stage. In UASB-A, the average effluent  $\text{NO}_3^-$  concentrations remained at 17.5 mg-N/L until the end of the enrichment experiment (Day 410).

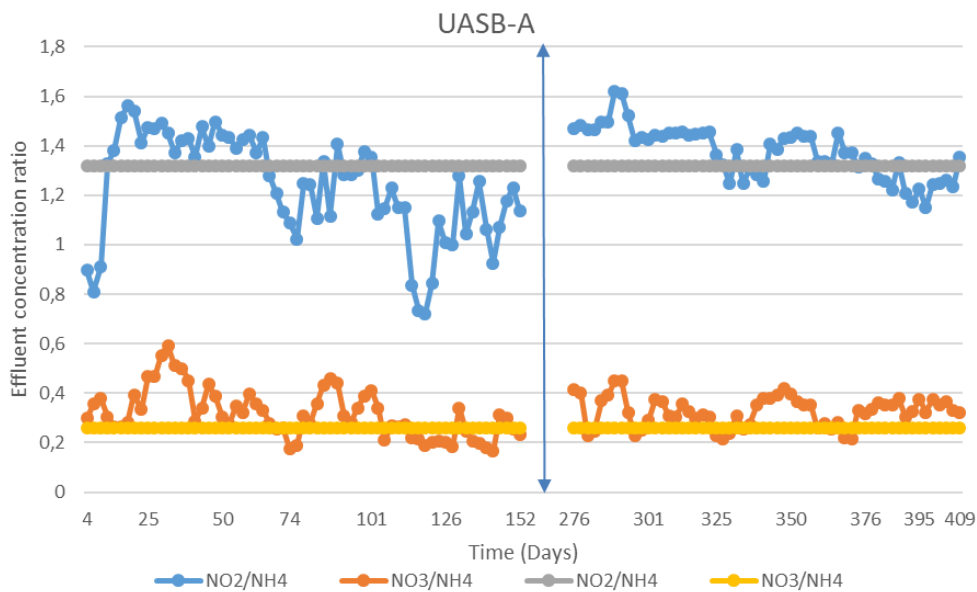


**Figure 3.4:** Percentage of Nitrogen removal in UASB-A and UASB-B during the enrichment phase. The blue line between days 152 – 276 indicates the lockdown period.

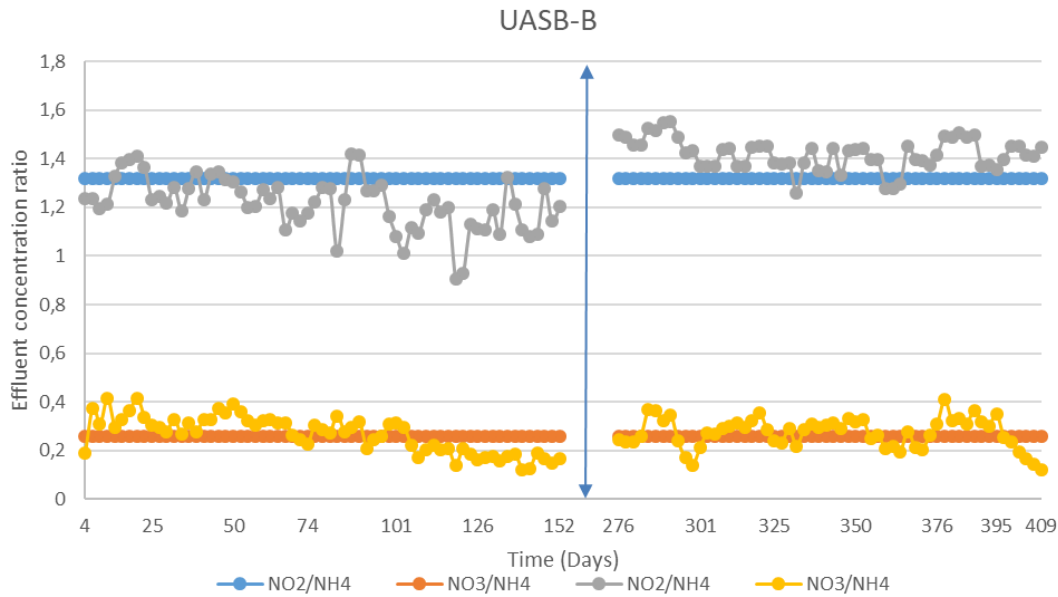
Nitrogen removal efficiencies were low (<50%) in both reactors in the first 10 days of the enrichment (Figure 3.4). During this phase, the NREs were also not constant and highly variable and the effluent contained higher levels of nitrogen (Figures 3.3(a) and 3.3(b)). In UASB-B, the average NRE increased from 84.11% to 91.34% between days 276 and 410, while UASB-A did not exhibit a significant change in NRE (78.7% to 79.78%). After the resumption of enrichment from day 276 (after lockdown), the highest average NRE of 84.1% was observed in UASB-B, while 78.7% NRE was observed in UASB-A.

### 3.3.1.2. Stoichiometry of nitrite consumption and nitrate production per ammonium consumption

The ratios of  $\text{NO}_2^-$  consumed per  $\text{NH}_4^+$  consumed ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and  $\text{NO}_3^-$  produced per  $\text{NH}_4^+$  consumed ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) consumed significantly varied in the reactors during the study period (Figure 3.5 and 3.6). The  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios from days 1-409 fluctuated from  $<0.09$  to  $>1.65$  in both UASB-A and UASB-B (Figure 3.5 and 3.6). On days 1-152, the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratio was lower than the expected stoichiometric ratios of ANAMMOX (1.32) and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratio was close to the expected stoichiometric ratio of 0.26 in all the reactors. A very sharp decrease of  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  to less than 0.8 was observed in UASB-A before day 152  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  which is far lower than the expected stoichiometric ratio of 1.32.



**Figure 3.5:** Nitrogen effluent concentration ratios of UASB-A during ANAMMOX enrichment. The blue line between days 152 – 276 indicates the lockdown period.



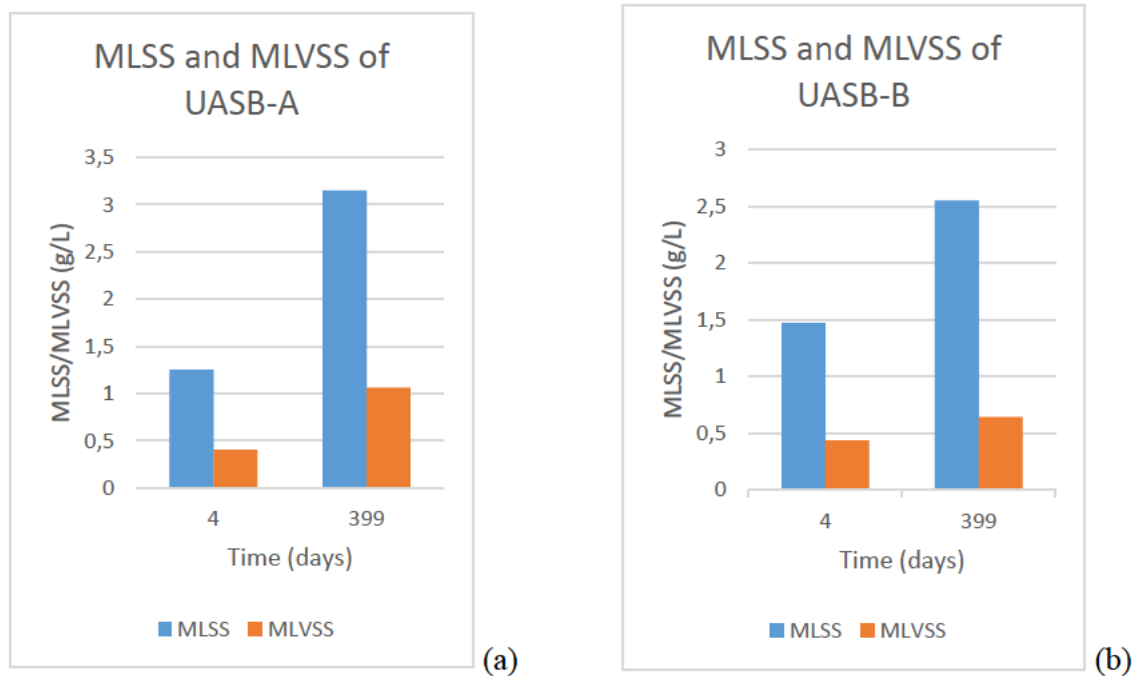
**Figure 3.6:** Nitrogen effluent concentration ratios of UASB-B during ANAMMOX enrichment. The blue line between days 152 – 276 indicates the lockdown period.

The UASB-A reactor showed a high variation in the ratios of NO<sub>2</sub><sup>-</sup> consumed per NH<sub>4</sub><sup>+</sup> consumed and NO<sub>3</sub><sup>-</sup> produced per NH<sub>4</sub><sup>+</sup> consumed after resuming reactor operation (on day 276) , with  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ranging from 1.15 to 1.6 and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ranging from 0.22 to 0.47. (Figure 3.5) (ANOVA,  $p < 0.005$ ). While, the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios in UASB-B varied from day 305 to day 376, from 1.28 to 1.43 and from 0.22 to 0.33, respectively, which gave better results than UASB-A. (Figure 3.6). Towards day 395, the calculated  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios in UASB-B approached the stoichiometric ratios expected of the ANAMMOX process (Figure 3.6). Conversely, towards day 395 in UASB-A, the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios were lower (1.15) than the stoichiometric ratios (1.32) expected of ANAMMOX process, while the  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios were higher (0.38) to the stoichiometric ratios of ANAMMOX process (Figure 3.5). From day 396 to 409 onwards, the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios in UASB-B remained close to the stoichiometric ratios of ANAMMOX

process, while in UASB-A the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios increased to 1.4 and the  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios were higher (0.35) than the stoichiometric ratio (0.26).

### 3.3.1.3. Biomass concentration changes during the enrichment period

The change in biomass concentration was monitored using MLSS and MLVSS values. The MLSS and MLVSS concentration in the initial inoculum was 1.25 g/L and 0.9 g/L, respectively. The MLSS concentration increased to ~3.15 g/L MLSS in UASB-A and ~2.55 g/L in UASB-B over the course of the enrichment, as shown in Figure 3.7(a) and 3.7(b).

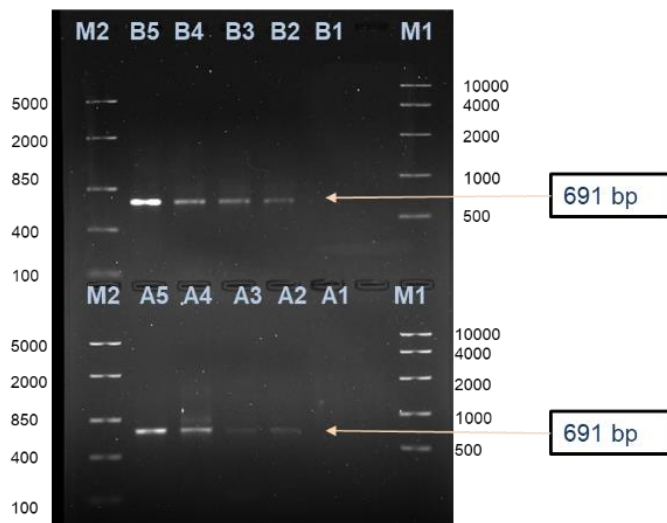


**Figure 3.7:** MLSS and MLVSS change within UASB-A (a) and UASB-B (b) after 399 days of enrichment.

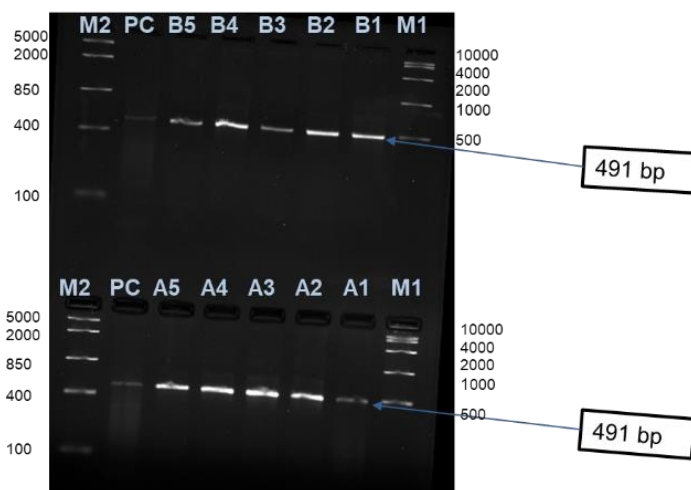
### 3.3.1.4. Detection of ANAMMOX and nitrifying bacteria

The key nitrifying bacterial groups such as ANAMMOX, AOB and NOB (*Nitrobacter spp.* & *Nitrospira spp.*) were detected from both the seed inoculum and the reactor samples using PCR. The amplified single bands in Figures 3.8, 3.9, 3.10, 3.11 and 3.12 show the simultaneous presence of ANAMMOX bacteria, AOB, the most common wastewater NOB genera

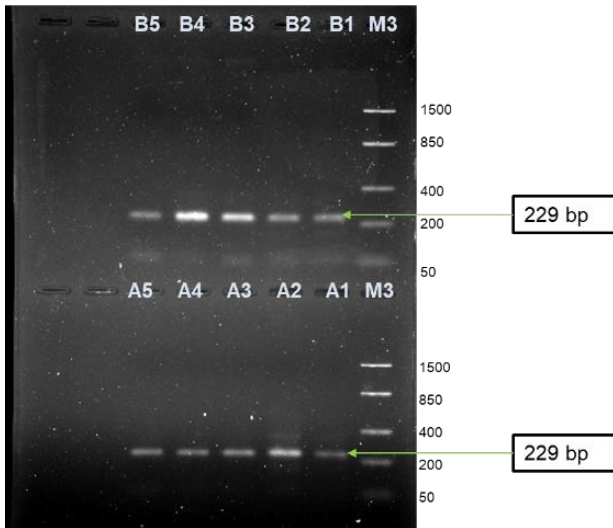
*Nitrobacter* and *Nitrospira*; and EUB, respectively, all coexisting within the UASB reactors from the final day of enrichment (day 410) and during 3 phases of C/N ratio.



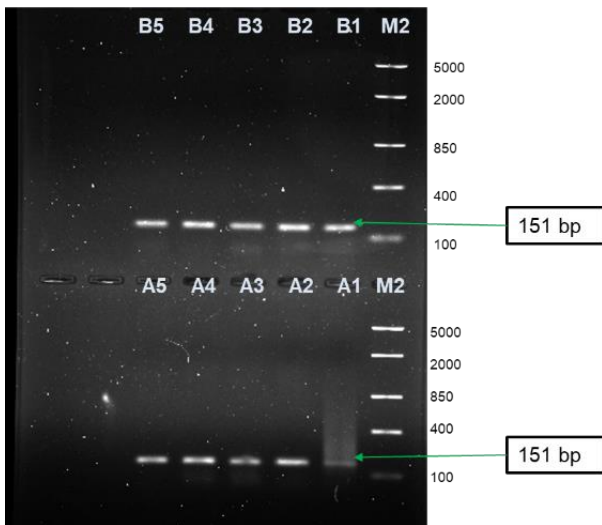
**Figure 3.8:** Agarose gel showing the 691 bp PCR fragments band for ANAMMOX. Lanes: M2 & M1: molecular weight-markers (middle range & high range DNA ladders, respectively), B1-B5: UASB-B samples A1-A5: UASB-A samples



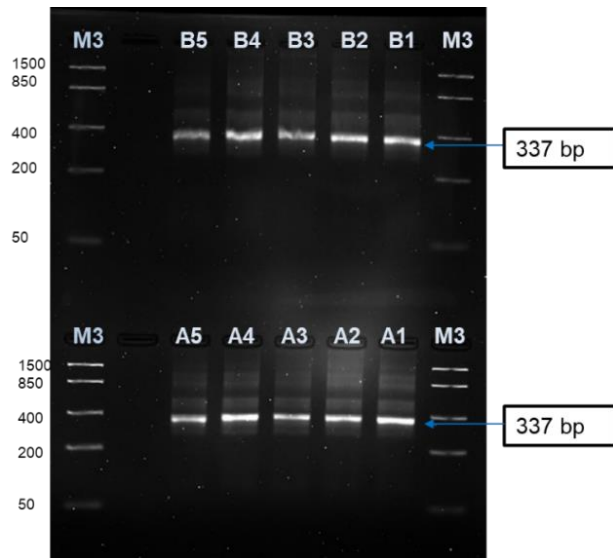
**Figure 3.9:** Agarose gel showing the 491 bp PCR fragments band AOB. Lanes: M2 & M1: molecular weight-markers (middle range & high range DNA ladders, respectively), B1-B5: UASB-B samples A1-A5: UASB-A samples



**Figure 3.10:** Agarose gel showing the 229 bp PCR fragments band for *Nitrobacter spp.* Lanes: M2 & M3: molecular weight-markers (middle range & low range DNA ladders, respectively), B1-B5: UASB-B samples A1-A5: UASB-A samples



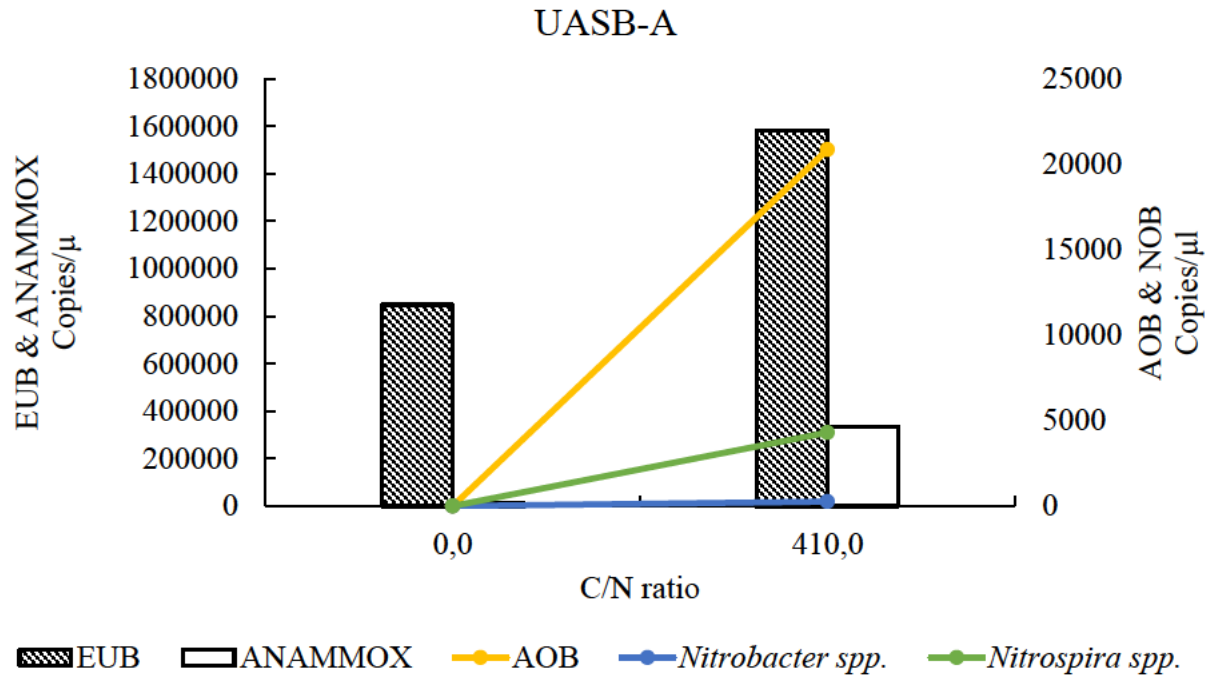
**Figure 3.11:** Agarose gel showing the 151 bp PCR fragments band for *Nitrospira spp.* Lanes: M2 & M3: molecular weight-markers (middle range & low range DNA ladders, respectively), B1-B5: UASB-B samples A1-A5: UASB-A samples



**Figure 3.12:** Agarose gel showing the 337 bp PCR fragments band for EUB. Lanes: M3: molecular weight-marker (low range DNA ladder), B1-B5: UASB-B samples A1-A5: UASB-A samples.

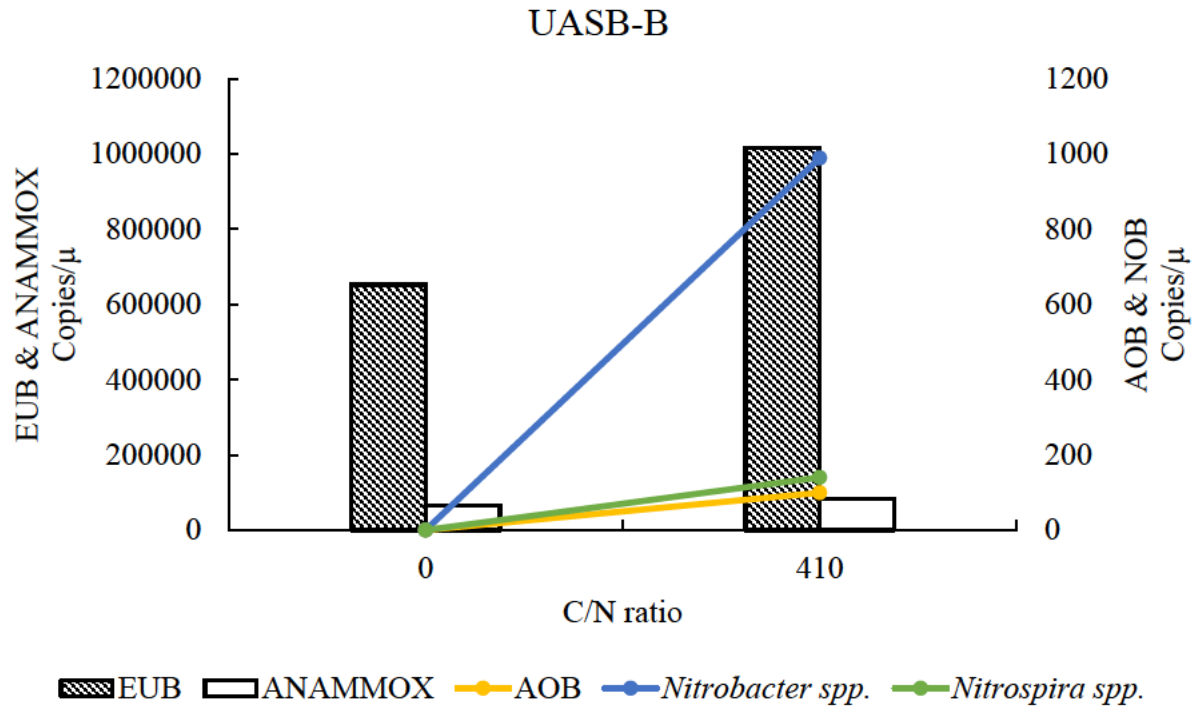
### 3.3.1.5. Quantification of ANAMMOX and nitrifying bacteria

Using quantitative PCR (QPCR), the absolute quantities of the ANAMMOX bacteria, AOB, NOBs (*Nitrobacter spp.* and *Nitrospira spp.*) and total Eubacterial populations were tracked from inoculum and over the 410-day enrichment period (Figure 3.13 and Figure 3.14).



**Figure 3.13:** Microbial analysis; qPCR of Total bacteria, ANAMMOX, AOB and NOB populations of UASB-A during the enrichment.

Figure 3.13 shows an increase of ANAMMOX and EUB populations observed in UASB-A from day 0 to day 410 of the enrichment period. ANAMMOX bacteria increased from  $1.30 \times 10^4$  copies/ $\mu\text{L}$  to  $3.34 \times 10^5$  copies/ $\mu\text{L}$ , while EUB population increased from  $8.47 \times 10^5$  copies/ $\mu\text{L}$  to  $1.58 \times 10^5$  copies/ $\mu\text{L}$  in UASB-A. AOB population displayed a fast increase towards the final day (day 410) of enrichment in UASB-A. The quantity of AOB population in UASB-A during day 410 was  $2.08 \times 10^4$  copies/ $\mu\text{L}$ . NOB populations also showed a slight increase from day 0 to day 410 with *Nitrospira spp.* ( $4.30 \times 10^3$  copies/ $\mu\text{L}$ ) dominating *Nitrobacter spp.* ( $2.58 \times 10^2$  copies/ $\mu\text{L}$ )



**Figure 3.14:** Microbial analysis; qPCR of Total bacteria, ANAMMOX, AOB and NOB populations of UASB-B during the enrichment.

Figure 3.14 shows an increase of EUB populations and a slight decrease of ANAMMOX population observed in UASB-B from day 0 to day 410 of enrichment period. EUB population increased from  $6.53 \times 10^5$  copies/ $\mu\text{L}$  to  $1.02 \times 10^6$  copies/ $\mu\text{L}$ , while ANAMMOX bacteria showed a slight increase from  $6.45 \times 10^4$  copies/ $\mu\text{L}$  to  $8.37 \times 10^4$  copies/ $\mu\text{L}$  in UASB-B. AOB population displayed a slight increase towards the final day (day 410) of enrichment in UASB-B, which was lower than that of UASB-A. The quantity of AOB population in UASB-B during day 410 was 99 copies/ $\mu\text{L}$ . NOB populations also showed an increase from day 0 to day 410. *Nitrobacter spp.* showed an increase of  $9.89 \times 10^2$  copies/ $\mu\text{L}$  compared to *Nitrospira spp.*, which was  $1.40 \times 10^2$  copies/ $\mu\text{L}$ .

### 3.4. Discussion

UASB reactors have been previously used for the successful enrichment of ANAMMOX bacteria due to their advantages such as high biomass concentration, rich microbial diversity, ease of operation, provides good sludge settling capability and produces high NRE (Wang *et al.*, 2020a). In this study, changes in the NRE and  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios were observed during the enrichment in both UASB-A and UASB-B. In spite of the initial NRE results being promising, the national lockdown (March 2020-July 2020) interfered with the operation of the reactors. The slow growing characteristics and sensitive nature of this bacteria presented a huge challenge for its recovery after the lockdown period, and as a result, the enrichment period took longer than expected. In the beginning of the enrichment, there was low % nitrogen removal in all the reactors (Figure 3.4). This could have been because the ANAMMOX bacteria were still adapting to the operating conditions in the reactors (Zhao *et al.*, 2018). At some instances, the effluent ammonia concentration was higher than the influent ammonia concentration. A possible reason for this may be the growth of fast-growing heterotrophic bacteria that are actively breaking down organic matter within the bacterial cells, such as dead bacteria, to release  $\text{NH}_4^+$  which may have contributed to the increased ammonia concentration in the effluent (Li *et al.*, 2021a). Additionally, there may be a competition between heterotrophic bacteria and ANAMMOX bacteria for  $\text{NO}_2^-$ , further limiting the slow growing ANAMMOX bacterial activities, thus affecting the removal of nitrogen in the reactors (Li *et al.*, 2018b). The performances of ANAMMOX during the enrichment were determined by monitoring several indicators, such as a change in suspended solids. The increase of MLSS in Figure 3.7(a) and Figure 3.7(b) indicated a significant total biomass growth over the enrichment period, despite the absence of organic carbon and active aeration. This implies that the increase in biomass was attributed primarily to the growth of autotrophic N-removal bacteria within the system. According to Ibrahim *et al.* (2015), ANAMMOX consumption

reaction involves acid; therefore, ANAMMOX activity can initially be indicated by a higher effluent pH than influent pH (Ibrahim *et al.*, 2015). Furthermore, the improvement of ANAMMOX enrichment was also measured by observing the changes in colour and gradual granulation of the biomass inside the reactors (Yang *et al.*, 2006). An orange-red colouration was observed as a confirmation of the presence of a stable ANAMMOX population in a bioreactor (Ibrahim *et al.*, 2015).

The stoichiometric ratio is generally used to measure the activity of ANAMMOX and nitrifiers in the community. A stoichiometry ratio of 1.32 is expected for  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and 0.26 is expected for  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  (Hassan and El-Gohary, 2021). Initially,  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratio was 0.9, which was very lower than the expected value of 1.32 and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratio was 0.37, which was a little bit higher than the expected value of 0.26. This was because of the initial start-up of the reactors; the ANAMMOX bacteria was still adjusting the conditions. From day 395 to 409 of enrichment, the  $\text{NO}_2^-/\text{NH}_4^+$  and  $\text{NO}_3^-/\text{NH}_4^+$  ratios of 1.36 and 0.25, respectively, were very close to the stoichiometric ratios expected (Hassan and El-Gohary, 2021) of ANAMMOX process in UASB-B. However, in UASB-A,  $\text{NO}_2^-/\text{NH}_4^+$  ratios were very lower than 1.32, and  $\text{NO}_3^-/\text{NH}_4^+$  ratios were higher than 0.26 (expected of the ANAMMOX process). This is probably because of competition for  $\text{NO}_2^-$  between ANAMMOX bacteria, heterotrophic bacteria and NOB as there were a few leakages that might have permitted oxygen inside the reactors, allowing the DO concentration to rise above 0.5 mgO<sub>2</sub>/L (Laureni *et al.*, 2015). The PCR results have shown the concurrent presence of ANAMMOX, AOB, NOB (represented by the genera *Nitrobacter* and *Nitrospira*); and EUB in both UASB-A and UASB-B reactors. In addition, the results in Figure 3.11 and Figure 3.12 show that there was a significant increase in ANAMMOX population from day 0 to the final day of enrichment (day 410). Although there was an increase in AOB population in UASB-A, it remained very low compared to ANAMMOX population. Also, an increase of *Nitrobacter spp.* population in UASB-B had no

significant effect on the stability of the reactor performance. 79.78 % and 91.34 % NRE were observed in the final enrichment stage within UASB-A and UASB-B, respectively. This was supported by the high ANAMMOX population observed in qPCR results on the final day of enrichment. The stoichiometric ratios of  $\text{NO}_2^-/\text{NH}_4^+$  and  $\text{NO}_3^-/\text{NH}_4^+$  remained closed to the expected values of 1.32 and 0.26, respectively, in UASB-B, while UASB-A had a  $\text{NO}_3^-/\text{NH}_4^+$  ratio of 0.35 which was higher than the expected value. Therefore, the presence of these populations provides an explanation for the high NRE observed within UASB-B during the final stage of enrichment. This could imply the successful enrichment of an ANAMMOX-nitrification groups for autotrophic ammonium removal. The PCR results indicated the presence of ANAMMOX bacteria, AOB and NOB (*Nitrobacter spp.* and *Nitrospira spp.*) in the inoculum as well as reactor samples. All these species reportedly contribute to N removal. As discussed in previous studies, AOB and NOB thrives under aerobic, whereas ANAMMOX under anaerobic conditions (Wang *et al.*, 2019a). These groups can compete with each other for substrate as well as electron acceptors. For example, NOB compete with ANAMMOX for  $\text{NO}_2^-$  under low DO conditions and AOB can compete with ANAMMOX for ammonia. Therefore, DO control is paramount in ANAMMOX reactors. The presence of both the AOB and NOB implies that the leakages in the reactor allowed the entrance of oxygen which might have increased the DO within UASB-A and UASB-B systems during the enrichment period.

The quantitative PCR analysis for each of the key N-removing populations in both UASB-A and UASB-B displayed a separate change in average population densities over the enrichment period. The initial phase of anaerobic bioreactors often exhibits low total biomass and low microbial population diversity due to the seed inoculum having to adjust to the new environment within the reactor. Both the reactors during the enrichment stage were operated anaerobically under conditions of no organic carbon; therefore, a shift towards an autotrophic N-removal population was expected. As the total eubacteria seemed to stabilize over the course

of the remainder of the enrichment period, the fluctuations were displayed by *Nitrospira* spp., *Nitrobacter* spp., and the AOB over the 410-day enrichment period. The relationship between ANAMMOX and AOB populations, when co-cultured, is reported to complement each other metabolically (Yin *et al.*, 2021). The increase in the AOB population in UASB-B might be because of operation errors such as pump failure which allowed the entrance of oxygen; therefore AOB population was favoured. On the other hand, under the limited DO conditions within the reactor, the AOB would have oxidized the provided  $\text{NH}_4^+$  to  $\text{NO}_2^-$ , while the ANAMMOX bacteria utilize the produced  $\text{NO}_2^-$  and any remaining  $\text{NH}_4^+$  to produce  $\text{N}_2$  gas. It is possible that despite the conditions provided within the reactor being suitable for both ANAMMOX and AOB propagation, they still represented such small fractions of the total population that their metabolic contributions would be minimal. NOB population also showed an increase towards the end of enrichment in UASB-B and correlated with a leak of oxygen into the reactor system. This oxygen leak caused the DO to increase from less than 0.2 mg/L to  $\sim 1.8 \text{ mg/L} \pm 0.5 \text{ mg/L}$  (measured on Day 395). The DO entrance provided an accumulative advantage to NOBs and favoured these species to outcompete the AOB within UASB-B (Gani *et al.*, 2020). The excess DO in UASB-B suppressed the growth of the ANAMMOX population. This situation was controlled by sparging of Argon or Nitrogen gas to minimize the DO within the reactor.

### 3.5. Conclusion

- The NRE increased from 84.11% to 91.34% between days 276 and 410 of the enrichment stage in UASB-B, while UASB-A did not display a significant change from 78.7% to 79.78%.
- The  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios in UASB-B remained close to the stoichiometric ratios (1.32) of the ANAMMOX process, while in UASB-A the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios increased to

1.4 and the  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios were higher (0.35) than the expected of ANAMMOX process stoichiometric ratio (0.26).

- PCR and qPCR results confirmed the presence and dominance of a mixed population of autotrophic N-removing organisms, including ANAMMOX and AOB, within the reactors after 410 days of enrichment.

## CHAPTER 4:

# THE EFFECT OF C/N RATIO ON THE PERFORMANCE OF ANAMMOX-MEDIATED SYSTEM AND MICROBIAL COMMUNITY STRUCTURE

### 4.1. Introduction

ANAMMOX has been reported to be affected negatively by organic matter, which is often present in wastewater along with  $\text{NH}_4^+$ . This has caused widespread concern for the application of ANAMMOX in the main stream. (Cho *et al.*, 2019). Inorganic carbon sources such as  $\text{CO}_2$  and  $\text{HCO}$  are particularly important for the cultivation of ANAMMOX bacteria and favor the growth and activity of these microorganisms (Xie *et al.*, 2017, Wang *et al.*, 2022b). However, organic compounds may adversely affect ANAMMOX bacteria and may impair the activity of ANAMMOX, especially at high concentrations (Bonassa *et al.*, 2021). Organic compounds can inhibit the ANAMMOX process by inactivating enzymes and may result in irreversible cell death. (Chen *et al.*, 2021). It has also been suggested that the competition between ANAMMOX bacteria and denitrifying heterotrophic bacteria for the electron acceptor nitrite causes ANAMMOX impairment. Since heterotrophic bacteria are able to grow faster than autotrophic ones, they outcompete the ANAMMOX bacteria and hinder their chemical reactions (Ni *et al.*, 2012, He *et al.*, 2015). Some reports state that a low organic matter concentration in ANAMMOX systems can improve the NRE (NRE) by promoting synergism between the ANAMMOX and denitrification processes (Xiong *et al.*, 2022). However, COD concentrations above 300 mg/L or a C/N ratio of 2 and above were reported to fully inhibit ANAMMOX reaction and concurrently favor the activity of denitrifying heterotrophic bacteria (He *et al.*, 2015). Ni *et al.* (2012) reported that at C/N ratios above 4 the ANAMMOX process

was impaired. Currently, there is no general agreement in the literature regarding the organic matter concentration and C/N ratio that influence or inhibit the ANAMMOX process. There may be a number of reasons for this, including the different types of reactors used in the ANAMMOX process, the operational conditions, and the microbial community structure, among others. Additionally, the composition of organic matter may also be responsible for these inconsistencies since denitrifying bacteria and heterotrophic bacteria utilize organic matter at different rates. (Xie *et al.*, 2012). There is a synergism that exists among the specialist N-removing microbial groups wherein the metabolic by-products of one microbial group form the primary growth substrates for another (Awolusi *et al.*, 2020). The complete characterization of ANAMMOX bacteria and their growth requirements has not yet been achieved due to the difficulty of isolating them in pure cultures. Therefore, all ANAMMOX bacterial species have the taxonomical status 'Candidatus' (Kartal *et al.*, 2013). Besides ANAMMOX bacteria, it has been reported that AOB, NOB, and heterotrophic bacteria are core community members of ANAMMOX-mediated systems (Park, 2011). Apart from this, other microbial groups, such as viruses, archaea and protozoans, have also been reported in ANAMMOX-mediated systems (Kosgey *et al.*, 2021). Among these, the activities of AOB and AOA (ammonia oxidizing archaea) could benefit the ANAMMOX process as both groups could generate  $\text{NO}_2^-$  (Zhang and Okabe, 2020), while the activities of heterotrophic bacteria and NOB could be detrimental to ANAMMOX process as they present competition to ANAMMOX bacteria for  $\text{NO}_2^-$ , which acts as an electron acceptor (You *et al.*, 2020). Similarly, some of the protozoans present in ANAMMOX-mediated systems could graze on autotrophic bacteria, consequently influencing nitrogen removal (Hoekstra *et al.*, 2018). Characterisation of bacterial communities in biological systems could thus be an important step in the description of process performance in ANAMMOX-mediated systems.

To date, several techniques have been employed for characterising microbial communities from ANAMMOX-mediated reactors, including polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), 16S rRNA sequencing, fluorescence in situ hybridization (FISH) (Hoekstra *et al.*, 2018) and metagenomics sequencing (Ji *et al.*, 2021, Guo *et al.*, 2022a). Microbial analyses that were done in previous studies provide crucial information relating to the impact of growth morphology and inoculum on microbial community richness, structures and diversities in ANAMMOX-mediated systems. However, more studies under baseline conditions are still necessary to understand the impact of C/N ratio on the prevailing microbial communities and their impact on nitrogen removal performance. This phase of the study was undertaken in order to understand the impact of the C/N ratio on the nitrogen removal performance and microbial activity of the reactors. This was achieved by increasing the C/N ratio of one of the reactors (UASB-B) after the enrichment period (chapter 3). Under varying C/N ratios (1.0, 1.5 and 2.0), ammonia removal performance, the dominance of key nitrifier communities, and levels of their expression of functional genes were studied.

## **4.2. Materials and methods**

### **4.2.1. Operation of the reactors (varying C/N ratio).**

After the enrichment period, UASB-B reactor was selected for the investigation of C/N ratio effects in 3 phases (Table 6), where each phase operated for a period of 60 days. In this phase, the UASB-A served as a control reactor (with no changes to the influent composition). In UASB-B, the influent concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$ , were kept constant, except for the organic carbon concentration (Table 4.1). In Phase I, the influent contained 139.2 mg/L of organic carbon (C/N=1), which was increased to 208.8 mg/L (C/N=1.5) in phase II, and thereafter to 278.4 mg/L (C/N=2) in phase III (Table 4.1). For all experimental runs, other factors were approximately maintained as follows: temperature 35 °C, pH between 7 and 8,

and dissolved oxygen (DO)  $\leq 0.2$  mg/L in both reactors. Both reactors were operated continuously at a flow rate of 5 mL/min.

**Table 4.1:** Summary of experimental setup C/N ratio phases

<i>Operation phase</i>	<b>C/N ratio</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg/L)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg/L)</b>	<b>COD (mg/L)</b>
<i>Phase I</i>	1	60	79.2	139.2
<i>Phase II</i>	1.5	60	79.2	208.8
<i>Phase III</i>	2	60	79.2	278.4

#### **4.2.2. MLSS and MLVSS and Chemical analysis**

Samples were collected weekly for biomass determination and at three times intervals per week for analytical procedures, including COD and nitrogen analysis. All the analysis was performed as shown in the previous chapter (Chapter 3, section 3.2.4.).

#### **4.2.3. Quantitative polymerase chain reaction (qPCR)**

Samples were collected from the reactor during different experimental runs monthly at different (C/N ratio) phases of the operation as well as from the control reactor. Genomic DNA was extracted from mixed liquor samples, as outlined in Chapter 3. The effect of the C/N ratio on the growth of microbial populations was assessed by quantifying the changes in 16S rRNA gene copies of Eubacteria, ANAMMOX, AOB and NOB (the *Nitrospira spp.* and the *Nitrobacter spp.*) using qPCR. The qPCR reactions and the primers used (Table 3.3) were prepared as outlined in Chapter 3. The Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) to carry out the qPCR reactions according to specific primer sets (Table 3.3).

#### **4.2.4. RNA extraction**

Samples were collected from the reactor during different experimental runs monthly at different (C/N ratio) phases of the operation as well as from the control reactor. The collected samples were centrifuged at 10,000 g for 5 min, washed with distilled water and the total RNA was extracted using the MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit according to the manufacturer's instructions. MEGAclean™ Kit (Thermo Fisher Scientific, USA) was used for the purification of transcription reactions according to the manufacturer's instructions. Bacterial mRNA was purified by removing rRNA from total RNA using MICROBExpress™ Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific, USA). The concentration and purity of the extracted RNA were determined using Implen NanoPhotometer NP 80 (Implen GmbH, Munich, Germany) and the quality of the extracted RNA was evaluated on 1% agarose gel. The extracted RNA was stored at -20 °C for further analysis.

#### **4.2.5. cDNA synthesis**

The reverse transcription reaction mixtures were prepared by combining the components of the GoScript™ Reverse Transcription System (Promega, USA) in a sterile microcentrifuge tube on ice. cDNA synthesis reactions were performed according to the manufacturer's instructions.

#### **4.2.6. cDNA quantification**

The effect of C/N ratio on the performance of ANAMMOX process was assessed by quantifying the changes cDNA copy number using the quantification PCR method. The Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) was used to carry out the qPCR reactions using primers in Table 3.3. The qPCR reactions were performed in a total volume of 10 µL containing 4 µL of Sso Advanced™ Universal SYBR® Green Supermix (BIO-RAD, USA), 0.4 µL of forward primer, 0.4 µL of reverse primer, 2 µL of template RNA, and 3.2 µL sterile distilled water.

#### **4.2.7. Functional gene expression analysis**

Functional gene expression encoding for hydrazine oxidoreductase (*hzo*), ammonia oxidation (*amoA*), nitrite oxidation (*nxr A* and *nxr B*) in ANAMMOX, AOB and NOB, respectively, were quantified using RT-qPCR to understand the activity of nitrifiers in a given condition. The mRNA concentrations of the selected functional genes were normalized using 16S rRNA as a reference gene specific to each targeted group (Table 3.3). The RT-qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystem), using qPCR as described in section 4.2.3. Gene expression was determined using  $\Delta\Delta C_T$  Method (Mughal *et al.*, 2018).

#### **4.2.8. Statistical analysis**

Statistical measures were used in capturing how two data series move together or relate over varied C/N ratios, to determine whether the nitrogen removal performance and change in microbial community behaviour at different C/N ratios was significant or not. Statistical analyses of reactor performance were performed in Microsoft excel using a single factor analysis of variance (ANOVA).

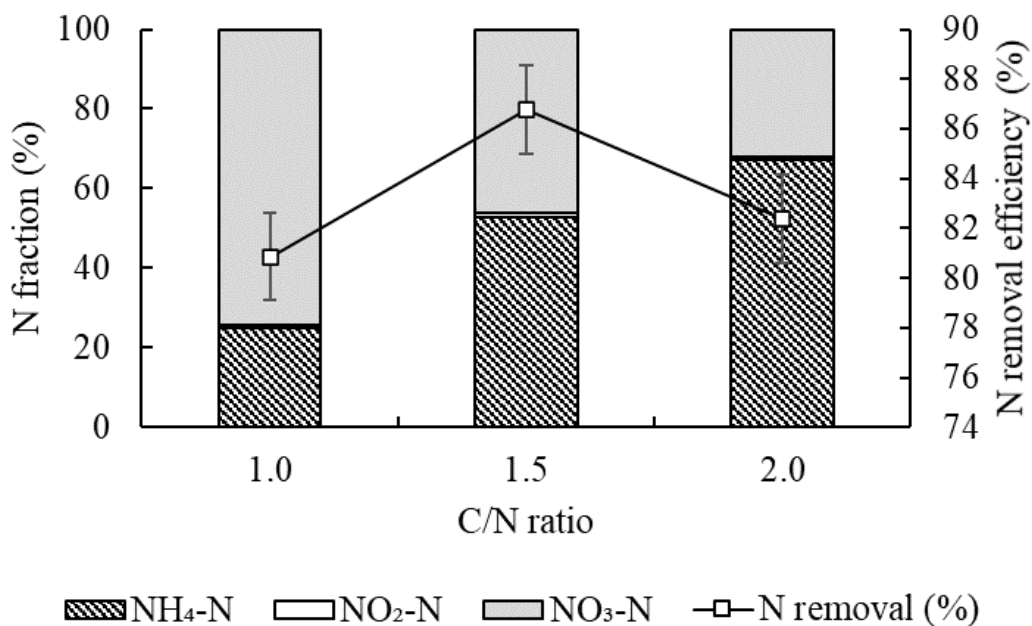
### **4.3. Results**

#### **4.3.1. Effect of C/N ratio on bioreactor performance**

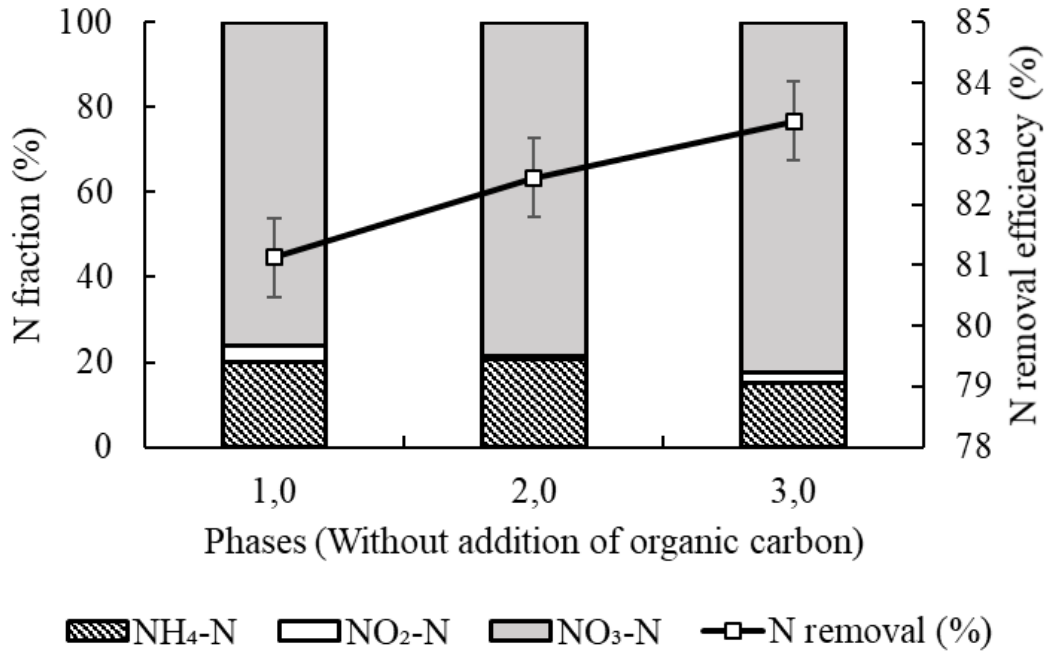
##### **4.3.1.1. Effect of C/N ratio on Nitrogen removal**

In UASB-B (experimental reactor), the introduction of low concentration of sodium acetate (139.2 mg/L) (C/N=1, Phase 1) caused an increase in average %N-removal to approximately 82% with high  $\text{NO}_3^-$ -N (74%) and low  $\text{NH}_4^+$ -N (25%) effluent concentrations (Figure 4.1). At this time, the control reactor (UASB-A) had an average %N-removal of approximately 81%, which was almost similar to UASB-B (Figure 4.2). It was observed that the %N-removal in UASB-B increased to 88% in Phase II when the C/N ratio was increased to 1.5, while there

was no significant change in the control reactor, which only showed a slight increase (82%). The theoretical ratios in UASB-B showed an increase in  $\text{NO}_2^-$ -N removal efficiency and a decrease in  $\text{NH}_4^+$ -N removal efficiency. However, a further increase in the C/N ratio to 2 caused a decrease in %N-removal to approximately 83 % in UASB-B. On the other hand, the control reactor showed consistent N removal performance with a slight increase during this time (Figure 4.2). Overall, significant variations in NREs were observed in the UASB-B reactor during the study period (ANOVA,  $p < 0.005$ ), compared to UASB-A (control).



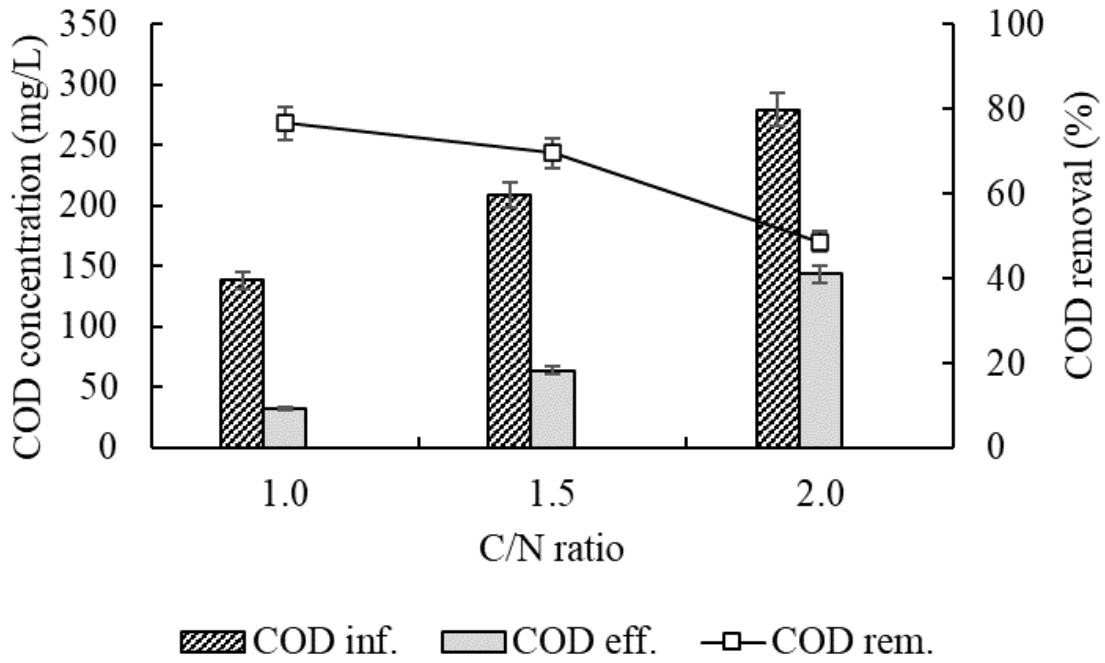
**Figure 4.1:** Effect of C/N ratio on N removal in UASB-B



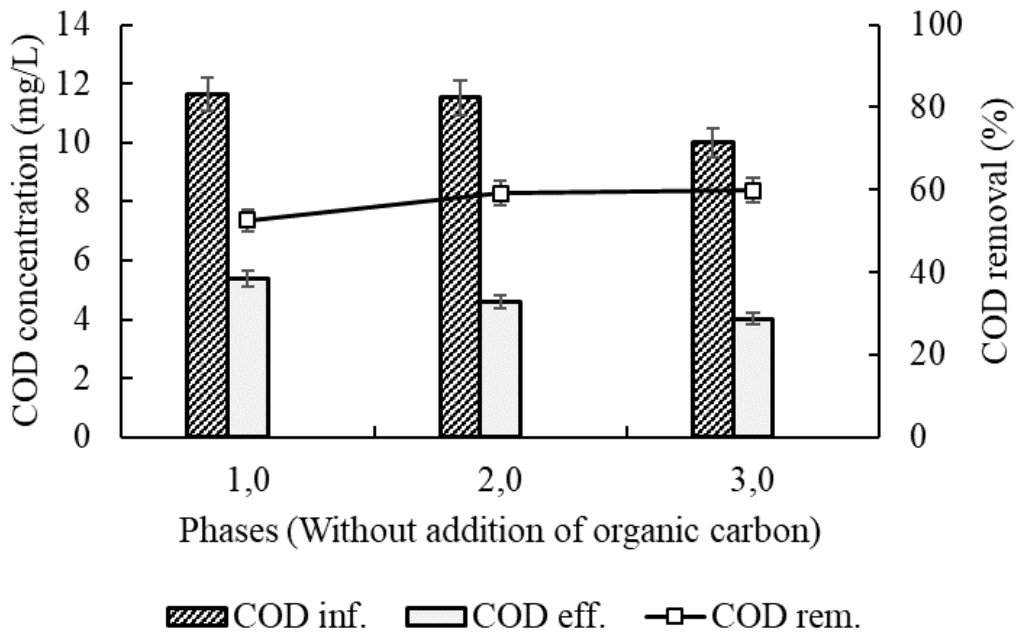
**Figure 4.2:** N removal performance of ANAMMOX control reactor (UASB-A)

#### 4.3.1.2. Effect of C/N ratio on COD removal

The effect of the C/N ratio on COD removal performance is shown in Figure 4.3. A reduction of approximately 85.5% COD was obtained in phase 1 (C/N=1) of the study. During this period, the average effluent COD concentration was ~30 mg/L. When sodium acetate concentration was increased from 139.2 mg/L to 208.8 mg/L in phase 2 (C/N=1.5), effluent COD concentration was slightly increased to an average of  $\geq 50$  mg/L. The %COD removal also slightly decreased with increasing organic matter concentrations (Figure 4.3). In phase 3 (C/N=2), when the influent sodium acetate concentration was increased to 278.4 mg/L, there was also a sharp increase of effluent COD concentration to an average of  $\geq 130$  mg/L. In addition, % of COD removal continued to decrease to approximately 45% with an increase of organic matter.



**Figure 4.3:** Effect of C/N ratio on COD removal (UASB-B)



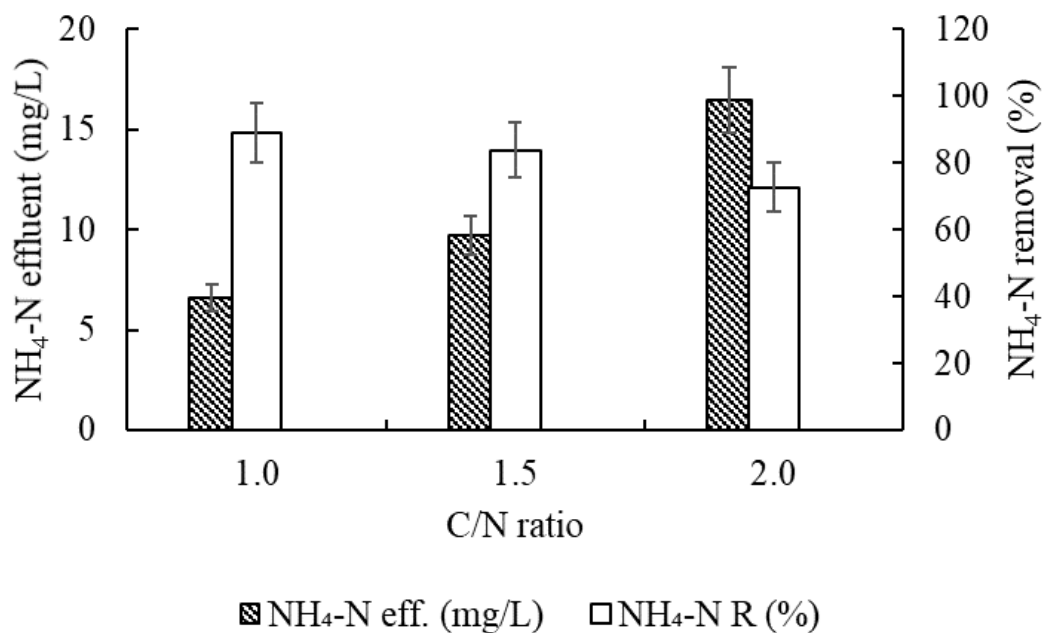
**Figure 4.4:** COD removal performance of ANAMMOX control reactor (UASB-A)

Though COD results were measured in UASB-A (control), there were very low or no COD concentrations detected because no organic carbon was introduced into the influent media

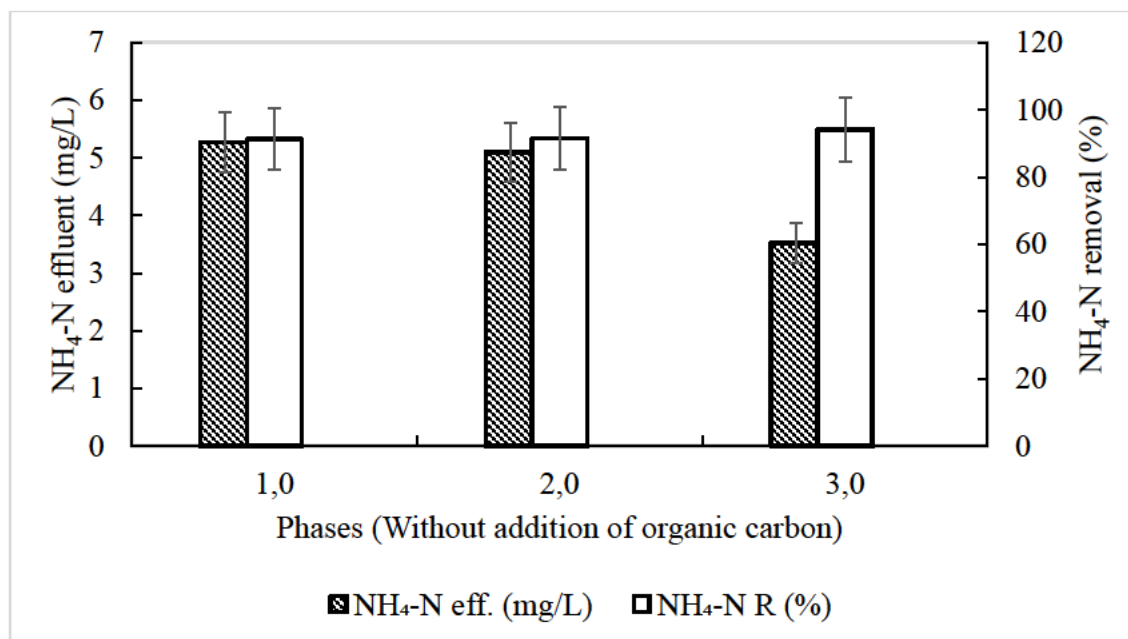
(Figure 4.4). Overall, significant variation in COD removal was observed in UASB-B (experimental reactor) during the study period (ANOVA,  $p < 0.005$ ).

#### 4.3.1.3. Effect of C/N ratio on $\text{NH}_4\text{-N}$ removal

Figure 4.5 shows the effect of the C/N ratio on  $\text{NH}_4\text{-N}$  removal. In phase 1 (C/N=1) at 139.2 mg/L influent concentration of sodium acetate, approximately 92 % of  $\text{NH}_4\text{-N}$  removal was observed. At this point, the average effluent  $\text{NH}_4\text{-N}$  concentration was 6.5 mg/L in UASB B. However, an increase of the C/N ratio to 1.5 in phase 2 caused a decrease in % $\text{NH}_4^+\text{-N}$  removal to 83 % as well as an increase in effluent  $\text{NH}_4\text{-N}$  concentration with an average of 9.5 mg/L in UASB B. In the control reactor (UASB A), % $\text{NH}_4^+\text{-N}$  removal remained at 91%, while an effluent  $\text{NH}_4\text{-N}$  concentration showed a slight decrease to 5 mg/L during this period. In phase 3, when the C/N ratio was further increased to 2,0 a sharp increase in effluent  $\text{NH}_4^+$  concentration to 16.45 mg/L and a decrease in % $\text{NH}_4^+\text{-N}$  removal to 72 % was observed in UASB B. The control reactor (UASB-A) showed a high % $\text{NH}_4^+\text{-N}$  removal of 94% in the last phase, compared to UASB-B (72 %).  $\text{NH}_4^+$  effluent concentration was also decreased to 16.45 mg/L in the control reactor (UASB-A).



**Figure 4.5:** Effect of C/N ratio on  $\text{NH}_4^+$ -N removal in ANAMMOX process (UASB-B)



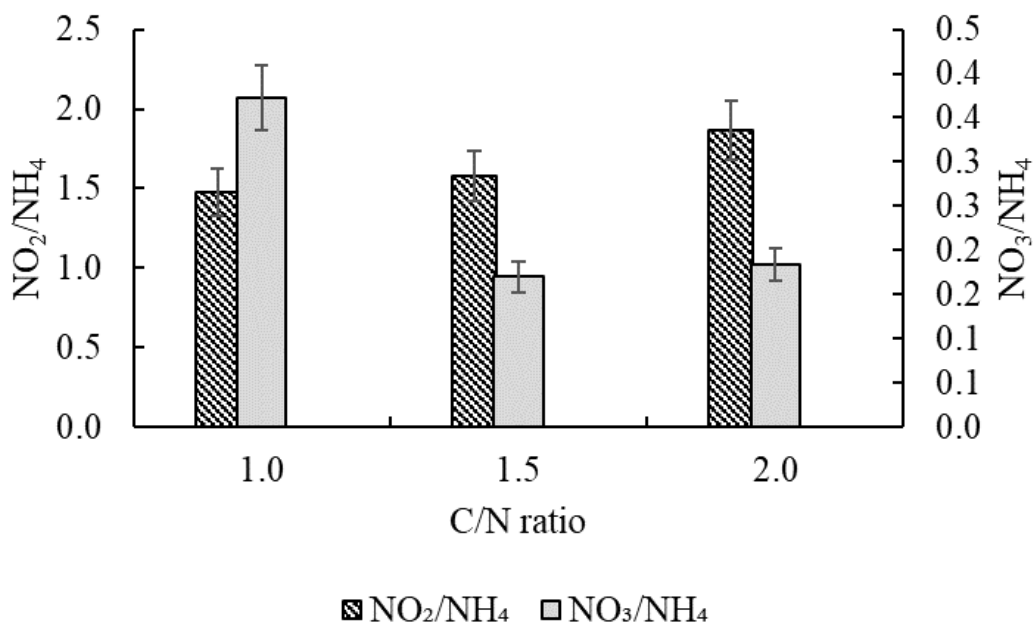
**Figure 4.6:**  $\text{NH}_4^+$ -N removal performance in ANAMMOX control reactor (UASB-A)

Overall, significant variation in  $\text{NH}_4^+$ -N removal was observed in UASB-B reactor during the study period (ANOVA,  $p < 0.005$ ), compared to UASB-A (control).

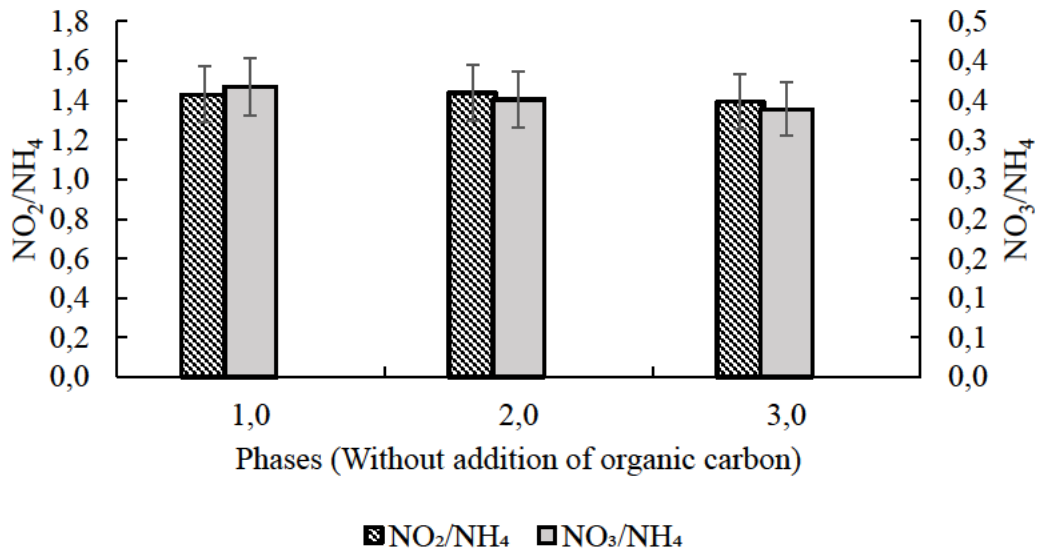
#### 4.3.1.4. Effect of C/N ratio on $\text{NO}_2^-/\text{NO}_3^-$ profiles

The  $\text{NO}_2^-/\text{NO}_3^-$  profiles under different C/N ratio in UASB-B is shown in Figure 4.7. The average ratios of  $\text{NO}_2^-$  consumed per  $\text{NH}_4^+$  consumed ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and  $\text{NO}_3^-$  produced per  $\text{NH}_4^+$  ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) consumed significantly varied in all 3 phases (ANOVA,  $p < 0.005$ ). The  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios during Phase 1 (C/N=1) fluctuated from  $>1.25$  to  $<1.6$  and from  $>0.35$  to  $<0.45$ , respectively. The average ratios of  $\text{NO}_2^-$  consumed per  $\text{NH}_4^+$  consumed ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and  $\text{NO}_3^-$  produced per  $\text{NH}_4^+$  ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) consumed had no significant variation in the control reactor (Figure 4.11) (ANOVA,  $p > 0.005$ ). In the control reactor (UASB-A), phase 1, the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratio was 1.43, and the  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  was 0.37 (Figure 4.8). In phase 2 of UASB-B (C/N=1.5), when organic carbon was increased to 208.8 mg/L (C/N=1.5),  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios approached the stoichiometric ratios

expected of ANAMMOX process. The  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  fluctuated from  $<1.4$  to  $>1.7$ , while  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios fluctuated from  $>0.22$  to  $<0.28$ . The  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  ratios of the control reactor in phase 2 remained close to the stoichiometric ratios of the ANAMMOX process. In phase 3 (C/N=2), a decrease in the  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  ratios were observed, reaching  $0.22\pm 0$ . The  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  average ratio was slightly lower than the stoichiometric ratio for ANAMMOX (0.26), while the  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  average ratio was higher than the expected stoichiometry ratio (1.32).



**Figure 4.7:** Effect of C/N ratio on the ratios of  $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$  and  $\text{NO}_3^- \text{-N}/\text{NH}_4^+ \text{-N}$  (UASB-B).

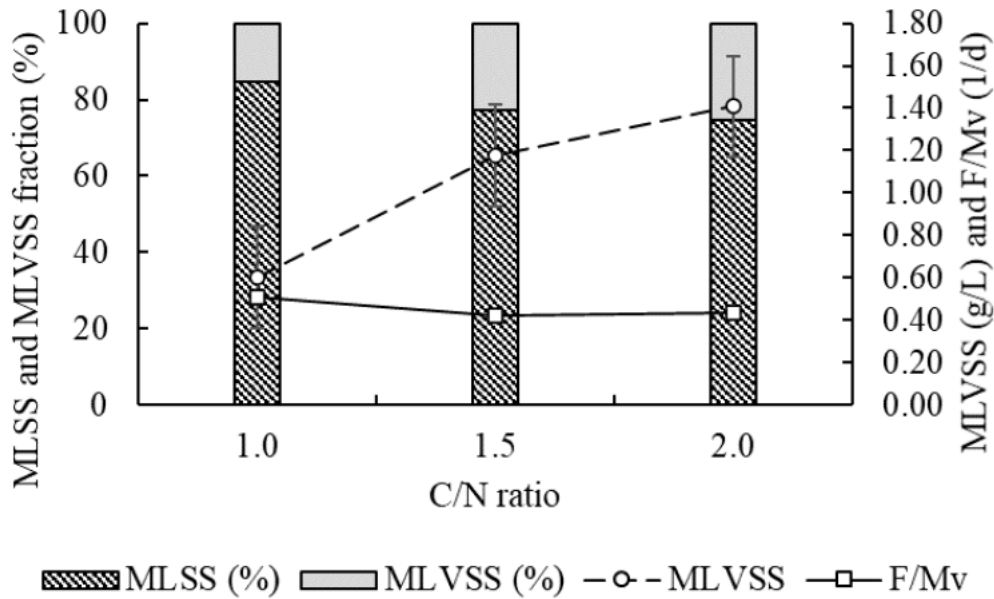


**Figure 4.8:** The ratios of  $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$  and  $\text{NO}_3^- \text{-N}/\text{NH}_4^+ \text{-N}$  in ANAMMOX control reactor (UASB-A).

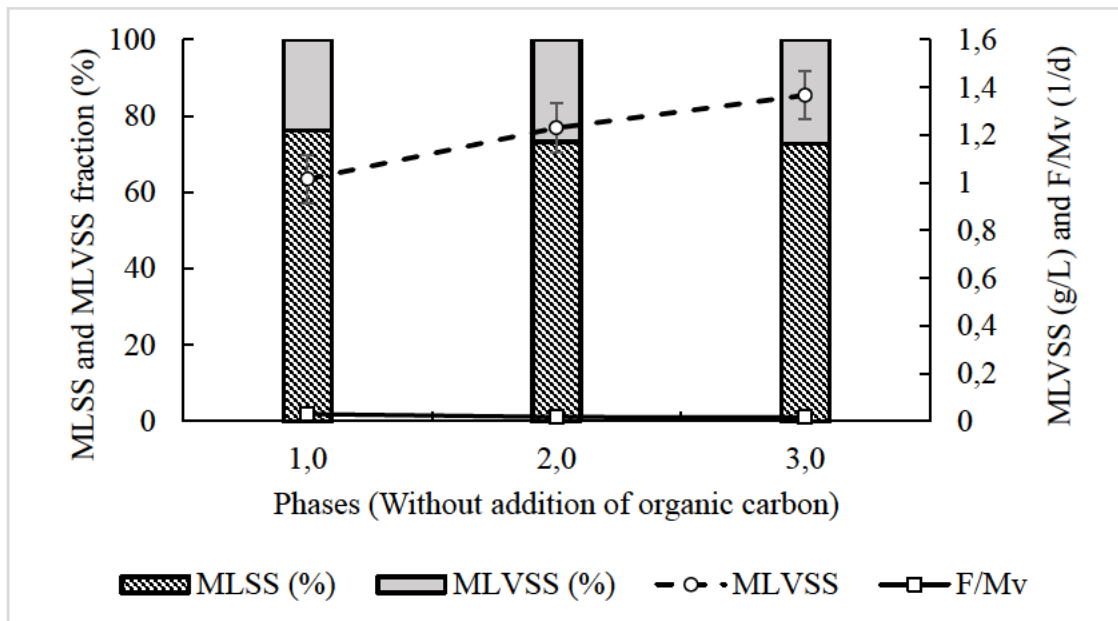
The control reactor had no significant variation in  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$  through all the phases of the C/N ratio (ANOVA,  $p > 0.005$ ), while there was significance in UASB-B reactor  $\text{NO}_2/\text{NO}_3$  profiles variations (ANOVA,  $p < 0.005$ ).

#### 4.3.1.5. Effect of C/N ratio on biomass concentration

The effect of the C/N ratio on biomass concentration is shown in Figure 4.9. Approximately 85% of MLSS and 15% of MLVSS were obtained in phase 1 of the C/N ratio (C/N=1). The control reactor, on the other hand, had 76% of MLSS and 24% MLVSS. In phase 2, an increase of the C/N ratio to 1.5 showed a slight decrease of %MLSS to less than 80% but greater than 75%, while MLVSS showed an increase to 22%. Similar behaviour was observed in the control reactor, which showed a slight decrease in MLSS while there was a slight increase in MLVSS (Figure 4.10). In phase 3, a further increase of the C/N ratio to 2 caused a further decrease of MLSS to approximately 72%, while MLVSS continued to increase slightly to approximately 28%.



**Figure 4.9:** Effect of C/N ratio on biomass concentration (MLSS and MLVSS) in UASB-B

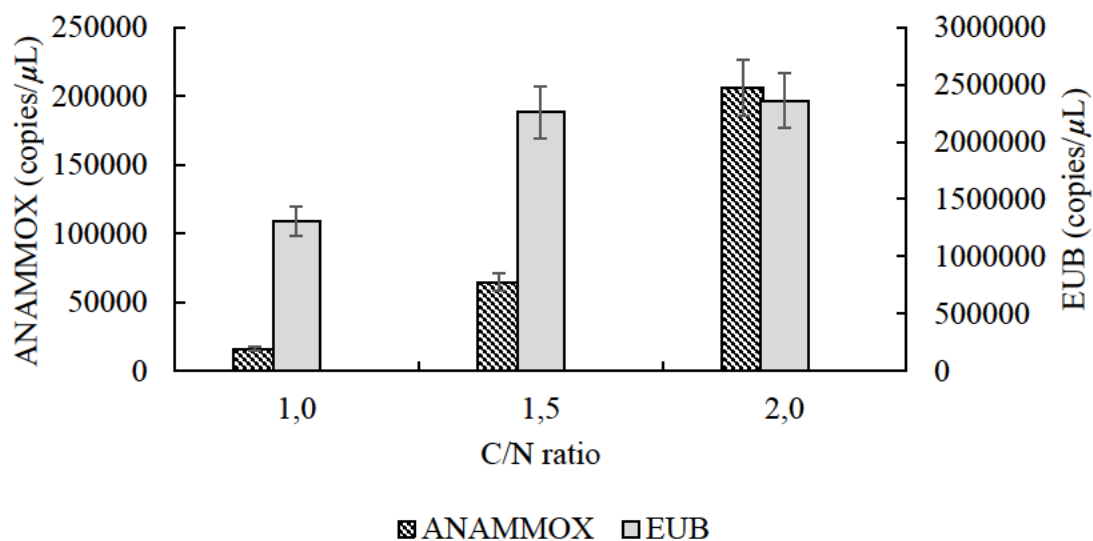


**Figure 4.10:** Effect of C/N ratio on biomass concentration (MLSS and MLVSS) in control reactor (UASB-A)

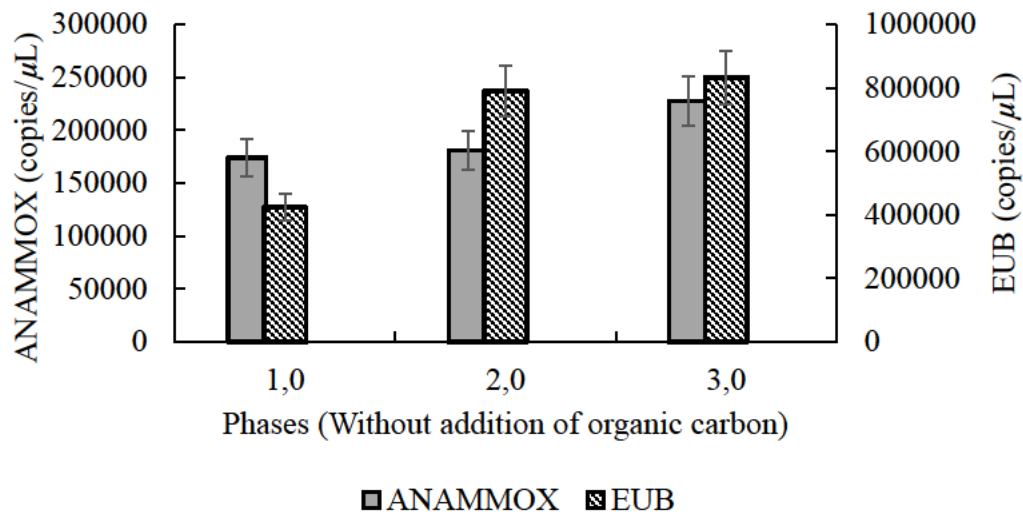
#### 4.3.2. Microbial analysis using quantitative PCR

Quantitative PCR was performed to track the dominance of ANAMMOX, AOB, NOB and EUB or heterotrophic bacterial populations during the 3 phases of reactor operation using

group-specific gene targets. Microbial analysis in the control (UASB-A) reactor is shown in Figure 4.12 and Figure 4.14. In the control reactor, the average total eubacterial load showed an increase from  $4.2 \times 10^5$  copies/ $\mu\text{L}$  to  $7.9 \times 10^5$  copies/ $\mu\text{L}$  from the first 60 days to 120 days, respectively. ANAMMOX bacteria also showed an increase from  $1.7 \times 10^5$  copies/ $\mu\text{L}$  to  $1.8 \times 10^5$  copies/ $\mu\text{L}$  in the control reactor during the first 60 days (Figure 4.12). A slight increase in the average EUB population to  $8.3 \times 10^5$  copies/ $\mu\text{L}$  was observed in phase 3. A further slight increase in the average ANAMMOX bacterial population to  $2.1 \times 10^5$  copies/ $\mu\text{L}$  was also observed during this phase. In the experimental reactor (UASB B), the average total eubacterial load showed a sharp increase from  $1.3 \times 10^6$  copies/ $\mu\text{L}$  to  $2.3 \times 10^6$  copies/ $\mu\text{L}$  when the C/N ratio was increased from 1 to 1.5 in phase 2, respectively (Figure 4.11). ANAMMOX bacteria also showed an increase from 16 copies/ $\mu\text{L}$  to  $6.5 \times 10^2$  copies/ $\mu\text{L}$  during this period (Figure 4.11). Thereafter a slight increase in the average EUB population to  $2.4 \times 10^6$  copies/ $\mu\text{L}$  was observed when the C/N ratio was further increased to 2 in phase 3. A very sharp increase in the average ANAMMOX bacterial population to  $2.1 \times 10^4$  copies/ $\mu\text{L}$  was also observed during this phase. Overall, significant variations of ANAMMOX and EUB abundances in 3 phases of the C/N ratio were observed (ANOVA,  $p < 0.005$ ) compared to the control reactor, which had no significant alterations.

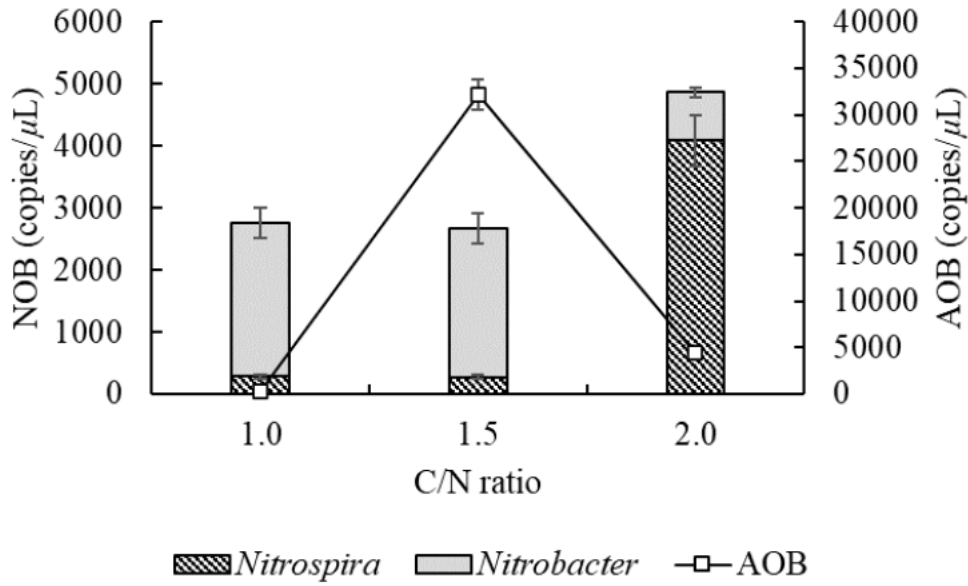


**Figure 4.11:** Effect of C/N ration on microbial analysis; qPCR of ANAMMOX and EUB populations.

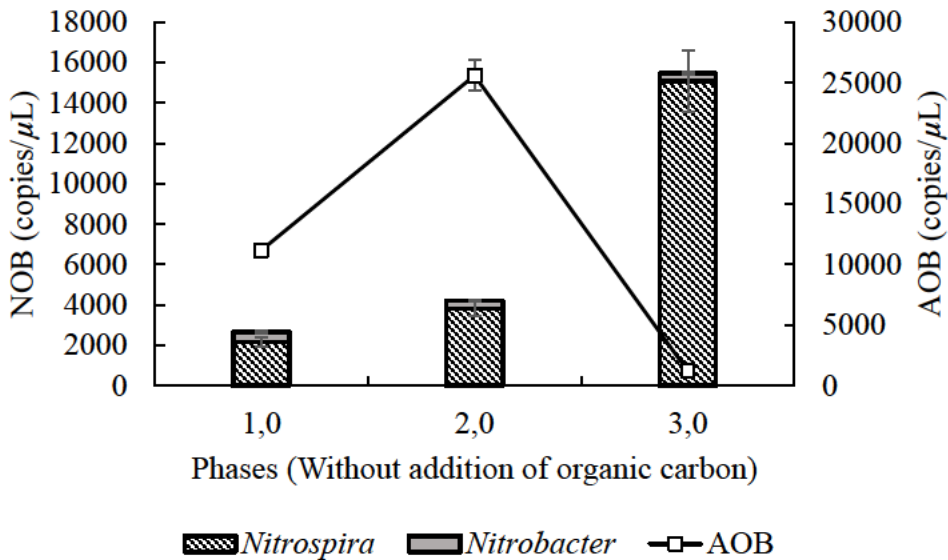


**Figure 4.12:** Microbial analysis; qPCR of ANAMMOX and EUB populations in ANAMMOX control reactor.

Among NOBs, *Nitrobacter spp.* showed a decrease from phase 1 to phase 2, as observed with other populations when the C/N ratio was altered from 1 to 1.5 (Figure 4.13); however, it should be noted that the starting quantity of *Nitrobacter spp.* was already higher than the other nitrifiers. The *Nitrobacter spp.* population decreased from  $2.5 \times 10^3$  copies/μL to  $2.4 \times 10^3$  copies/μL after the C/N ratio adjustment from 1 to 1.5, respectively. The *Nitrospira spp.* population also displayed a characteristic decrease, slightly, from 280 copies/μL to 276 copies/μL when the C/N ratio was adjusted from 1 to 1.5, respectively, while AOB showed a sharp increase from  $2.3 \times 10^2$  copies/μL to  $3.2 \times 10^4$  copies/μL, respectively. Further adjustment of the C/N ratio to 2 in phase 3 caused a further decrease of *Nitrobacter spp.* population to 780 copies/μl. The *Nitrospira spp.* population, however displayed an increase to  $4.1 \times 10^3$  copies/μL after the C/N ratio was further increased to 2. A sharp decrease in AOB to  $4.5 \times 10^3$  copies/μL was observed in phase 3 after the C/N ratio was adjusted to 2.



**Figure 4.13:** Microbial analysis; qPCR of *Nitrobacter spp.*, *Nitrospira spp.* and AOB populations.



**Figure 4.14:** Microbial analysis; qPCR of *Nitrobacter spp.*, *Nitrospira spp.* and AOB populations in ANAMMOX control reactor.

The *Nitrobacter spp.* abundance was highest during the first phase (C/N=1) of the study, having about orders of magnitude above the AOB and *Nitrospira spp.* The changes in AOB, *Nitrospira*

*spp.* and *Nitrobacter spp.* abundance across the study period is shown in Figure 4.13 and Figure 4.14 for both UASB-B and control reactor, respectively.

### 4.3.3. cDNA quantification

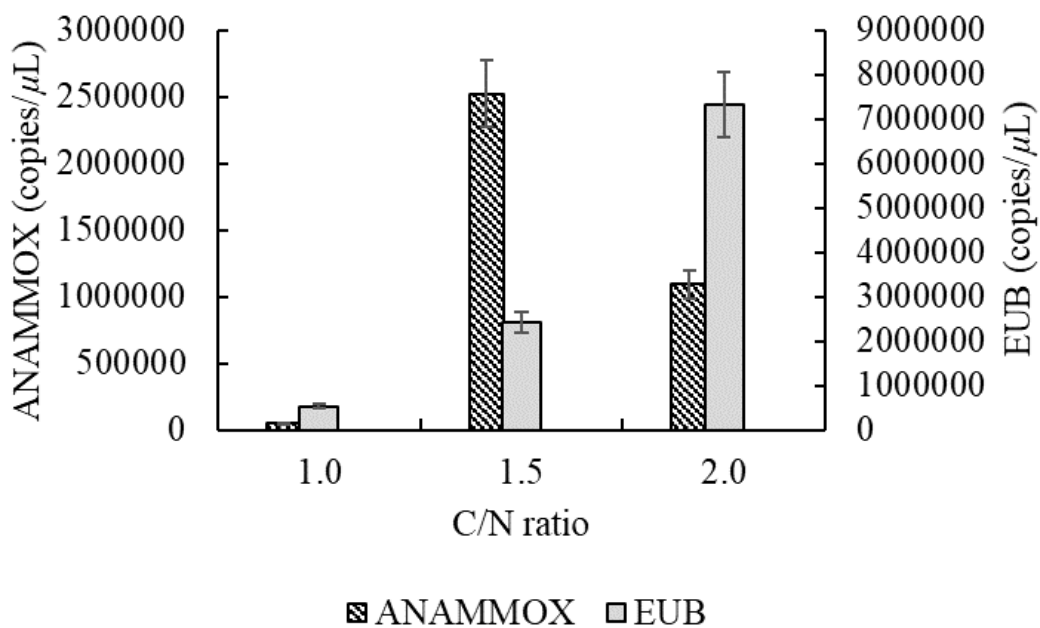
cDNA quantification was performed to track the quantities of transcripts abundances of ANAMMOX, AOB, NOB and total EUB populations during the 3 phases of the C/N ratio using primer sets in Table 3.3. To ensure the accuracy of the cDNA quantification, only data that matched the quality control parameters were used in the quantification for each population (Table 4.2).

**Table 4.2:** Summary of cDNA quantification qPCR standard curves and amplification quality for each of the key microbial populations

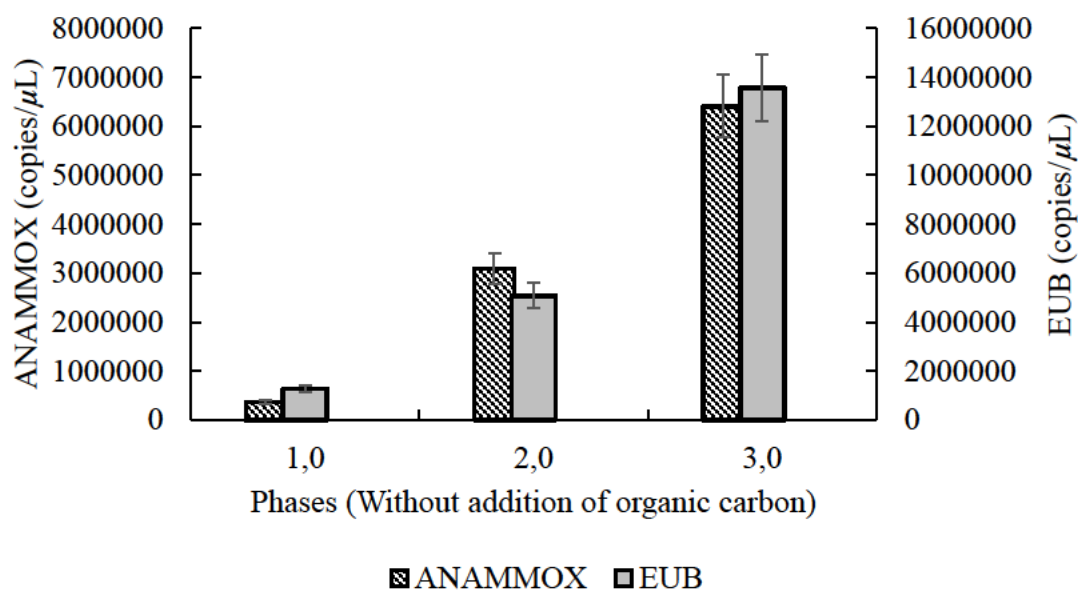
<i>Parameter</i>	<i>ANAMMOX</i>	<i>AOB</i>	<i>Nitrobacter spp.</i>	<i>Nitrospira spp.</i>	<i>EUB bacteria</i>
<i>Efficiency (%)</i>	86.4	98.2	95.8	99.0	100.3
<i>R<sup>2</sup></i>	1.000	0.997	0.997	0.999	0.971
<i>Slope</i>	-3.696	-3.365	-3.427	-3.345	-3.315
<i>y-intercept</i>	35.175	33.201	35.734	35.584	34.760

An increasing transcript abundance of total bacteria and ANAMMOX bacteria was observed from phase 1 to phase 2 of the experimental reactor (UASB-B) when the C/N ratio was changed from 1 to 1.5, respectively (Figure 4.15). The control reactor (UASB A) also showed an increase of ANAMMOX bacteria and EUB transcript abundances between these two phases (Figure 4.16). In UASB-B, the abundance of ANAMMOX bacteria showed a sharp increase from an average of  $4.6 \times 10^4$  copies/ $\mu\text{L}$  to approximately  $2.5 \times 10^6$  copies/ $\mu\text{L}$  when the C/N ratio was altered from 1 to 1.5, and EUB also showed an increase after the organic carbon was increased. The abundance of EUB increased from an average of  $5.2 \times 10^5$  copies/ $\mu\text{L}$  to approximately  $2.4 \times 10^6$  copies/ $\mu\text{L}$  when the C/N ratio was altered from 1 to 1.5 (Figure 4.15).

In the control reactor, the abundance of ANAMMOX bacteria showed an increase from an average of  $3.6 \times 10^5$  copies/ $\mu\text{L}$  to approximately  $3.0 \times 10^6$  copies/ $\mu\text{L}$  from phase 1 to phase 2, and EUB also showed an increase from  $1.3 \times 10^6$  copies/ $\mu\text{L}$  to approximately  $5.1 \times 10^6$  copies/ $\mu\text{L}$  (Figure 4.16). However, a sharp decrease of ANAMMOX bacteria transcript abundance to an average of approximately  $1.1 \times 10^6$  copies/ $\mu\text{L}$  was observed when the C/N ratio was further increased to 2 in phase 3. EUB transcript abundance continued to show a great increase to approximately  $7.3 \times 10^6$  copies/ $\mu\text{L}$ , in phase 3 (C/N=2). ANAMMOX bacteria and EUB in the control reactor, however continued to increase in phase 3 to an average of  $6.4 \times 10^6$  copies/ $\mu\text{L}$  and  $1.4 \times 10^7$  copies/ $\mu\text{L}$ , respectively.



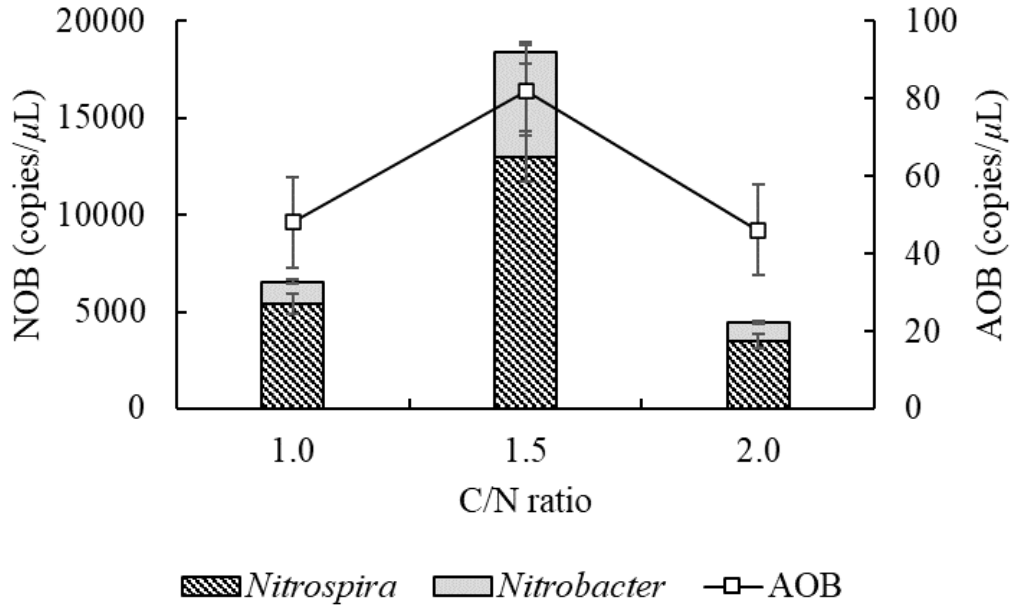
**Figure 4.15:** The effect of C/N ratio on transcript abundance of ANAMMOX and EUB populations



**Figure 4.16:** Transcript abundance of ANAMMOX and EUB populations in ANAMMOX control reactor

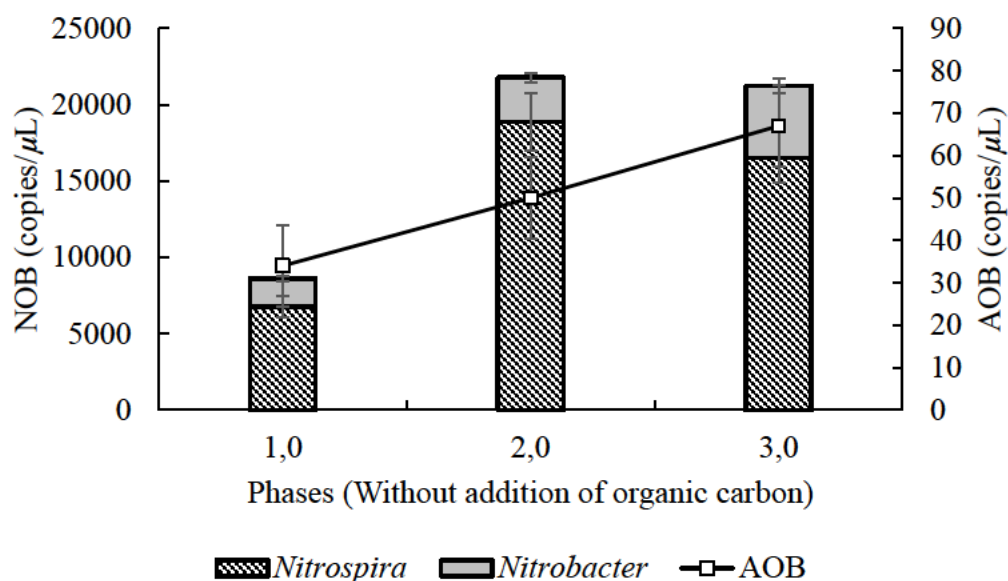
Overall, a significant variation of ANAMMOX and EUB transcript abundances in UASB-B was observed (ANOVA,  $p < 0.005$ ) under different C/N ratios, compared to the control reactor (UASB-A).

The *Nitrobacter spp.*, *Nitrospira spp.* and AOB population also displayed the characteristic fluctuation of transcript abundance when the sodium acetate concentration to nitrogen (C/N) ratio was altered from 1 to 1.5, then to 2 in respective of phase 1, phase2 and phase 3 (Figure 4.17) . The average transcript abundance of *Nitrobacter spp.*, *Nitrospira spp.* and AOB increased from  $1.2 \times 10^3$  copies/μL to  $5.4 \times 10^3$  copies/μL, from  $5.4 \times 10^3$  copies/μL to  $1.3 \times 10^4$  copies/μL and from 48 copies/μL to 82 copies/μL, respectively, when C/N ratio was altered from 1 to 1.5. When the C/N ratio was further increased to 2, the abundance of *Nitrobacter spp.*, *Nitrospira spp.* and AOB populations showed a decrease to  $9.8 \times 10^2$  copies/μL,  $3.5 \times 10^3$  copies/μL and 46 copies/μL, respectively.



**Figure 4.17:** The effect of C/N ratio on transcript abundance of *Nitrobacter spp.*, *Nitrospira spp.* and AOB populations.

In the control reactor, the *Nitrobacter spp.*, *Nitrospira spp.* and AOB population also displayed the characteristic fluctuation of transcript abundance during the operation of the UASB reactor without organic carbon (Figure 4.18). The average transcript abundance of *Nitrobacter spp.*, *Nitrospira spp.* and AOB increased from  $1.8 \times 10^3$  copies/μL to  $2.9 \times 10^3$  copies/μL,  $6.8 \times 10^3$  copies/μL to  $1.9 \times 10^4$  copies/μL, and 34 copies/μL to 50 copies/μL, respectively, from phase 1 to phase 2. Further operation of the control reactor to phase 2, the *Nitrobacter spp.* and AOB populations transcript abundance continued with an increase to an average of  $4.7 \times 10^3$  copies/μL and 67 copies/μL, respectively. A decrease in *Nitrospira spp.* transcript abundance to an average of  $1.7 \times 10^2$  copies/μL. Overall, significant variations of NOB and AOB transcript abundances in 3 phases of the C/N ratio and in the control reactor was observed (ANOVA,  $p < 0.005$ ).



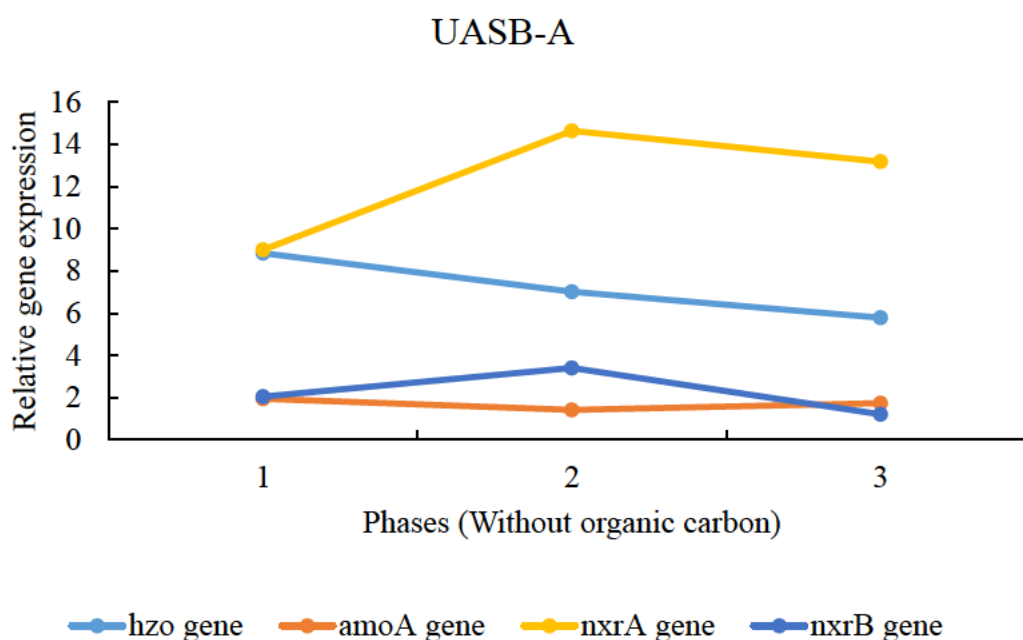
**Figure 4.18:** Transcript abundance of *Nitrobacter spp.*, *Nitrospira spp.* and AOB populations in ANAMMOX control reactor.

#### 4.3.4. Reverse Transcriptase-qPCR (RT-qPCR)

The relationship between nitrogen removal and functional gene expression was investigated using 16S rRNA as a reference gene and target functional genes for ANAMMOX bacteria, AOB and NOB (*Nitrobacter spp.* and *Nitrospira spp.*). Both housekeeping and functional gene primers for these nitrifying bacteria are listed in Table 3.3 of Chapter 3. The relative gene expression of microbial populations in the control reactor (UASB-A) and experimental reactor (UASB-B) is shown in Figure 4.19 and Figure 4.20, respectively.

In the control reactor, hzo gene expression for ANAMMOX had a level 8.8 folds of expression in phase 1, almost equal to nxr A gene for *Nitrobacter spp.*, which was 9 folds. Nxr B and amoA genes for *Nitrospira spp.* and AOB were 2 and 1.9 folds, respectively (Figure 4.19). There were no significant changes in hzo, amoA and nxrB genes throughout phase 2 and phase 3 of the study. nxrA showed a slight increase to 14.6 folds in phase 2, which was expressed more than all other studied genes. In the last phase, nxrA showed a slight decrease to 13.2 folds, while nxrB and amoA remained at 1.2 and 1.7 folds which had no significant difference

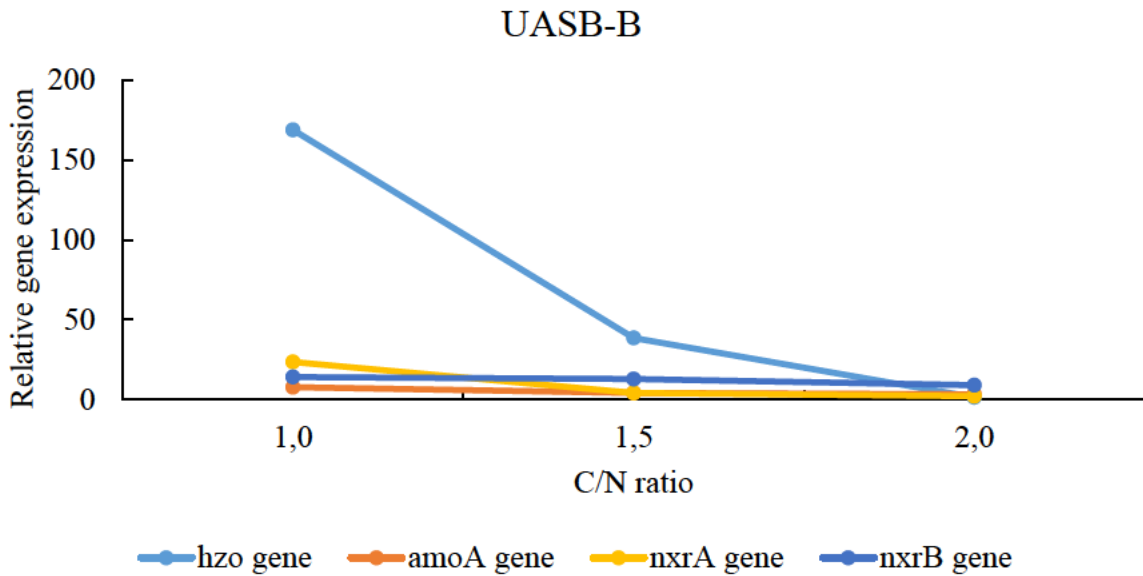
throughout the study. The *hzo* gene expression showed a slight decrease to 5.8 folds towards the end of phase 3, but the change was insignificant.



**Figure 4.19:** RT-qPCR temporal changes in functional genes *hzo*, *amoA*, *nxrA* and *nxrB* in control reactor (UASB-A) during this study.

In the experimental reactor (UASB-B), the expression level *hzo* (ANAMMOX) was 168.9 folds in phase 1, compared to the control reactor. This is supported by the high nitrogen removal performance observed in Figure 4.1 during the initial phase of the C/N ratio (C/N=1). The level of *nxrA*, *nxrB* and *amoA* genes for *Nitrobacter spp.*, *Nitrospira spp.* and AOB were 23.56, 14.12 and 7.78 folds, respectively, which were very low compared to *hzo* gene expression in phase 1. When the C/N ratio was increased to 1.5 *hzo* gene decreased to 38.59 folds of expression. *nxrA*, *nxrB* and *amoA* genes had no significant change at C/N=1.5 but showed a decrease to 4.17, 12.91 and 4.23 folds of expression. When the C/N ratio was further increased to 2 in phase 3, the *hzo* gene displayed near inhibition as it decreased to 1.47 folds of expression. The *nxrA*, *nxrB* and *amoA* genes had no significant variation throughout 3 phases

of C/N ratio but they showed a slight decrease to 2.14, 9.06 and 3.33 folds of expression in phase 3 (C/N=2).



**Figure 4.20:** RT-qPCR temporal changes in functional genes hzo, amoA, nxrA and nxrB in experimental reactor (UASB-B) during 3 phases of C/N ratio.

## 4.4. Discussion

### 4.4.1. Effect of C/N ratio on the operation of UASB reactor

The effect of sodium acetate as a source of organic carbon in synthetic wastewater on nitrogen removal performance is presented in this chapter. Ni *et al.* (2012) reported that organic matter is believed to cause some adverse effects on ANAMMOX bacteria. Another report by Jin *et al.* (2012) stated that the organic carbon compounds are toxic to ANAMMOX bacteria even at low concentrations. However, in this study, the addition of an initial low concentration of organic carbon in phase 1 (C/N=1), caused an increase of %N-removal with high  $\text{NO}_3^-$ -N and low  $\text{NH}_4^+$ -N (Figure 4.5) in the effluents, indicating a rise in ANAMMOX activity. Almost similar %N-removal was observed in the control reactor (Figure 4.2). During this period in the experimental reactor (UASB-B), the  $\Delta\text{NO}_2/\Delta\text{NH}_4^+$  ratio was 1.35, which was close to the

theoretical value of 1.32 for ANAMMOX bacteria (Wang *et al.*, 2020b). In this case, it may be because organic carbon introduced to the ANAMMOX bioreactor at lower concentrations had no adverse effects on the bacteria in the bioreactor. Also, approximately 92 % of  $\text{NH}_4^-\text{N}$  removal was observed in phase 1 of the experimental reactor, and high effluent nitrate indicated a good ANAMMOX process in the system. These findings confirm that a lower concentration of organic matter in the ANAMMOX process could support their activity. In addition, a high COD removal in phase 1 following the addition of sodium acetate was observed, which might be because of organic carbon consumption by denitrification (Leal *et al.*, 2016). The presence of low organic matter in the bioreactor is believed to stimulate denitrifying bacterial growth (Bonassa *et al.*, 2021). He *et al.* (2018) reported that organic matter added to the ANAMMOX system increased NRE due to the synergistic interaction between denitrifying bacteria and ANAMMOX. It has been demonstrated that denitrifying bacteria can convert produced  $\text{NO}_3^-\text{N}$  to  $\text{NO}_2^-\text{N}$  at low organic matter concentrations and that ANAMMOX bacteria can then utilize the produced  $\text{NO}_2^-\text{N}$  together with  $\text{NH}_4^+\text{N}$  as electron donors (Li *et al.*, 2017). Significant changes in MLSS and MLVSS levels were also observed. MLVSS and MLSS increased with increasing C/N ratios, indicating rapid biomass growth after introducing sodium acetate (Krishna Mohan *et al.*, 2016). When the C/N ratio was increased to 1.5 in phase 2, the %N-removal continued to increase, and the theoretical ratios showed an increase in  $\text{NO}_2^-\text{N}$  removal efficiency and a decrease in  $\text{NH}_4^+\text{N}$  removal efficiency. Also, a decrease of  $\text{NO}_3^-\text{N}$  concentration in the effluent was observed in this phase, indicating a possible consumption of  $\text{NO}_3^-\text{N}$  by denitrifying bacteria (He *et al.*, 2018). A gradual decrease in the COD removal efficiency was also observed, with a decrease in  $\text{NH}_4^+\text{N}$  removal. A reduction of the  $\text{NO}_3^-\text{N}/\text{NH}_4^+\text{N}$  ratio was observed following an increase of the C/N ratio to 1.5, while a gradual increase of  $\text{NO}_2^-\text{N}/\text{NH}_4^+\text{N}$  was also observed.

The ratio of  $\text{NO}_2^- \text{-N} / \text{NH}_4^+ \text{-N}$  increased to 1.49, which continued to be higher than the theoretical value of 1.32, and the ratio of  $\text{NO}_3^- \text{-N} / \text{NH}_4^+ \text{-N}$  decreased to 0.26, which was close to the expected stoichiometric ratio in phase 2 ( $\text{C/N}=1.5$ ). This is because the denitrifying bacteria converted  $\text{NO}_3^- \text{-N}$  produced by the ANAMMOX process to  $\text{N}_2$  gas in the presence of organic matter (Eq. 2.2). Although there was a slight increase of  $\text{NO}_2^- \text{-N} / \text{NH}_4^+ \text{-N}$  ratio in phase 2, the  $\text{NH}_4^+ \text{-N}$  removal and NRE were 84% and 94%, respectively (Figure 4.5 and Figure 4.1). This is because although part of  $\text{NO}_2^- \text{-N}$  may have been converted by denitrifying bacteria, the ANAMMOX bacteria was still contributing to nitrogen removal (Li *et al.*, 2017). These findings correspond to the previous study performed by He *et al.* (2018), who found that although the ANAMMOX process remained the main nitrogen removal reaction operating in this system, heterotrophic denitrification was also observed to play an important role in utilizing organic carbon to produce nitrogen gas, using nitrite as an electron acceptor.

A decrease in the average MLSS was observed from phase 1 to phase 2 when the sodium acetate concentration or C/N ratio was increased. Although the performance of the ANAMMOX process was good at this phase, the decrease in overall MLSS can also be attributed to the loss of biomass during sample collection for different analyses. A slight increase in MLVSS was observed, which might be due to the insufficient wasting of solids leading to the accumulation of inorganic solids (Guo *et al.*, 2022b). This could also be because of the fact that the reactor was supplemented with carrier particles to prevent the biomass washout (Figure 3.1).

The increase of the C/N ratio to 2 in phase 3 caused a sudden decrease of %N-removal. The ANAMMOX activity has been reported to be inhibited at high concentrations of organic matter (Chen *et al.*, 2016, Li *et al.*, 2017, He *et al.*, 2018). Chen *et al.* (2016) observed a decrease in  $\text{NH}_4^+ \text{-N}$  removal efficiency and NRE to about 20% and 65%, respectively, at COD/N of 1.71. Similarly, He *et al.* (2018) observed a decrease in  $\text{NH}_4^+ \text{-N}$  removal efficiency and NRE to as

low as  $55.57 \pm 3.76\%$  and  $82.02 \pm 3.14\%$ , respectively, under elevated COD/N ratio. When organic matter is high, it is possible for fast-growing heterotrophic bacteria to outcompete ANAMMOX bacteria (Wang *et al.*, 2019c), or the substrate may be toxic at higher concentrations. A study by Yang *et al.* (2019) indicates that organic matter can inhibit the ANAMMOX process through a number of different mechanisms: non-toxic organic matter stimulates rapid growth of denitrifying bacteria, which competes with ANAMMOX bacteria for the substrate nitrite, and toxic inhibition, where the ANAMMOX bacteria are directly inhibited by toxic organic matter in the reactor. Similarly, the COD removal dropped to 48.5%. Furthermore, it is important to note that throughout the different phases of the reactor, the influent  $\text{NO}_2^-$ -N concentration was fixed at 79.2 mg/L (Table 4.1), which was sufficient for the ANAMMOX process; consequently, the electron acceptor did not support the growth of denitrifying bacteria, resulting in a reduction in COD removal (He *et al.*, 2018). Likewise, the HRT of the reactor is also kept constant at different phases, which may also explain a low performance under a high C/N ratio (Kaźmierczak *et al.*, 2017). The organic load and biomass ratio are usually used to calculate the HRT when there is a higher organic load and if there is a need for increasing HRT. In this study, the HRT is usually kept high enough to support the growth of AOB while lowering NOB growth (van der Star *et al.*, 2007). At high HRT, the response of the microbial N-transformation pathways to the varied operational conditions imposed by the different C/N ratio is reported to be distinct (Suhr *et al.*, 2013).

In a study conducted by He *et al.* (2018) on the effect of the C/N ratio on COD removal, it was reported that the C/N ratio of 0.53 resulted in a high-efficiency reduction in COD removal. However, when sodium acetate concentration was increased to C/N=1.59, COD removal efficiency declined rapidly and then dropped to 44.21% when the ratio was increased further (He *et al.*, 2018). Likewise, another study by Wang *et al.* (2016) showed the COD removal efficiency was about 96.2% at C/N=0.33. When the C/N ratio was increased from 0.33 to 0.50,

the COD removal efficiencies increased to 97.9%. However, they observed a decrease in COD removal to 87.2% when the C/N ratio was further increased to 0.67 (COD up to 200 mg L<sup>-1</sup>)(Wang *et al.*, 2016). Furthermore, this study observed a decrease in ammonium removal from 83% to 70% under a high concentration of organic matter. The increasing organic matter has also decreased the NO<sub>3</sub><sup>-</sup>-N concentrations in the effluent because of a possible denitrification reaction (Ni *et al.*, 2012). Ni *et al.* (2012) also observed a decrease in NH<sub>4</sub><sup>+</sup>-N removal from almost 100% to 89.3 ± 1.4 when the organic matter concentration was increased to 200 mg/L. Few other studies have also reported that the ANAMMOX bacteria was affected by the presence of organic matter, leading to less ammonia removal (Nguyen and Tran, 2021). Similarly, Chen *et al.* (2016) observed that the NH<sub>4</sub><sup>+</sup>-N removal efficiency decreased to about 20% at a C/N of 1.71. He *et al.* (2018) observed that the NH<sub>4</sub><sup>+</sup>-N removal efficiency decreased sharply to as low as 55.57 ± 3.76% at C/N of 1.59 (945.85 mg/L sodium acetate concentration). All these studies have clearly indicated that the addition of organic matter promotes the shifting of the nitrogen removal process from ANAMMOX to denitrification (Chen *et al.*, 2016). In addition, the ratio of NO<sub>2</sub><sup>-</sup>-N/NH<sub>4</sub><sup>+</sup>-N was far higher than the theoretical ratio (1.32), getting to 1.8 ± 0.25. The use of NO<sub>2</sub><sup>-</sup>-N as an electron acceptor is favoured at high COD concentrations. This supports the findings by previous studies that the ANAMMOX process, to some extent, can be inhibited at elevated C/N ratio up to a certain level. A pH increase of approximately 8.9 was also observed during reactor operation under high C/N ratio, which was higher than the reported pH range of 6.5 to 8.0 for good ANAMMOX growth (Ibrahim *et al.*, 2015). The increase in pH in this study may be another factor contributing to the inhibition of biomass growth (He *et al.*, 2018), resulting in a further decrease in MLSS to 72% as well as an overall reduction in the performance of the reactor. The increase in pH could also lead to the loss of NH<sub>4</sub><sup>+</sup>-N through volatilization.

#### 4.4.2. The shift in key microbial activities within the reactor

Real-time PCR (qPCR) allows quantifying microbial populations by measuring gene copy numbers. qPCR can also estimate transcript abundance when combined with reverse transcription (RT), providing data on functional microbial activity (Postollec *et al.*, 2011). Currently, qPCR and Reverse Transcriptase qPCR (RT-qPCR) have become the methods of choice to quantify genes and gene expression or transcript abundance quantification, respectively (Lu *et al.*, 2018a). However, not many studies have been conducted on using qPCR and RT-qPCR to study the effect of the C/N ratio on ANAMMOX performance. The quantitative PCR results obtained in all 3 phases have shown that the increase in the C/N ratio caused a quick rise in the total bacteria, indicating a fast growth of heterotrophic bacteria. It was also observed that the ANAMMOX bacteria displayed an increase in phase 2 (C/N=1.5), indicating their ability to survive in low organic carbon concentrations competing for nitrite with denitrifying bacteria. This observation is also in line with the chemical data. Few other studies have also reported this observation (Bi *et al.*, 2015, Wang *et al.*, 2016, Wang *et al.*, 2019b). Nevertheless, such an environment could promote mutualistic growth of both Anammox and denitrifying bacteria. Denitrifiers can utilize the nitrate produced during the ANAMMOX reaction, and ANAMMOX bacteria may benefit from the nitrite produced by denitrifiers (Wang *et al.*, 2022a). In this study, the highest nitrogen removal was observed in phase 2, which also showed an increase in both the genomic (DNA) and transcript (cDNA) abundance of ANAMMOX bacteria and eubacteria. This beneficial combination has been explored in new ANAMMOX-based systems such as DEAMOX (Cao *et al.*, 2016).

A C/N ratio of 2 (phase 3) increased the genomic copy of ANAMMOX bacteria, but the cDNA quantities decreased in accordance with the nitrogen removal performance of the reactor. Increasing the C/N ratio to 2 decreased the ammonia removal, showing its effectiveness in decreasing key microbial activity such as ANAMMOX. In a similar study by Wang *et al.*

(2019), nitrogen removal rates increased with an increase in the C/N ratio, but ANAMMOX dominance gradually declined over denitrification. Furthermore, it has been observed that the NOBs (*Nitrospira* spp. and *Nitrobacter* spp.) are more abundant at a C/N ratio of 2. As a result, nitrate may have been produced in the reactor, which may have been utilised by denitrifying bacteria, given the higher organic carbon sources present in the reactor (Guo *et al.*, 2020). The increase in the NOB population will negatively affect the ANAMMOX bacteria as they compete for nitrite (Laureni *et al.*, 2019). Further, it was also noted that the biomass colour changed from reddish brown to black, indicating a dead ANAMMOX biomass in the reactor when 278.4 mg/L of sodium acetate was added (Furukawa *et al.*, 2006, Li *et al.*, 2012).

Targeted functional gene experiments further verified the above microbial community changes (Sheng *et al.*, 2018). As an alternative to quantitative PCR, quantifying cDNA (transcript abundance) has been described as a means of understanding the functional status of an organism (Yang *et al.*, 2020, Wang, 2021). In this study, as opposed to genomic and cDNA quantification, the expression of the *hzo* gene was found to decrease as the C/N ratio increased. *Hzo* gene encodes for hydrazine dehydrogenase (HDH) in ANAMMOX bacteria, which catalyses the oxidization of the unique ANAMMOX intermediate hydrazine to N<sub>2</sub> (Yang *et al.*, 2020). Therefore, the decrease in *hzo* gene expression in Figure 4.20 could represent a decrease in ANAMMOX activity when the C/N ratio was increased from 1.0 to 2.0. The results also align with the chemical data and the reported literature, where an increase in the C/N ratio was found to affect the activity of ANAMMOX bacteria. Similarly, the *amoA* gene targets the ammonia monooxygenase (AMO) enzyme subunit in AOB (Wu *et al.*, 2020). Although the operational conditions were strictly anaerobic, a small amount of dissolved oxygen was detected, which could be why some AOB genes were expressed. The increase of AOB in phase 2 could contribute to the increase of NH<sub>4</sub><sup>+</sup>-N removal (Figure 4.5) and NOBs abundance (Figure 4.13) because more nitrite was made available for nitrification. A slight decrease of

amoA gene when the C/N ratio was increased to 2 is in line with an increase of effluent  $\text{NH}_4^+$ -N concentration in the experimental reactor. Therefore a decrease of both hzo and amoA genes made more  $\text{NH}_4^+$ -N available in the effluent as the ANAMMOX process showed an inhibition. Both nxr genes (nxrA and nxrB) target nitrite oxidoreductase (NXR), which is the key enzyme in NOBs for  $\text{NO}_2^-$  oxidation (Sun *et al.*, 2022). The abundance of NOBs in the initial phase might be because of bioreactor leaking which also supported the availability of AOB bacteria. The increase of the C/N ratio caused a decrease in ANAMMOX bacteria, therefore, more nitrite continued to be available for NOBs as they showed proliferation in the last phase.

#### **4.5. Conclusion**

- The results showed that acetate greatly impacted the competition among the microbial populations within the ANAMMOX systems.
- An increase in the C/N ratio to 1.5 showed an improvement in NRE; however, a slight decrease in % $\text{NH}_4^+$ -N removal was observed.
- Based on quantitative PCR, a C/N ratio above 1.5 was found to cause a decrease in ANAMMOX bacteria abundance while an increase in the EUB population.
- The gene expression studies based on targeted functional genes further confirmed the inhibition of ANAMMOX and other autotrophic nitrifying bacteria under an elevated C/N ratio. For instance, the ANAMMOX hzo gene showed minimal activity under a C/N ratio above 1.5.

## CHAPTER 5:

### GENERAL CONCLUSION AND RECOMMENDATIONS

#### 5.1. General Conclusions

This study examined the effect of the C/N ratio on the ANAMMOX process performance and microbial community structure using a laboratory-scale UASB reactor. A combination of PCR, quantitative polymerase chain reaction (qPCR) and reverse transcriptase qPCR (RT-qPCR) was applied to assess the impact of the C/N ratio on key autotrophic nitrogen-converting bacteria.

- The enrichment of the ANAMMOX was successfully achieved in two UASB reactors after 410 days of operation. A significant increase in NRE was evident in both reactors from the day 0 to 410 days indicating an increase in microbial activity within the reactors.
- The presence and dominance of a mixed population autotrophic N-removing bacteria such as ANAMMOX, AOB and NOB were confirmed in both the reactors using PCR and qPCR.
- The addition of acetate (organic carbon) at a lower concentration (C/N ratio of 1 and 1.5) in the experimental phase increased NRE of the experimental reactor, indicating an increase in nitrogen removal in the presence of organic carbon at lower concentrations.
- A further increase of the C/N ratio to 2, however, showed a sharp decrease in NRE and %NH<sub>4</sub><sup>+</sup>-N removal, demonstrating a shift in the microbial population in the presence of elevated organic carbon
- The qPCR analysis indicated a shift in the dominance of eubacteria and key nitrifying bacteria, including ANAMMOX, when the C/N ratio was varied between 1, 1.5 and 2.

From phase 2 (C/N=1.5) to phase 3 (C/N=2) ANAMMOX bacteria genomic copy number displayed an increase, but the cDNA quantities decreased in agreement with the performance of the reactor in nitrogen removal.

- The gene expression studies based on reverse transcriptase PCR further confirmed the inhibition of ANAMMOX and other autotrophic nitrifying bacteria under an elevated C/N ratio. An increase in C/N ratio to 1.5, significantly affected the expression of key enzymes such as hzo (ANAMMOX) and amoA (AOB). Furthermore, almost complete inhibition of hzo gene was observed when the C/N ratio was further increased to 2.

## **5.2. Recommendations**

- As of yet, there is no consensus on the most suitable type of reactor to be used for anammox enrichment under an elevated C/N ratio, and therefore a comparison of different configurations may be required in order to assess the performance of ANAMMOX-based systems for such applications. Biofilm-based reactors, for example, have been found to be more stable under elevated C/N conditions than suspended reactors.
- A further evaluation of the impact of operating conditions such as HRT and SRT should be conducted in order to gain a better understanding of how an anammox reactor will perform under elevated C/N ratios.
- In the same vein, the size of the reactors could impact the stability of the reactor, as large reactors are known to be more stable than small reactors. To gain a deeper understanding of their long-term performance under these conditions, pilotscale studies are required.
- Utilizing advanced molecular platforms, such as metagenomics and metatranscriptomics, it is necessary to assess the relationship and competition between different functional microorganisms under different C/N ratios.

## 6. References

- Adams, M., Xie, J., Xie, J., Chang, Y., Guo, M., Chen, C. & Zhang, T. C.** 2020. The effect of carrier addition on Anammox start-up and microbial community: a review. *Reviews in Environmental Science and Bio/Technology*, **19**, 355-368.
- Agrawal, S., Karst, S. M., Gilbert, E. M., Horn, H., Nielsen, P. H. & Lackner, S.** 2017. The role of inoculum and reactor configuration for microbial community composition and dynamics in mainstream partial nitritation anammox reactors. *Microbiologyopen*, **6**.
- An, P., Xu, X., Yang, F. & Li, Z.** 2013. Comparison of the characteristics of anammox granules of different sizes. *Biotechnology and Bioprocess Engineering*, **18**, 446-454.
- Anjali, G. & Sabumon, P. C.** 2014. Unprecedented development of anammox in presence of organic carbon using seed biomass from a tannery Common Effluent Treatment Plant (CETP). *Bioresour Technol*, **153**, 30-8.
- Annabhajjala, M. K., Kapoor, V., Santo-Domingo, J. & Chandran, K.** 2018. Comammox functionality identified in diverse engineered biological wastewater treatment systems. *Environmental science & technology letters*, **5**, 110-116.
- Awolusi, O. O., Kosgey, E. K., Gokal, J., Kumari, S. & Bux, F.** 2020. Development of a Two-Stage Nitritation-Anammox Process for Improved Ammonia Removal from Wastewater. Water Research Commission, Available at: <https://www.wrc.org.za/?mdocs-file=60541>.
- Azimi, S. C., Shirini, F. & Pendashteh, A.** 2019. Evaluation of COD and turbidity removal from woodchips wastewater using biologically sequenced batch reactor. *Process Safety and Environmental Protection*, **128**, 211-227.

- Bandosz, T. J. & Block, K. A.** 2006. Removal of hydrogen sulfide on composite sewage sludge-industrial sludge-based adsorbents. *Industrial & engineering chemistry research*, **45**, 3666-3672.
- Banihani, Q., Hadadin, N. & Jamrah, A.** 2012a. Start-up of anaerobic ammonium oxidation (anammox) from conventional return activated sludge in up-flow anaerobic sludge blanket (UASB) reactor for autotrophic nitrogen removal from wastewater. *Jordan journal of civil engineering*, **6**, 17-27.
- Banihani, Q., Hadadin, N. & Jamrah, A.** 2012b. *Start-up of Anaerobic Ammonium Oxidation (Anammox) from Conventional Return Activated Sludge in Up-Flow Anaerobic Sludge Blanket (UASB) Reactor for Autotrophic Nitrogen Removal from Wastewater* \Jordan Journal of Civil Engineering.-2012, Vol. 6, Jordan University of Science and Technology.
- Bertino, A.** 2011. Study on one-stage Partial Nitritation-Anammox process in Moving Bed Biofilm Reactors: a sustainable nitrogen removal. TRITA LWR Degree Project. Available at: <https://www.diva-portal.org/smash/record.jsf?pid=diva2:530273>.
- Bhattacharjee, A. S., Wu, S., Lawson, C. E., Jetten, M. S. M., Kapoor, V., Domingo, J. W. S., McMahon, K. D., Noguera, D. R. & Goel, R.** 2017. Whole-Community Metagenomics in Two Different Anammox Configurations: Process Performance and Community Structure. *Environ Sci Technol*, **51**, 4317-4327.
- Bi, Z., Takekawa, M., Park, G., Soda, S., Zhou, J., Qiao, S. & Ike, M.** 2015. Effects of the C/N ratio and bacterial populations on nitrogen removal in the simultaneous anammox and heterotrophic denitrification process: mathematic modeling and batch experiments. *Chemical Engineering Journal*, **280**, 606-613.

- Bock, E., Schmidt, I., Stüven, R. & Zart, D.** 1995. Nitrogen loss caused by denitrifying Nitrosomonas cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. *Archives of Microbiology*, **163**, 16-20.
- Bonassa, G., Bolsan, A. C., Hollas, C. E., Venturin, B., Candido, D., Chini, A., De Pra, M. C., Antes, F. G., Campos, J. L. & Kunz, A.** 2021. Organic carbon bioavailability: Is it a good driver to choose the best biological nitrogen removal process? *Sci Total Environ*, **786**, 147390.
- Bowden, G., Tsuchihashi, R. & Stensel, H.** 2015. Technologies for Sidestream Nitrogen Removal, WERF Research Report Series.
- Burrell, P. C., Keller, J. & Blackall, L. L.** 1998. Microbiology of a nitrite-oxidizing bioreactor. *Applied and Environmental Microbiology*, **64**, 1878-1883.
- Cao, S., Peng, Y., Du, R. & Wang, S.** 2016. Feasibility of enhancing the DENitrifying AMmonium OXidation (DEAMOX) process for nitrogen removal by seeding partial denitrification sludge. *Chemosphere*, **148**, 403-7.
- Cao, Y., van Loosdrecht, M. C. & Daigger, G. T.** 2017. Mainstream partial nitrification-anammox in municipal wastewater treatment: status, bottlenecks, and further studies. *Appl Microbiol Biotechnol*, **101**, 1365-1383.
- Chamchoi, N., Nitorisavut, S. & Schmidt, J. E.** 2008. Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation (ANAMMOX) and denitrification. *Bioresour Technol*, **99**, 3331-6.
- Chen, C., Jiang, Y., Liu, J., Adams, M., Chang, Y., Guo, M., Xie, J. & Xie, J.** 2021. The structure of anammox granular sludge under varying long-term organic matter stress: Performance, physiochemical and microbial community. *Journal of Cleaner Production*, **323**, 129117.

- Chen, C., Sun, F., Zhang, H., Wang, J., Shen, Y. & Liang, X.** 2016. Evaluation of COD effect on anammox process and microbial communities in the anaerobic baffled reactor (ABR). *Bioresource Technology*, **216**, 571-578.
- Cho, S., Kambey, C. & Nguyen, V.** 2019. Performance of Anammox Processes for Wastewater Treatment: A Critical Review on Effects of Operational Conditions and Environmental Stresses. *Water*, **12**, 20.
- Choi, D., Cho, K. & Jung, J.** 2019. Optimization of nitrogen removal performance in a single-stage SBR based on partial nitrification and ANAMMOX. *Water Res*, **162**, 105-114.
- Ciudad, G., Rubilar, O., Muñoz, P., Ruiz, G., Chamy, R., Vergara, C. & Jeison, D.** 2005. Partial nitrification of high ammonia concentration wastewater as a part of a shortcut biological nitrogen removal process. *Process Biochemistry*, **40**, 1715-1719.
- Cotto, I., Dai, Z., Huo, L., Anderson, C. L., Vilardi, K. J., Ijaz, U., Khunjar, W., Wilson, C., De Clippeleir, H. & Gilmore, K.** 2020. Long solids retention times and attached growth phase favor prevalence of comammox bacteria in nitrogen removal systems. *Water research*, **169**, 115268.
- Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J. & Bulaev, A.** 2015. Complete nitrification by Nitrospira bacteria. *Nature*, **528**, 504-509.
- Dehestaniathar, S., Nesari, S., Borzooei, S. & Abolfathi, S.** 2021. Application of natural biodegradable fiber as biofilm medium and carbon source in DENitrifying AMmonium OXidation (DEAMOX) process for nitrogen removal from wastewater. *Journal of the Taiwan Institute of Chemical Engineers*, **119**, 108-114.
- di Biase, A., Kowalski, M. S., Devlin, T. R. & Oleszkiewicz, J. A.** 2019. Moving bed biofilm reactor technology in municipal wastewater treatment: A review. *J Environ Manage*, **247**, 849-866.

- Díaz, C., Belmonte, M., Campos, J. L., Franchi, O., Faúndez, M., Vidal, G., Argiz, L., Pedrouso, A., Val del Rio, A. & Mosquera-Corral, A.** 2020. Limits of the anammox process in granular systems to remove nitrogen at low temperature and nitrogen concentration. *Process Safety and Environmental Protection*, **138**, 349-355.
- Ding, C., Enyi, F. O. & Adrian, L.** 2018. Anaerobic Ammonium Oxidation (Anammox) with Planktonic Cells in a Redox-Stable Semicontinuous Stirred-Tank Reactor. *Environ Sci Technol*, **52**, 5671-5681.
- Dionisi, H. M., Layton, A. C., Harms, G., Gregory, I. R., Robinson, K. G. & Sayler, G. S.** 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. *Applied and environmental microbiology*, **68**, 245-253.
- Dosta, J., Fernandez, I., Vazquez-Padin, J. R., Mosquera-Corral, A., Campos, J. L., Mata-Alvarez, J. & Mendez, R.** 2008. Short- and long-term effects of temperature on the Anammox process. *J Hazard Mater*, **154**, 688-93.
- Engström, P., Dalsgaard, T., Hulth, S. & Aller, R. C.** 2005. Anaerobic ammonium oxidation by nitrite (anammox): Implications for N<sub>2</sub> production in coastal marine sediments. *Geochimica et Cosmochimica Acta*, **69**, 2057-2065.
- Fowler, S. J., Palomo, A., Dechesne, A., Mines, P. D. & Smets, B. F.** 2018. Comammox *Nitrospira* are abundant ammonia oxidizers in diverse groundwater-fed rapid sand filter communities. *Environmental microbiology*, **20**, 1002-1015.
- Fuerst, J. A.** 2013. *Planctomycetes: cell structure, origins and biology*. Springer Science & Business Media.
- Fumasoli, A., Burgmann, H., Weissbrodt, D. G., Wells, G. F., Beck, K., Mohn, J., Morgenroth, E. & Udert, K. M.** 2017. Growth of *Nitrosococcus*-Related Ammonia

Oxidizing Bacteria Coincides with Extremely Low pH Values in Wastewater with High Ammonia Content. *Environ Sci Technol*, **51**, 6857-6866.

**Furukawa, K., Lieu, P. K., Tokitoh, H. & Fujii, T.** 2006. Development of single-stage nitrogen removal using anammox and partial nitritation (SNAP) and its treatment performances. *Water Sci Technol*, **53**, 83-90.

**Gani, K. M., Awolusi, O. O., Khan, A. A., Kumari, S. & Bux, F.** 2020. Potential strategies for the mainstream application of anammox in treatment of anaerobic effluents - A review. *Critical Reviews in Environmental Science and Technology*, **51**, 2567-2594.

**Gilbert, E. M., Muller, E., Horn, H. & Lackner, S.** 2013. Microbial activity of suspended biomass from a nitritation-anammox SBR in dependence of operational condition and size fraction. *Appl Microbiol Biotechnol*, **97**, 8795-804.

**Gonzalez-Martinez, A., Rodriguez-Sanchez, A., Garcia-Ruiz, M. J., Muñoz-Palazon, B., Cortes-Lorenzo, C., Osorio, F. & Vahala, R.** 2016. Performance and bacterial community dynamics of a CANON bioreactor acclimated from high to low operational temperatures. *Chemical Engineering Journal*, **287**, 557-567.

**Guo, J., Peng, Y., Fan, L., Zhang, L., Ni, B. J., Kartal, B., Feng, X., Jetten, M. S. & Yuan, Z.** 2016. Metagenomic analysis of anammox communities in three different microbial aggregates. *Environ Microbiol*, **18**, 2979-93.

**Guo, M., Jiang, Y., Xie, J., Cao, Q., Zhang, Q., Mabruk, A. & Chen, C.** 2022a. Bamboo charcoal addition enhanced the nitrogen removal of anammox granular sludge with COD: Performance, physicochemical characteristics and microbial community. *J Environ Sci (China)*, **115**, 55-64.

**Guo, Y., Niu, Q., Sugano, T. & Li, Y. Y.** 2020. Biodegradable organic matter-containing ammonium wastewater treatment through simultaneous partial nitritation, anammox, denitrification and COD oxidization process. *Sci Total Environ*, **714**, 136740.

- Guo, Y., Qian, Y., Shen, J., Qin, Y. & Li, Y. Y.** 2022b. The startup of the partial nitrification/anammox-hydroxyapatite process based on reconciling biomass and mineral to form the novel granule sludge. *Bioresour Technol*, **347**, 126692.
- Gutwiński, P., Cema, G., Ziemińska-Buczyńska, A., Wszyńska, K. & Surmacz-Górska, J.** 2021. Long-term effect of heavy metals Cr(III), Zn(II), Cd(II), Cu(II), Ni(II), Pb(II) on the anammox process performance. *Journal of Water Process Engineering*, **39**, 101668.
- Hassan, G. K. & El-Gohary, F. A.** 2021. Evaluation of Partial Nitrification/Anammox Process for Reduction of Pollutants from Sanitary Landfill Leachate. *Water, Air, & Soil Pollution*, **232**.
- He, S., Niu, Q., Ma, H., Zhang, Y. & Li, Y.-Y.** 2015. The Treatment Performance and the Bacteria Preservation of Anammox: A Review. *Water, Air, & Soil Pollution*, **226**.
- He, S., Yang, W., Qin, M., Mao, Z., Niu, Q. & Han, M.** 2018. Performance and microbial community of anammox in presence of micro-molecule carbon source. *Chemosphere*, **205**, 545-552.
- Hendrickx, T. L. G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H. & Buisman, C. J. N.** 2012. Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research*, **46**, 2187-2193.
- Hoekstra, M., de Weerd, F. A., Kleerebezem, R. & van Loosdrecht, M. C. M.** 2018. Deterioration of the anammox process at decreasing temperatures and long SRTs. *Environ Technol*, **39**, 658-668.
- Hu, B. L., Shen, L. D., Liu, S., Cai, C., Chen, T. T., Kartal, B., Harhangi, H. R., Op den Camp, H. J., Lou, L. P., Xu, X. Y., Zheng, P. & Jetten, M. S.** 2013a. Enrichment of an anammox bacterial community from a flooded paddy soil. *Environ Microbiol Rep*, **5**, 483-9.

- Hu, Z., Lotti, T., van Loosdrecht, M. & Kartal, B.** 2013b. Nitrogen removal with the anaerobic ammonium oxidation process. *Biotechnol Lett*, **35**, 1145-54.
- Hubaux, N., Wells, G. & Morgenroth, E.** 2015. Impact of coexistence of flocs and biofilm on performance of combined nitrification-anammox granular sludge reactors. *Water Res*, **68**, 127-39.
- Ibrahim, M., Yusof, N., Mohd Yusoff, M. Z. & Hassan, M. A.** 2015. Enrichment of anaerobic ammonium oxidation (anammox) bacteria for short start-up of the anammox process: a review. *Desalination and Water Treatment*, **57**, 13958-13978.
- Izadi, P., Izadi, P. & Eldyasti, A.** 2022. Development of long-term dynamic BioWin(R) model simulation for ANAMMOX UASB micro-granular process. *Chemosphere*, **286**, 131859.
- Jensen, M. M., Thamdrup, B. & Dalsgaard, T.** 2007. Effects of specific inhibitors on anammox and denitrification in marine sediments. *Appl Environ Microbiol*, **73**, 3151-8.
- Jetten, M. S.** 2008. The microbial nitrogen cycle. *Environ Microbiol*, **10**, 2903-9.
- Ji, B.** 2022. Towards environment-sustainable wastewater treatment and reclamation by the non-aerated microalgal-bacterial granular sludge process: Recent advances and future directions. *Sci Total Environ*, **806**, 150707.
- Ji, X.-M., Zheng, C., Wang, Y.-L. & Jin, R.-C.** 2021. Decoding the interspecies interaction in anammox process with inorganic feeding through metagenomic and metatranscriptomic analysis. *Journal of Cleaner Production*, **288**, 125691.
- Jin, R.-C., Yang, G.-F., Yu, J.-J. & Zheng, P.** 2012. The inhibition of the Anammox process: A review. *Chemical Engineering Journal*, **197**, 67-79.

- Kalyuzhnyi, S., Gladchenko, M., Mulder, A. & Versprille, B.** 2007. Comparison of quasisteady-state performance of the DEAMOX process under intermittent and continuous feeding and different nitrogen loading rates. *Biotechnol J*, **2**, 894-900.
- Kartal, B., de Almeida, N. M., Maalcke, W. J., Op den Camp, H. J., Jetten, M. S. & Keltjens, J. T.** 2013. How to make a living from anaerobic ammonium oxidation. *FEMS Microbiol Rev*, **37**, 428-61.
- Kartal, B., Keltjens, J. T. & Jetten, M. S.** 2008. The metabolism of anammox. *eLS*.
- Kartal, B., van Niftrik, L., Keltjens, J. T., Op den Camp, H. J. & Jetten, M. S.** 2012. Anammox--growth physiology, cell biology, and metabolism. *Adv Microb Physiol*, **60**, 211-62.
- Kaźmierczak, B., Ismail, S., Tawfik, A., Kutylowska, M., Piekarska, K., Jouhara, H. & Danielewicz, J.** 2017. Comprehensive study for Anammox process via multistage anaerobic baffled reactors. *E3S Web of Conferences*, **22**, 00068.
- Khin, T. & Annachhatre, A. P.** 2004. Novel microbial nitrogen removal processes. *Biotechnol Adv*, **22**, 519-32.
- Kilonzo, P. M. & Margaritis, A.** 2004. The effects of non-Newtonian fermentation broth viscosity and small bubble segregation on oxygen mass transfer in gas-lift bioreactors: a critical review. *Biochemical Engineering Journal*, **17**, 27-40.
- Kimura, Y. & Isaka, K.** 2014. Evaluation of inhibitory effects of heavy metals on anaerobic ammonium oxidation (anammox) by continuous feeding tests. *Appl Microbiol Biotechnol*, **98**, 6965-72.
- Kits, K. D., Sedlacek, C. J., Lebedeva, E. V., Han, P., Bulaev, A., Pjevac, P., Daebeler, A., Romano, S., Albertsen, M. & Stein, L. Y.** 2017. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature*, **549**, 269-272.

- Kosgey, K., Chandran, K., Gokal, J., Kiambi, S. L., Bux, F. & Kumari, S.** 2021. Critical Analysis of Biomass Retention Strategies in Mainstream and Sidestream ANAMMOX-Mediated Nitrogen Removal Systems. *Environ Sci Technol*, **55**, 9-24.
- Krishna Mohan, T. V., Nancharaiah, Y. V., Venugopalan, V. P. & Satya Sai, P. M.** 2016. Effect of C/N ratio on denitrification of high-strength nitrate wastewater in anoxic granular sludge sequencing batch reactors. *Ecological Engineering*, **91**, 441-448.
- Kuenen, J. G.** 2008. Anammox bacteria: from discovery to application. *Nature Reviews Microbiology*, **6**, 320-326.
- Kumar, M. & Lin, J. G.** 2010. Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal--Strategies and issues. *J Hazard Mater*, **178**, 1-9.
- Laureni, M., Weissbrodt, D. G., Szivak, I., Robin, O., Nielsen, J. L., Morgenroth, E. & Joss, A.** 2015. Activity and growth of anammox biomass on aerobically pre-treated municipal wastewater. *Water Res*, **80**, 325-36.
- Laureni, M., Weissbrodt, D. G., Villez, K., Robin, O., de Jonge, N., Rosenthal, A., Wells, G., Nielsen, J. L., Morgenroth, E. & Joss, A.** 2019. Biomass segregation between biofilm and flocs improves the control of nitrite-oxidizing bacteria in mainstream partial nitrification and anammox processes. *Water Res*, **154**, 104-116.
- Law, Y., Ye, L., Pan, Y. & Yuan, Z.** 2012. Nitrous oxide emissions from wastewater treatment processes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 1265-1277.
- Leal, C. D., Pereira, A. D., Nunes, F. T., Ferreira, L. O., Coelho, A. C., Bicalho, S. K., Mac Conell, E. F., Ribeiro, T. B., de Lemos Chernicharo, C. A. & de Araujo, J. C.** 2016. Anammox for nitrogen removal from anaerobically pre-treated municipal wastewater: Effect of COD/N ratios on process performance and bacterial community structure. *Bioresour Technol*, **211**, 257-66.

- Lee, K. H., Wang, Y. F., Zhang, G. X. & Gu, J. D.** 2014. Distribution patterns of ammonia-oxidizing bacteria and anammox bacteria in the freshwater marsh of Honghe wetland in Northeast China. *Ecotoxicology*, **23**, 1930-42.
- Li, H., Zhou, S., Ma, W., Huang, G. & Xu, B.** 2012. Fast start-up of ANAMMOX reactor: Operational strategy and some characteristics as indicators of reactor performance. *Desalination*, **286**, 436-441.
- Li, J., Li, J., Gao, R., Wang, M., Yang, L., Wang, X., Zhang, L. & Peng, Y.** 2018a. A critical review of one-stage anammox processes for treating industrial wastewater: Optimization strategies based on key functional microorganisms. *Bioresour Technol*, **265**, 498-505.
- Li, J., Zhang, L., Peng, Y. & Zhang, Q.** 2017. Effect of low COD/N ratios on stability of single-stage partial nitrification/anammox (SPN/A) process in a long-term operation. *Bioresour Technol*, **244**, 192-197.
- Li, X., Klaus, S., Bott, C. & He, Z.** 2018b. Status, Challenges, and Perspectives of Mainstream Nitrification-Anammox for Wastewater Treatment. *Water Environ Res*, **90**, 634-649.
- Li, Y.-y., Huang, X.-w. & Li, X.-y.** 2021a. Use of a packed-bed biofilm reactor to achieve rapid formation of anammox biofilms for high-rate nitrogen removal. *Journal of Cleaner Production*, **321**, 128999.
- Li, Y., Liu, Y., Luo, J., Li, Y. Y. & Liu, J.** 2021b. Emerging onsite electron donors for advanced nitrogen removal from anammox effluent of leachate treatment: A review and future applications. *Bioresour Technol*, **341**, 125905.
- Liu, T., Hu, S. & Guo, J.** 2019. Enhancing mainstream nitrogen removal by employing nitrate/nitrite-dependent anaerobic methane oxidation processes. *Crit Rev Biotechnol*, **39**, 732-745.

- Lotti, T., van der Star, W. R., Kleerebezem, R., Lubello, C. & van Loosdrecht, M. C.** 2012. The effect of nitrite inhibition on the anammox process. *Water Res*, **46**, 2559-69.
- Lu, J., Yang, C., Zhang, Y. & Pan, H.** 2018a. Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review. *Front Physiol*, **9**, 1560.
- Lu, L., Guest, J. S., Peters, C. A., Zhu, X., Rau, G. H. & Ren, Z. J.** 2018b. Wastewater treatment for carbon capture and utilization. *Nature Sustainability*, **1**, 750-758.
- Lucker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T., Damste, J. S., Spieck, E., Le Paslier, D. & Daims, H.** 2010. A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proc Natl Acad Sci U S A*, **107**, 13479-84.
- Ma, X. & Wang, Y.** 2018. Anammox bacteria exhibit capacity to withstand long-term starvation stress: A proteomic-based investigation of survival mechanisms. *Chemosphere*, **211**, 952-961.
- Magri, A., Beline, F. & Dabert, P.** 2013. Feasibility and interest of the anammox process as treatment alternative for anaerobic digester supernatants in manure processing--an overview. *J Environ Manage*, **131**, 170-84.
- Maktabifard, M., Zaborowska, E. & Makinia, J.** 2018. Achieving energy neutrality in wastewater treatment plants through energy savings and enhancing renewable energy production. *Reviews in Environmental Science and Bio/Technology*, **17**, 655-689.
- Manasa, R. L. & Mehta, A.** 2021. Current perspectives of anoxic ammonia removal and blending of partial nitrifying and denitrifying bacteria for ammonia reduction in wastewater treatment. *Journal of Water Process Engineering*, **41**, 102085.

- Mao, X., Myavagh, P. H., Lotfikatouli, S., Hsiao, B. S. & Walker, H. W.** 2020. Membrane Bioreactors for Nitrogen Removal from Wastewater: A Review. *Journal of Environmental Engineering*, **146**, 03120002.
- Molinuevo, B., Garcia, M. C., Karakashev, D. & Angelidaki, I.** 2009. Anammox for ammonia removal from pig manure effluents: effect of organic matter content on process performance. *Bioresour Technol*, **100**, 2171-5.
- Mughal, B. B., Leemans, M., Spirhanzlova, P., Demeneix, B. & Fini, J. B.** 2018. Reference gene identification and validation for quantitative real-time PCR studies in developing *Xenopus laevis*. *Sci Rep*, **8**, 496.
- Nguyen, H. T. M. & Tran, H. T. H.** 2021. Effect of organic matter on nitrogen removal through the anammox process. *Water Environ Res*, **93**, 608-619.
- Ni, S. Q., Gao, B. Y., Wang, C. C., Lin, J. G. & Sung, S.** 2011. Fast start-up, performance and microbial community in a pilot-scale anammox reactor seeded with exotic mature granules. *Bioresour Technol*, **102**, 2448-54.
- Ni, S. Q., Ni, J. Y., Hu, D. L. & Sung, S.** 2012. Effect of organic matter on the performance of granular anammox process. *Bioresour Technol*, **110**, 701-5.
- Nie, W. B., Xie, G. J., Ding, J., Peng, L., Lu, Y., Tan, X., Yue, H., Liu, B. F., Xing, D. F., Meng, J., Han, H. J. & Ren, N. Q.** 2020. Operation strategies of n-DAMO and Anammox process based on microbial interactions for high rate nitrogen removal from landfill leachate. *Environ Int*, **139**, 105596.
- Nozhevnikova, A. N., Botchkova, E. A. & Plakunov, V. K.** 2015. Multi-species biofilms in ecology, medicine, and biotechnology. *Microbiology*, **84**, 731-750.
- Orschler, L., Agrawal, S. & Lackner, S.** 2019. On resolving ambiguities in microbial community analysis of partial nitrification anammox reactors. *Sci Rep*, **9**, 6954.

- Oshiki, M., Awata, T., Kindaichi, T., Satoh, H. & Okabe, S.** 2013. Cultivation of planktonic anaerobic ammonium oxidation (anammox) bacteria using membrane bioreactor. *Microbes Environ*, **28**, 436-43.
- Palomo, A., Jane Fowler, S., Gülay, A., Rasmussen, S., Sicheritz-Ponten, T. & Smets, B. F.** 2016. Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of *Nitrospira* spp. *The ISME journal*, **10**, 2569-2581.
- Park, H.** 2011. *Microbial ecology, activity and abundance of aerobic and anaerobic ammonium oxidizing bacteria in engineered drinking water and wastewater systems*, Columbia University.
- Park, H., Rosenthal, A., Jezek, R., Ramalingam, K., Fillos, J. & Chandran, K.** 2010. Impact of inocula and growth mode on the molecular microbial ecology of anaerobic ammonia oxidation (anammox) bioreactor communities. *Water Res*, **44**, 5005-13.
- Park, H., Sundar, S., Ma, Y. & Chandran, K.** 2015. Differentiation in the microbial ecology and activity of suspended and attached bacteria in a nitrification-anammox process. *Biotechnology and bioengineering*, **112**, 272-279.
- Park, M. R., Park, H. & Chandran, K.** 2017. Molecular and Kinetic Characterization of Planktonic *Nitrospira* spp. Selectively Enriched from Activated Sludge. *Environ Sci Technol*, **51**, 2720-2728.
- Peng, M. W., Qi, J., Yan, P., Guan, Y., Liu, Y. Y., Sun, Z. H., Zhang, L. J., Weng, X., Shen, Y., Fang, F., Guo, J. S. & Chen, Y. P.** 2022a. Insight into the structure and metabolic function of iron-rich nanoparticles in anammox bacteria. *Sci Total Environ*, **806**, 150879.
- Peng, Z., Lei, Y., Liu, Y., Wan, X., Yang, B. & Pan, X.** 2022b. Fast start-up and reactivation of anammox process using polyurethane sponge. *Biochemical Engineering Journal*, **177**, 108249.

- Pereira, A. D., Cabezas, A., Etchebehere, C., Chernicharo, C. A. d. L. & de Araújo, J. C.** 2017. Microbial communities in anammox reactors: a review. *Environmental Technology Reviews*, **6**, 74-93.
- Pereira, T. D. S., Spindola, R. H., Rabelo, C., Silveira, N. C., Adorno, M. A. T., Kunz, A., Pires, E. C. & Damianovic, M.** 2021. A predictive model for N<sub>2</sub>O production in anammox-granular sludge reactors: Combined effects of nitrite/ammonium ratio and organic matter concentration. *J Environ Manage*, **297**, 113295.
- Perez-Garcia, O., Bashan, Y. & Esther Puente, M.** 2011. Organic Carbon Supplementation of Sterilized Municipal Wastewater Is Essential for Heterotrophic Growth and Removing Ammonium by the Microalga *Chlorella Vulgaris*1. *Journal of Phycology*, **47**, 190-199.
- Pinto, A. J., Marcus, D. N., Ijaz, U. Z., Bautista-de Lose Santos, Q. M., Dick, G. J. & Raskin, L.** 2016. Metagenomic Evidence for the Presence of Comammox Nitrospirilla-Like Bacteria in a Drinking Water System. *mSphere*, **1**.
- Postollec, F., Falentin, H., Pavan, S., Combrisson, J. & Sohier, D.** 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiol*, **28**, 848-61.
- Qian, Y., Ding, Y., Ma, H., Chi, Y., Yuan, H., Li, Y. Y., Tian, S. & Zhang, B.** 2021. Startup and performance of a novel single-stage partial nitrification/anammox system for reject water treatment. *Bioresour Technol*, **321**, 124432.
- Rahimi, S., Modin, O. & Mijakovic, I.** 2020. Technologies for biological removal and recovery of nitrogen from wastewater. *Biotechnol Adv*, **43**, 107570.
- Ratray, J. E., van de Vossenberg, J., Hopmans, E. C., Kartal, B., van Niftrik, L., Rijpstra, W. I., Strous, M., Jetten, M. S., Schouten, S. & Sinninghe Damste, J. S.** 2008.

- Ladderane lipid distribution in four genera of anammox bacteria. *Arch Microbiol*, **190**, 51-66.
- Ren, Z. Q., Wang, H., Zhang, L. G., Du, X. N., Huang, B. C. & Jin, R. C.** 2022. A review of anammox-based nitrogen removal technology: From microbial diversity to engineering applications. *Bioresour Technol*, **363**, 127896.
- Sabumon, P. C.** 2007. Anaerobic ammonia removal in presence of organic matter: a novel route. *J Hazard Mater*, **149**, 49-59.
- Saijai, S., Ando, A., Inukai, R., Shinohara, M. & Ogawa, J.** 2016. Analysis of microbial community and nitrogen transition with enriched nitrifying soil microbes for organic hydroponics. *Biosci Biotechnol Biochem*, **80**, 2247-2254.
- Schmid, M. C., Hooper, A. B., Klotz, M. G., Woebken, D., Lam, P., Kuypers, M. M., Pommerening-Roeser, A., Op den Camp, H. J. & Jetten, M. S.** 2008. Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. *Environ Microbiol*, **10**, 3140-9.
- Sheng, S., Liu, B., Hou, X., Liang, Z., Sun, X., Du, L. & Wang, D.** 2018. Effects of different carbon sources and C/N ratios on the simultaneous anammox and denitrification process. *International Biodeterioration & Biodegradation*, **127**, 26-34.
- Singh, V., Ormeci, B., Mishra, S. & Hussain, A.** 2022. Simultaneous partial Nitrification, ANAMMOX and denitrification (SNAD) – A review of critical operating parameters and reactor configurations. *Chemical Engineering Journal*, **433**, 133677.
- Strous, M., Heijnen, J., Kuenen, J. G. & Jetten, M.** 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied microbiology and biotechnology*, **50**, 589-596.
- Suarez, C. 2017. Microbial communities in anammox and nitrifying biofilms. Available at: <https://gupea.ub.gu.se/handle/2077/52688>.

- Suhr, K. I., Pedersen, P. B. & Arvin, E.** 2013. End-of-pipe denitrification using RAS effluent waste streams: Effect of C/N-ratio and hydraulic retention time. *Aquacultural Engineering*, **53**, 57-64.
- Sun, H., Zhang, H., Zhang, F., Yang, H., Lu, J., Ge, S., Ding, J. & Liu, Y.** 2022. Response of substrate kinetics and biological mechanisms to various pH constrains for cultured Nitrobacter and Nitrospira in nitrifying bioreactor. *J Environ Manage*, **307**, 114499.
- Sun, P., Zhao, Z., Fan, P., Chen, W., Ruan, Y. & Wang, Q.** 2021. Ammonia- and Nitrite-Oxidizing Bacteria are Dominant in Nitrification of Maize Rhizosphere Soil Following Combined Application of Biochar and Chemical Fertilizer. *Front Microbiol*, **12**, 715070.
- Tal, Y., Watts, J. & Schreier, H.** 2006. Characterization and abundance of anaerobic ammonia oxidizing (anammox) bacteria in biofilters of recirculating aquaculture systems. *Appl Environ Microbiol*, **72**, 2896-2904.
- Talan, A., Tyagi, R. D. & Drogui, P.** 2021. Critical review on insight into the impacts of different inhibitors and performance inhibition of anammox process with control strategies. *Environmental Technology & Innovation*, **23**, 101553.
- Tian, X., Schopf, A., Amaral-Stewart, B., Christensson, M., Morgan-Sagastume, F., Vincent, S. & Delatolla, R.** 2020. Anammox attachment and biofilm development on surface-modified carriers with planktonic- and biofilm-based inoculation. *Bioresour Technol*, **317**, 124030.
- Tomaszewski, M., Cema, G. & Ziembinska-Buczynska, A.** 2017. Influence of temperature and pH on the anammox process: A review and meta-analysis. *Chemosphere*, **182**, 203-214.
- Trigo, C., Campos, J. L., Garrido, J. M. & Mendez, R.** 2006. Start-up of the Anammox process in a membrane bioreactor. *J Biotechnol*, **126**, 475-87.

- Val del Río, Á., Campos Gómez, J. L. & Mosquera Corral, A. 2016. *Technologies for the treatment and recovery of nutrients from industrial wastewater*, IGI Global.
- Van de Graaf, A. A., de Bruijn, P., Robertson, L. A., Jetten, M. S. & Kuenen, J. G.** 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology*, **142**, 2187-2196.
- van der Star, W. R., Abma, W. R., Blommers, D., Mulder, J. W., Tokutomi, T., Strous, M., Picioreanu, C. & van Loosdrecht, M. C.** 2007. Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. *Water Res*, **41**, 4149-63.
- van der Star, W. R., Miclea, A. I., van Dongen, U. G., Muyzer, G., Picioreanu, C. & van Loosdrecht, M. C.** 2008. The membrane bioreactor: a novel tool to grow anammox bacteria as free cells. *Biotechnol Bioeng*, **101**, 286-94.
- Van Kessel, M. A., Speth, D. R., Albertsen, M., Nielsen, P. H., Op den Camp, H. J., Kartal, B., Jetten, M. S. & Lücker, S.** 2015. Complete nitrification by a single microorganism. *Nature*, **528**, 555-559.
- van Kessel, M. A., Stultiens, K., Slegers, M. F., Guerrero Cruz, S., Jetten, M. S., Kartal, B. & Op den Camp, H. J.** 2018. Current perspectives on the application of N-damo and anammox in wastewater treatment. *Curr Opin Biotechnol*, **50**, 222-227.
- Van Loosdrecht, M., Hao, X., Jetten, M. & Abma, W.** 2004. Use of Anammox in urban wastewater treatment. *Water Science and Technology: Water Supply*, **4**, 87-94.
- van Niftrik, L., Geerts, W. J., van Donselaar, E. G., Humbel, B. M., Yakushevska, A., Verkleij, A. J., Jetten, M. S. & Strous, M.** 2008. Combined structural and chemical analysis of the anammoxosome: a membrane-bounded intracytoplasmic compartment in anammox bacteria. *J Struct Biol*, **161**, 401-10.

- van Niftrik, L. A., Fuerst, J. A., Sinninghe Damste, J. S., Kuenen, J. G., Jetten, M. S. & Strous, M.** 2004. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *FEMS Microbiol Lett*, **233**, 7-13.
- van Teeseling, M. C., Mesman, R. J., Kuru, E., Espaillet, A., Cava, F., Brun, Y. V., VanNieuwenhze, M. S., Kartal, B. & van Niftrik, L.** 2015. Anammox Planctomycetes have a peptidoglycan cell wall. *Nat Commun*, **6**, 6878.
- Vandekerckhove, T. G. L., Props, R., Carvajal-Arroyo, J. M., Boon, N. & Vlaeminck, S. E.** 2020. Adaptation and characterization of thermophilic anammox in bioreactors. *Water Res*, **172**, 115462.
- Vimala, R. T. V., Lija Escaline, J., Murugan, K. & Sivaramakrishnan, S.** 2020. An overview of organic matters in municipal wastewater: Removal via self-assembly flocculating mechanism and the molecular level characterization. *J Environ Manage*, **266**, 110572.
- Wang, D., Huang, K., He, X., Zhang, X. X. & Meng, Y.** 2022a. Varied interspecies interactions between anammox and denitrifying bacteria enhanced nitrogen removal in a single-stage simultaneous anammox and denitrification system. *Sci Total Environ*, **813**, 152519.
- Wang, F.** 2021. Semi-quantitative RT-PCR: An effective method to explore the regulation of gene transcription level affected by environmental pollutants. *Environmental Toxicology and Toxicogenomics*. Springer.
- Wang, G., Xu, X., Gong, Z., Gao, F., Yang, F. & Zhang, H.** 2016. Study of simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) process in an intermittent aeration membrane bioreactor. *Process Biochemistry*, **51**, 632-641.
- Wang, J., Lei, Z., Wang, L., Yang, S., Zhao, Y., Li, Y. Y. & Chen, R.** 2020a. Insight into using up-flow anaerobic sludge blanket-anammox to remove nitrogen from an

- anaerobic membrane reactor during mainstream wastewater treatment. *Bioresour Technol*, **314**, 123710.
- Wang, J., Rong, H. & Zhang, C.** 2018. Evaluation of the impact of dissolved oxygen concentration on biofilm microbial community in sequencing batch biofilm reactor. *J Biosci Bioeng*, **125**, 532-542.
- Wang, S., Liu, Y., Niu, Q., Ji, J., Hojo, T. & Li, Y. Y.** 2017. Nitrogen removal performance and loading capacity of a novel single-stage nitrification-anammox system with syntrophic micro-granules. *Bioresour Technol*, **236**, 119-128.
- Wang, W., Wang, Y., Wang, X., Zhang, Y. & Yan, Y.** 2019a. Dissolved oxygen microelectrode measurements to develop a more sophisticated intermittent aeration regime control strategy for biofilm-based CANON systems. *Chemical Engineering Journal*, **365**, 165-174.
- Wang, W., Zhu, J., Xiong, D., Su, Y., Li, Y. & Fu, J.** 2022b. Comparison between two anammox fiber fillers under load impact and the effect of  $\text{HCO}_3^-$  concentration. *RSC Advances*, **12**, 24-31.
- Wang, X., Yang, H., Su, Y. & Liu, X.** 2022c. Effects of sludge morphology on the anammox process: Analysis from the perspectives of performance, structure, and microbial community. *Chemosphere*, **288**, 132390.
- Wang, X., Yang, R., Guo, Y., Zhang, Z., Kao, C. M. & Chen, S.** 2019b. Investigation of COD and COD/N ratio for the dominance of anammox pathway for nitrogen removal via isotope labelling technique and the relevant bacteria. *J Hazard Mater*, **366**, 606-614.
- Wang, Z., Ji, Y., Yan, L., Yan, Y., Zhang, H., Gao, P. & Li, S.** 2020b. Simultaneous anammox and denitrification process shifted from the anammox process in response to

- C/N ratios: Performance, sludge granulation, and microbial community. *J Biosci Bioeng*, **130**, 319-326.
- Winkler, M. K., Kleerebezem, R. & van Loosdrecht, M. C.** 2012. Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. *Water Res*, **46**, 136-44.
- Wiszniewski, J., Robert, D., Surmacz-Gorska, J., Miksch, K. & Weber, J. V.** 2006. Landfill leachate treatment methods: A review. *Environmental Chemistry Letters*, **4**, 51-61.
- Wu, X., Ren, L., Zhang, J. & Peng, H.** 2020. Effects of Zeolite and Biochar Addition on Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea Communities during Agricultural Waste Composting. *Sustainability*, **12**, 6336.
- Xiao, Y., Zeng, G. M., Yang, Z. H., Liu, Y., Ma, Y. H., Yang, L., Wang, R. J. & Xu Zh, Y.** 2009. Coexistence of nitrifiers, denitrifiers and Anammox bacteria in a sequencing batch biofilm reactor as revealed by PCR-DGGE. *J Appl Microbiol*, **106**, 496-505.
- Xie, H., Ji, D. & Zang, L.** 2017. Effects of Inhibition Conditions on Anammox process. *IOP Conference Series: Earth and Environmental Science*, **100**, 012149.
- Xie, L., Chen, J., Wang, R. & Zhou, Q.** 2012. Effect of carbon source and COD/NO<sub>3</sub>(-)-N ratio on anaerobic simultaneous denitrification and methanogenesis for high-strength wastewater treatment. *J Biosci Bioeng*, **113**, 759-64.
- Xiong, H., Yang, G., Shan, X. & Miao, L.** 2022. Unveiling the effect of acetate on the interactions of functional bacteria in an anammox biofilm system. *Chemosphere*, **305**, 135408.
- Yang, Q., Peng, Y., Liu, X., Zeng, W., Mino, T. & Satoh, H.** 2007. Nitrogen removal via nitrite from municipal wastewater at low temperatures using real-time control to optimize nitrifying communities. *Environmental science & technology*, **41**, 8159-8164.

- Yang, Y., Li, M., Li, H., Li, X. Y., Lin, J. G., Denecke, M. & Gu, J. D.** 2020. Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes. *Sci Total Environ*, **734**, 139387.
- Yang, Y., Zuo, J., Quan, Z., Lee, S., Shen, P. & Gu, X.** 2006. Study on performance of granular ANAMMOX process and characterization of the microbial community in sludge. *Water Sci Technol*, **54**, 197-207.
- Yin, Q., Feng, Z., Hu, Y., Zhan, X. & Wu, G.** 2021. Microbial Interactions in Pollution Control Ecosystems. *Current Pollution Reports*, **7**, 104-114.
- You, Q. G., Wang, J. H., Qi, G. X., Zhou, Y. M., Guo, Z. W., Shen, Y. & Gao, X.** 2020. Anammox and partial denitrification coupling: a review. *RSC Adv*, **10**, 12554-12572.
- Zhang, J., Miao, Y., Sun, Y., Zhang, Q., Dai, J. & Peng, Y.** 2021. An effective strategy for in situ start-up of mainstream anammox process treating domestic sewage. *Bioresour Technol*, **339**, 125525.
- Zhang, L. & Okabe, S.** 2020. Ecological niche differentiation among anammox bacteria. *Water Res*, **171**, 115468.
- Zhang, L., Zheng, P., Tang, C. J. & Jin, R. C.** 2008. Anaerobic ammonium oxidation for treatment of ammonium-rich wastewaters. *J Zhejiang Univ Sci B*, **9**, 416-26.
- Zhang, Q., Lin, J. G., Kong, Z. & Zhang, Y.** 2022. A critical review of exogenous additives for improving the anammox process. *Sci Total Environ*, **833**, 155074.
- Zhang, X., Li, J., Yu, Y., Xu, R. & Wu, Z.** 2016. Biofilm characteristics in natural ventilation trickling filters (NVTfS) for municipal wastewater treatment: Comparison of three kinds of biofilm carriers. *Biochemical Engineering Journal*, **106**, 87-96.
- Zhao, Y., Liu, S., Jiang, B., Feng, Y., Zhu, T., Tao, H., Tang, X. & Liu, S.** 2018. Genome-Centered Metagenomics Analysis Reveals the Symbiotic Organisms Possessing Ability

to Cross-Feed with Anammox Bacteria in Anammox Consortia. *Environ Sci Technol*, **52**, 11285-11296.

**Zhu, G., Peng, Y., Li, B., Guo, J., Yang, Q. & Wang, S.** 2008. Biological Removal of Nitrogen from Wastewater. *Reviews of Environmental Contamination and Toxicology*, 159–195.