Evaluation of the effect of influent ammonium: nitrite ratio on anammox reactor efficiency

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ABSTRACT

The anaerobic ammonium oxidation (anammox) process has been recognized as an energy-efficient and cost-effective alternative to the conventional nitrification-denitrification route. The anammox process offers many advantages over the conventional processes such as less oxygen demand, non-requirement of external carbon source, and low operational cost. However, the major limitation of this process is the extremely slow growth rate of anammox bacteria and the need for stringent metabolic and reactor conditions leading to a long start-up period, which hinders its application in wastewater treatment.

This study focused on evaluating the effect of key substrates (ammonium and nitrite) on anammox performance (nitrogen (N) removal) and community structure in anaerobic sequencing batch reactors (ASBR). For this, three 1L reactors containing different ammonium: nitrite ratios namely; Reactor 1 (1 NH_4^+-N: 1.32 NO_2^--N), Reactor 2 (2 NH_4^+-N: 1 NO_2^--N) and Reactor 3 (1 NH_4^+-N: 2 NO_2^--N) were operated for 320 days using enriched anammox bacterial seed inoculum. The N removal performance of the reactors was assessed over time based on chemical and microbial analysis.

From the results, the highest nitrogen removal efficiency (NRE) was observed in Reactor 3 containing high NO_2^--N (68.1 ± 7.7 %), followed by Reactor 1 containing the reported anammox stoichiometric substrate ratio (66.3 ± 13.3 %) and Reactor 2 containing high NH_4^+-N (64.1 ± 7.2 %) on the 320th day of reactor operation. By using different substrate ratios, a significant variation (α= 0.05; P= 0.0004) in NRE in the three reactors was observed. Overall, the observed NO_2^--N (consumed)/NH_4^+-N (removed), NO_3^--N (produced)/NH_4^+-N (removed) ratios in Reactor 3 (1.38 ± 0.35 and 0.51 ± 0.34) was closer to the reported anammox stoichiometry ratio compared to Reactor 1 (0.88 ± 0.35 and 0.91 ± 0.48) and Reactor 2 (0.69 ± 0.32 and 0.72 ± 0.26) indicating a better anammox enrichment in Reactor 3.

The inhibitory impact of free ammonia (FA) and free nitrous acid (FNA) concentration was monitored throughout the operational period. The FA concentration did not have a negative effect on anammox bacteria and AOB since the observed FA inhibitory concentration was below the reported inhibitory

concentration of 1700 µg/L for anammox bacteria in all three reactors. As for FNA, Reactor 3 recorded the highest FNA concentrations (27.3 – 27.4 µg HNO_2^--N/L) throughout the study period. This FNA concentration did not negatively affect anammox bacteria on the 170th day, since anammox population was increased. However, long-term exposure resulted in anammox inhibition on the 320th day indicated by reduction of anammox bacteria. Whereas, nitrite oxidising bacteria (NOB) were not negatively affected by the observed FNA concentration, since their activity and growth was observed throughout the operation. As for Reactor 1 and 2, the FNA concentration (5.5 – 5.9 µg HNO_2^--N/L) was below inhibitory concentration on the 170th day. However, on the 320th day, the FNA concentration (6.2 – 7.3 µg HNO_2^--N/L) was above the reported inhibitory value resulting in anammox inhibition.

A detailed exploration of the changes in the microbial community structures within the three reactors were studied by quantitative polymerase chain reaction (qPCR), sequencing and phylogenetic analyses. Using qPCR, Reactor 3 (1:2) with high NH_4^+-N concentration showed high abundance of anammox bacteria followed by Reactor 2 (2:1) with high NO_2^--N concentration and Reactor 1 (1:1.32) having balanced NH_4^+-N: NO_2^--N respectively on the 170th day. Thereafter, a shift from anammox bacteria abundance towards proliferation of AOB and NOB was observed on the 320th day. The AOB population was favoured by the fluctuating DO concentrations (0.39 ± 0.19 – 0.49 ± 0.20 mg/L). High AOB population observed in Reactor 1 (1:1.32) followed by Reactor 3 (1:2) and Reactor 2 (2:1) on 170th and 320th day. The NOB population was high in Reactor 3 (1:2) followed by Reactor 1 (1:1.32) and Reactor 2 (2:1) respectively throughout the operational period.

High throughput sequencing analysis further showed a shift in the microbial community structure on 170th day with an increase in phylum *Planctomycetes* population from 0.76 % to 3.30 % in Reactor 1, 21. 32 % in Reactor 2 and 22.26 % in Reactor 3. The population of *Proteobacteria* increased from 6.38 % to 6.70 % in Reactor 1, 21.63 % in Reactor 2 and 21.73 % in Reactor 3. On the 320th day, *Planctomycetes* population decreased drastically to 2.84 %, 0.36 % and 4.91 % in Reactors 1, 2 and 3, respectively. Whereas *Proteobacteria* population further increased to 28.95 %, 24.15 % and 23.86 % in Reactors 1, 2 and 3,

respectively. The *Nitrospira* population were below 0.10 % on the 170th day, however, an increase was observed on the 320th day from 0.01 % to 2.84 %, 7.38 % and 1.09 % in Reactors 1, 2 and 3, respectively which are in accordance with the qPCR results.

In conclusion, different substrate ratios showed a significant influence on the overall N removal performance as well as on the selection of nitrifiers during the initial 170 days of operation. However, the long term operation of the reactors negatively affected the performance as well as community structure irrespective of the ratio used. Furthermore, the intermittent spike in DO and FNA concentrations (above inhibitory levels) could have affected the growth of anammox bacteria adversely. A further study based on continuous reactor operation is recommended for further verification of the results and prediction of unstable reactor episodes and possible process inhibitions in real-time.

DECLARATION

I Nomalanga Petronella Gasa, hereby declare that the work provided in this dissertation is, to the best of my knowledge, original (except where cited) and that this work has never been submitted for another degree at this or any other tertiary institution.

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I hereby approve the final submission of the following thesis.

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CONTENTS

DEDICATION

This thesis is dedicated to

My Family

My late mother, who has always encouraged to further my studies.

My sisters, Nontobeko and Charlotte, for their ongoing support throughout my studies and unconditional love.

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LIST OF ABBREVIATIONS

Anammox	Anaerobic ammonium oxidation
AOB	Ammonia oxidizing bacteria
NOB	Nitrite oxidizing bacteria
N	Nitrogen
N_2	Nitrogen gas
NO_2^-	Nitrite
NH_4^+	Ammonium
NH_3	Ammonia
NO_3^-	Nitrate
NRE	Nitrogen removal efficiency
FA	Free ammonia
FNA	Free nitrous acid
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
WWTP	Wastewater treatment plant
SBR	Sequencing batch reactor
ASBR	Anaerobic sequencing reactor
DO	Dissolved oxygen
FISH	Fluorescent *in-situ* hybridization
DAPI	4′-6-diamino-2-phenylindole
Tris-HCl	Tris hydrochloride
BLAST	Basic Local Alignment Search Tool
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
PBS	Phosphate saline buffer

PCR	Polymerase chain reaction
qPCR	Quantitative real-time
TE	Tris-EDTA

PREFACE

Research outputs

- **Nomalanga P. Gasa**, Chika F. Nnadozie, Kosgey Kiprotich, Faizal Bux and Sheena Kumari. Effect of ammonium to nitrite ratio on reactor performance and microbial population structure in anammox reactors DOI: 10.1080/09593330.2019.1610076

- **Nomalanga P. Gasa**, Chika F. Nnadozie, Faizal Bux and Sheena Kumari. Evaluation of the effect of infeed ammonium: nitrite ratio on the performance of anammox reactor. Water Institute of Southern Africa (WISA) Biennial Conference & Exhibition, International Convention Centre, Cape Town South Africa, June 2018 (Oral Presentation).

CHAPTER 1: INTRODUCTION

The biological nitrogen (N) removal process known as anaerobic ammonium oxidation (anammox) process has steadily gained popularity ever since it was discovered in the 1990s (Strous *et al*., 1998, Cao *et al*., 2016). The anammox process offers many advantages such as high N removal, reduced energy demand for aeration (up to 60-90 %), no organic carbon requirement for denitrification, minimal sludge production, reduced carbon dioxide (CO_2) and nitrous oxide (N_2O) emission (Kuenen. 2008).

The anammox bacteria are capable of oxidising ammonium (NH_4^+) directly to dinitrogen (N_2) gas using nitrite (NO_2^-) as the electron acceptor under anoxic conditions (Connan *et al*., 2016, Strous *et al*., 1998, Hu *et al*., 2011). The anammox process is a cost-effective and sustainable technology for N removal over the conventional nitrification-denitrification process. Despite all these advantages, successful implementation of the anammox process can still be limited due to its sensitivity to operational and environmental conditions (Costa *et al*., 2014, Shu *et al*., 2015, Chen *et al*., 2017). Furthermore, anammox bacteria are difficult to culture with very low cellular yield and growth rates; hence it requires long start-up periods (Kuenen. 2008). The doubling time for anammox bacteria is reported to be approximately 2 – 12 days (Trigo *et al*., 2006, Gao *et al*., 2013, Bagchi *et al*. 2010). Wang *et al.* (2012) reported an average of 101 days as anammox start-up in a SBR.

The stability of the anammox process can be affected by various factors including biomass composition, substrate concentration, reactor configuration, dissolved oxygen (DO), pH and temperature (Chen *et al*., 2017, Zhang *et al*., 2016). Based on earlier studies, the substrate ratio of 1 NH_4^+-N: 1.32 NO_2^--N has been widely accepted as the optimum anammox process stoichiometry (Bai *et al*. 2019, Strous *et al*. 1998). Both NH_4^+ and NO_2^- function as substrates, however, high concentrations of either can disrupt anammox stability and reduce anammox activity (Fernandez *et al*., 2012, Connan *et al*., 2016). Studies have reported that the anammox process is constrained by NH_4^+ concentration greater than 1000 mg N/L (Fernandez *et al*., 2012, Dapena-Mora *et al*., 2007, Strous *et al*., 1999). Nitrite inhibition is also widely studied in the anammox process and has been observed to be a critical factor for the stability of anammox process (Jin *et al*.,

2012, Raudkivi *et al*., 2017). Complete loss of anammox activity was reported at NO_2^- concentration of 100 mgN/L (Ma *et al*., 2016, Dapena-Mora *et al*., 2007). Other studies also recognised free ammonia (FA) and free nitrous acid (FNA) as true inhibitors rather than NH_4^+ and NO_2 (Raudkivi *et al*., 2017, Liu *et al*., 2019, Jaroszynski *et al*., 2012). Therefore studies suggest that there is a need to properly understand the influence of these substrates on the overall performance of the anammox process.

Temperature has also been reported amongst the key factor that affects the anammox bacterial growth, metabolism, and abundance. Anammox bacteria have been detected in environments with a broad range of temperatures (de Almeida *et al*., 2018, He *et al*., 2018, Dosta *et al*., 2007). According to literature, anammox bacteria could adapt to low-temperature conditions (around 20 °C) by acclimatisation, while higher temperatures (above 45 °C) could cause an irreversible decline in anammox activity (Dosta *et al*., 2008, Egli *et al*., 2001). However, the optimal temperatures reported for anammox growth are between 25 and 40 °C (Tikilili. 2016, Tomaszewski *et al*., 2017b). Similarly, high pH has been reported to increase FA concentration whilst low pH increase FNA concentration, therefore, to ensure anammox growth and stability, the pH should be kept within a range of 7 and 8 (Banihani *et al*., 2012, Mosquera-Corral *et al*., 2005, Bäckman. 2013). Additionally, anammox bacteria are sensitive to DO, and reversible anammox inhibition has been reported at low DO concentration (< 0.17 mg/L) (Jung *et al*., 2007, Egli *et al*., 2001).

Anammox bacteria have been reported to be present in different terrestrial and oceanic water environments (Kawagoshi *et al*., 2009). However, many studies suggest that anammox bacteria exist with other bacterial groups since no pure cultures have been obtained yet (Ali *et al*., 2015, Connan *et al*., 2016, Ibrahim *et al*., 2015). Studies have discovered the presence of anammox bacteria and other bacteria including ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and denitrifiers in the same reactor under controlled conditions (Yang *et al*., 2018, Xie *et al*., 2018, Zeng *et al*., 2016). Therefore, an anammox reactor is prone to contain other functional microbial groups due to similar substrate requirements (Third *et al*., 2001, Yan *et al*., 2010). Furthermore, studies targeting 16S rRNA metagenomic have detected several microorganisms including

Chloroflexi, *Bacteroidetes*, *Acidobacteria* etc. (Gonzalez-Martinez *et al*., 2016, Pereira *et al*., 2017, Connan *et al*., 2016, Carvajal-Arroyo *et al*., 2013). Therefore, it is imperative to study the microbial structure for better understanding of the anammox process.

Since anammox bacteria do not exist alone in nature, different bioreactors have been engineered in order to provide conditions that selectively enrich and stimulate them successfully (Connan *et al*., 2016). Anammox process has been implemented in different types of bioreactors including membrane bioreactor (MBR), upflow biofilter (UBF) reactor, gas lift reactor, sequencing batch reactor (SBR), rotating biological contactor (RBC), upflow anaerobic sludge blanket (UASB) reactor, fluidized bed reactor (FBR) and moving bed biofilm reactor (MBBR) (Laureni *et al*., 2015, Connan *et al*., 2016). The SBR has been reported to be efficient for anammox enrichment with its advantages of consistent biomass and substrate distribution (to prevent substrate inhibition and prevents NO_2^- accumulation), efficient biomass retention, simple set-up and reliable operation (stable more than one year) (Strous *et al*., 1998, Xin *et al*., 2016, Zhang *et al*., 2016, Reino and Carrera, 2017). Studies have shown the enrichment of anammox bacteria up to 74 % in SBR (Dapena-Mora *et al.*, 2004, Jin *et al*., 2012, Wang *et al*., 2019).

Anammox process can be improved by selectively enriching the anammox bacteria whilst inhibiting the activity of other microbial groups such as NOB and/or AOB. However, the effect of different ammonium/ nitrite substrate ratios on the synergy of these microbial is not known. The substrate concentration (NH_4^+ and NO_2^-) can encourage or reduce the performance of the anammox and other related bacteria during the operation of the reactors. Thus, the effect of different substrate ratios on the performance of the anammox process including microbial community structure requires consideration. This study therefore aimed at developing and operating three anammox based ASBRs, understanding the effect of different key anammox substrate ratios (ammonium: nitrite) on the performance (N conversion) of the anammox based ASBRs. The reactors were inoculated with microbial consortia (preliminary identified to contain anammox bacteria, AOB and NOB) and operated under different substrate ratios. Each reactor performance was monitored by measuring the relevant N compounds

(NO_2^- and NH_4^+ removal and NO_3^- and N_2 gas production) and formation of inhibitory compounds (FA and FNA). Finally, the microbial community structure within these anammox reactors was also assessed using molecular techniques.

1.1 Aim and objectives of the study

Aim

To assess the effect of ammonium: nitrite ratio on reactor performance and microbial community structure of anammox based anaerobic sequencing batch reactors (ASBRs).

Objectives

The key objectives are to:

i. Develop and operate a laboratory-scale anammox based ASBR and evaluate the reactor performance at different substrate concentration by measuring the ammonium and nitrite removal and nitrate formation.
ii. Assess the formation of inhibitory compounds FNA and FA under different substrate ratios.
iii. Evaluate the effect of selected substrate ratios on the microbial community structure within the reactors.

Thesis Outline

Chapter one: This chapter presents the introduction of the topic, the research aim, objectives and the outline of the thesis.

Chapter two: Presents a literature review on anammox process, anammox microbiology, factors influencing the anammox process and anammox application in wastewater treatment. Additionally, chapter two highlights the molecular techniques used to detect and quantify anammox bacteria.

Chapter three: Presents the start-up and operation of three ASBRs using anammox biomass under different substrate ratios. Furthermore, full details on how each reactor's performance was monitored in terms of N removal and inhibitory compounds formation (Objective 1 and 2).

Chapter four: Presents the details of the microbial community structure and its shift during the operation of the reactors using qPCR and high-throughput 16S rRNA sequencing and analysis (Objective 3).

Chapter five: Consists of the conclusions, recommendations of the study and concludes with the references cited in this thesis and appendices.

CHAPTER 2: LITERATURE REVIEW

2.1 Anammox process

The anammox process is a cost-effective and sustainable technology with maximum potential for treating wastewater with high ammonium (NH_4^+) concentrations (Kuenen. 2008, de Almeida Fernandes *et al*., 2018, Ma *et al*., 2011). The anammox reaction was first detected in a denitrifying pilot-scale reactor experiment in Delft (Netherlands) 20 years ago. In this reactor, NH_4^+ and nitrite (NO_2^-) simultaneously disappeared from an anaerobic reactor followed by a minor production of dinitrogen gas (N_2) and nitrate (NO_3^-) (Ahn, 2006, Egli, 2003). Research on anammox activity was later reported worldwide following this first discovery (Schmid *et al*., 2005, Ibrahim *et al*., 2015).

The anammox process has been reported in various natural and engineered environments such as wastewater treatment reactors, marine environments and laboratory-scale bioreactors (Connan *et al*., 2016). In the marine environments, the anammox process was revealed to contribute approximately 79 % of N_2 production, thus supporting the anammox significance in the global N cycle (Dalsgaard *et al*., 2005). de Almeida Fernandes *et al*. (2018) reported that the average N removal efficiency of 96 % was observed in a reactor inoculated with anaerobic pre-treated municipal wastewater. The anammox process overcomes drawbacks and eliminates all the enzymatic steps associated with conventional nitrification/ denitrification processes (Figure 2.1) (Du *et al*., 2015, Jaroszynski *et al*., 2011, Jetten *et al*., 1998). The biochemical reaction of the anammox process involves the oxidation of NH_4^+ to N_2 gas with NO_2^- as an electron acceptor under anoxic conditions (equation 1) (Strous *et al*., 1998). The molar ratios for NH_4^+ consumed, NO_2^- removed, N_2 produced, and NO_3^- produced are 1.00:1.32:1.02:0.26, respectively.

$$1\,NH_4^+\ 1.32\,NO_2^- + 0.66\,HCO_3^- + 0.13\,H^+ = 1.02N_{\ 2} + 0.26\,NO_3^- + 0.066\,CH_2O_{0.5}N_{0.15} + 1.03\,H_2O \quad (1)$$

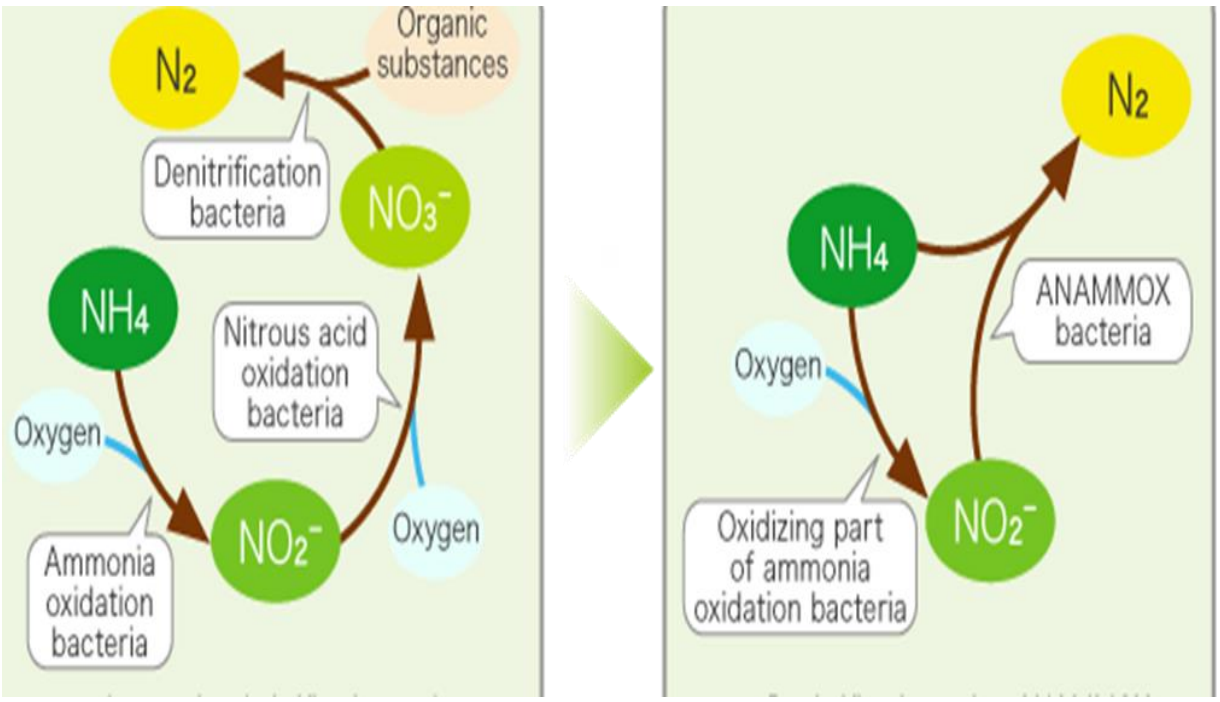

Figure 2.1: Conventional nitrification/ denitrification processes (left) compared with anammox process (right) (Kurita. 2017).

The first full-scale application of the anammox process was reported at Rotterdam (Netherlands) WWTP to remove excess NH_4^+ from an anaerobic sludge digester effluent (Mulder and Hellinga, 2001). Currently, more than 200 full-scale WWTPs using an anammox process have been implemented worldwide (Li, 2018, Tomaszewski *et al*., 2017b). These full-scale systems are developed mainly to treat industrial wastewater (that are rich N compounds and low C:N ratio) viz: winery industries, anaerobic digestion reject water, tannery, fermentation yeast, food processing, semiconductor and distillery (Vlaeminck *et al*., 2012, Lackner *et al*., 2014, Cao *et al*., 2017, van Hulle *et al*., 2010). Sidestream application of anammox process has also been reported in Gaobeidian WWTP in Beijing, China (Zheng *et al*., 2016), Water Reclamation Plant in Changi, Singapore (Yeshi *et al*., 2016) and Dokhaven WWTP in the Netherlands (van der Star *et al*., 2007).

2.2 Anammox bacteria biodiversity

The anammox process is mediated by chemolithoautotrophic bacteria belonging to the order *Planctomycetales* (Jetten *et al*., 2001). Six *Candidatus* genera and 13 species of these bacteria are identified. These include *Brocadia* (*fulgida, anammoxidans*, *sinica*), *Scalindua* (*marina, brodae*, *wagneri*, *arabica, sorokinii*, *profunda*), *Kuenenia* (*stuttgartiensis*), *Jettenia* (*asiatica*, *caeni*), *Anammoxomicrobium* (*moscowii*) and *Anammoxoglobus* (*propionicus*) (Li *et al*., 2018, Kuenen, 2020, Abbas *et al*., 2019). Studies have reported the ubiquitous occurrence of different anammox species in natural and engineered environments (Jiang *et al*., 2017, Shu *et al*., 2015, Zhang *et al*., 2017). Strous *et al*. (1999) were the first to identify *Candidatus Brocadia*, followed by Schmid *et al*. (2000) who identified *Kuenenia* (*stuttgartiensis*) from a fluidized bed reactor. Anammox bacterial groups are considered to have distinct specific habitats. The genus *Scalindua* is commonly identified in WWTPs, marine environment, and freshwater worldwide (Yokota *et al*., 2018, Ibrahim *et al*., 2015). While *Kuenenia*, *Brocadia*, *Jettenia*, *Anammoxoglobus* and *Anammoxomicrobium* genera were identified in both wastewater treatment plants and freshwater (Yang *et al*., 2018, Tomaszewski *et al*., 2017, Ma *et al*., 2016). Moreover, the genus *Jettenia* was also retrieved from agricultural soil (Hu *et al*., 2012, Ali *et al*., 2015). Furthermore, various studies have revealed that different anammox bacteria have a versatile

metabolism (Fu *et al*., 2015, Zheng *et al*., 2016, Bettazzi *et al*., 2010). de Almeida Fernandes *et al*. (2018) observed high N (NH_4^+ and NO_2^-) removal efficiency by *Candidatus Brocadia* and *Candidatus Anammoximicrobium.* The growth and substrate affinity of different anammox species differ i.e. *Brocadia* dominated at lower substrate affinity than *Kuenenia* (Puyol *et al*., 2014, Zhang *et al*., 2016). Studies on microbial competition among anammox bacteria revealed substrate (NO_2^-) competition among *Brocadia* (*sinica*), *Jettenia* (*caeni*) and *Kuenenia* (*stuttgartiensis*) (Zhang *et al*., 2017, Oshiki *et al*., 2016).

Anammox bacteria (*Kuenenia*, *Brocadia* and *Anammoxoglubus*) can use alternative electron donors including propionate, formate and acetate beside NH_4^+ (van Niftrik and Jetten. 2012, Shu *et al*., 2015). Jiang *et al*. (2017) reported salinity driven shifts in the activity, diversity, and abundance of anammox bacteria from *Scalindua* (*wagneri*, *marina* and *brodae*) to *Kuenenia* (*stuttgartiensis*). Furthermore, different doubling times have been reported regarding different anammox bacteria i.e. 7 days for *Brocadia* and 8 – 11 days for *Kuenenia* (Lotti *et al*., 2015, van der Star *et al*., 2008). The anammox bacteria 16S rRNA revealed *Scalindua* was the most abundant, followed by *Brocadia* and *Kuenenia* as second and third most abundant genus respectively and *Jettenia* was the lowest abundant among all anammox bacteria (Zhang *et al*., 2017, Kuenen, 2020).

2.3 Phylogeny of anammox bacteria

Anammox bacteria belong to the phylum *Planctomycete* and order *Brocadiales*. The 16S rRNA sequences phylogenetic analyses of anammox bacteria show that they form a monophyletic branch. This branch consists of five genera with about 90 % sequence similarity between different families of anammox bacteria (Kuenen, 2008). Furthermore, an anammox 16S rRNA gene phylogenetic tree revealed global anammox bacterial distribution across a wide range of habitats (Pereira *et al*., 2017, Sanchez-Melsio *et al*., 2009).

2.4 Structure of anammox bacteria

The *Planctomycete* (anammox bacterium) possesses unique membrane-bound sub-cellular compartments namely: (i) paryphoplasm, (ii) riboplasm and (iii) anammoxosome (Figure 2.2) (Ibrahim *et al*., 2015, Kuenen, 2008). The cell wall is composed of outermost membrane together with peptidoglycan layer, the second membrane surrounds the cytoplasm and the largest part of the cell comprises a vacuolar cell organelle (Kartal and Keltjens. 2016).

Paryphoplasm membrane is the outermost compartment with nearly 20 % average of the total anammox cell size (van Niftrik, 2013). The paryphoplasm membrane was reported to play a crucial role in regulating the osmosis of the cell and constraining the hyperosmotic contents of the anammox cell wall (Neumann *et al*., 2014). The riboplasm (surrounded by an intracytoplasmic membrane) is the middle compartment and contains ribosomes where ATP is produced (van Niftrik, 2013). The anammoxosome (tubule-like structure) is an important compartment dedicated to anammox metabolism (Figure 2.2) which is made up of more than 30 % of the cell volume (Niftrik *et al*., 2004). Exclusive unique ladderane membrane lipids surrounding the anammoxosome protect the cell from toxic intermediates (van Niftrik and Jetten, 2012, Kartal *et al*., 2004).

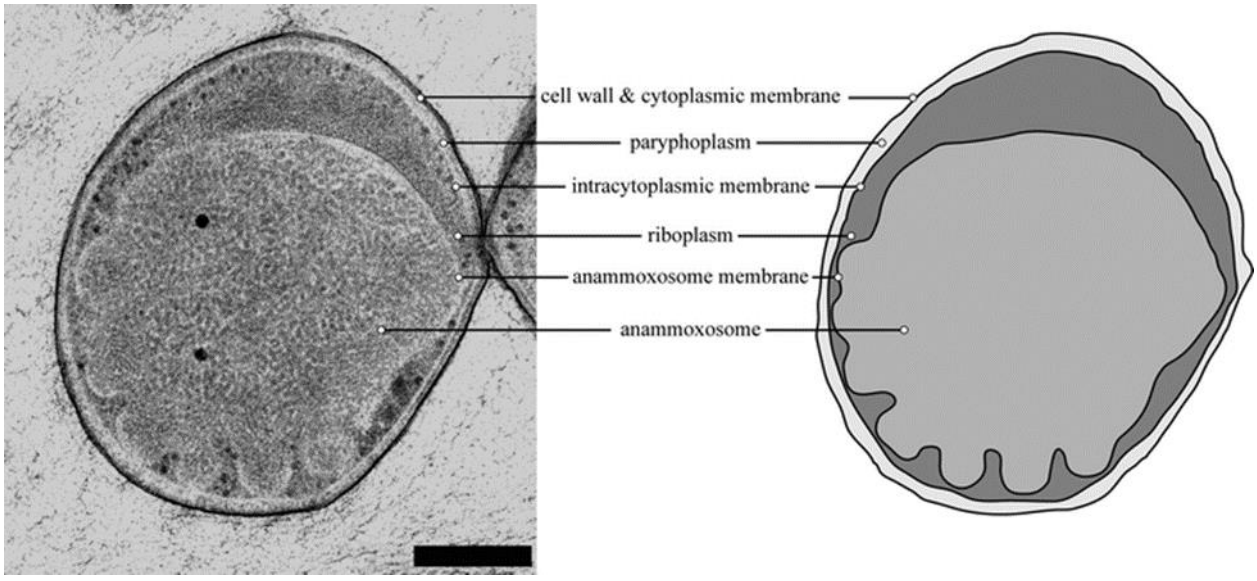

Figure 2.2: Electron micrograph and line drawing of the anammox cell compartments (van Niftrik, 2013).

2.5 Metabolism of anammox bacteria

Nitric oxide (NO) and hydrazine (N_2H_4) are two key intermediates in the anammox metabolic activity (Figure 2.3) (Kartal and Keltjens, 2016). The conversion of the substrate involves three main reaction steps. The first step is NO_2^- reduction to NO by nitrite reductase (nir). The second step is the combination of NO with NH_4^+ to produce hydrazine by hydrazine synthase (hzs), this step requires three electrons provided by electrons from menaquinone through postulated electron transport module located in the membrane. The final step is the oxidation of hydrazine to N_2 gas by hydrazine dehydrogenase (Kuenen. 2020). Four electrons are derived from this oxidation and are transferred to soluble cytochrome c electron carriers (de Almeida *et al*., 2016). Menaquinone (MK) is an electron carrier used to transfer electrons from hydrazine by hydrazine dehydrogenation to membrane-bound protein complexes (Ali *et al*., 2015).

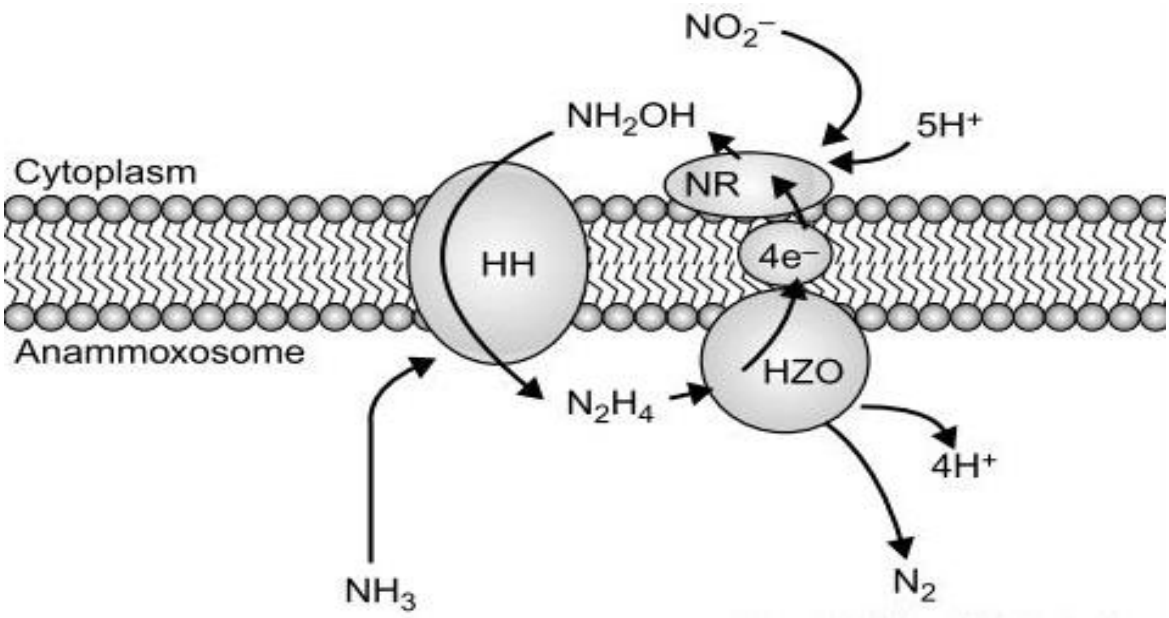

Figure 2.3: Depiction of the metabolic pathway of anammox reaction in the anammoxosome membrane (Jetten *et al*., 2001).

2.6 Anammox enrichment

Successful application of the anammox process in wastewater treatment systems relies on the interaction of anammox bacteria, operational parameters and environmental conditions, which enables efficient anammox activity and N removal (Chen *et al*., 2017). Presently no anammox bacteria have been isolated in pure culture (Ali *et al*., 2015, Connan *et al*., 2016, Ibrahim *et al*., 2015). Different bacterial species are known to coexist with anammox bacteria in a typical anammox biomass which also includes nitrifiers, denitrifiers, heterotrophic and filamentous bacteria (Wang *et al*., 2016, Costa *et al*., 2014) depending on the substrate and operational conditions. The operational conditions viz., pH, temperature, DO, substrate concentration etc., have a direct impact on microbial community structure (Zhang *et al*., 2016).

Various strategies have been employed to shorten the start-up period of anammox process and exclusively enrich anammox bacteria from mixed consortia to achieve suitable N removal within anammox system (Trigo *et al*., 2006, Gao *et al*., 2013, Connan *et al*., 2016, Uyanik *et al*., 2011). These strategies include but not limited to designing appropriate bioreactors, using seed inoculum that already contains dominant anammox bacteria, manipulating environmental conditions (i.e. pH, temperature, substrate concentration, DO and inhibitors) to favour the cultivation of anammox bacteria (Chen *et al*., 2017). Furthermore, maintaining substrate (NH_4^+ and NO_2^-) concentration below the inhibitory level is crucial to prevent substrate inhibition (Connan *et al*., 2016).

Designing a reactor with optimum reactor conditions suitable for anammox bacterial growth and activity is in demand to reduce the challenge of a long start-up period. Additionally, the selection of a reactor is very important in the anammox process because it must be appropriate for continuous enrichment, cultivation and quantitative analysis (Strous *et al*., 1998, Chamchoi and Nitisoravut, 2007). Different type of reactors including membrane bioreactor (Li *et al*., 2014, Li *et al*., 2015), gas lift reactor (Dapena-Mora *et al*., 2004), fluidised reactor (Mulder and Hendriks, 2014), SBR and rotating biological contactor (Strous *et al*., 1998) were employed for anammox bacterial enrichment.

Studies have revealed SBR to be suitable for anammox enrichment because of the consistent mixture of biomass, long term operation, reduced substrate

inhibition (high NO_2^- concentration) and efficient biomass retention time (Strous *et al*., 1998). The SBR operates under a series of cycles or periods namely: fill, react, settle, decant, and idle periods. These periods can be manipulated to achieve desired biological nutrient removal treatment (Singh *et al*, 2011). Furthermore, the SBR is flexible and can be designed with the ability to treat a wide range of influent volumes (Al-Rekabi *et al*., 2007). Sequencing batch reactor, therefore, meets the demand of long start-up period, cultivation and quantitative analysis for slow-growing anammox bacteria (Kuenen. 2008). According to earlier studies, using SBR, successful enrichment of anammox bacteria can be established within 60 days with a high nitrogen removal efficiency (82 %) (Ibrahim *et al*., 2015, Dapena-Mora *et al*., 2004).

2.7 Factors influencing anammox enrichment in bioreactors

The widespread industrial-scale application of the anammox process is hindered by the slow growth of the anammox bacteria. Previous studies have shown that the anammox process is more stable when the anammox bacteria are dominant in the system (Chen *et al*., 2017, Cao *et al*., 2016, Shu *et al*., 2015). However, the growth of anammox bacteria is dependent on various factors including influent substrate composition, DO, pH, temperature, sludge retention time and the presence of organic compounds (Connan *et al*., 2016). Therefore, controlling the above key parameters is vital in maintaining the stability of the anammox process (Du *et al*., 2014).

2.7.1 Influent substrate concentration

An optimum level of NH_4^+ and NO_2^- is key for the growth and metabolism of anammox bacteria. The substrate theoretical stoichiometric ratios of 1 NH_4^+ and 1.32 NO_2^- are used to analyse the anammox process (Strous *et al*., 1998). This anammox ratio is widely used in various anammox studies (Dapena-Mora *et al*., 2007, Connan *et al*., 2016, Lotti *et al*., 2014, Zhang *et al*., 2016). However, incomplete consumption will lead to poor effluent quality, inhibition of anammox bacteria and a decrease in N removal efficiency (Jin *et al*., 2013). Higher or lower substrate concentration could subsequently reduce the metabolic function of anammox bacteria (Tang *et al*., 2010, Ibrahim *et al*., 2015). Nitrite may cause severe irreversible inhibition at high concentrations (Puyol *et al*., 2014, Ma *et al*.,

2016, Lotti *et al*., 2012). Complete loss of anammox activity has been observed at NO_2^- concentration of 100 mg N/L (Strous *et al*., 1999). Few other studies have reported varying NO_2^- inhibitory concentration under different experimental conditions. Egli *et al*. (2001), observed anammox deactivation when NO_2^- concentration was above 185 mg N/L. Furthermore, a 50 % decrease in anammox activity was observed by Dapena-Mora *et al*. (2007) when NO_2^- concentration was above 350 mg N/L. However, Isaka *et al*. (2007) noted that NO_2^- concentration below 280 mg N/L was suitable for stable anammox activity. On the other hand, too low NO_2^- concentration can also have inhibitory effect on the anammox process. Few reports indicate that low NO_2^- concentration between 5 and 40 mg N/L strongly inhibits anammox activity (Wett, 2007, Lotti *et al*., 2012). Nitrite concentration greater than 100 mg N/L was reported to suppress anammox bacteria (Dapena-Mora *et al*., 2007).

Anammox process is reportedly inhibited by NH_4^+ concentrations above 1 g N/L (Strous *et al*., 1999, Jin *et al*., 2012). However, Dapena-Mora *et al*. (2007) indicated a 50 % loss of anammox activity at NH_4^+ concentration of 770 mg N/L. Unlike NO_2^- inhibition, there is very limited literature on NH_4^+ inhibition of the anammox process (Jaroszynski *et al*., 2012).

2.7.2 pH

pH is a crucial parameter for anammox bacterial activity and growth in the anammox process, due to the sensitivity of anammox bacteria (Tomaszewski *et al*., 2017b). The optimum pH for anammox bacteria enrichment was reported in the range of 6.7 – 8.3 (Tomaszewski *et al*., 2017b). However, anammox activity has been also reported over a wide pH range i.e. from 6.5 to 9.3 (Jin *et al*., 2012, Jaroszynski *et al*., 2011). Anammox bacterial inhibition is caused by pH values above and below optimal value. Tang *et al*. (2010), observed loss of anammox activity in an up-flow biofilm reactor when the pH range was 8.7 - 9.05. The pH also influences the concentration of toxic forms of NH_4^+ and NO_2^- i.e. FA and FNA. Loss of anammox activity associated with FA and FNA have been reported by many studies (Lotti *et al*., 2012, Jung *et al*., 2007, Fernandez *et al*., 2012, Puyol *et al*., 2014).

2.7.3 Temperature

Temperature is an essential factor for the growth of anammox bacteria and can directly affect their activity (Lotti *et al*., 2015, Ma *et al*., 2016). The anammox bacteria presence and activity has been identified in a wide range of temperatures (Jin *et al*., 2011). Temperatures between 25 °C and 40 °C have been reported optimum for anammox bacteria (Tomaszewski *et al*., 2017b). de Almeida Fernandes *et al*. (2018) achieved a high average NH_4^+ (96 %) and NO_2^- (98 %) removal efficiency in an anammox reactor operated at temperatures between 20 and 35 °C. Also, high NH_4^+ removal (90 %) was observed at a temperature below 25 °C in an anammox MBR (Hu *et al*., 2012). On the other hand, a 40 % NH_4^+ removal was reported in a partial nitritation/anammox reactor operated at 13 °C (Gonzalez-Martinez *et al*., 2016). Lotti *et al*. (2015) and Laureni *et al*. (2015) have reported the dominance of genera *Brocadia* at a low-temperature range of 6 – 15 °C. At a low temperature of 12 °C Laureni *et al*. (2015) observed N removal rate above 0.04 g N $L^{-1}d^{-1}$ in an SBR.

Furthermore, controlling temperature and pH is crucial in the anammox process since it can have an influence on the formation and concentration of free nitrous acid (FNA) and FA concentrations within the reactor. The simultaneous effect of temperature and pH can significantly determine the FA and FNA concentrations in the reactors (Jin *et al*., 2012).

2.7.4 Free ammonia and free nitrous acid

The FA concentration increases at high pH and decreases at low pH, while the FNA concentration increases at low pH and decreases at high pH (Puyol *et al*., 2014). The concentration of FA and FNA is dependent on the substrate concentration, pH and temperature within the anammox reactor. Furthermore, the concentration of FA and FNA can have either reversible or irreversible effects on the anammox process (Niu *et al*., 2016). Free ammonia can easily diffuse across the anammox cell membrane, neutralize the intracellular pH and cause cell death (Jaroszynski *et al*., 2011, Laloo and Bond. 2018). The lowest FA inhibitory concentration reported for anammox was 1700 µg/L (Jung *et al*., 2007). Destabilisation of the anammox process was observed when pH levels were

between 8.7 and 9.1 and the recorded FA concentration was between 67 and 73 mg/L (Tang *et al*., 2009).

pH can also affect the equilibrium of FNA concentration thus inhibiting the anammox process. According to Jin *et al*. (2012), a stable anammox process was observed when the FNA concentration was below 0.5 µg/L. However, loss of *Kuenenia stuttgartiensis* activity was observed when FNA concentration was 0.04 mg N/L, whereas *Brocadia anammoxidans* was completely inhibited by FNA concentration of 0.006 mg N/L (Strous *et al*., 1999, Egli *et al*., 2001).

2.7.5 Dissolved oxygen (DO)

Studies have shown that DO can negatively affect the anammox microbial community structure as well as N removal performance (Chen *et al*., 2012). Anammox bacteria are sensitive to oxygen, therefore, it is vital to strictly control DO concentration to avoid reversible or even irreversible anammox inhibition (Ibrahim *et al*., 2015). The obligate anaerobic anammox bacteria can be inhibited by DO concentrations above 0.5 mg/L. However, even lower DO concentrations (0.08 – 0.1 mg/L) have inhibitory effect on anammox bacteria (Schmid *et al*., 2003, Egli *et al*., 2001,Yin *et al*., 2016). Exposure to DO is difficult to avoid in bioreactors due to oxygen leakage, which can cause deterioration of anammox performance (Third *et al*., 2005). Elevated DO concentration in the reactor can promote the activity of other microbial groups such as AOB and NOB (Chen *et al*., 2017). Anammox genus *Brocadia* and *Kuenenia* were observed in bioreactors containing with <63 mmol and <200 mmol of DO concentration respectively (Oshiki *et al*., 2011, van der Star *et al*., 2008). Overall control of substrate in combination with pH, temperature and DO is paramount in order to maintain stable anammox process and achieve efficiency of N removal.

2.7.6 Inoculum source

Inoculum source containing anammox bacteria is essential during the start-up period of the anammox process (Li, 2018). Numerous biomass sources have been used to inoculate anammox reactors for a rapid start-up including anammox biomass, activated sludge, marine sediments, nitrifying sludge, denitrifying sludge, aerobic sludge, mixed seeding sludge and anaerobic sludge (Costa *et al.*, 2014, Chen *et al*., 2016, Li, 2018). Ni *et al*. (2011) demonstrated that

inoculating with matured anammox biomass accelerated the start-up period to two weeks. A successful start-up period of 50 days was achieved in the MBBR reactor seeded with granular biomass (Kowalski *et al*., 2018). Furthermore, rapid start-up times of approximately three months were achieved in an SBR inoculated with mixed culture (Date *et al*., 2009, Chamchoi and Nitisoravut, 2007).

2.8 Application of anammox process in wastewater treatment

The anammox process application in biological N removal in WWTPs has increased rapidly since its first discovery. Successful application of the anammox process for treating various N-rich wastewaters such as digester liquor, landfill leachate, turtle breeding and pig manure effluents wastewater has been reported (Ruscalleda *et al*., 2008, Ali and Okabe, 2015, Monballiu *et al*., 2013, Chen *et al*., 2012, Fux and Siegrist, 2004). Adaptation of anammox bacteria to new reactor conditions is paramount for an effective and rapid application of the anammox process in wastewater treatment systems. Acclimatisation could either promote the existing bacterial population to the new environment or a substantial shift in the microbial community. Many research strategies have developed to enhance the activity and growth of anammox bacteria, thus making it feasible to implement anammox process in mainstream (de Almeida *et al*., 2018, Li, 2018). Nevertheless, the mainstream application is very limited due to numerous differences in wastewater characteristics.

Since the discovery of anammox bacteria, novel technologies for N removal in wastewater treatment have been established. However, like any other technology, the anammox suffers some drawbacks, which include the production of approximately 11 % NO_3^- in the effluent. The chemical oxygen demand (COD) in wastewater can disrupt the anammox process thus decreasing the N removal efficiency. Lastly, the presence of NOB can restrain anammox bacteria due to inadequate supply of NO_2^- since NOB can rapidly consume NO_2^- (Zhang *et al*., 2017). Researchers have developed partial nitrification and anammox technologies to effectively solve these drawbacks. In partial nitrification and anammox process, a portion of NH_4^+ is oxidised to NO_2^- by AOB in wastewater, the remaining NH_4^+ and NO_2^- produced is reduced to N_2 gas by anammox bacteria (Yang *et al*., 2018, Xu *et al*., 2014, Wang *et al*., 2017). Partial nitrification

and anammox technologies include completely autotrophic nitrogen removal over nitrite (CANON) (Gonzalez-Martinez *et al*., 2016), single reactor for high activity ammonium removal over nitrite (SHARON) (Shalini and Joseph, 2018), oxygen-limited autotrophic nitrification-denitrification (OLAND) (Monballiu *et al*., 2013, Hien *et al*., 2017) and partial nitrification, anammox and denitrification (SNAD) (Zhang *et al*., 2017). Partial nitrification and anammox process has been successfully applied in NH_4^+-rich wastewater (Wang *et al*., 2018, Ibrahim *et al*., 2015).

2.9 Interaction between ammonia oxidizing bacteria, nitrite oxidizing bacteria and anammox bacteria

The biological N cycle involves interaction between various microorganisms catalysing distinct reactions (Stein and Klotz, 2016). Conventional nitrification/ denitrification processes rely on the sequential interaction of nitrification/ denitrification bacteria. Contrary, the anammox process is performed by only anammox bacteria that convert NH_4^+ and NO_2^- to produce NO_3^- and N_2. Anammox bacteria and AOB are capable of oxidizing NH_4^+ to NO_2^-, whereas anammox bacteria and NOB utilise NO_2^- to produce NO_3^-. Therefore, the presence of anammox bacteria, AOB and NOB in the same niche may result in substrate competition (Dai *et al*., 2019). Ammonia oxidizing bacteria and NOB have a faster metabolic rate compared to anammox bacteria which may cause substrate competition, thus allowing anammox bacteria to be outcompeted (Gao *et al*., 2013). A combination of AOB and anammox bacteria in a single reactor displayed better microbial activity and economic stability than separate anammox and AOB processes (Dai *et al*., 2019). Ammonia oxidizing bacteria can oxidise NH_4^+ to NO_2^- and provide oxidised NO_2^- to anammox bacteria under oxygen-limited conditions (Lam *et al*., 2009). On the other hand, simultaneous enrichment of NOB could lead to NO_3^-accumulation and deterioration N removal and anammox inhibition. Nitrite available in the reactor would be consumed rapidly by NOB and anammox inhibition would be prompted by lack of NO_2^- (Xu *et al*., 2014). Therefore, controlling NOB in a reactor is crucial for effective anammox bacterial activity. The partial nitrification-anammox process was developed as an effective technology that can inhibit NOB growth in a reactor.

Intermittent aeration successfully inhibited NOB activity due to lag phase resulting from aerobic conditions in a process called SNAD. Zhang *et al*. (2017), adopted the SNAD process and achieved significant total N removal efficiency of 99 % in a SBR. In the same study a decrease in NOB population was observed throughout the operational period.

2.10 Detection and quantification of anammox bacteria using molecular techniques

Molecular methods have been used as essential and valuable tools in identifying and understanding microbial communities in complex natural and engineered anammox environments (Pereira *et al*., 2017, Chu *et al*., 2015). Specific DNA/ RNA-based molecular techniques can be used to determine the distribution of anammox in natural and engineered environments (Pereira *et al*., 2017). Previously only a few microorganisms were erroneously thought to be responsible for the N cycle (Juretschko *et al*., 1998). Monitoring a microbial population or specific group using only one technique cannot yield conclusive results as each technique has its own advantages and disadvantages. Therefore, a combination of qualitative and quantitative methods could be used to overcome the limitations of one technique (Park *et al*., 2010). Various molecular techniques used to detect and quantify anammox bacteria and other microbial communities in anammox systems include polymerase chain reaction (PCR), quantitative real-time polymerase chain reaction (qPCR), fluorescent in-situ hybridization (FISH), Sanger sequencing and next generation sequencing (NGS) (Figure 2.4).

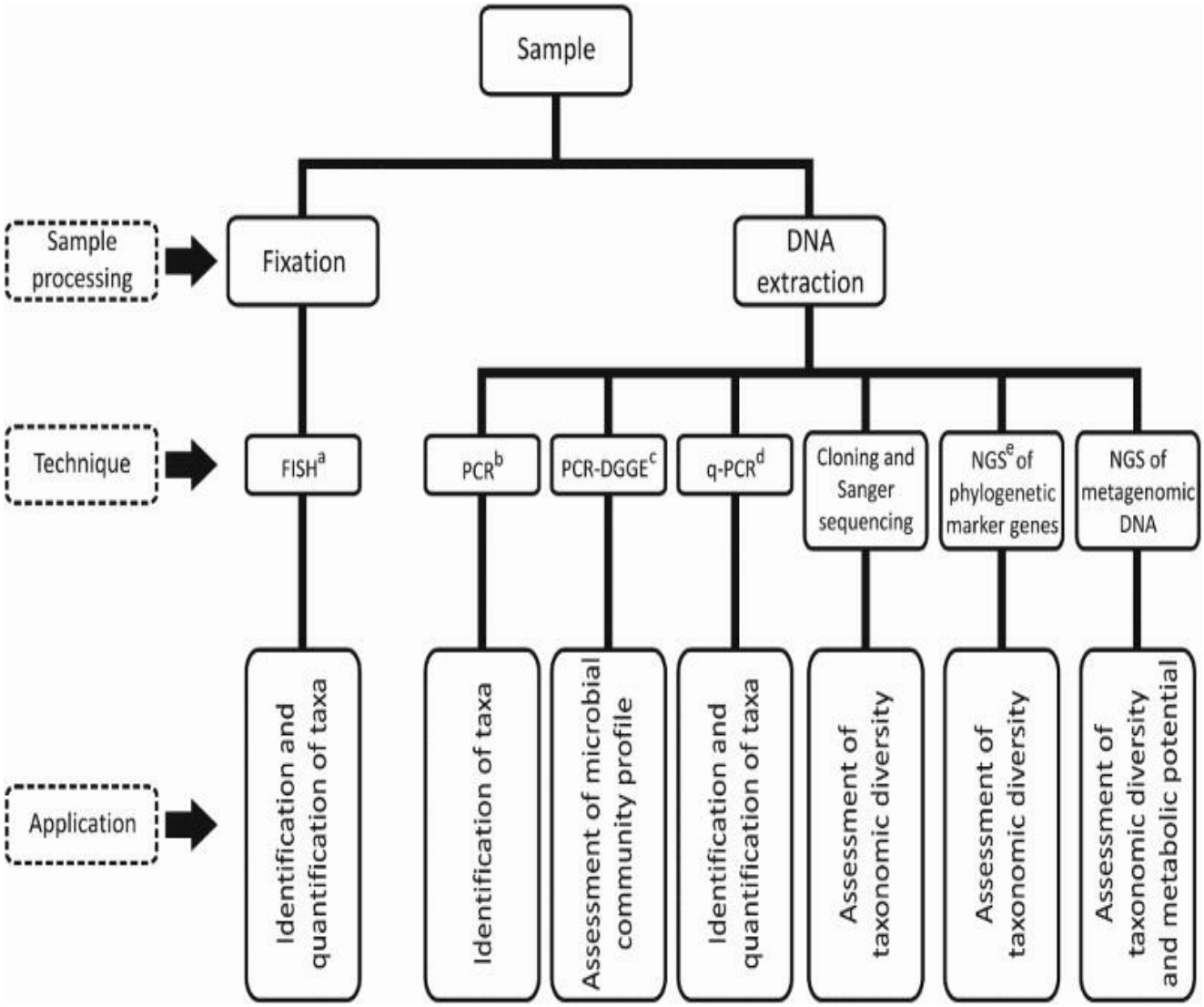

Figure 2.4: Molecular techniques commonly employed to identify and quantify microbial communities in anammox reactors (Pereira *et al*., 2017).

2.10.1 Polymerase chain reaction (PCR) and real-time polymerase chain reaction (qPCR)

Polymerase chain reaction (PCR) and quantitative real-time polymerase chain reaction (qPCR) techniques are reported to be effective for screening anammox bacteria without isolation and cultivation. This is helpful during the early stages of start-up to confirm the presence of anammox bacteria in the biomass. The PCR and qPCR techniques are carried out by PCR specific primer targeting 16S rRNA and functional gene of anammox bacteria. Studies have revealed that the 16S rRNA anammox gene is affiliated with *Planctomycetes* phylum (Hao *et al*., 2009). Using PCR and qPCR, all six anammox genera (*Brocadia*, *Scalindua*, *Kuenenia*, *Jettenia*, *Anammoxomicrobium* and *Anammoxoglobus*) have been quantified in many different environments including marine environments (Yokota *et al*., 2018), lab-scale reactors (Connan *et al*., 2016, Ma *et al*., 2016), marine oxygen minimum zones (Pereira *et al*., 2017) and wastewater treatment reactors (Bae *et al*., 2010, Reino and Carrera, 2017). Recently, several specific functional biomarkers have been developed to identify, quantify and characterise anammox bacteria. Anammox functional gene include hydrazine synthase (*hzs*), nitrite reductase (*nir*) and hydrazine oxidoreductase (*hzo*) (Shu *et al*., 2015). A list of 16S rRNA and functional gene primers used in previous studies for anammox detection and quantification is provided in Table 2.1.

Van der Star *et al*. (2002) were one of the earliest to report the use of qPCR to quantify anammox 16S rRNA gene. Wang *et al*. (2016) used qPCR to quantify the relationship between 16S rRNA and functional gene in anammox process and their association with N removal. Furthermore, Wang *et al*. (2016) concluded that anammox bacterium cell contains more than one copy of the 16S rRNA and functional genes. Real-time PCR has previously been used to measure the maximum specific growth rate (μ max) of anammox bacteria during the initial stages of the anammox process and in enrichment culture (van der Star *et al*., 2007, Bae *et al*., 2010). Furthermore, Zhang *et al*. (2017) evaluated the μ max of anammox bacterial species (*Candidatus Brocadia sinica*, *Candidatus Scalindua japonica* and *Candidatus Jettenia caeni*) by measuring exponential increase in 16S rRNA gene copy numbers.

Table 2.1: List of 16S rRNA and functional gene primers used in previous studies for anammox detection and quantification.

Primer	Specificity	Sequence (5' – 3'	Annealing temperature (°C)	Reference
Pla46F	*Planctomycetes*	GGATTAGGCATGCAAGTC	55	(van der Star *et al*., 2007)
Amx368F	Anammox bacteria	CCTTTCGGGCATTGCGAA	57	(Schmid *et al*., 2003)
Amx 820R	*Brocadia* and *Kuenenia*	AAAACCCCTCTACTTAGTGCCC	56	(Schmid *et al*., 2000)
BS 820R	*Scalindua*	TAATTCCCTCTACTTAGTGCCC	56	(Kuypers *et al*., 2003)
HzoCl1F	*hzo* gene	TGYAAGACYTGYCAYTGG	48	(Schmid *et al*., 2008)
HzoCl1R		ACTCCAGATRTGCTGACC		
AnnirS379F	*nirS* gene	TCTATCGTTGCATCGCATTT	45	(Li *et al*., 2011)
AnnirS821R		GGATGGGTCTTGATAAACA		
hzsA1597F	*hzs* hene	WTYGGKTATCARTATGTAG	42	(Harhangi *et al*., 2012)
hzsA1857R		AAABGGYGAATCATARTGGC		

2.10.2 Fluorescent in situ hybridization

Fluorescent *in-situ* hybridization (FISH) enables simultaneous identification and quantification of anammox bacteria through fluorescent microscopy and analysis software (Ibrahim *et al*., 2015). Identification of anammox bacteria involves hybridization of specific 16S rRNA oligonucleotide probe with target DNA or RNA sequence. A list of 16S rRNA-targeted oligonucleotide probe used in previous studies for anammox quantification is provided in Table 2.2. Quantification of anammox bacteria using FISH has been successfully performed by various studies (Casagrande *et al*., 2013, Wang *et al*., 2011, Schmid *et al*., 2005, Ge *et al*., 2015). Wang *et al*. (2011) revealed that anammox bacteria were the dominant population in MBR after 90 days of enrichment using FISH analysis. Another study by Hu *et al*. (2010) confirmed that the anammox bacterium (*Candidatus Brocadia sinica*) dominated the microbial community of eight different wastewater treatment systems. Additionally, biomass from FBR reveald that approximately 60 % of anammox bacteria comprised of *Candidatus Brocadia anammoxidans* and 40 % belonged to *Candidatus Kuenenia stuttgartiensis* (Engelbrecht *et al*., 2018).

Table 2.2: List of 16S rRNA-targeted oligonucleotide probe used in previous studies for anammox quantification

Probe	Specificity	Sequence (5'-3')	Reference
AMX368	Anammox bacteria	CCTTTCGGGCATTGCGAA	(Schmid *et al*., 2003)
Pla46	*Planctomycetales*	GACTTGCATGCCTAATCC	(Egli *et al*., 2001)
AMX820	*Brocadia*, *Kuenenia*	AAAACCCCTCTACTTAGTGCCC	(Schmid *et al*., 2005)
BS 820	*Scalindua*	TAATTCCCTCTACTTAGTGCCC	(Kuypers *et al*., 2003)
Kst 1275	*Kuenenia*	TCGGCTTTATAGGTTTCGCA	(Schmid *et al*., 2003)
Apr 820	*Anammoxoglobus*, *Jettenia*	AAACCCCTCTACCGAGTGCCC	(Kartal *et al*., 2008)

2.10.3 Next-generation sequencing (NGS)

Next-generation sequencing has revolutionized biological science in which advanced new technologies are rapidly developed to accelerate ecological and environmental research. There are different commercially available NGS platforms today, among which 454-pyrosequencing and Illumina/Solexa sequencing techniques are most widely used in the wastewater treatment system.

High-throughput sequencing techniques exhibit complex profiling of the entire microbial community structure qualitatively and quantitatively. The first high-throughput sequencing was performed to investigate the microbial community using 454 pyrosequencing technique in different municipal WWTP activated sludges (Hu *et al*., 2012). The application of high-throughput sequencing has been demonstrated by many researchers to explore the underlying community structure in anammox processes (Ye and Zhang. 2013, Yang *et al*., 2018). Microbial characterisation of mature anammox granules obtained from SBR revealed approximately 44 % of 16S rRNA reads were *Planctomycetes* (Sobotka *et al*., 2017). Other comprehensive research studies on the anammox process confirmed the coexistence of anammox bacteria with other bacterial groups including nitrifying, denitrifying bacteria and other heterotrophic bacteria (Yang *et al*., 2018, Ding *et al*., 2019). Chen *et al*. (2017) characterised microbial community structure using Illumina high-throughput sequencing technology and confirmed the presence of *Proteobacteria*, *Planctomycetes*, *Chlorobi*, *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* in reactor inoculated with anammox sludge. Similarly, high throughput sequencing was used to determine the diversity and abundance of microbial structure of anammox systems (Zhang *et al*., 2017, Zhang *et al*., 2014, Zeng *et al*., 2016). Furthermore, high throughput sequencing revealed that the presence of microbial competition for common substrate (NO_2^-) since similar bacterial groups (*Proteobacteria*, *Chlroflexi*, *Chlorobi* etc.) are commonly found in anammox reactors (Costa *et al*., 2014).

Although various strategies have been developed and applied to anammox bacterial enrichment, very little work has compared the effect of these different substrate ratios on both anammox and on the synergistic microbial populations co-existing within the same reactor. Since these populations will all be subjected

to similar environmental conditions, determining the optimum substrate ratio that encourages anammox enrichment will be critical to the development of a stable, cost-effective, anammox wastewater treatment system.

CHAPTER 3: START-UP OF ASBR AND EVALUATION OF REACTOR PERFORMANCE USING DIFFERENT INFLUENT AMMONIUM: NITRITE SUBSTRATE RATIO

3.1 Introduction

The anammox process and its application in nitrogen removal from ammonium-rich wastewater have been the centre of attention for in the last few decades (Kuenen. 2020). However, slow-growing anammox bacteria result in long start-up periods which has restricted its widespread application in full-scale systems (Guo *et al*., 2016). Various factors including seed inoculum, substrate (ammonia: nitrite) ratio, temperature, sludge retention time, pH and dissolved oxygen (DO) are reported to influence the growth rate and metabolic activity of anammox bacteria (Oshiki *et al*., 2011, Awata *et al*., 2013, Jaroszynski *et al*., 2012, Carvajal-Arroyo *et al*., 2013, Connan *et al*., 2016, Narita *et al*., 2017, Bai *et al*., 2015). Many studies have attempted to reduce the start-up period by various means such as using mature anammox seed inoculum, use of various types of carriers and different reactor configurations (Egli *et al*., 2001, Dosta *et al*., 2008, Li *et al*., 2015, Ali and Okabe, 2015, Ma *et al*., 2016, Schmid *et al*., 2003, Mosquera-Corral *et al*., 2005). For example, upflow anaerobic sludge bed reactor (UASB) inoculated with mature anammox granule reached a steady-state after 26 days of operation (Cao *et al*. 2016).

On the other hand, few other studies reported that the anammox start-up period comprises of five distinct phases (sludge lysis, lag phase, propagation phase, stationary phase and inhibition phase) which occurs for approximately 130 days (Banihani *et al*., 2012, Tang *et al*. 2009). One of the critical limitations of the anammox process is substrate concentration (NO_2^- and NH_4^+). Low NO_2^- can reduce the growth while NO_2^- concentration above 100 mg/L are toxic to the anammox bacteria and can ultimately inhibit the process (Zhang *et al*., 2014). However, other researchers confirmed that free nitrous acid (FNA) was the true inhibitor rather than NO_2^- (Zhou *et al*., 2011, Puyol *et al*., 2014).

As for NH_4^+ inhibition, Strous *et al*. (1999) stated that NH_4^+ concentration of 1000 mg N/L did not suppress anammox activity. Contrary, Dapena-Mora *et al*. (2007) observed loss of anammox activity (50 % loss of activity, IC50 value) when NH_4^+

concentration was 770 mg N/L. Furthermore, free ammonia (FA) has been recognised to inhibit the anammox process (Jung *et al*., 2007, Fernandez *et al*., 2012, Tomaszewski *et al*., 2017a). Jung *et al*. (2007), reported the lowest FA inhibitory concentration of 1700 µg/L.

Therefore, the effect of substrate concentrations and associated inhibitions caused by FA and FNA on the anammox process requires in-depth understanding. This chapter focuses on evaluating the effect of different substrate (ammonia: nitrite) ratios on anaerobic SBR (ASBR) reactor performance. The reactor performance was determined by measuring the concentration of N compounds (NH_4^+ and NO_2^- removal, NO_3^- production, N_2 gas production and substrates/ product conversion ratios) within each reactor.

3.2 Methodology

3.2.1 Seed inoculum

The seed inoculum for the lab-scale ASBRs was obtained from an ongoing lab-scale anammox reactor operated for a period of six months (preliminary molecular analysis revealed the dominance of anammox bacteria (*Candidatus Kuenenia* and *Candidatus Jettenia*), AOB (*Nitrosospira, Nitrosococcus* and *Nitrosomonas*) and NOB (*Nitrospira*) in the samples. The biomass concentration was measured according to standard methods (APHA, 2012) as mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS). The biomass concentration in the inoculum was 154 mg/L (MLSS) and 116 mg/L (MLVSS). To remove any available contaminants in the inoculum and maintain optimum pH, the biomass was re-suspended in phosphate buffer (140 mg/L KH_2PO_4 and 750 mg/L K_2HPO_4) before inoculating into the reactors as previously described (Dosta *et al*., 2008, Dapena-Mora *et al*., 2007).

3.2.2 Synthetic medium

The composition of synthetic growth medium and trace elements for anammox bacteria was initially depicted by van de Graaf *et al*. (1995). To promote anammox activity, the key substrates (NH_4^+-N and NO_2^--N) were added as $(NH_4)_2SO_4$ and $NaNO_2$ respectively and trace element solution was kept constant (Table 3.1). The reactors contained different ammonium: nitrite ratios namely; Reactor 1 (1 NH_4^+-N: 1.32 NO_2^--N), Reactor 2 (2 NH_4^+-N: 1 NO_2^--N) and Reactor 3 (1 NH_4^+-N: 2 NO_2^--N). In Reactor 1, the substrate ratio used represents the reported anammox substrate ratio of 1: 1.32 (Ibrahim *et al*., 2015, Uyanik *et al*., 2011). Reactors 2 and 3 were used to evaluate the individual effects of NH_4^+ and NO_2^- on reactor performance (N removal and inhibitory compounds formation) as well as microbial (AOB, NOB and anammox bacteria) selection. In this regard, Reactor 2 contained high NH_4^+ and low NO_2^- concentrations and Reactor 3 contained low NH_4^+ and high NO_2^- concentrations. The substrate concentrations were kept below 100 mg N/L, to avoid the potential of these compounds reaching inhibitory levels thus impairing the stable growth of anammox populations. The medium pH was kept between 7 and 8, using 2M HCl and 5M NaOH solutions respectively. The synthetic medium was sparged with gas Argon/CO_2 (95 % /5 %) for 15 minutes to maintain anaerobic conditions.

Table 3.1: Composition of synthetic media for anammox bacterial enrichment (van de Graaf *et al*., 1995)

Compound		Concentration (mg/L)	
	Ratio 1:1.32	Ratio 2:1	Ratio 1:2
$(NH_4)_2SO_4$	235	470	235
$NaNO_2$	310	240	490
$NaHCO_3$	3335	3335	3335
KH_2PO_4	27	27	27
$MgSO_4 \cdot 7H_2O$	320	320	320
$CaCl_2.2H_2O$	176	176	176
$FeSO_4$	5.1	5.1	5.1
EDTA	20	20	20
Trace element solution[1]	1.25 mL/L	1.25 mL/L	1.25 mL/L

[1] $ZnSO_4\,7H_2O$ 0.41 (mg/L), $CoCl_2\,6H_2O$ 0.237 (mg/L), $MnCl_2\,4H_2O$ 0.99 (mg/L), $CuSO_4\,5H_2O$ 0.25 (mg/L), $NaMoO_4\,2H_2O$ 0.02 (mg/L), $NiSO_4\,6H_2O$ 0.021 (mg/L), H_3BO_3 0.011 (mg/L) previously depicted by van de Graaf *et al*. (1995).

3.2.3 Reactor operational setup

The three ASBRs (Reactor 1, 2 and 3) consisted of Disposable Spinner Flask (Corning, USA), each with a working volume of 1L and modified to include tubing (silicone) for inlet and outlet (Figure 3.1). The reactors were operated in 48-hour cycle time as follows: 3 h settling, 2 h decanting, 2h feeding and 41 h continuous mixing as previously described by Tikilili and Chirwa (2016), with modifications. The reactors were operated at ambient temperatures ranging from 24 to 30 °C and the pH was controlled between 7 and 8. Argon/CO_2 (95 % /5 %) was used to maintain anaerobic conditions in the reactors. Mixing in the reactors was attained by using a mechanical stirrer operated at a fixed rotating speed of 80 rpm. The reactors were fed with the growth medium once the main substrate (NH_4^+-N or NO_2^--N) concentrations were approximately 20 mg N/L. All other

operational conditions such as DO, pH and temperature were kept constant for all the reactors.

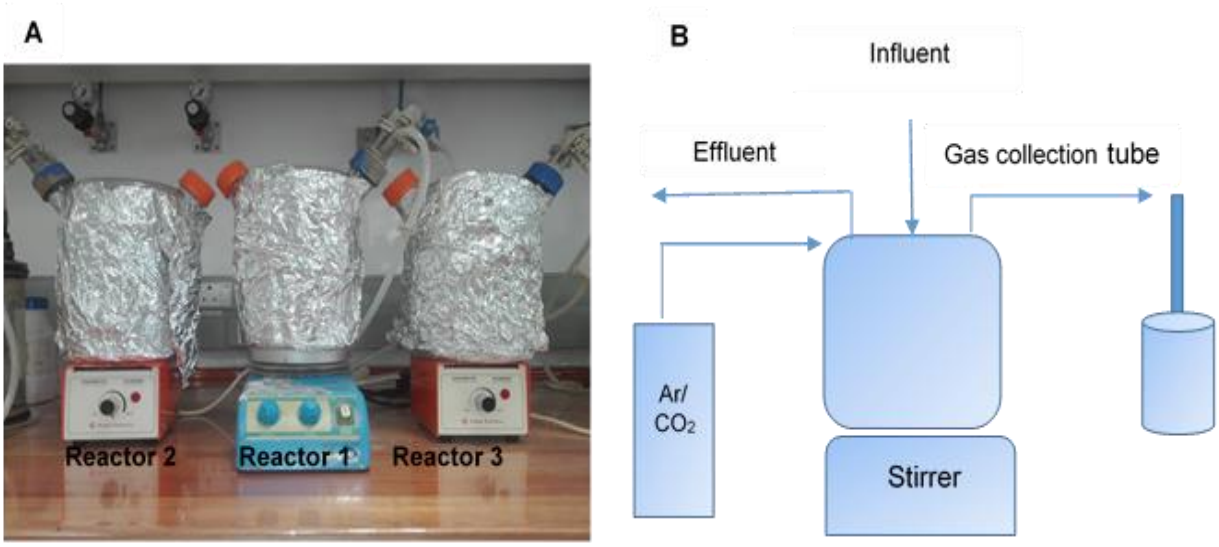

Figure 3.1: Experimental set-up of the anaerobic sequencing batch reactors (ASBRs) used in the study (A) and schematic diagram of ASBRs setup (B).

3.2.4 Analytical methods

Effluent samples were obtained directly from each reactor three times a week during the decanting phase. After the biomass has settled during the settling phase, decanting phase was achieved by decanting 300 mL of the supernatant using a 50 mL syringe. The samples were filtered through a 0.45 µm cellulose acetate syringe filter (Merck Millipore, USA) prior to analysis.

3.2.4.1 Nitrogen concentration

Reactor performance was continuously analysed by measuring N transformation using effluent samples, N compounds (NH_4^+-N, NO_2^--N and NO_3^--N) were measured colorimetrically at 655 nm and 585 nm and 400 nm respectively using the Hach DR900 Multiparameter Colorimeter. Nitrogen removal efficiency (NRE)

was calculated from analysis of N compounds results (the difference in the total nitrogen (TN) concentration between the influent and effluent) (Du *et al*., 2015).

$$NRE\ (\%) = \frac{(inf.\ TN - eff.\ TN)}{inf.\ TN} X\ 100 \quad (2)$$

3.2.4.2 Biomass Concentration

The MLSS and MLVSS were measured in each reactor according to the standard methods (APHA, 2012). Mixed liquor suspended solid was determined by collecting 50 mL of mixed liquid sample from the reactor and filtered through a pre-weighed 47 mm, 1.2 µM pore size glass-microfiber filter (Munktell Ahlstrom, Europe) and the biomass residue on the microfiber filter was dried overnight at 105 °C. The dried microfiber filter was weighed on Mettler-Toledo ME204 analytical balance (Mettler-Toledo International Inc., USA) to determine the dry biomass. To determine MLVSS, the microfiber filter was incinerated at 550 °C for 20 min. After incineration in a furnace, the microfiber filter was cooled in a desiccator containing silica gel and weighed on an analytical balance. The difference in grams between the empty microfiber filter and post-heating at 105 °C was recorded as MLSS and MLVSS was calculated as the difference (grams) between the microfiber filter and post-heating at 550 °C.

3.2.4.3 pH, temperature and dissolved oxygen

The pH in the synthetic media and reactors was controlled between 7 and 8 using 2 M HCl and 5 M NaOH solutions respectively. The DO concentration were reduced (< 0.5 mg/L) by continuously sparging the media and reactors with Argon/CO_2 (95 % /5 %). The reactors were operated at ambient temperatures ranging from 24 – 30 °C. Portable YSI meter (YSI Professional Plus) was used to measure the pH, temperature and DO in each reactors.

3.2.4.4. Nitrogen gas

Nitrogen gas (N_2) production was monitored weekly by using the fluid displacement method and the fluid displaced and gas collected was measured. The N_2 gas produced in each reactor was confirmed using gas chromatography (GC-2014, Japan) installed with a thermal conductivity detector (TCD) and packed column: GS-Gas Pro (30 m × 0.32 mm ID). The temperatures of the oven,

detector and injection port were 80, 250, and 120 °C respectively and helium gas was used as a baseline. A standard gas (Helium Baseline, Afrox, South Africa) was used for the calibration of the system. A 100 uL gas syringe (VICI, Precision Sampling Inc., USA) was used for gas collection (Zhong *et al*., 2011).

3.2.4.5 Free ammonia and free nitrous acid concentration

The FA and FNA concentration were calculated from the effluent liquid samples during each of the reactors decanting phases using equations (9) and (10) respectively (Park and Bae, 2009, Anthonisen *et al*., 1976) where: total ammonia is the actual concentration of NH_4^+-N, T is the temperaturen degrees centigrade and NO_2^--N is the actual concentration of NO_2^--N.

$$FA\ as\ NH_3\ (mg/L) = \frac{17}{14} X \frac{Total\ ammonia\ N\ (mg/L)\ X\ 10^{pH}}{e^{(\frac{6344}{273+T})} + 10^{pH}} \quad (3)$$

$$FNA\ as\ HNO_2\ (mg/L) = \frac{46}{14} X \frac{NO_2^- - N\ (mg/L)}{e^{(\frac{-2300}{273+T})} + 10^{pH}} \quad (4)$$

3.2.4.5 Statistical analysis

Data obtained was used calculate mean, ranges and standard deviation. Graphs were achieved using GraphPad Prism v 5.0 (GraphPad Software, San Diego California USA). Statistical analysis using the nonparametric Kruskal – Wallis test (α=5%) was performed with XLS stat v2017 tool in Excel, 2013.

3.3 Results

The effect of different key substrate ratios on reactor performance determined by measuring effluent NH_4^+, NO_2^-, NO_3^-, FA, FNA concentrations and N_2 production are presented below. The three ASBR contained different ammonium: nitrite ratios i.e. Reactor 1 (1:1.32), Reactor 2 (2:1), and Reactor 3 (1:2).

3.3.1 Substrate profile in Reactor 1

Balanced substrate ratio of 1 NH_4^+-N: 1.32 NO_2^--N in Reactor 1 showed a slight increase in effluent NH_4^+-N was observed for the first 9 days, thereafter a steady decrease in NH_4^+-N below 20 mg N/L was observed. The accumulation of NH_4^+-N can be caused by hydrolysis of N compound released from cell lysis. Furthermore, between 210th and 300th day NH_4^+-N increased above 30 mg N/L, and a sharp decline (below 10 mg N/L) was observed on the 320th day. The NO_2^--N concentration increased above 30 mg N/L for the first 30 days, showing activity of AOB. Thereafter a steady NO_2^--N removal between 20 and 30 mg N/L was observed up to day 300. Similarly, a sharp decrease (below 10 mg N/L) in NO_2^--N concentration was observed on the 320th day. Due to consistent oxygen leakage, AOB and anammox bacteria might have vastly reduced NO_2^--N. Initially, high NO_3^--N concentration above 50 mg N/L was observed, indicating high activity of NOB since NO_3^--N was not present in the synthetic medium. Thereafter a gradual decrease (below 30 mg N/L) was observed throughout the study period (Figure 3.2).

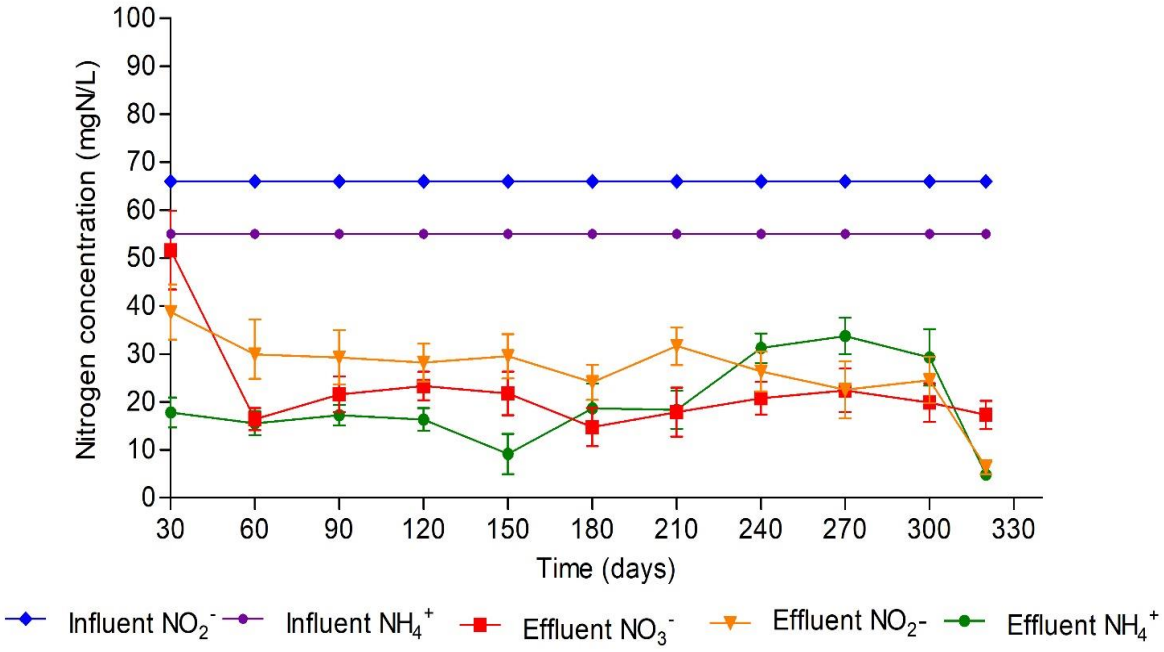

Figure 3.2: Reactor 1 (1:1.32) kinetic profiles of N compounds expressed as influent and effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations over 320 days of operation.

3.3.2 Substrate profile in Reactor 2

In Reactor 2 containing substrate ratio of 2 NH_4^+-N: 1 NO_2^--N ratio, the effluent NH_4^+-N fluctuation between 20 and 40 mg N/L was observed throughout the study period (Figure 3.3). Lower NO_2^--N concentration (below 20 mg N/L) was observed after 21 days of reactor operation, then stable effluent concentrations (between 10 and 20 mg N/L) were observed. After the 210th day, the NO_2^--N concentration increased above 20 mg N/L, indicating high activity of AOB. High NO_3^--N concentration (above 40 mg N/L) was observed for the first 30 days, thereafter NO_3^--N concentration fluctuated below 20 mg N/L throughout the study (Figure 3.3). Stable reactor performance was achieved after the 30th day up to the 210th day, indicating simultaneous activity of anammox bacteria, AOB and NOB.

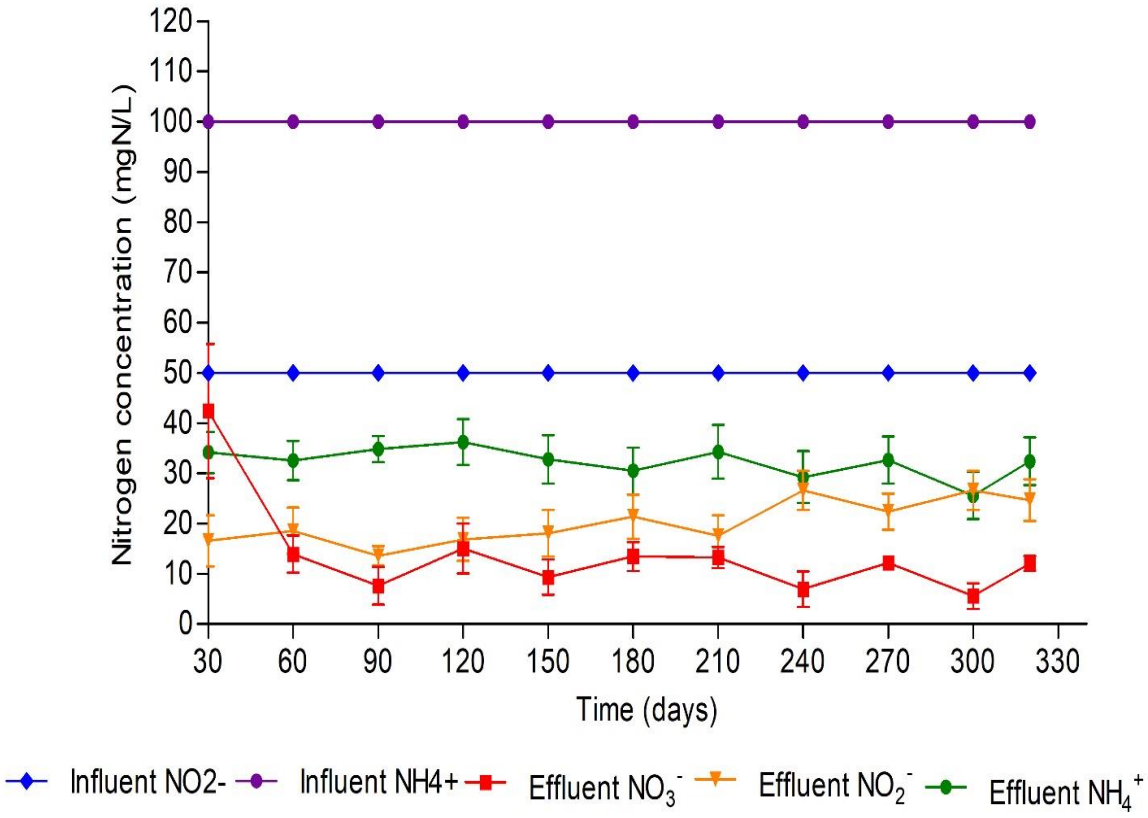

Figure 3.3: Reactor 2 (2:1) kinetic profiles of N compounds expressed as influent and effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations over 320 days of operation.

3.3.3 Substrate profile in Reactor 3

In Reactor 3 containing substrate ratio of 1 NH_4^+-N: 2 NO_2^--N, effluent NH_4^+-N maintained stable effluent concentration (below 20 mg N/L) for 240 days of operation, thereafter high NH_4^+-N concentration above 20 mg N/L was observed. Presumably indicating accumulation of NH_4^+-N from hydrolysis of N compound released after cell lysis. High NO_2^--N concentration above 90 mg N/L were observed during the initial phase of the study, showing high AOB activity. Subsequently, between 60 and 180 days a steady effluent NO_2^--N concentration was observed. Finally fluctuating NO_2^--N concentrations between 20 and 30 mg N/L were observed throughout the operational period. Initially, NOB activity might have been observed by high effluent NO_3^--N concentration. After the first 60 days

NO_3^--N concentration remained below 20 mg N/L for most of the study period, averaging approximately 15 mg N/L for the entire operational period (Figure 3.4).

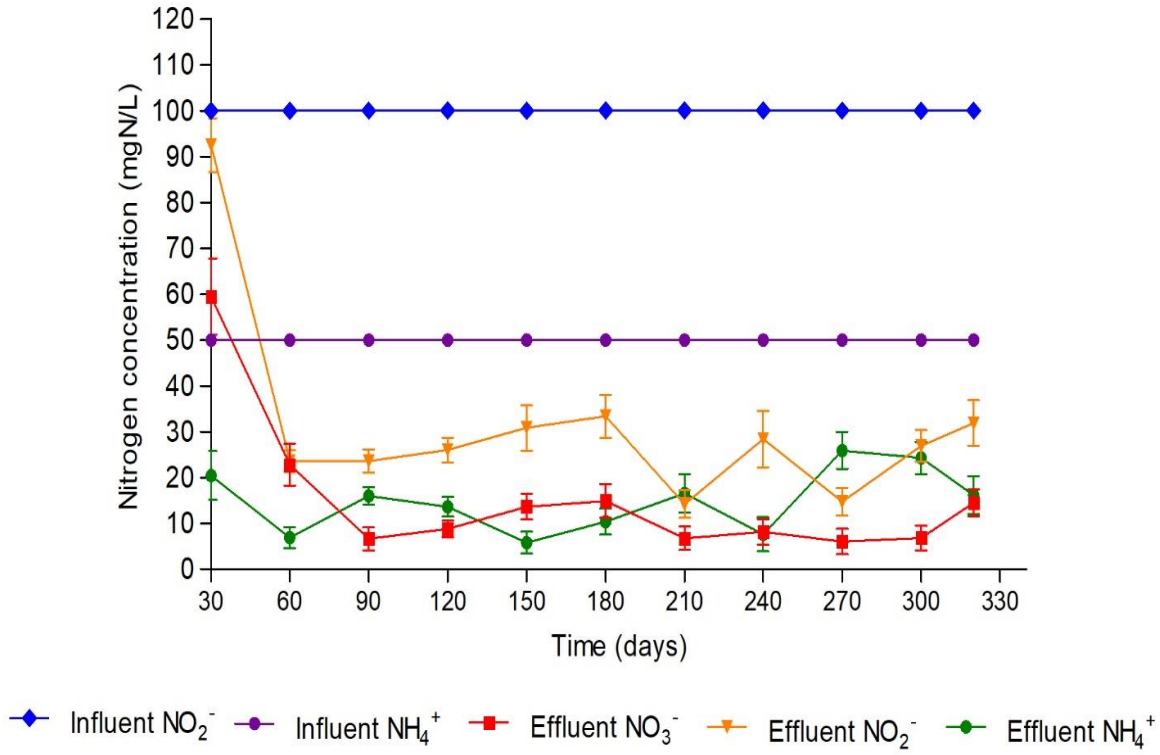

Figure 3.4: Reactor 3 (1:2) kinetic profiles of N compounds expressed as influent and effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations over 320 days of operation.

3.3.4 Effect of substrate ratio on accumulation of biomass by ASBRs

Biomass concentration in the reactors was monitored to determine the effect of substrate N on bacterial growth and biomass accumulation and/or washout. The initial inoculum biomass concentration obtained from the ongoing lab-scale reactor was 154 mg/L). On the 170th day after initiation of the three ASBRs, bacteria growth was observed when the biomass concentration increased to 210 mg/L, 285 mg/L and 345 mg/L in Reactors 1, 2 and 3 respectively. Thereafter a drastic decrease in the biomass concentration was observed to 125 mg/L, 95 mg/L, 120 mg/L in Reactors 1, 2 and 3 respectively was observed on 320th day of the study (Figure 3.5). A reduction in biomass might have been caused by cell

lysis and/or biomass washout, this a serious problem especially for slow growing anammox bacteria.

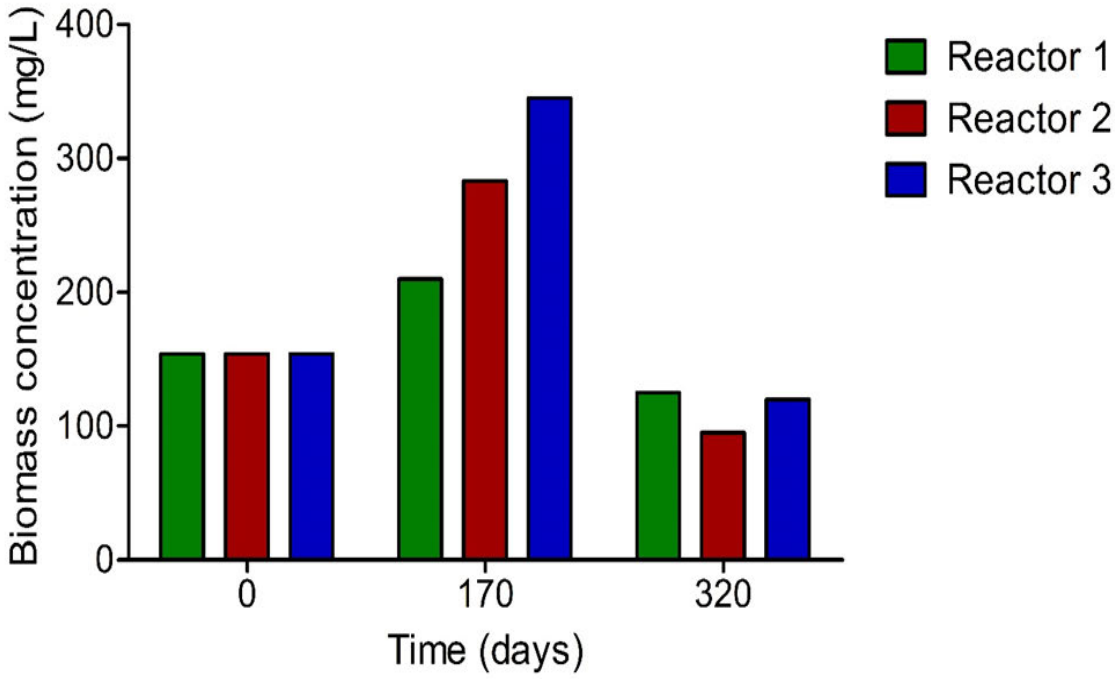

Figure 3.5: Biomass concentration (MLSS) in Reactor 1, Reactor 2 and Reactor 3 throughout the study period.

Biomass attachment to the sides of the reactor was observed in all the reactors (Figure 3.6). Biomass attachment occurred approximately three months of operation. Biomass attachment and biofilm formation is a distinct characteristic for anammox bacteria. Furthermore, anammox bacteria are characterised by a brown-reddish colour observed in all three reactors (Figure 3.6). Biomass colour change is a visual observation that can be used to identify successful anammox enrichment.

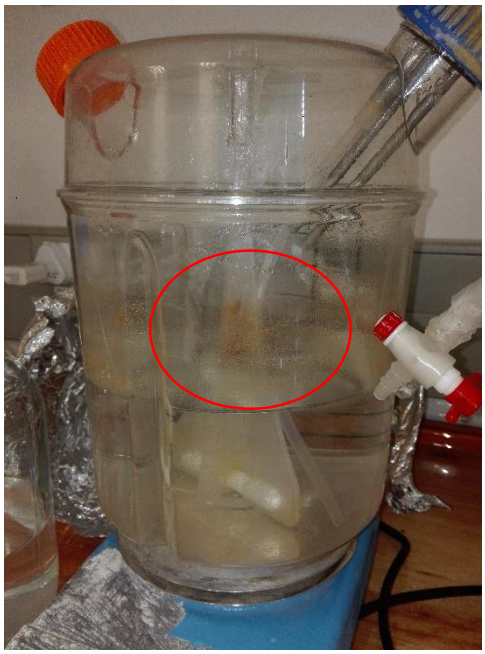

Figure 3.6: Visual observation of the reactor biomass

3.3.5 Process performance of the reactors

Dissolved oxygen concentration is a crucial parameter in the anammox process especially when the seed inoculum contains other bacterial groups such as NOB. During this study, frequent oxygen leakage was observed in all three reactors. The average DO concentration during the whole operational period was 0.49 ± 0.20 mg/L in Reactor 1, 0.39 ± 0.19 mg/L in Reactor 2 and 0.43 ± 0.16 mg/L in Reactor 3 (Figure 3.7 – 3.9).

The NO_2^--N (consumed)/ NH_4^+-N (removed) ratio and NO_3^--N (produced)/ NH_4^+-N (removed) ratio were different from the three treatment conditions. In Reactor 1, the overall NO_3^--N (produced)/NH_4^+-N (removed) ratios fluctuated above the reported anammox stoichiometric ratio of 0.26. The ratios of NO_2^--N (consumed)/NH_4^+-N (removed) constantly fluctuated below the reported anammox stoichiometric ratio of 1.32 throughout the study (Figure 3.7). This was not expected since the fed used contained standard anammox stoichiometry ratio of (1 NH_4^+-N: 1.32 NO_2^--N). The observed ratios implies the concurrent activity of AOB and NOB.

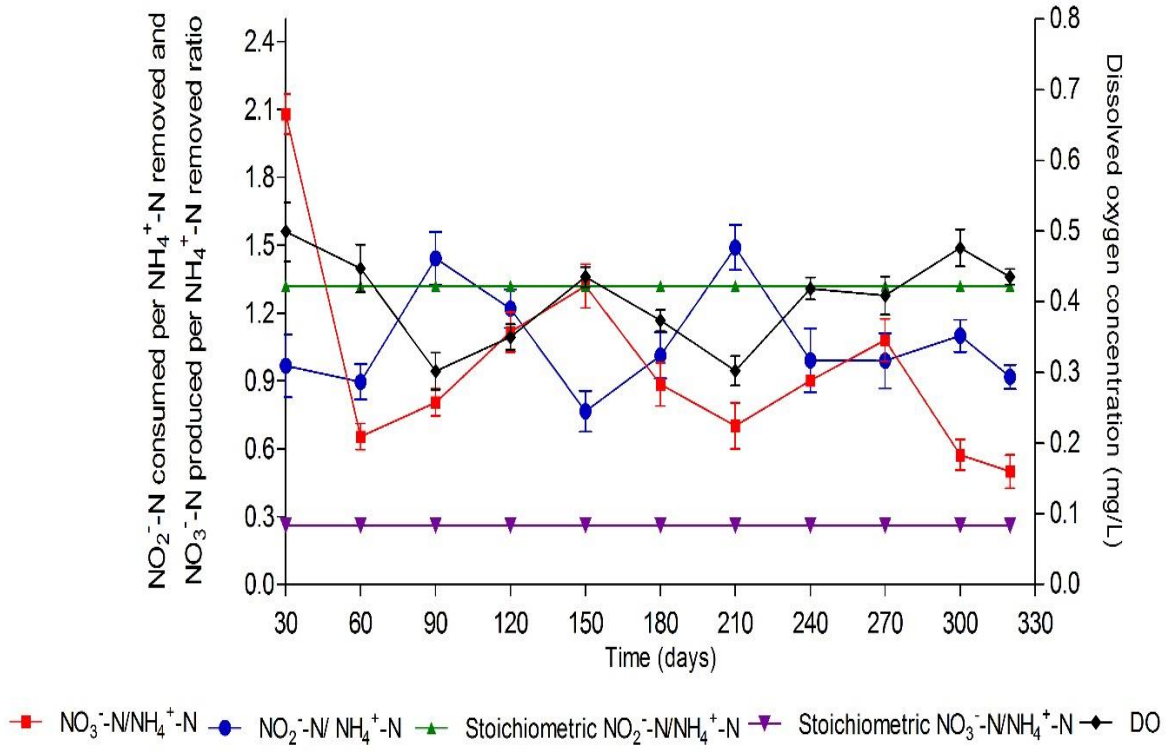

Figure 3.7: Profiles of NO_2^--N (consumed)/ NH_4^+-N (removed) ratio, NO_3^--N (produced)/ NH_4^+-N (removed) ratio, DO concentration in Reactor 1 and the theoretical anammox stoichiometric ratio in terms of NO_2^--N/ NH_4^+-N and NO_3^--N/ NH_4^+-N.

In Reactor 2, NO_2^--N (consumed)/NH_4^+-N (removed) ratios observed were lower than 1.32 for most of the study period whereas the ratio NO_3^--N (produced)/NH_4^+-N (removed) fluctuated above the anammox stoichiometric ratio of 0.26 (Figure 3.8). These results indicated the activity of other bacterial groups including AOB and NOB.

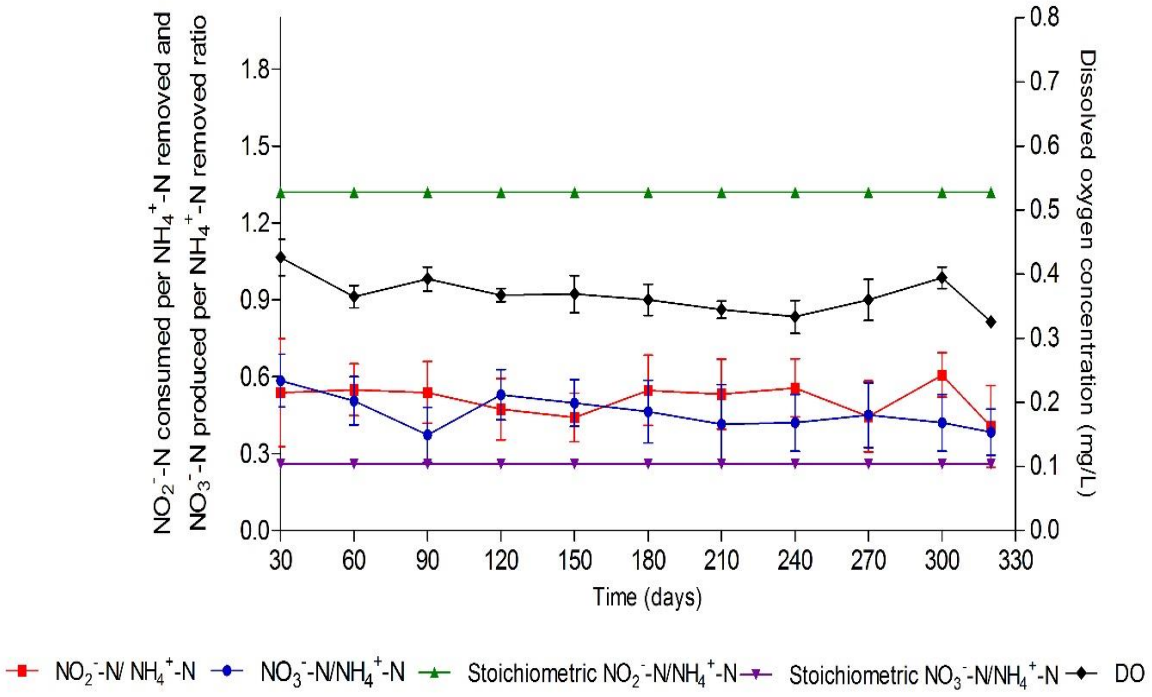

Figure 3.8: Profiles of NO_2^--N (consumed)/ NH_4^+-N (removed) ratio, NO_3^--N (produced)/ NH_4^+-N (removed) ratio, DO concentrations in Reactor 2 and the theoretical anammox stoichiometric ratio in terms of NO_2^--N/ NH_4^+-N and NO_3^--N/ NH_4^+-N.

In Reactor 3, NO_2^--N (consumed)/NH_4^+-N (removed) ratios were closer to the reported anammox stoichiometric ratio of 1.32 for 210 days of reactor operation, indicating establishment and domination of anammox activity inside the reactor. Thereafter, the NO_2^--N (consumed)/NH_4^+-N (removed) ratios were below 1.32 between 240 and 300 days, finally, higher (above 1.32) ratios were observed on 320th day. The NO_3^--N (produced)/ NH_4^+-N (removed) ratios were higher than 0.26 for the first 60 days. This might indicate that AOB or NOB were still active in the reactor. The NO_3^--N (produced)/ NH_4^+-N (removed) fluctuated closer to the reported anammox stoichiometric ratio of 0.26 (Figure 3.9). Overall, Reactor 3 indicated good anammox performance proved by NO_2^--N (consumed)/NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratios fairly closer to the reported anammox stoichiometry.

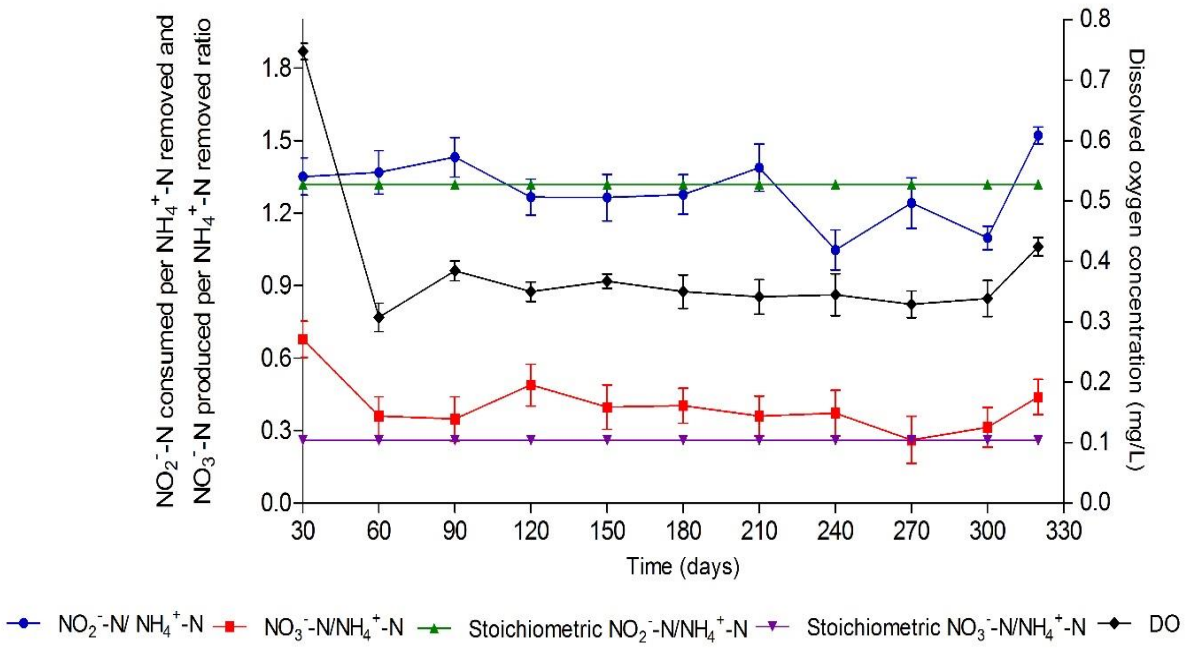

Figure 3.9: Profiles of NO_2^--N (consumed)/ NH_4^+-N (removed) ratio, NO_3^--N (produced)/ NH_4^+-N (removed) ratio, DO concentration in Reactor 3 and the theoretical anammox stoichiometric ratio in terms of NO_2^--N/ NH_4^+-N and NO_3^--N/ NH_4^+-N.

3.3.6 Nitrogen gas production

During this study, N_2 gas produced was measured in all three reactors and a different volume of N_2 gas production was observed. Throughout the study, Reactor 1 resulted in the lowest N_2 gas production, whilst the highest N_2 gas production was observed in Reactor 3 (Figure 3.8).

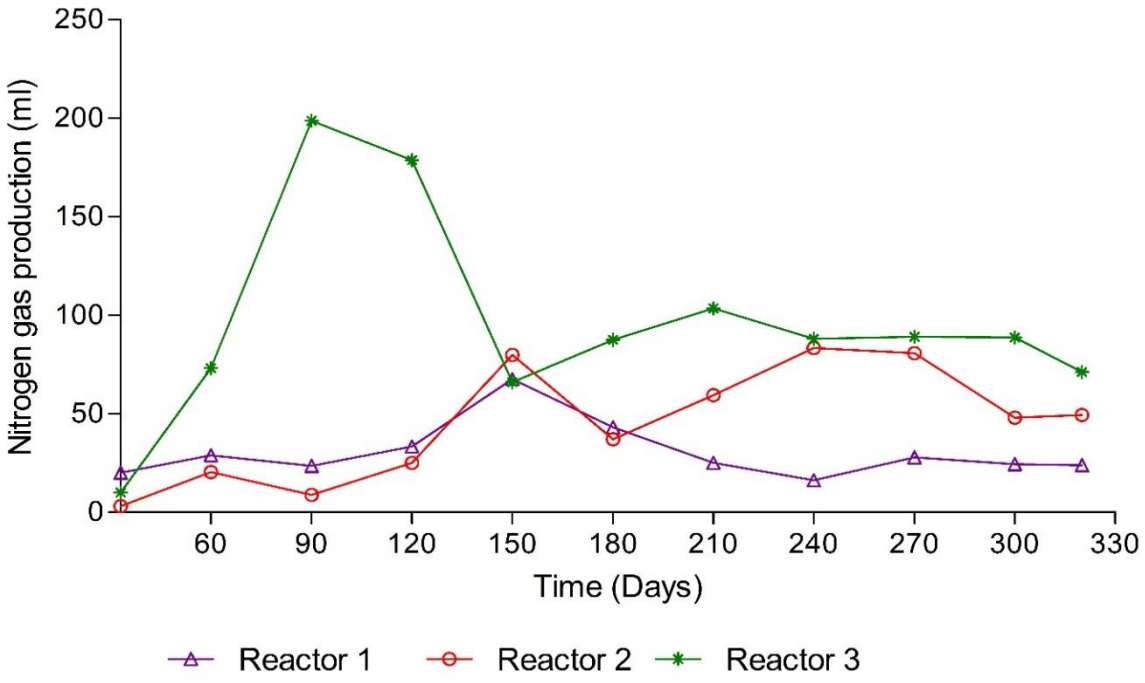

Figure 3.10: Dinitrogen gas (N_2) production

3.3.7. Free ammonia and free nitrous acid concentration

The effluent pH values ranged from 6.49 to 8.3 in Reactor 1, 6.5 to 8.0 in Reactor 2 and 6.6 to 8.3 in Reactor 3. The temperature ranged between 23.6 – 35.1 °C, 23.2 – 33.2 °C and 24.0 – 32.8 °C in Reactors 1, 2 and 3 respectively. Free ammonia and FNA are dependent on pH, temperature and substrate concentration. During the study, the FA concentrations were highest in Reactor 1 (891.2 ± 290.4 µg NH_3-N/L), followed by Reactor 2 (734.7 ± 350.5 µg NH_3-N/L) and Reactor 3 (453.3 ± 260.4 µg NH_3-N/L) on 170th day of operation. Thereafter on 320th day the average FA concentrations were estimated to be higher in Reactor 3 (832.1 ± 171.1 µg NH_3-N/L) followed by Reactor 2 (671.1 ± 270.0 µg NH_3-N/L) and low FA concentration was achieved in Reactor 1 (622.2 ± 350.1 µg NH_3-N/L). Suggesting that FA was eliminated as a potential inhibitor for the anammox process, since the observed concentrations were below the reported inhibitory concentration.

The average FNA concentrations were estimated to be highest in Reactor 3 (27.3 ± 16.1 µg HNO_2^--N/L), followed by Reactor 2 (5.9 ± 2.2 µg HNO_2^--N/L) and least in Reactor 1 (5.5 ± 2.5 µg HNO_2^--N/L) on 170th day of operation. On the 320th

day, average FNA concentrations were also estimated to be highest in Reactor 3 (27.4 ± 1.1 µg HNO_2^--N/L) and lower in Reactor 2 and 1 (7.3 ± 2.2 mg HNO_2^--N/L) and (6.2 ± 2.1 µg HNO_2^--N/L), respectively. This was assumed to contribute to the deterioration of reactor performance. Additionally, Reactor 3 attained the maximum NRE of 68.7 ± 7.7 %, followed by Reactor 1 with 66.3 ± 13.3 % and Reactor 2 achieved 64.1 ± 7.2 % (Table 3.2).

Table 3.2: Free ammonia (FA), free nitrous acid (FNA) and nitrogen removal efficiency (NRE) obtained in the ASBRs during operational period.

	FA (µg NH_3-N/L)[2]		FNA (µg HNO_2^--N/L)[2]		NRE (%)[2]
	170th day	320th day	170th day	320th day	
Reactor 1	891.2 ± 290.4	622.2 ± 350.1	5.5 ± 2.5	6.2 ± 2.1	66.3 ± 13.3
Reactor 2	734.7 ± 350.5	671.1 ± 270.0	5.9 ± 2.2	7.3 ± 2.2	64.1 ± 7.2
Reactor 3	453.3 ± 260.4	832.1 ± 171.1	27.3 ± 16.1	27.4 ± 1.1	68.7 ± 7.7

[2]Average ± standard deviation

3.4 Discussion

3.4.1 Nitrogen removal performance

Over 20 years after the first report on the anammox process, anammox bacteria are still considered to be very slow growing and no pure culture have been established yet (Ali *et al*., 2015, Connan *et al*., 2016, Ibrahim *et al*., 2015). In this study, the effect of different substrate ratios on three ASBRs each containing a mixed consortium of anammox bacteria, AOB and NOB was evaluated. The reactors were operated with a synthetic medium containing different substrate ratios as follows: balanced ratio of 1:1.32 (Reactor 1), high NH_4^+N ratio of 2:1 (Reactor 2) and high NO_2^-N ratio 1:2 (Reactor 3). The performance of each reactor was monitored by measuring relevant effluent NH_4^+ and NO_2^- removal, NO_3^- production concentration and formation of inhibitory compounds (FA and FNA).

An increase in effluent NH_4^+-N concentration initially observed in all three reactors could have been caused by death of obligate aerobic bacteria and other heterotrophic bacteria that were initially present in the seed inoculum. Cell lysis can cause organic N release in the form of NH_4^+-N, thus adding up NH_4^+-N to the reactors. Related observations were reported by other studies (Bae *et al*., 2010, Anjali and Sabumon, 2014, Wang *et al*., 2013). Thereafter, lower effluent NH_4^+-N concentration was noticed (Figure 3.2 to 3.4).

Similarly, an initial increase in effluent NO_2^--N concentration was observed for all reactors indicating that a fraction of NH_4^+-N was being converted to NO_2^--N. This occurs when AOB partially oxidise NH_4^+-N to produce NO_2^--N. Partial nitrification is occasionally observed in an anammox reactor due to possible oxygen leakage (Yan *et al*., 2010). Oxygen leakage was also observed in this study, evidenced by the increase in DO concentration above 0.5 mg/L irrespective of N substrate supply. Similar observations were reported previously by other researchers (Puyol *et al*., 2014, Zhou *et al*., 2011, Jung *et al*., 2007, Huang *et al*., 2016). Furthermore, gradual decrease in effluent concentration of NO_2^--N and NH_4^+-N later observed could indicate that the anammox bacteria have acclimatised to the new substrate concentration. Gutwiński *et al*. (2016) observed anammox microbial acclimatisation when N removal efficiency increased from 24.3 % to 77.2 % after 150 days of reactor operation.

The anammox process can be proven through N conversion ratios of NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) that follow the reported anammox stoichiometry. In this study, exposure to high NO_2^- concentration (Reactor 3) supported the proliferation of anammox bacteria, since the NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratios were within the reported anammox stoichiometric ratios of 1.32 and 0.26 obtained by Strous *et al*. (1999b). Whereas, in Reactor 1 and 2, the NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratios were either lower or higher than the reported anammox stoichiometric ratios, suggesting a simultaneous occurrence of anammox, nitrification and/or heterotrophic processes in the reactors (Wen *et al*., 2016). The existence of these microbial groups in the same reactors could encourage substrate competition, causing variation in NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) (Yan *et al*., 2010).

A decrease in NO_2^--N (consumed)/NH_4^+-N (removed) suggests that AOB was more active compared to anammox bacteria (Wen *et al*., 2016, Yin *et al*., 2016). This resulted in NO_2^- production caused by fluctuating DO concentration observed throughout the study period. Furthermore, Lotti *et al*. (2012) reported that exposure to high NH_4^+ concentrations (Reactor 2) resulted in a smaller amount of NH_4^+ removed. It is worth noting that in a complex system that involves a mixed microbial community, the variation in the measured ratios and anammox stoichiometric ratios could also be justified by the presence and activity of other microbial (heterotrophs) groups in the reactor (Anjali and Sabumon, 2014, Laureni *et al*., 2015). On one hand, AOB is capable of NH_4^+-N oxidation under oxygen-limited environments, and on the other hand, NOB is capable of NO_2^- oxidation to NO_3^- (Anjali and Sabumon, 2014). Therefore, the observed ratios of NO_3^--N (produced)/ NH_4^+-N (removed) might be indicative of the presence of active NOB communities, and the obtained NO_2^--N (consumed)/ NH_4^+-N (removed) ratio might be indicative of AOB activity (Li *et al*., 2009). Laureni *et al*. (2015) observed variation in the stoichiometric ratio during the enrichment of anammox bacteria using aerobically pre-treated municipal wastewater.

Studies have revealed FA to be a true inhibitor of the anammox process rather than NH_4^+ since unionized ammonia (FA) can penetrate through the cell

membrane and changes the cytoplasmic pH causing cell death (Zhang *et al*., 2016, Jaroszynski *et al*., 2012). Anammox bacteria and AOB were reported to be limited by FA concentrations of 1700 µg NH_3-N/L and 10000 µg NH_3-N/L respectively (Wang *et al*., 2017, Fernandez *et al*., 2012). Therefore, the performance of anammox bacteria and AOB were not affected by FA concentration, since the expected FA concentrations were below the inhibitory concentration in all the reactors. The NOB is reported to be more sensitive to FA concentration of 100 – 1000 µg NH_3-N/L, in this regard NOB, may have been inhibited as the estimated FA concentration in all the reactors were within the inhibitory concentration (Liu *et al*., 2019, Aktan *et al*., 2012).

Several studies have demonstrated that FNA was inhibitory to several key microbial groups involved in nutrient removal in WWTP (Zhang *et al*., 2017, Tomaszewski *et al*., 2017a Jin *et al*., 2012). Furthermore, various authors have claimed that FNA (protonated form of NO_2^-) is the actual inhibitor of the anammox process (Fernandez *et al*., 2012, Puyol *et al*., 2014, Zhou *et al*., 2011). The FNA concentration of 6 µg HNO_2^- N/L were reported to reduce 100% anammox bacteria (Yin *et al*., 2016, Zhang *et al*., 2017). The performance of AOB and anammox bacteria was not inhibited by the FNA concentration, since the FNA concentrations were lower than the reported inhibitory concentration for anammox bacteria and AOB in Reactor 1 and 2. However, FNA concentrations (between 190th – 230th and 320th day) above 6 µg HNO2- N/L, suggest that anammox activity was inhibited after the 170th day.

On the contrary, the FNA concentration did not affect the N removal in Reactor 3 since it showed strong N removal performance. Previous research has demonstrated 87.4 % N removal in the presence of 70 µg HNO_2^- N/L FNA concentrations (Zheng *et al*., 2017). In Reactor 3, it is was anticipated that the population of NOB would be lower, since the FNA concentration revealed in this reactor was within the range of inhibitory concentrations reported (Zhang *et al*., 2017). Although FNA inhibition of NOB could be required for the anammox process, unrestricted concentrations of FNA could also limit anammox bacteria (Zhou *et al*., 2011). Notably, inhibition of FNA and FA is reversible and AOB recuperated faster than NOB (Sun *et al*., 2015, Qian *et al*., 2017). Additionally, FA inhibition to anammox bacteria was reported reversible (Liu *et al*., 2019).

3.5 Conclusion

In this study, high NO_2^--N concentration in Reactor 3 achieved sufficient N removal and anammox activity, as evidenced by NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratio closer to the reported anammox stoichiometric ratios. Whereas, the substrate ratios in Reactor 1 and 2 had a negative influence on anammox activity, since the observed NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratios were not within the reported anammox stoichiometric ratios. Furthermore, high NO_2^--N concentration (1: 2) achieved the highest N removal followed by balanced ratio (1:1.32) and high NH_4^+-N (2:1) respectively.

The FA concentrations were below the reported inhibitory concentration for anammox bacteria and AOB in all the reactors throughout the study period. However, the FA were above the reported inhibitory concentration for NOB in all the reactors.

The FNA concentrations varied across the reactors throughout the study period. However, the observed FNA did not negatively affect anammox activity in Reactor 3, since anammox activity was evident through NO_2^--N (consumed)/ NH_4^+-N (removed) and NO_3^--N (produced)/ NH_4^+-N (removed) ratio. The results proved that N removal was a result of the activity of total microbial consortia initially present in the inoculum (AOB, NOB and anammox bacteria). Furthermore, the outcome proves that it is difficult to manage anammox bacteria alone in the same reactor that contain different microbial groups that compete for the same substrate under different substrate ratios.

CHAPTER 4: MOLECULAR ANALYSIS OF THE ANAEROBIC SEQUENCING BATCH REACTORS

4.1 Introduction

The performance and sustainability of biological nitrogen removal in wastewater treatment is significantly influenced by microbial community structure and environmental dynamics. Nitrogen removal in WWTPs is accomplished by numerous biological processes namely: anammox, nitrification, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) and ammonification (Wongkiew *et al*., 2017, Langone, 2013, Li *et al*., 2018, Carrera *et al*., 2003). These processes are mediated by either a single bacterium or multiple bacterial groups. Furthermore, influent composition, environmental and operational conditions can greatly influence the predominant microbial community structure.

No pure anammox cultures have been isolated yet and studies have shown that the anammox bacteria co-exist with other microbial groups that have similar metabolic requirements (Ali *et al*., 2015, Connan *et al.*, 2016). Several bacterial groups such as *Proteobacteria*, *Chloroflexi*, *Bacteroidetes* and *Planctomycetes* are frequently reported from the anammox reactors (Shu *et al*., 2015, Chen *et al*., 2017, Shu *et al*., 2016). Dominance by any one species is predominantly due to operational conditions within the reactors

Ammonium and nitrite are the key substrates for anammox bacteria as well as other nitrifying bacteria. The ratio between these two substrates, therefore, plays a major role in the selection of anammox and nitrifying bacteria apart from other operational conditions. A high concentration of the substrates can inhibit the anammox process. Therefore, understanding the microbial diversity within the anammox system under different substrate (NH_4^+-N: NO_2^--N) concentration is crucial in the effort to improve anammox process. More precise microbial community structure detection and quantification techniques (real-time PCR, FISH and metagenomics analysis) are suitable for evaluating the effect of operational conditions on the microbial community structure of anammox systems (Pereira *et al*., 2017, Zhu and Al-Moniee, 2017, Shalini and Joseph, 2018, Bassin *et al*., 2018). This chapter is aimed at evaluating the effect of

varying key substrate (NH_4^+-N: NO_2^--N) ratios on the microbial community structure within the ASBR using advanced molecular techniques.

4.2 Methodology

4.2.1 Fluorescence *in-situ* Hybridization (FISH)

4.2.1.1 Sample fixation

The preliminary screening and characterization of anammox bacteria was performed using FISH as described by Amann *et al*. (1995). For this, biomass samples were fixed using paraformaldehyde fixation. The biomass samples were suspended in a 1:3 (v/v) ratio of 1 x phosphate buffered saline (PBS) solution: 4 % paraformaldehyde, and incubated overnight at 4 °C. Thereafter, the biomass samples were washed twice with 1x PBS, re-suspended in a 1:1 solution of 1 x PBS: absolute ethanol, and thereafter stored at -20 °C until further analysis.

4.2.1.2 Sample pre-treatment and hybridization

The fixed samples were pre-treated with 10 µL of lysozyme (10 mg/mL) for 30 min at 37 °C, 100 µL of sterile water was used to dilute the sample and vortexed at maximum speed for 5 min (Amann *et al*., 1995). Pre-treated sample volume (10 µL) were fixed on poly-L-lysine coated slides and air-dried at 48 °C. Followed by dehydration of samples by a series of ethanol (50, 80 and 100 % respectively) washes at 3 min interval. The samples were then hybridized by the addition of 9 µL of hybridization solution containing 1 M Tris/HCl (pH 8), 10 % SDS, 5 M NaCl and a required formamide volume (Table 4.1), 1 µL probe and incubated overnight at 46 °C. The oligonucleotide probes were labelled with CAL Fluor Red 590 dye at the 5'-end respectively (Table 4.1). Thereafter the samples were rinsed with pre-warmed wash buffer containing 1 M Tris/HCl (pH 8), 10 % SDS, 5 M NaCl and 0.5 M EDTA (Table 4.1) for 10 min at 48 °C. Additionally, the samples were rinsed with distilled water and air-dried at ambient temperature. The sample was stained with 4´-6-diamino-2-phenylindole (DAPI) at a working concentration of 0.25 µg/mL and incubated for 10 min at room temperature, rinsed in pre-warmed distilled water and air-dried in the dark. An anti-fading solution was used to mount the slides (Vectashield, Vector Laboratories, Inc. Burlingame).

4.2.1.3 Microscopic image analysis

Axiolab Apotome microscope (Carl Zeiss, Germany) equipped with essential filters (FLUOR fluorochrome) specific for TAMRA and FAM was used to analyse the slides. The Zeiss AxioCam MRC camera captured the images and analysed using Zeiss Axio vision Release 4.8 imaging software.

Table 4.1: 16S rRNA oligonucleotide probes with the corresponding formamide stringency and NaCl concentrations used in this study.

Probe name	**Target group**	**Probe Sequence (5'-3')**	**Formamide concentration (%)/ NaCl (µl)**	**Reference**
Amx 368	Anammox	CCTTTCGGGCATTGCGAA	15/ 300	(Schmid *et al*., 2003)
EUB338		GCTGCCTCCCGTAGGAGT	15/ 300	(Amann *et al*., 1995)
EUB338 II	Eubacteria	GCAGCCACCCGTAGGTGT	15/ 300	(Daims *et al*., 1999)
EUB338 III		GCTGCCACCCGTAGGTGT	15/ 300	(Daims *et al*., 1999)

4.2.2 Sample collection and genomic DNA extraction

Mixed liquor samples (50 mL) were collected at different sampling intervals (0, 60, 90, 170 and 320 days) and centrifuged at 5000x g for 10 min at 4 ˚C. The supernatant was discarded and biomass was collected. Approximately 0.25 g of biomass carefully scraped using a spatula and transferred in 2 mL Eppendorf tube for DNA isolation. Genomic DNA was then extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. The concentration and purity of the extracted DNA

were determined spectrophotometrically using NanoDrop ND-1000 (NanoDrop Technologies, USA). The quality of the extracted genomic DNA was further determined using 1 % agarose gel pre-stained with ethidium bromide. The obtained genomic DNA was stored at -20 ˚C until further analysis.

4.2.3 Polymerase chain reaction (PCR)

The genomic DNA was amplified using the Thermo Scientific PCR master mix in a T100 Thermal Cycler (Bio-Rad). Each PCR amplification reaction contained a total working volume 25 µL, including 12.5 µL of the Thermo Scientific PCR master mix, 1 µL of each primer (0.5 µM), 2 µL of diluted DNA (10 ng/L) and 8.5 µL of sterile water was used. The PCR amplification conditions were optimized for each primer set. The modified PCR amplification conditions of each primer are represented in (Table 4.2).

4.2.4 Agarose gel electrophoresis detection of genomic DNA and PCR products

The extracted genomic DNA and PCR amplicons were electrophoresed in 1 % (w/v) Tris-borate EDTA agarose gel (2.5 mM EDTA, pH 8.0, 10 mM Tris-HCl, 10 mM boric acid). The electrophoresis was carried out and optimized at 80 volts for 60 min. An appropriate size DNA ladder was included on each gel as a standard and the gel was captured below UV light using G: Box gel imaging and analysis system (Vacutec, South Africa).

4.2.5 Plasmid preparation

The PCR amplicons from the gel with the expected size were purified using GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instruction. Concentration (ng/µL) of the purified DNA (purified 16S rRNA gene fragments) used as templates for the standard was measured using the NanoDrop ND-1000 (NanoDrop Technologies, USA). Thereafter, the concentration was used for calculating the copy numbers, based on the formula which includes the Avogadro's number and molecular weight size (Trivedi *et al*., 2009) as shown below:

$$Number\ of\ Copies = \frac{Amount\ in\ ng\ X\ Avogadro's\ number}{Lenght\ in\ bp\ X\ 1\ X\ 10^9 X\ 650}$$

Table 4.2: Primers used for PCR and qPCR and optimized annealing temperature used in this study

Name	Sequence 5'-3'	Specificity	Annealing Temperature (°C)	Fragment size (bp)	Reference
hzocl1F1	TGYAAGACYTGYCAYTGG	*hzo* gene	59	470	(Schmid *et al*., 2008)
hzocl1R2	CTCCAGATRTGCTGACC				
hzsA_526F	TAYTTTGAAGGDGACTGG	*hzs* gene	52	526	(Harhangi *et al*., 2012)
hzsA_1829R	TCATACCACCARTTGTA				
AnnirS379F	TCTATCGTTGCATCGCATTT	*nirS* gene	53	442	(Li *et al*., 2011)
nirS821R	GGATGGGTCTTGATAAACA				
amoA-1F	GGGGTTTCTACTGGTGGT	AOB	58	491	(Jin *et al*., 2011)
amoA-2R	CCCCTCKGSAAAGCCTTCTTC				
NSR 1113F	CCTGCTTTCAGTTGCTACCG	*Nitrospira*	55	151	(Dionisi *et al*., 2002)
NSR 1264R	GTTTGCAGCGCTTTGTACCG				

4.2.6 Quantitative time-real PCR (qPCR)

Quantification of gene copy numbers in the extracted genomic DNA was carried out using real-time PCR (C-1000 Touch, CFX 96, Bio-Rad Laboratories Pty Ltd, USA). The primer sets targeting different species of AOB, NOB and anammox functional genes were used (Table 4.2). The qPCR total reaction volume of 10 µL comprised of 4 µL of SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories Pty Ltd, USA), 0.4 µL of each primer (0.4 µM), 2 µL of template DNA (1 ng/L) and 3.2 µL sterile distilled water. The qPCR protocols were optimized for each set of primers accordingly. The accuracy of each qPCR assay was established by melting curve analysis and agarose gel electrophoresis as described by Leal *et al*. (2016). Negative controls (qPCR reactions with no genomic DNA) were used to eliminate possible contamination/ carry-over for all qPCR experiments.

4.2.7 Sequencing and phylogenetic analysis

The PCR amplicons were submitted to a commercial lab (Inqaba Biotechnical Industries (Pty), South Africa) for sequencing and analysis. The obtained sequences were edited using Finch TV software. Based on the similarity score, sequences with greater than 95 % identity were grouped into a single operational taxonomic unit (OTU) for analysis. The sequences obtained were checked against the National Centre for the Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) to confirm phylogenetic affiliations. The nucleotide sequences from this study and those obtained from the NCBI database were aligned with CLUSTALX in BioEdit (Hall, 1999). The aligned sequences were transferred into MEGA6 to branch matrices of evolutionary distances (Tamura *et al*., 2013). Phylogenetic trees were checked and constructed via bootstrap analysis (based on 1,000 replicates) (Tamura *et al*., 2011).

4.2.8 Analysis of the bacterial communities by Illumina

Microbial community structure was established through high through-put sequencing of biomass sampled from: initial biomass (inoculum used to seed the reactors) and from the reactors during its operation (170 and 320 days).The extracted genomic DNA was sent to Inqaba Biotech (South Africa) for high-

throughput sequencing using Illumina MiSeq platform at Inqaba Biotechnical Industries (Pty), South Africa. The V1-V3 region: 27F 5' (CTATCCC CTGTGTGCCTTGGCAGTCTCAG) 3', in which the tag was included, and 518R 5' (CCATCTCATCCCTGCGTGTCTCCGA CTCAG) 3' primers were used to amplify 16S rRNA genes (Okubo., *et al*. 2009). Quantitative Insights Into Microbial Ecology (QIIME) software was used for sequence analysis (Caporaso *et al*., 2010). Less than 200 sequence bases, less than 30 quality coefficient, greater than 6 homopolymer size and ambiguous bases were removed. Operational taxonomic units (OTUs) were classified using UCLUST algorithm (Edgar, 2010) based on 97 % identity. Classification of reads using suggested boot-strap cut off set at 50 % was accomplished using the Classifier tool from the Ribosomal Database Project (http://edp.cme.msu.edu/classifier/classifier.jps). Rarefaction and alpha diversity analyses were done using R statistical calculation software package vegan (Oksanen *et al*., 2018). Abundance-based coverage estimator indices (ACE) and Chao 1 were used to assess the microbial diversity and richness within the reactors at different enrichment periods (0, 170 and 320 days).

4.3 Results

4.3.1 Characterisation with FISH

The initial seed inoculum was characterized using FISH for visual confirmation of the presence of anammox bacteria. The anammox bacteria were detected in all three reactors using Amx 368 probe labelled with CAL Fluor Red 590. The FISH micrographs of the samples hybridized with DAPI and CAL Fluor Red 590 positive with anammox bacteria are shown as follows initial inoculum [A1 and A2], Reactor 1 [B1 and B2], Reactor 2 [C1 and C2] and Reactor 3 [D1 and D2] (Figure 4.1).

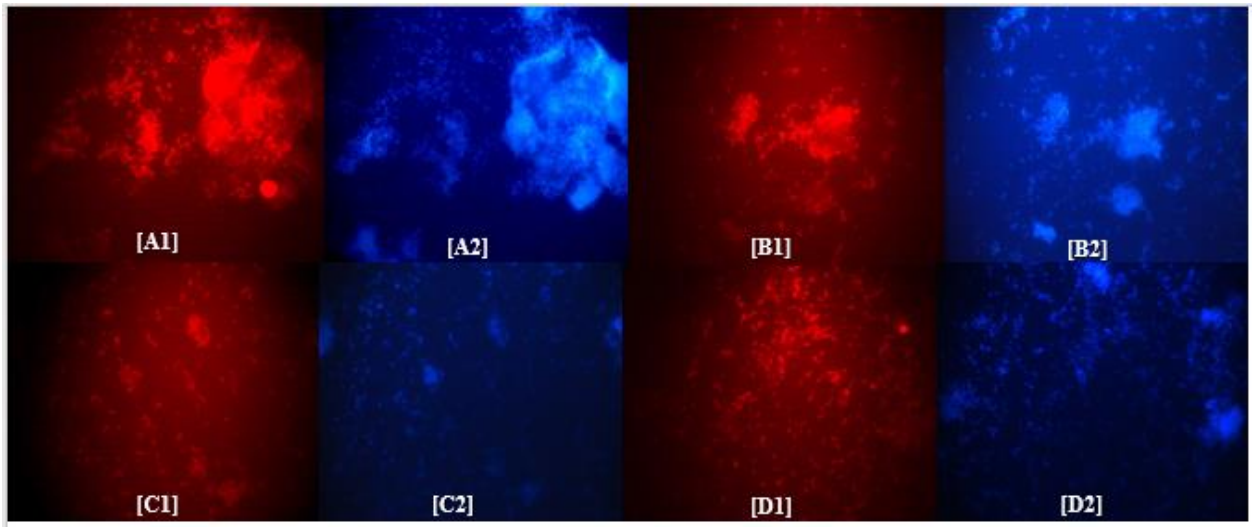

Figure 4.1: Fluorescence *in situ* hybridization images of samples in the reactors. All microorganisms stained with DAPI (blue). Samples hybridized with AMX 368 (anammox bacteria) oligonucleotide probe labelled with Cal Fluor Red 590 (red). DAPI and Cal Fluor Red 590 couples represent the same fields of microscopic view.

4.3.2 Quantification of the microbial population in each reactor

The qPCR was used to track and quantify the microbial populations in all the reactors and to establish the correlation between microbial populations with the N performance within each reactor. The abundance of anammox functional genes (*hzo*, *hzs* and *nirS*), AOB and NOB were quantified in each reactor over the operational period of 320 days. Standard curves for qPCR were obtained by preparing 10-fold dilutions of plasmids fragment amplified (PCR) with each primer set (Table 4.2). All standard curves showed a linear range between 10^8 and 10^2 copies, with a slope between -3.268 and -3.577 for all primer sets. The regression analysis correlation coefficient (R^2) values for all the qPCR assays ranged between 0.995 and 0.999. The calculated qPCR efficiencies for each primer assays were between 89 % and 106 %. Quantification of both AOB and NOB (*Nitrospira*) was done based on gene copy numbers. The abundance of anammox bacteria functional were quantified using specific functional gene (*hzs, hzo* and *nirS*) primers.

In Reactor 1, the AOB population decreased from 5.99 x 10^4 (inoculum) to 4.69 x 10^2 copies/µL (60^{th} day) followed by an increase to 5.48 x 10^2 (90^{th} day) and 7.11 x 10^3 copies/µL (170^{th} day), thereafter a final increase to 8.00 x 10^3 copies/µL was observed on 320^{th} day. Thus, implying that AOB population was actively involved in N removal throughout the study. The anammox bacterial functional gene copy numbers showed variation during this study period. A reduction in *hzs* gene was initially observed from 4.40 x 10^3 in the initial inoculum to 2.71 x 10^3 copies/µL on the 60^{th} day and further increased to 4.63 x 10^3 copies/µL (90^{th} day) and decreased again to 5.69 x 10^2 copies/µL (170^{th} – 320^{th} day). Similarly, *hzo* genes revealed differences in abundance throughout the study. The *hzo* were found to be 1.17 x 10^9 copies/µL in the inoculum and increased to 8.31 x10^9 copies/µL (60^{th} day) followed by a decrease to 5.55 x 10^9 copies/µL (90^{th} day), an increase to 1.53 x 10^{10} copies/µL (170^{th} day) and a decrease to 2.60 x 10^9 copies/µL (320^{th} day). The *nirS* genes presented a steady decrease throughout the operational period from 9.39 x 10^7 copies/µL in the inoculum to 2.88 x 10^5 copies/µL on 320^{th} day. These results indicate that the substrate ratio of 1 NH_4^+-N: 1.32 NO_2^--N did not favour activity and growth of anammox bacteria. Additionally, these results were not anticipated since the

substrate (NH_4^+-N and NO_2^--N) ratio of 1: 1.32 used is the reported stoichiometric ratio for the anammox reaction. The *Nitrospira* population showed an increase on the 60th day from 2.31 x 10^1 to 1.46 x 10^5 copies/µL and further decreased on the 90th day to 3.06 x 10^4 copies/µL, then on the 170th and 320th day the population increased to 7.85 x 10^5 copies/µL (Figure 4.2). Suggesting that the reported anammox stoichiometric ratio (1: 1.32) favoured NOB activity.

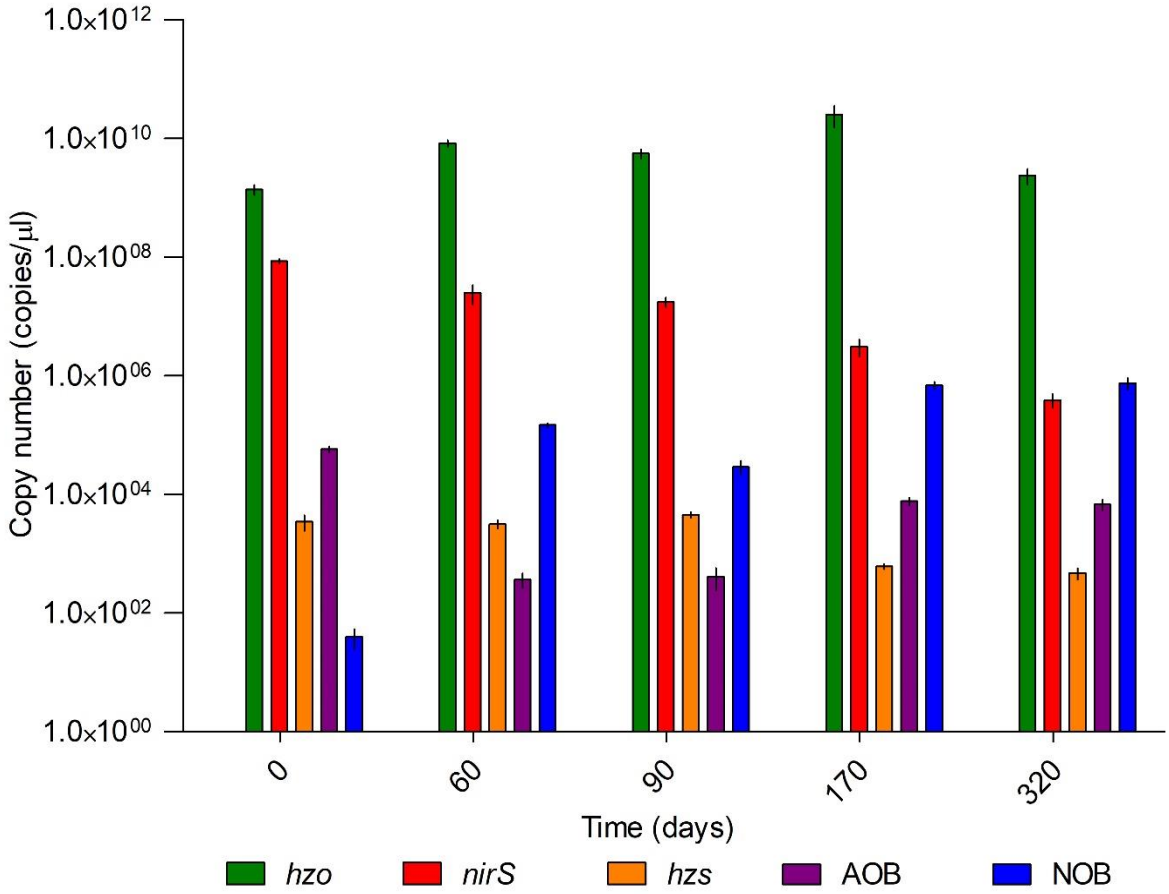

Figure 4.2: Real-time quantification analysis of anammox functional genes biomarkers (*hzo, nirS* and *hzs*), AOB and NOB (*Nitrospira*) in Reactor 1 during the operation period of 320 days.

In Reactor 2, the abundance of AOB increased from 1.20 x 10^2 (inoculum) to 9.50 x 10^2 (170th day) copies/µL and increased to 1.22 x 10^3 copies/µL (on the 320th day). Indicating AOB activity throughout the operational period. The copy numbers of *hzs*, *hzo* and *nirS* genes increased from 6.98 x 10^4, 5.45 x 10^{11} and 2.73 x 10^7 (inoculum) to 2.89 x 10^5, 7.21 x 10^{11} and 4.99 x 10^7 (60th day)

copies/µL, respectively. By the 90th day, the *hzs*, *hzo* and *nirS* genes had decreased to 1.39 x 10^4, 4.6 x 10^{10} and 4.75 x 10^6 copies/µL, respectively, but then increased to 4.59 x 10^5 (*hzs* genes), 3.25 x 10^{12} (*hzo* genes) and 4.79 x 10^7 (*nirS* genes) copies/µL by the 170th day. However, a decrease was observed in *hzs*, *hzo* and *nirS* genes to 4.41 x 10^5, 2.96 x 10^{10} and 3.57 x 10^6 copies/µL respectively by the 320th day (Figure 4.5). Implying that anammox bacteria were active up to the 170th day, thereafter loss of anammox activity was observed. The *Nitrospira* population showed variation across the study with an increase from 2.31 x 10^1 to 2.91 x 10^4 copies/µL was observed on the 60th day, a decrease in population to 1.45 x 10^4 copies/µL (90th day), an increase in the population as observed on the 170th day to 4.66 x 10^5 copies/µL and an increase was observed on the 320th day to 4.99 x 10^5 copies/µL (Figure 4.3). Similarly, high NH_4^+-N favoured activity of NOB.

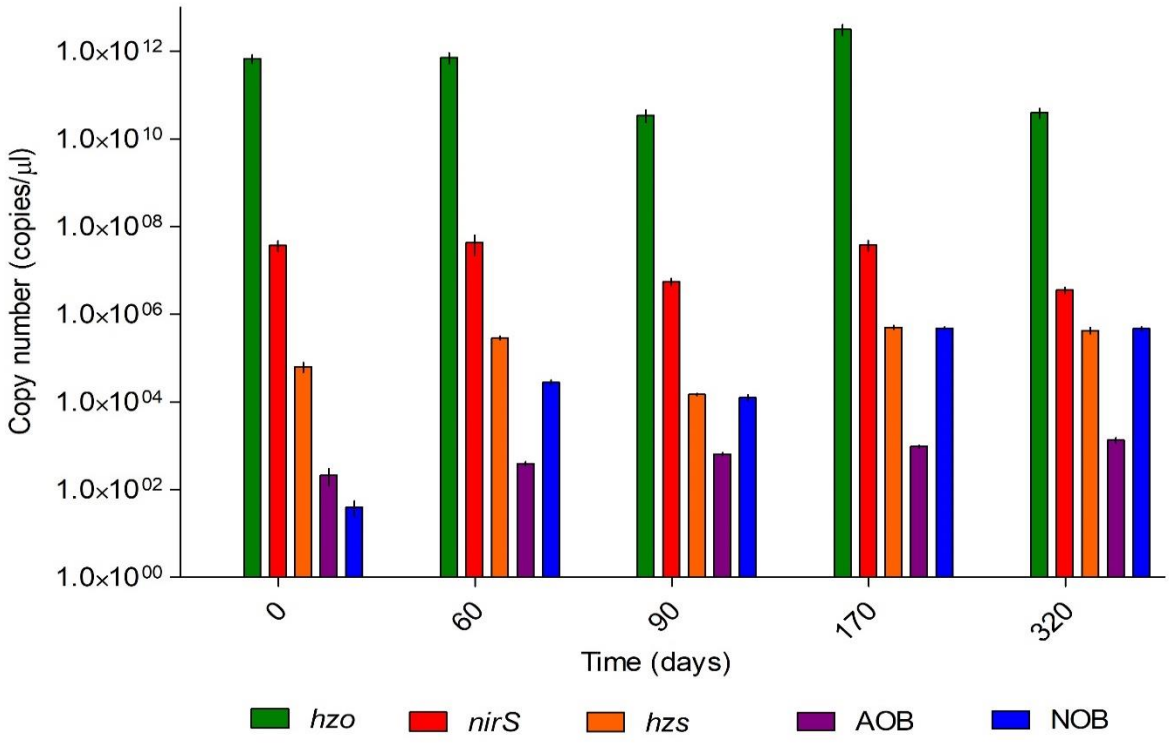

Figure 4.3: Real-time quantification analysis of anammox functional genes biomarkers (*hzo, nirS* and *hzs*), AOB and NOB (*Nitrospira*) in Reactor 2 during the operation period of 320 days.

In Reactor 3, AOB population fluctuation was observed during the study, on the 60th day, AOB increased from 1.20 x 10^2 to 1.05 x 10^3 copies/µL followed by a decrease to 6.70 x 10^2 copies/µL on the 90th day and later increased to 5.48 x 10^3 copies/µL by 320th day. Indicating that AOB activity was also favoured by high NO_2^--N concentration. In addition, the *hzs* genes increased from 6.98 x 10^4 copies/µL (inoculum) to 2.52 x 10^5 copies/µL (60th day) and subsequently decreased during the study period (up to the 320th day). The *hzo* genes decreased from 5.45 x 10^{11} to 2.02 x 10^9 copies/µL between the 60th and 90th day and increased to 1.77 x 10^{11} copies/µL on 170th day and a decrease to 1.23 x 10^9 copies/µl on the 320th day was observed. The *nirS* genes continuously decreased throughout the study period from 2.73 x 10^7 copies/µL in the inoculum to 1.27 x 10^5 copies/µL by the 320th day. In a similar trend, *Nitrospira* population in this reactor fluctuated throughout the study period as it was observed in Reactor 2. There was an increase in the *Nitrospira* population from 2.31 x 10^1 to 1.46 x 10^5 copies/µL by the 60th day and decreased to 3.06 x 10^4 copies/µL by the 90th day. Furthermore, by day 320, the population increased to 3.15 x 10^6 copies/µl at the end of the study period (Figure 4.4).

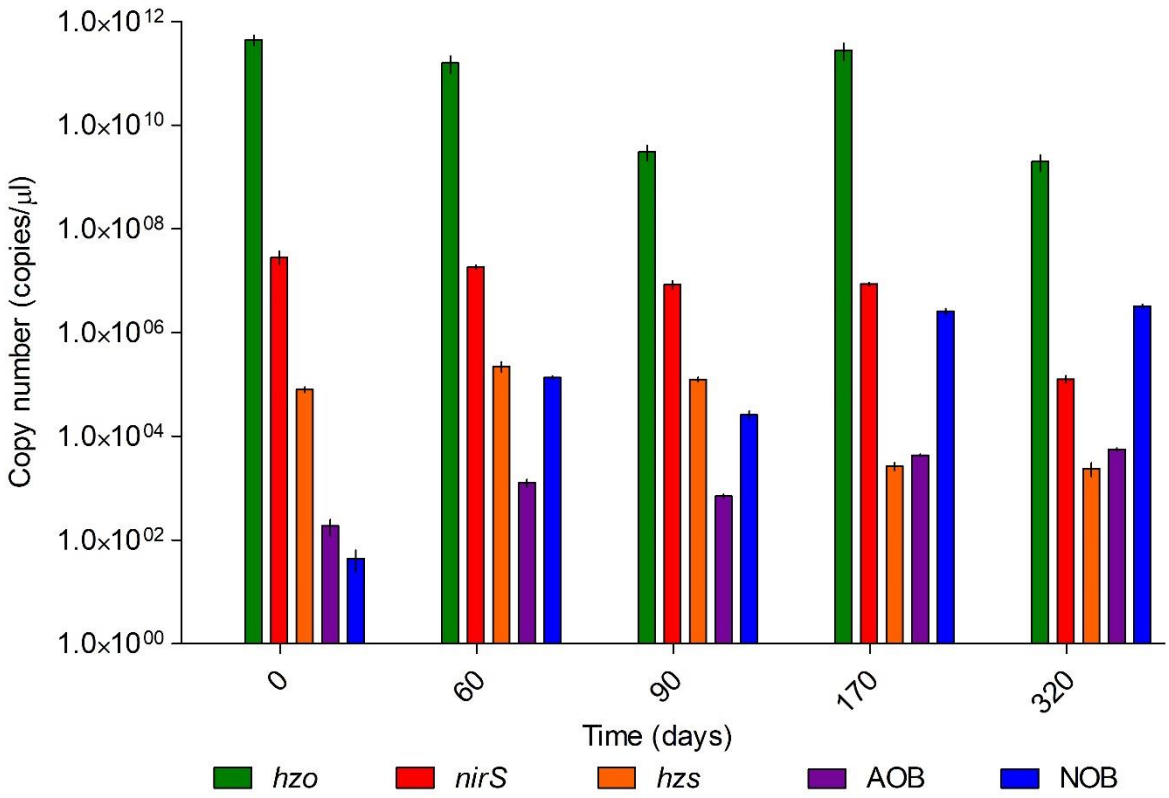

Figure 4.4: Real-time quantification analysis of anammox functional genes biomarkers (*hzo, nirS* and *hzs*), AOB and NOB (*Nitrospira*) in Reactor 3 during the operation period of 320 days.

Table 4.3: Description of qPCR standard curves parameters for anammox functional genes (*hzs*, *hzo* and *nirS*), AOB and NOB primers optimized for analysis during this study.

Parameters	*hzs* gene	*hzo* gene	*nirS* gene	AOB	NOB
Efficiency (%)	97.65 ± 0.07	103.61 ± 3.92	94.80 ± 2.43	98.00 ± 3.26	101.52 ± 4.30
R^2	0.996 ± 0.001	0.997 ± 0.002	0.999 ± 0.000	0.997 ± 0.005	0.996 ± 0.001
Slope	-3.41 ± 0.06	-3.32 ± 0.16	-3.52 ± 0.08	-3.37 ± 0.08	-3.29 ± 0.15
y-intercept	38.39 ± 0.08	37.94 ± 0.69	41.64 ± 0.79	34.41 ± 3.00	0.93

4.3.3 Phylogenetic analysis

Phylogenetic analysis of the microbial population in the reactors was performed using specific primer sets (Table 4.1), the anammox functional genes, AOB and NOB were amplified from samples obtained at 170 and 320 days. The phylogenetic analysis of 16S rRNA sequences revealed that the dominant anammox bacteria sequences were related to two common *Planctomycete* (anammox) species namely; *Candidatus Kuenenia* and *Candidatus Jettenia* (95 – 99 % similarity) in all three reactors (Figure 4.5 – 4.7). The phylogenetic trees of amoA (AOB) are shown in Figure 4.8 – 4.10 from Reactor 1, 2 and 3 respectively. Reactor 1 showed the presence of *Nitrosospira*. In Reactor 2 and 3, the presence of *Nitrosococcus* and *Nitrosomonas*, with 16S rRNA sequence similarity of 95 – 99 % was observed. Thus suggesting a high AOB species diversity. Lastly, Figure 4.11 – 4.13 shows the constructed phylogenetic tree of NSR (NOB) gene sequences. The reactors (Reactors 1, 2 and 3) detected the presence of *Nitrospira* with a 16S rRNA sequence similarity of 100 %.

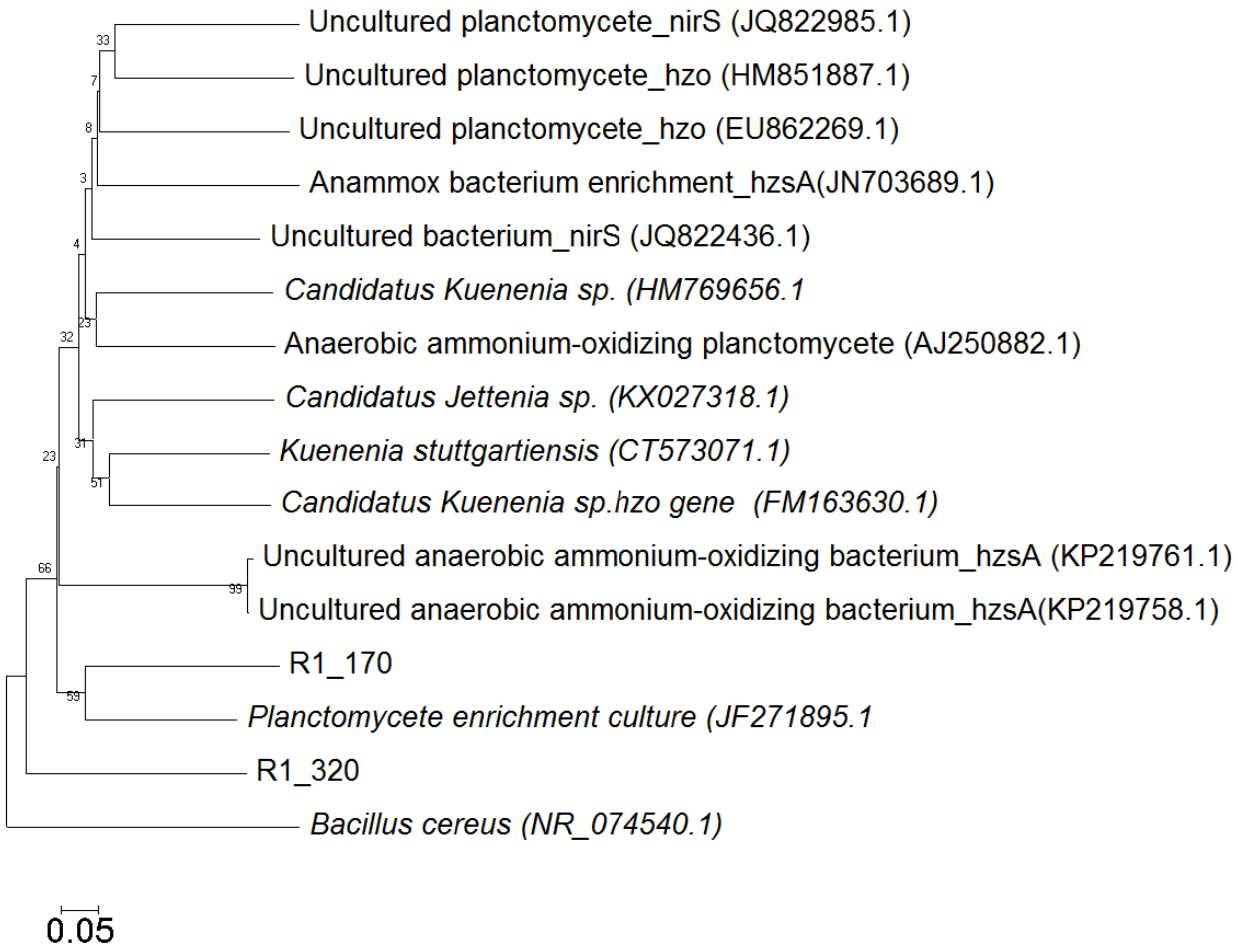

Figure 4.5: Neighbor-joining phylogenetic tree retrieved with anammox functional genes (*hzs*, *hzo* and *nirS*) primer sets from Reactor 1 at 170 (R1_170) and 320 (R1_320) days.

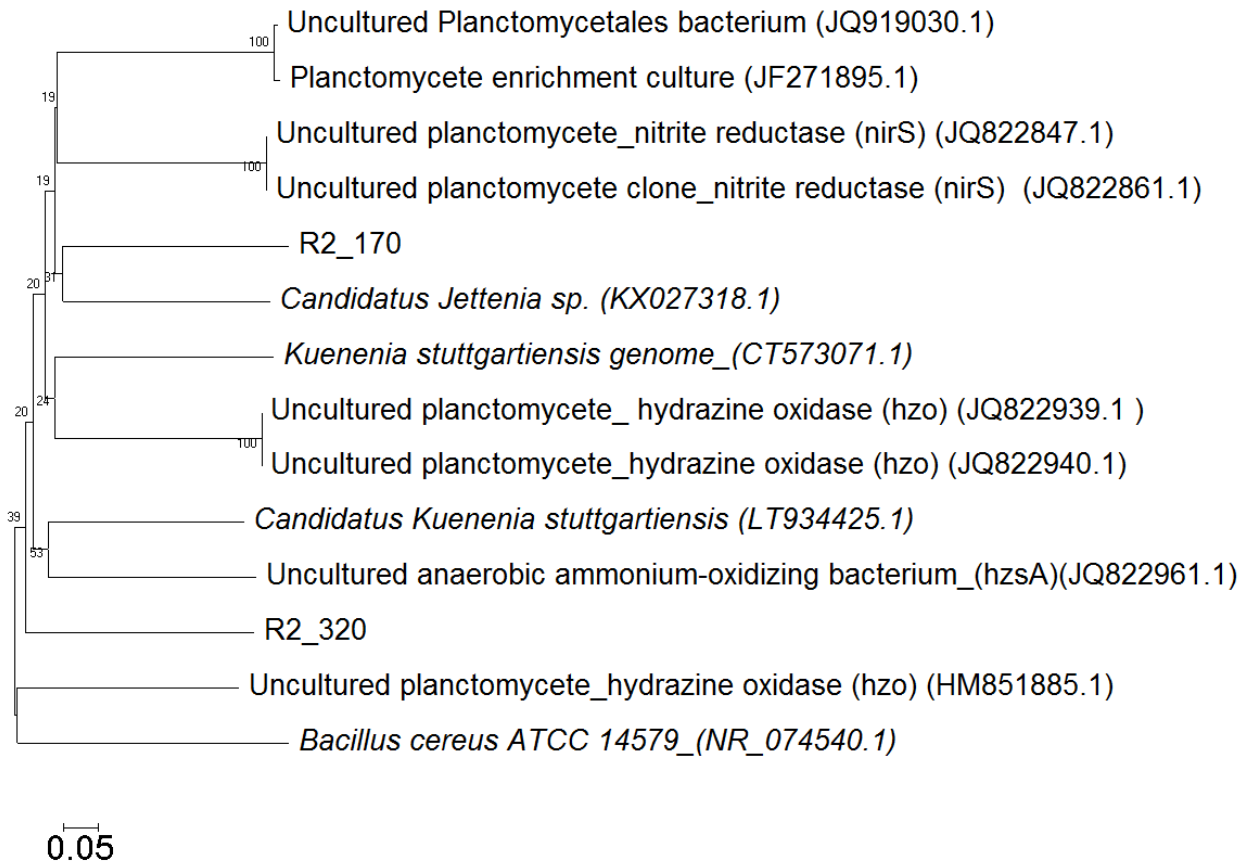

Figure 4.6: Neighbor-joining phylogenetic tree retrieved with anammox functional genes (*hzs*, *hzo* and *nirS*) primer sets from Reactor 2 at 170 (R2_170) and 320 (R2_320) days.

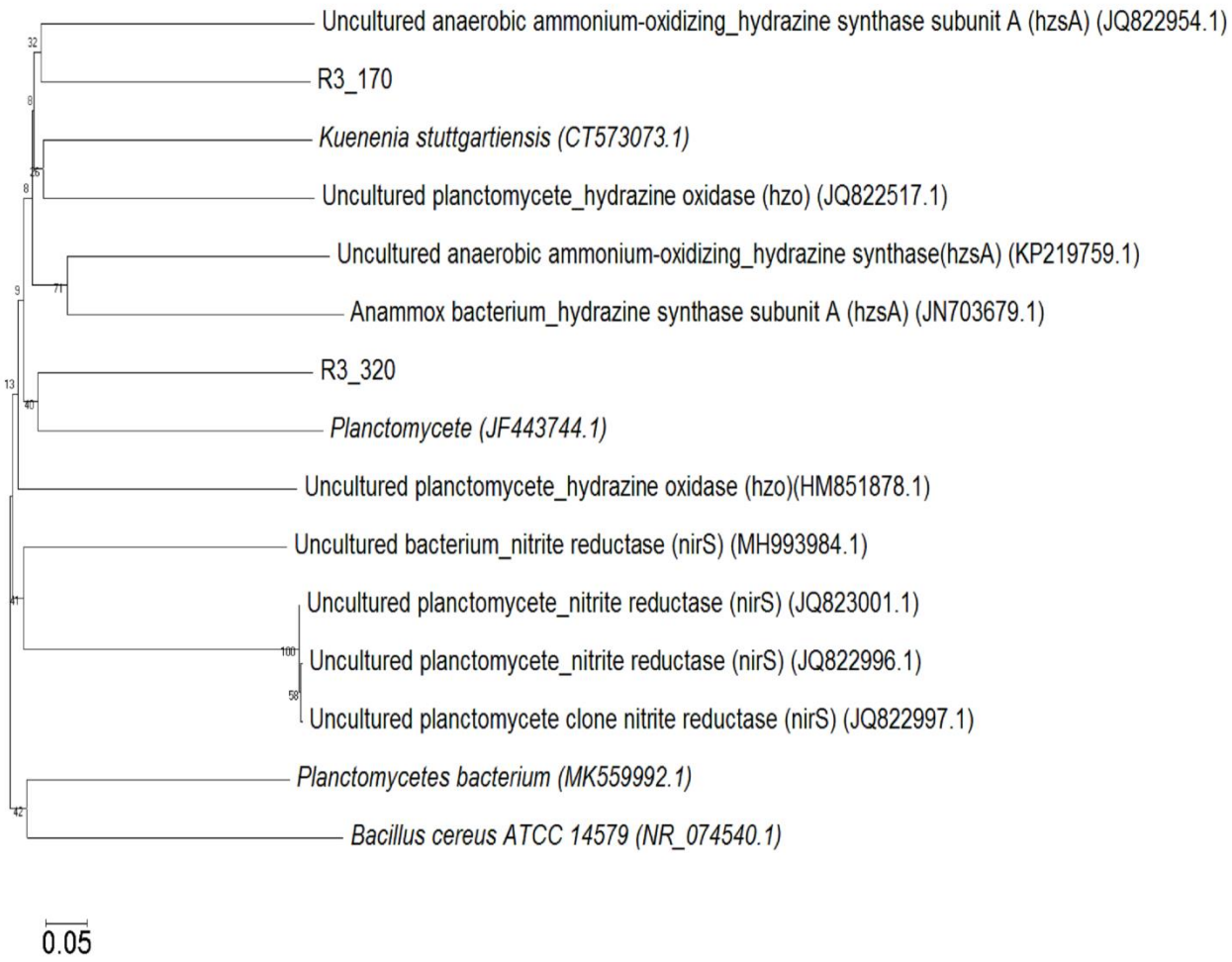

Figure 4.7: Neighbor-joining phylogenetic tree retrieved with anammox functional genes (*hzs*, *hzo* and *nirS*) primer sets from Reactor 3 at 170 (R3_170) and 320 (R3_320) days.

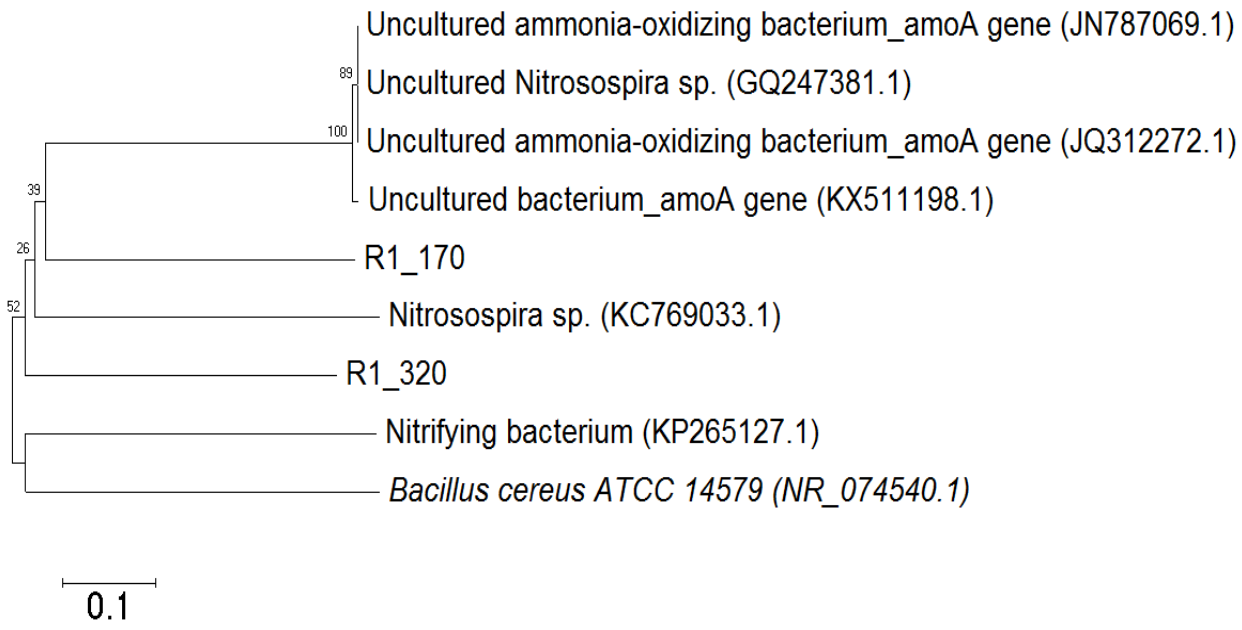

Figure 4.8: Phylogenetic neighbour-joining retrieved with primer sets amoA-1F/ amoA-2R (AOB) sequences from Reactor 1 at 170 (R1_170) and 320 (R1_320) days.

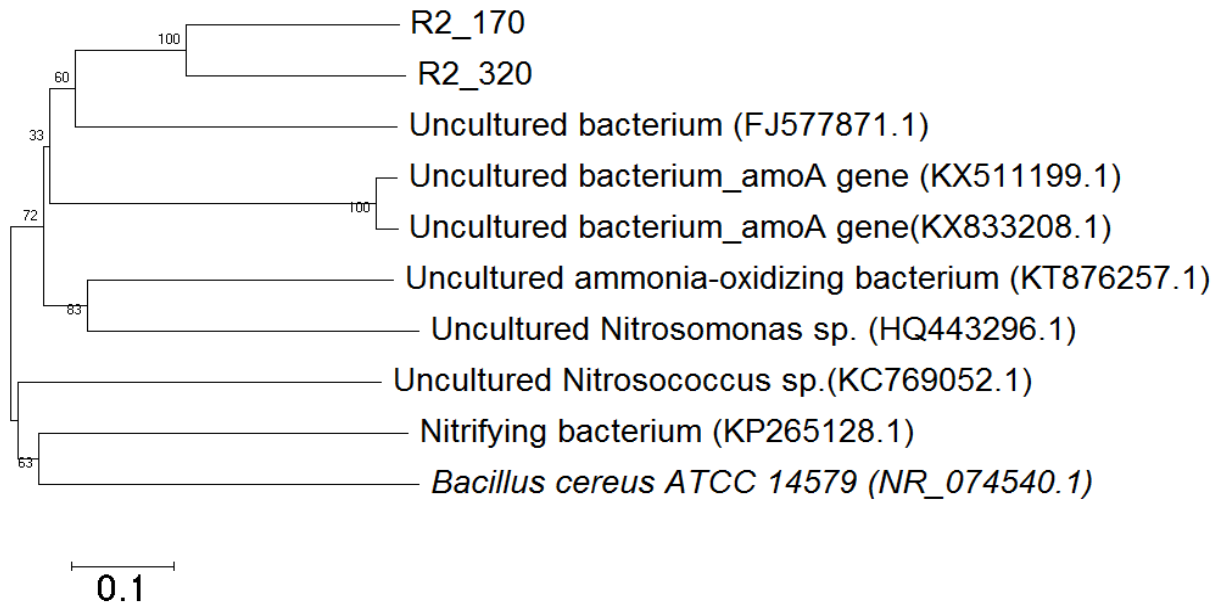

Figure 4.9: Phylogenetic neighbour-joining tree retrieved with primer sets amoA-1F/ amoA-2R (AOB) sequences from Reactor 2 at 170 (R2_170) and 320 (R2_320) days.

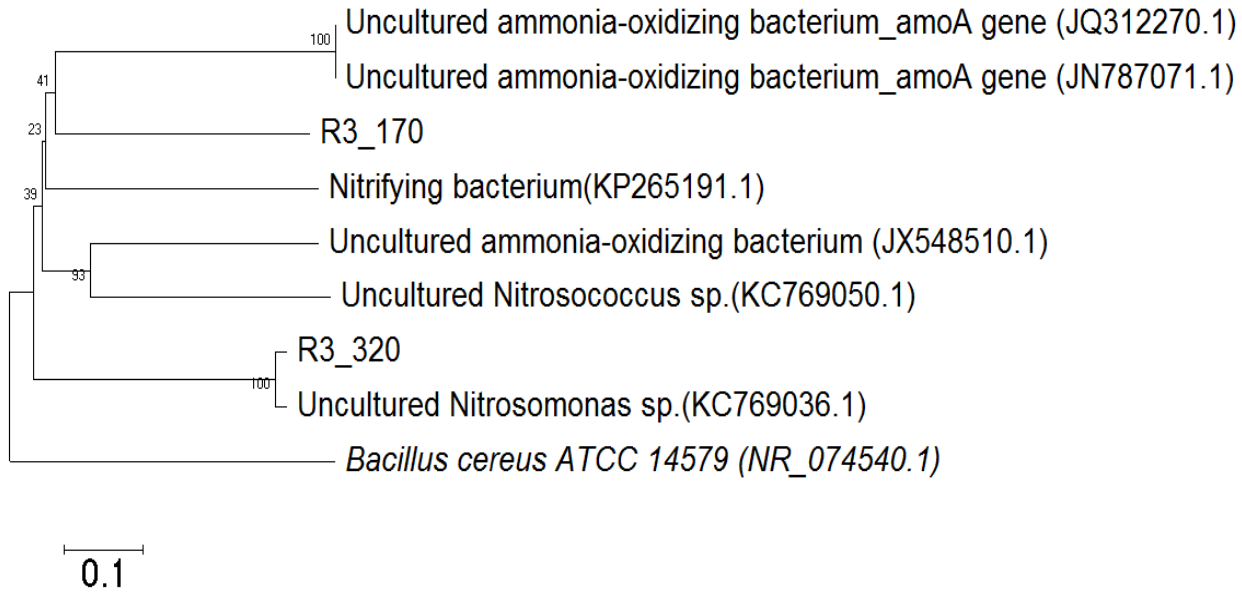

Figure 4.10: Phylogenetic neighbour-joining tree retrieved with primer sets amoA-1F/ amoA-2R (AOB) sequences from Reactor 3 at 170 (R3_170) and 320 (R3_320) days.

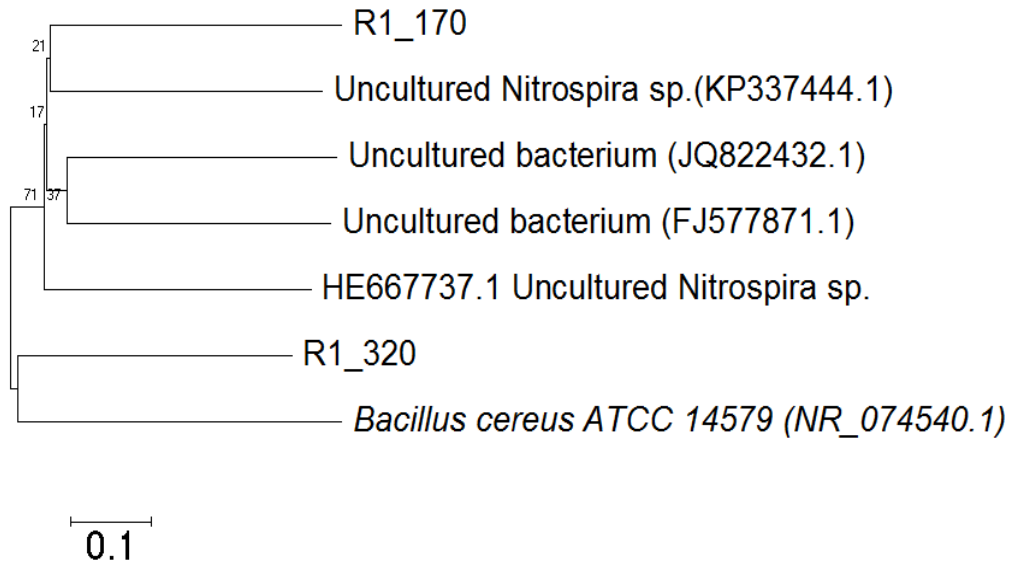

Figure 4.11: Neighbor-joining phylogenetic tree retrieved with primer sets NSR113/ NSR1264R (NOB) sequences from Reactor 1 at 170 (R1_170) and 320 (R1_320) days.

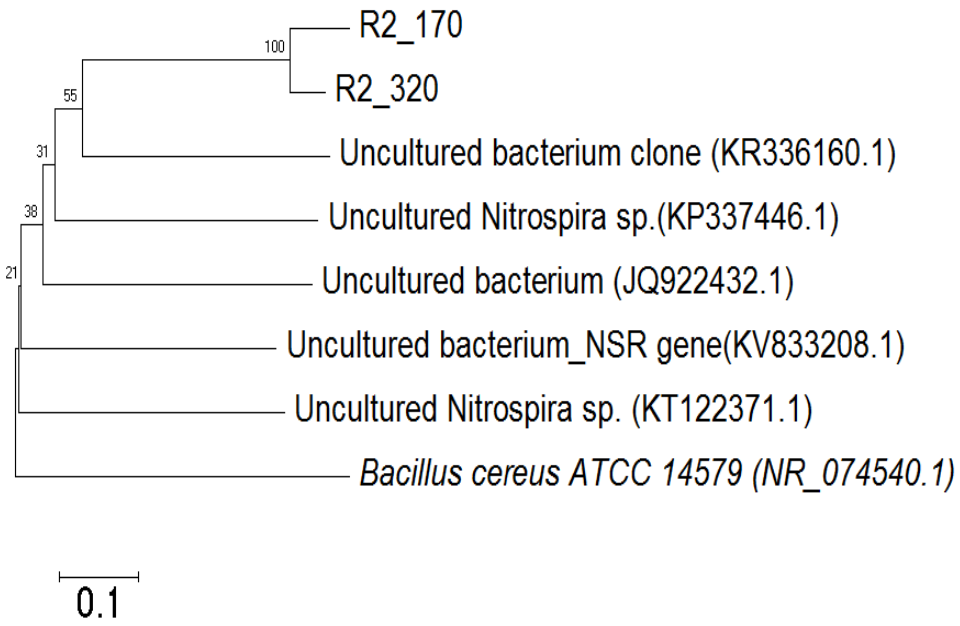

Figure 4.12: Neighbor-joining phylogenetic tree retrieved with primer sets NSR113/ NSR1264R (NOB) sequences from Reactor 2 at 170 (R2_170) and 320 (R2_320) days.

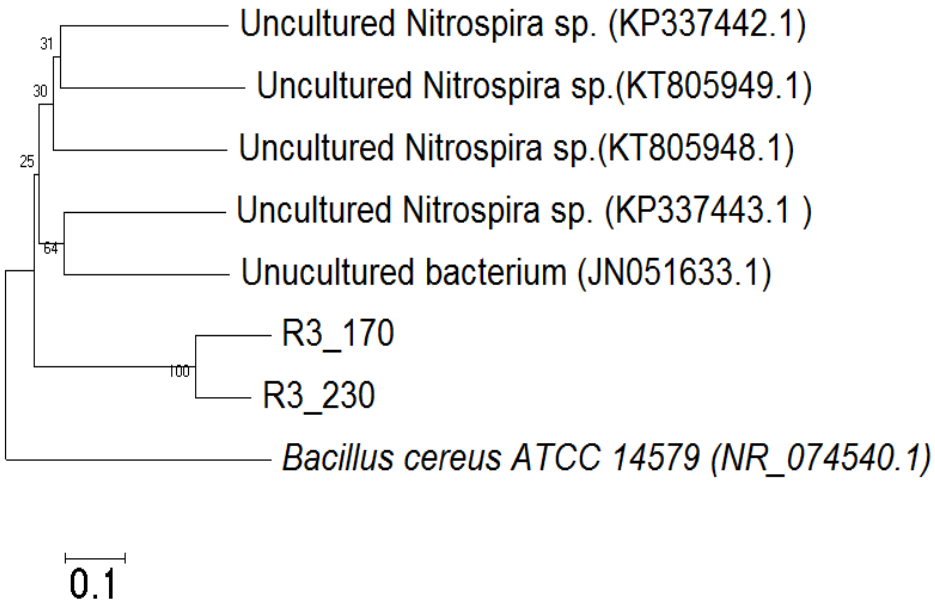

Figure 4.13: Neighbor-joining phylogenetic tree retrieved with primer sets NSR113/ NSR1264R (NOB) sequences from Reactor 3 at 170 (R3_170) and 320 (R3_320) days.

4.3.4 The total microbial community diversity as detected by Illumina sequencing analysis

The rarefaction curve can be used to evaluate if the sequencing depth was enough to fully cover the biodiversity. The rarefaction curve of Reactor 1, 2 and 3 as shown in Figure 4.14. The rarefaction curves of samples from Reactor 1 (320 days), Reactor 2 (320 days), Reactor 3 (320 days) at 0.03 distance suggested that the sequencing depths were not sufficient as they did not reach a plateau, hence the actual bacterial diversity was not fully covered. The rarefaction curves for initial inoculum, Reactor 1 (170 days) and Reactor 2 (170 days) showed a plateau, hence the sequencing depths were sufficient enough to fully reflect the diversity in microbial communities.

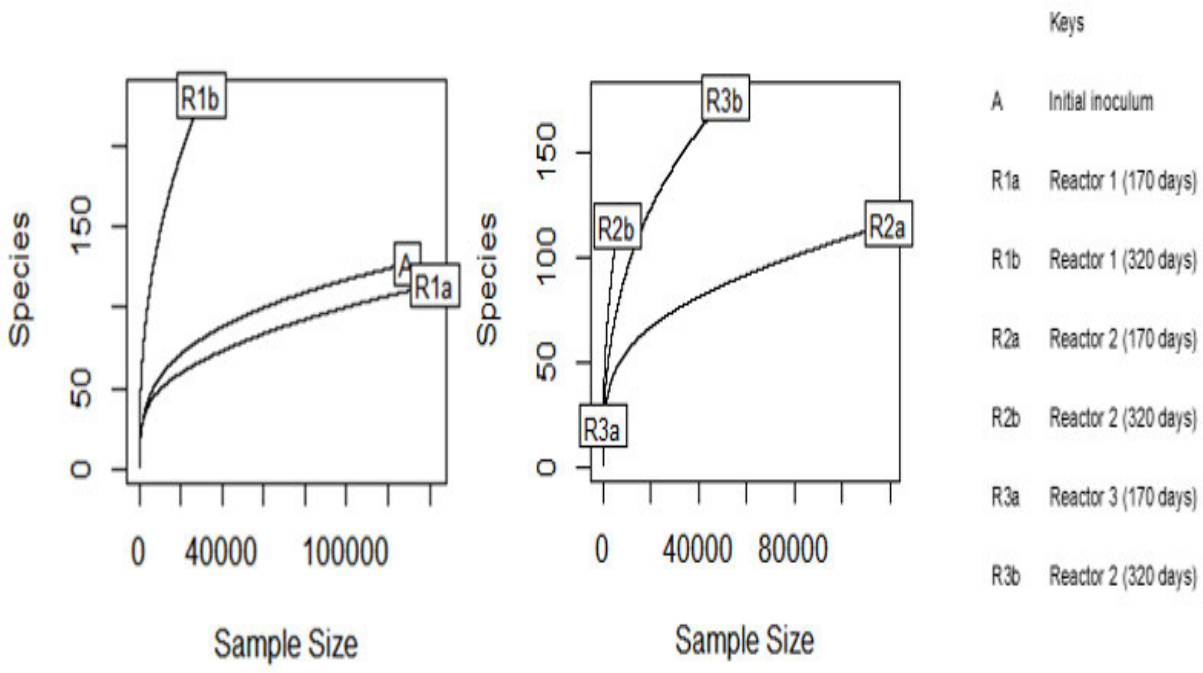

Figure 4.14: The rarefaction curve, plotting the number of observed OTUs (at a similarity cut off of 97 %) as a function of the number of sequences, was computed using vegan package in R.

The alpha diversity was used to analyse complexity of species diversity for each reactor. Alpha-diversity indices, including Shannon, Simpson, Chao1, ACE and Pilou Evenness were performed for each sample using vegan package in R version 2.5 – 2 (Oksanen *et al*., 2018). The Shannon and Simpson indices depict the community diversity whilst the Chao1 and ACE indicate the community richness. The alpha-diversity indices obtained in Reactors 1, 2 and 3 over the study period are summarized in Table 4.4.

The species richness estimated by Chao 1 in Reactor 1 decrease after 170 days from 187 (initial inoculum) to 168 and the highest species richness of 441 was recorded after 320 days of the study in the same reactor. In Reactor 2, the species richness increased from the initial 187 to 305 by the 170th day which drastically decreased to 197 on 320th day. Reactor 3 was dominated by a few species (*Proteobacteria*, *Planctomycetes* and *Firmicutes*), with its richness reduced to 47 by the 170th day, however, after 320 days of the study increased to 270. The Abundance-based Coverage Estimator (ACE) also showed trend similar to Chao1 (Table 4.4). The bacterial community from Reactor 3 (170th day) showed the highest evenness followed by Reactor 2 on days 170 and 320, whilst the inoculum was the least even.

Table 4.4: Microbial richness and diversity of Reactor 1, 2 and 3.

Group	OTU	Diversity		Pilou Evenness (J)	Richness	
		Shannon-Wiener Index (H')	Simpson's Index (λ)		Chao1	ACE
Inoculum	127	0.85	0.32	0.06	187	180
Reactor 1 (170 days)	114	1.36	0.58	0.12	168	186
Reactor 1 (320 days)	231	2.26	0.72	0.13	441	417
Reactor 2 (170 days)	116	1.87	0.76	0.16	305	200
Reactor 2 (320 days)	115	2.14	0.73	0.15	197	213
Reactor 3 (170 days)	20	1.52	0.67	0.22	47	37
Reactor 3 (320 days)	176	1.94	0.68	0.13	270	264

Variation in the microbial community diversity of the reactors (containing varying substrate ratio) was observed throughout the study. In total, 7 dominant bacterial phyla were identified in Reactors 1, 2 and 3 respectively. *Proteobacteria*, *Planctomycetes*, *Actinobacteria*, *Acidobacteria*, *Nitrospira*, *Bacteroidetes* and *Euryarchaeota* phyla were common in all the reactors and unclassified sequences were also detected in the reactors. The majority of sequences from all the reactors could not be classified even at the phylum level. Variation of the unknown phyla was observed in the inoculum, on the 170th and 320th day. In Reactor 1, the unknown phyla were 92.48 % (inoculum), 89.33 % (170 day) and 63.80 % (320 day), in Reactor 2 the unknown phyla were 89.33 % (inoculum), 51.29 % (170 day) and 62.14 % and in Reactor 3 the unknown phyla were 92.48 % (inoculum), 53.65 % (170 day) and 61.37 % (320 day). In the seed inoculum, *Proteobacteria* (6.38 %) was second dominant phyla followed by *Planctomycetes* (0.76 %), *Acidobacteria* (0.05 %), *Bacteroidetes* (0.03 %), *Euryarchaeota* (0.02 %) and *Nitrospira* (0.01 %) whilst *Actinobacteria* were below the detection level.

In Reactor 1, succeeding the start-up on 170th day, the proportion of the phylum increased in *Proteobacteria* (6.70 %), *Planctomycetes* (3.30 %), *Acidobacteria* (0.16 %), *Actinobacteria* (0.09 %) and *Nitrospira* (0.02 %) was revealed. The proportion of *Euryarchaeota* (0.02 %) remained constant, whilst *Bacteroidetes* was not detected. After 320 days of the study period, there was an increased in *Proteobacteria* (28.95 %), *Nitrospira* (2.84 %), *Bacteroidetes* (0.31 %) and *Actinobacteria* (0.67 %), whereas there was a decrease in *Planctomycetes* (2.04 %) and *Acidobacteria* (0.02 %) (Figure 4.15).

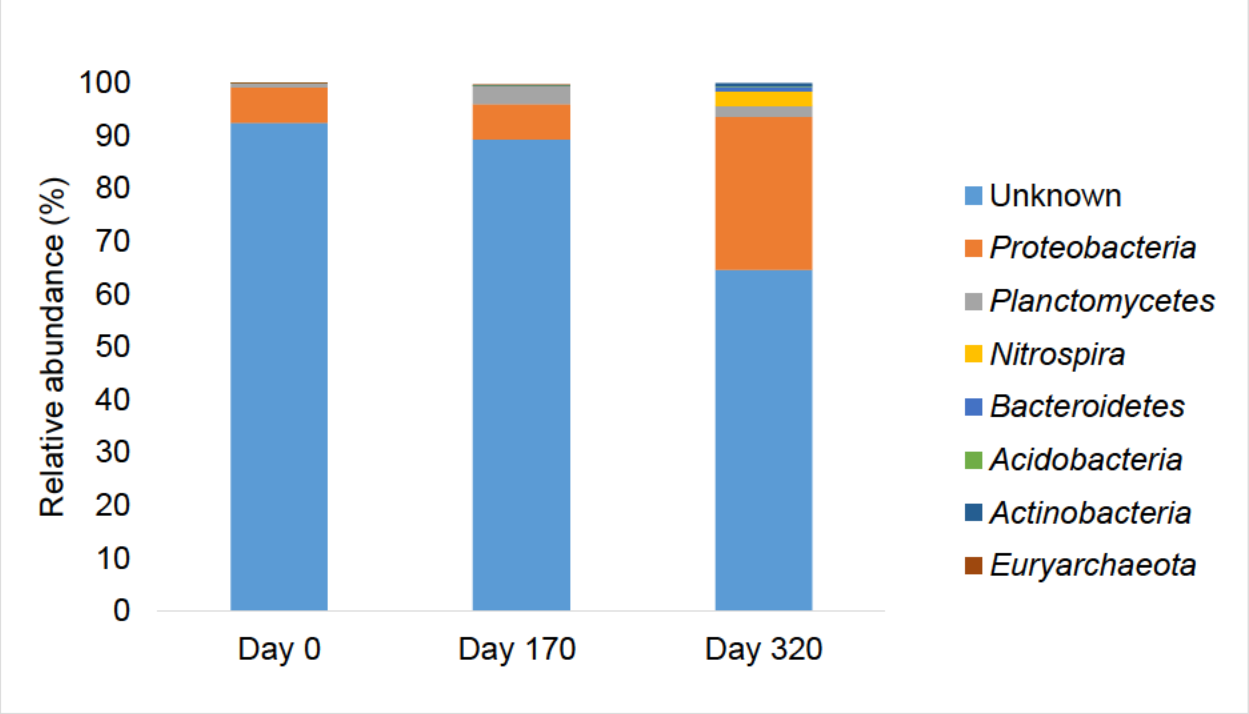

Figure 4.15: Relative abundance (%) of the microbial community structure at phylum level from day 0, day 170 and day 320 present in Reactor 1 (1:1.32 ratio).

In Reactor 2 containing ammonium: nitrite ratio of 2:1, the most dominant phyla apart from unknown sequences included *Proteobacteria* (21.63 %) followed by *Planctomycetes* (21.32 %), *Actinobacteria* (1.67 %), *Acidobacteria* (0.37 %), *Bacteroidetes* (0.20 %), *Nitrospira* (0.05 %) and *Euryarchaeota* (0.05 %) on the 170th days of the study. On 320th day of the study period, the proportion of the phylum increased in *Proteobacteria* (24.15 %), *Nitrospira* (7.38 %), *Actinobacteria* (3.04 %), *Acidobacteria* (2.05 %) and *Bacteroidetes* (0.48 %), with a significant decrease in proportion on *Planctomycetes* (0.36 %) (Figure 4.16).

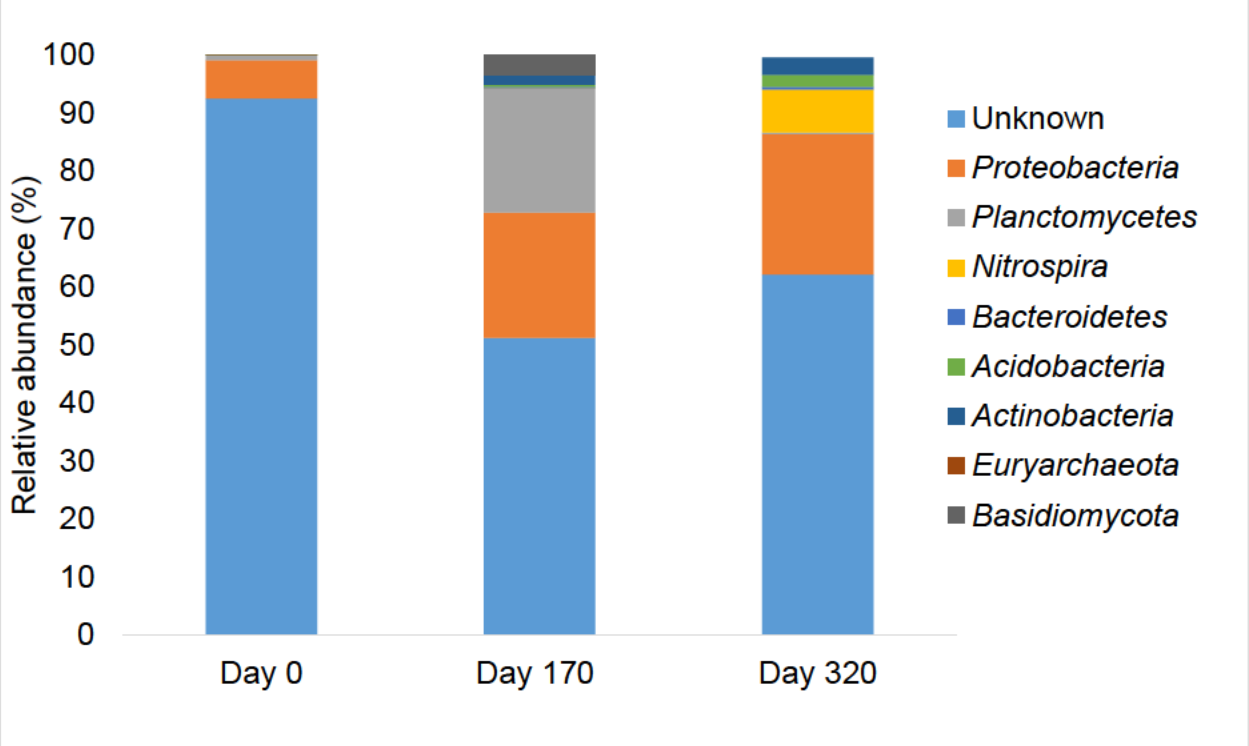

Figure 4.16: Relative abundance (%) of the microbial community structure at phylum level from day 0, say 170 and day 320 present in Reactor 2 (2:1 ratio).

Reactor 3 also showed variation in the phyla proportion by the 170th day. Apart from the unknown, only 3 phyla were detected including *Proteobacteria* (23.72 %), *Planctomycetes* (22.26 %) and *Firmicutes* (0.36 %). However, after 320 days there was an increase in *Proteobacteria* (23.86 %), *Actinobacteria* (4.09 %), *Euryarchaeota* (2.51 %), *Bacteroidetes* (1.12 %), *Nitrospira* (1.09 %) and *Acidobacteria* (0.92 %), whereas a decrease in *Planctomycetes* (4.91 %) was observed (Figure 4.17).

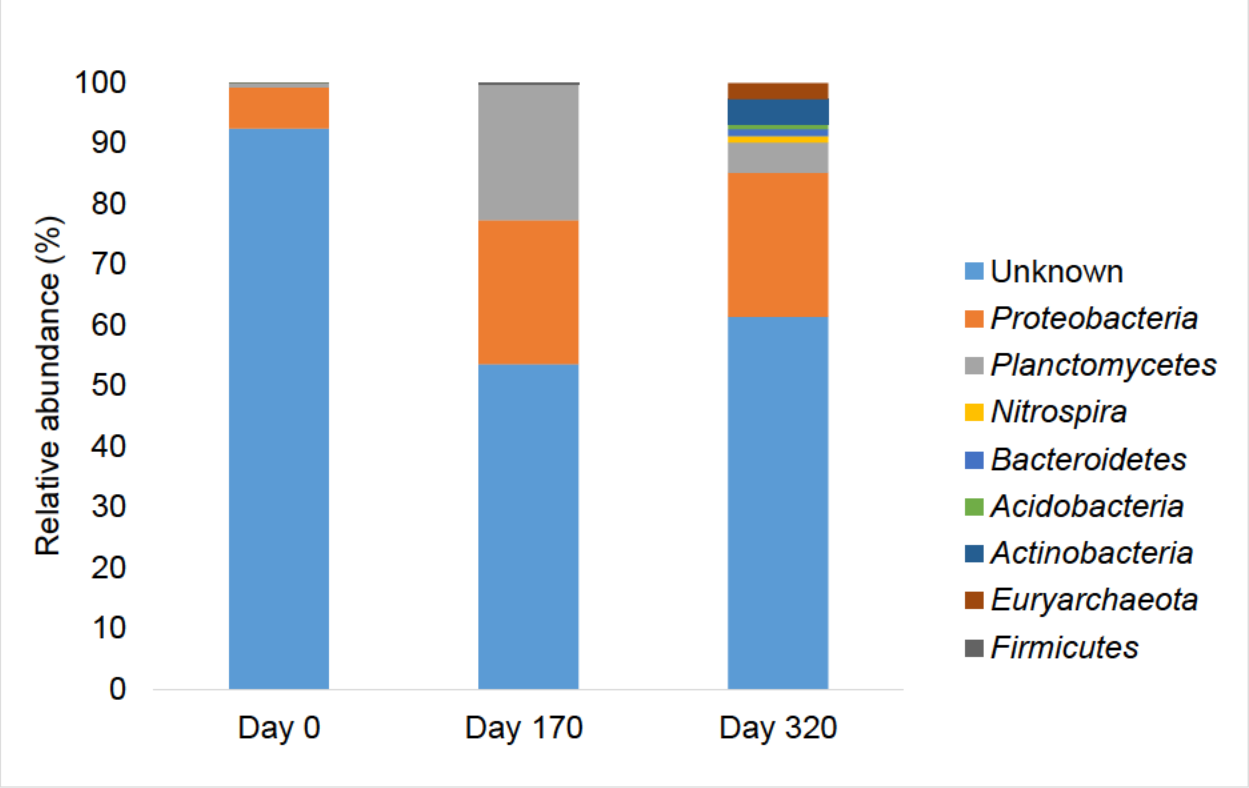

Figure 4.17: Relative abundance (%) of the microbial community structure at phylum level from day 0, say 170 and day 320 present in Reactor 3 (1:2 ratio).

The class level showed a similar high proportion of the unknown class population throughout the operational period. Apart from the unknown, the proportion of the class level in the initial seed inoculum was found to be *Betaproteobacteria* (5.38 %) as a dominant class, followed *Planctomycetacia* (0.76 %), *Gammaproteobacteria* (0.49 %), *Alphaproteobacteria* (0.47 %), *Acidobacteria* (0.05 %) and *Nitrospira* (0.01 %). In Reactor 1, on the 170th day, the proportion of *Alphaproteobacteria* (4.70 %) increased followed by *Planctomycetacia* (3.30 %), *Acidobacteria* (0.16 %) and *Nitrospira* (0.02 %), with a decrease in the proportion of *Betaproteobacteria* (2.10 %) and *Gammaproteobacteria* (0.09 %). On the 320th day of operation, the proportion of *Gammaproteobacteria* (16.34 %) increased significantly, followed by *Betaproteobacteria* (6.84 %), *Alphaproteobacteria* (5.77 %), *Nitrospira* (2.84 %), while a decrease in *Planctomycetacia* (2.10 %) and *Acidobacteria* (0.02 %) was observed (Figure 4.18).

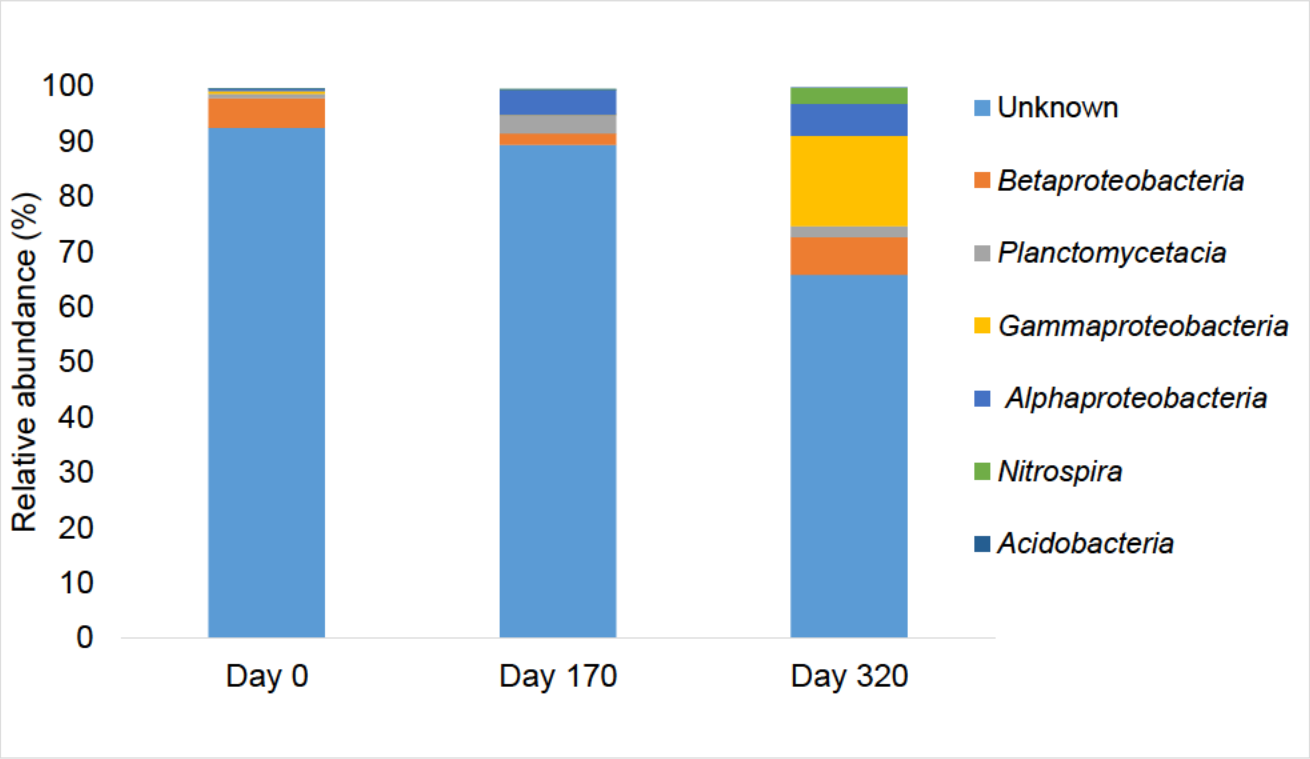

Figure 4.18: Relative abundance (%) of the microbial community structure at class level from day 0, day 170 and day 320 present in Reactor 1 (1:1.32 ratio).

In Reactor 2 containing ammonium: nitrite ratio of 2:1, an increase was observed in the proportion of *Planctomycetia* 21.32 % followed by *Betapreteobacteria* 19.89 %, *Alphaproteobacteria;* 1.59 %, *Gammaproteobacteria* 0.16 %, *Nitrospira* 0.05 %, *Actinobacteria* 1.67 %, *Acidobacteria* 0.37 % and *Bacteroidetes* 0.05 % on the 170th day of the study (Figure 4.14). On the 320th day of reactor operation the proportion of *Gammaproteobacteria* increased significantly to 11.99 %, followed by *Alphaproteobacteria* 8.66 %, *Nitrospira* 7.38 %, *Actinobacteria* 3.04 %, *Acidobacteria* 2.05 % and *Chloroflexi* 0.07 %. Whereas a decrease in *Betapreteobacteria* 3.43 %, *Planctomycetia* 0.36 % and *Bacteroidetes* 0.02 % was observed (Figure 4.19).

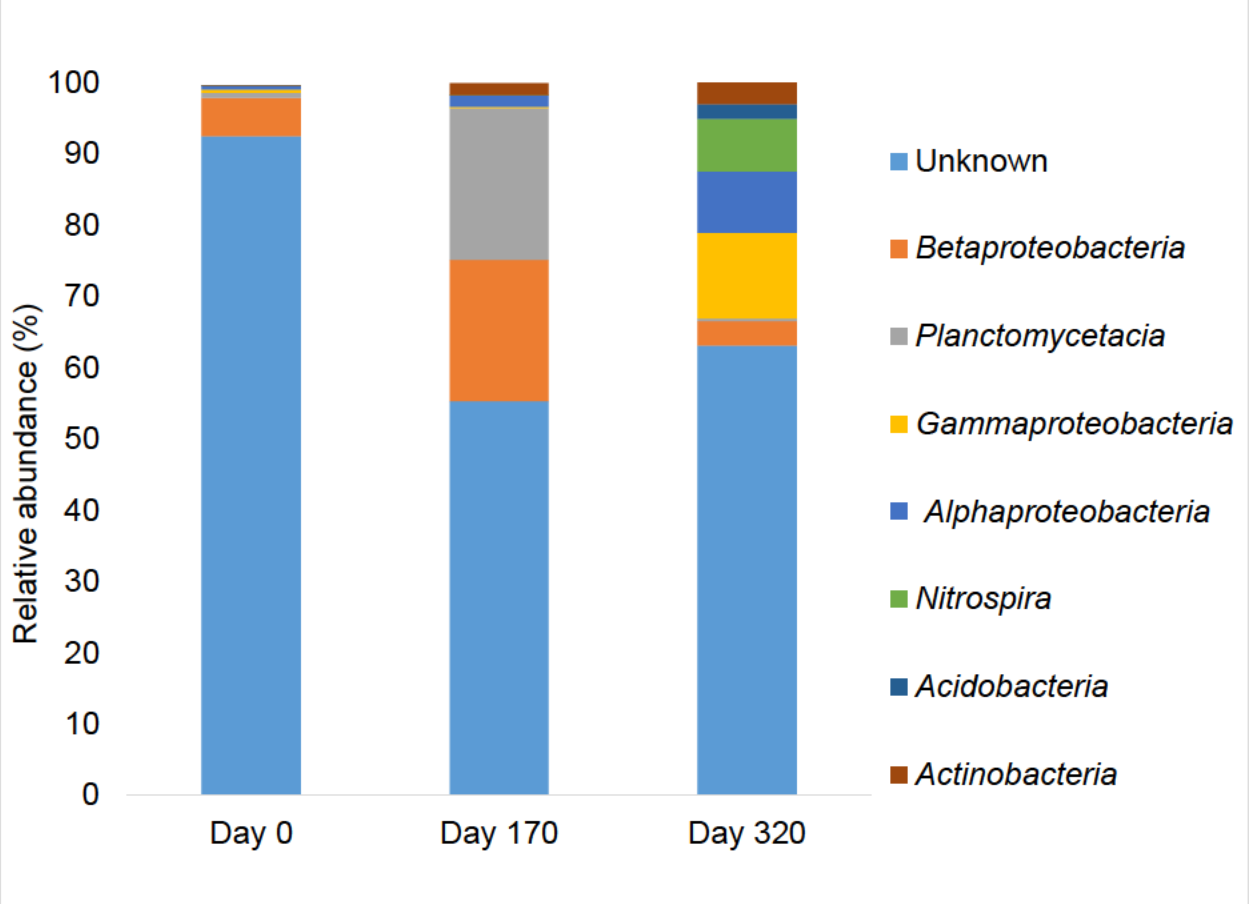

Figure 4.19: Relative abundance (%) of the microbial community structure at class level from day 0, day 170 and day 320 present in Reactor 2 (2:1 ratio).

In Reactor 3 containing ammonium: nitrite ratio of 1:2, an increase was observed in the proportion of *Planctomycetia* 22.26 %, *Betapreteobacteria* 21.17 %, *Alphaproteobacteria* 1.09 % and *Gammaproteobacteria* 1.46 % on the 170th days. On the 320th day of reactor operation, an increase in the proportion of *Alphaproteobacteria* 15.91 % followed by *Actinobacteria* 4.09 %, *Nitrospira* 1.09 %, *Acidobacteria* 0.92 %, *Bacteroidetes* 0.79 %, whereas a decrease in *Planctomycetia* 4.91 % and *Betapreteobacteria* 4.49 % was observed (Figure 4.20). Overall, these results indicated that only *Planctomycetes* were not favoured by different substrate ratios used in all the reactors. Furthemore, other microbial groups such as

Proteobacteria and *Nitrospira* might have out competed the anammox bacteria, resulting in deterioration of anammox bacteria from lack of substrate in the reactor.

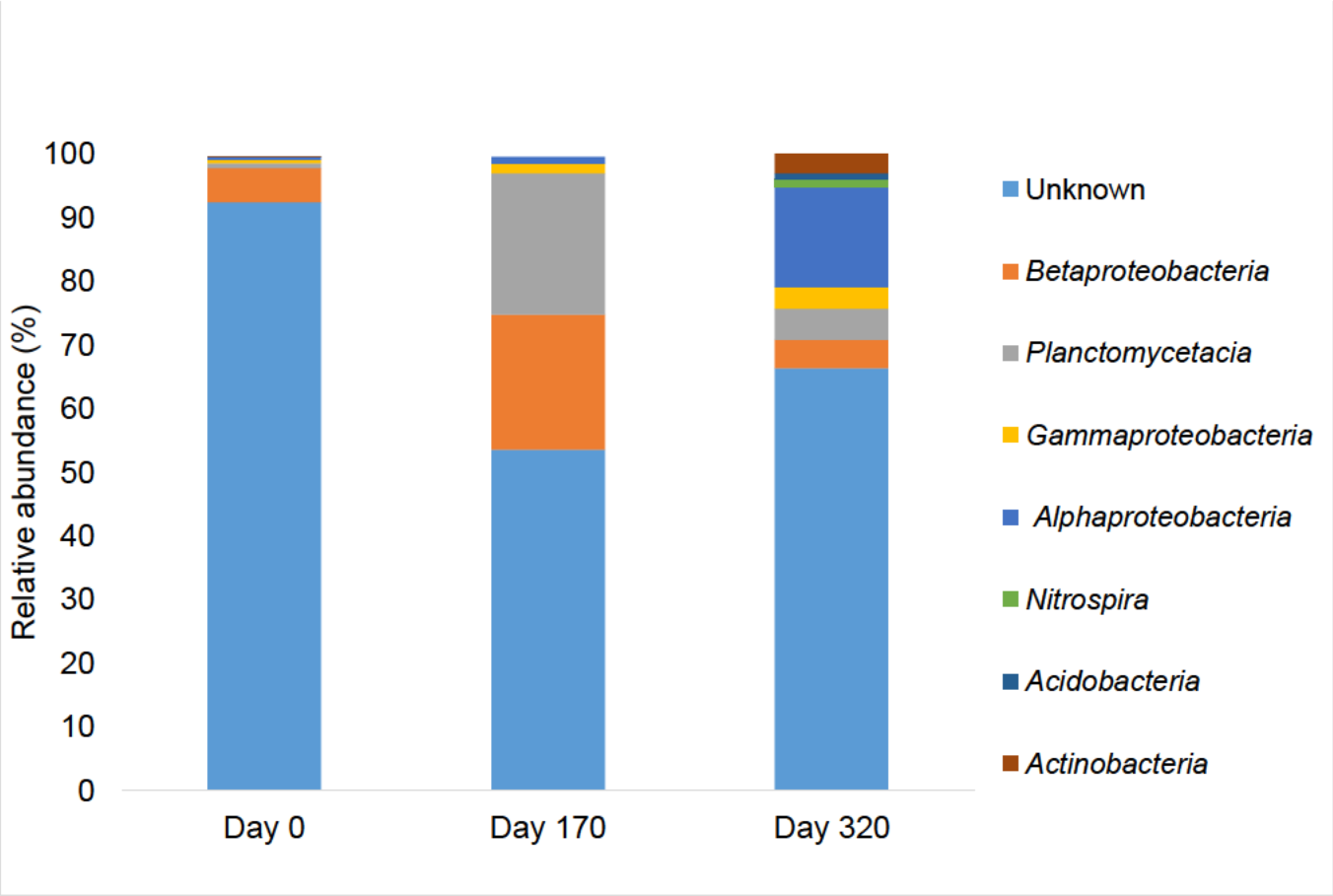

Figure 4.20: Relative abundance (%) of the microbial community structure at class level from day 0, day 170 and day 320 present in Reactor 3 (1:2 ratio).

The sequence reads were classified taxonomically which showed high diversity with 15 orders across the three reactors. The most dominant and common orders among the reactors were *Nitrosomonadales*, *Burkholderiales*, *Planctomycetales*, *Pseudomonadales*, *Rhodobacterales*, *Alteromonadales*, *Nitrospirales* and *Actinomycetales*. The analysis based on diversity and relative abundance of different species revealed a strong difference among the bacterial communities within each reactor. At the species level, more variations were identified in each

reactor throughout the study period. Amongst the common species that were identified across all the reactors were uncultured *Planctomycetes*, *Brevundimonas* species, *Ralstonia pickettii*, *Alicycliphilus denitrificans* and uncultured *Nitrospira* with relatively low abundances (Figure 4.21, 4.22 and 4.23). The overall patterns in the microbial community diversity of the three reactors were illustrated with heatmap using Heatplus and Vegan packages in R (Figure 4.24). Differences among sites were characterized using Bray-Curtis distances and differences among taxa were characterized using Euclidean distances.

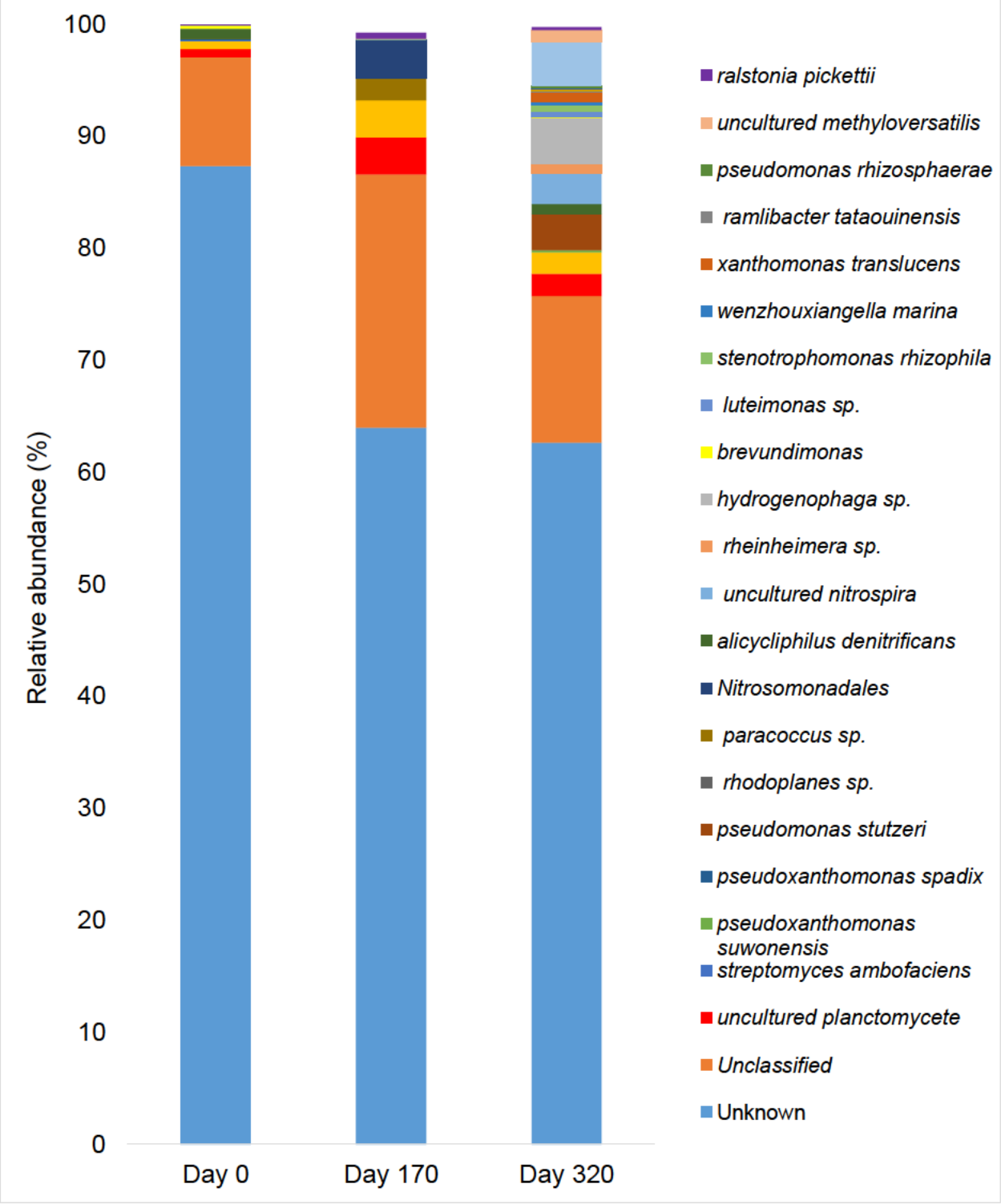

Figure 4.21: Relative abundance (%) of the microbial community structure species level from day 0, day 170 and day 320 present in Reactor 1 (1:1.32 ratio).

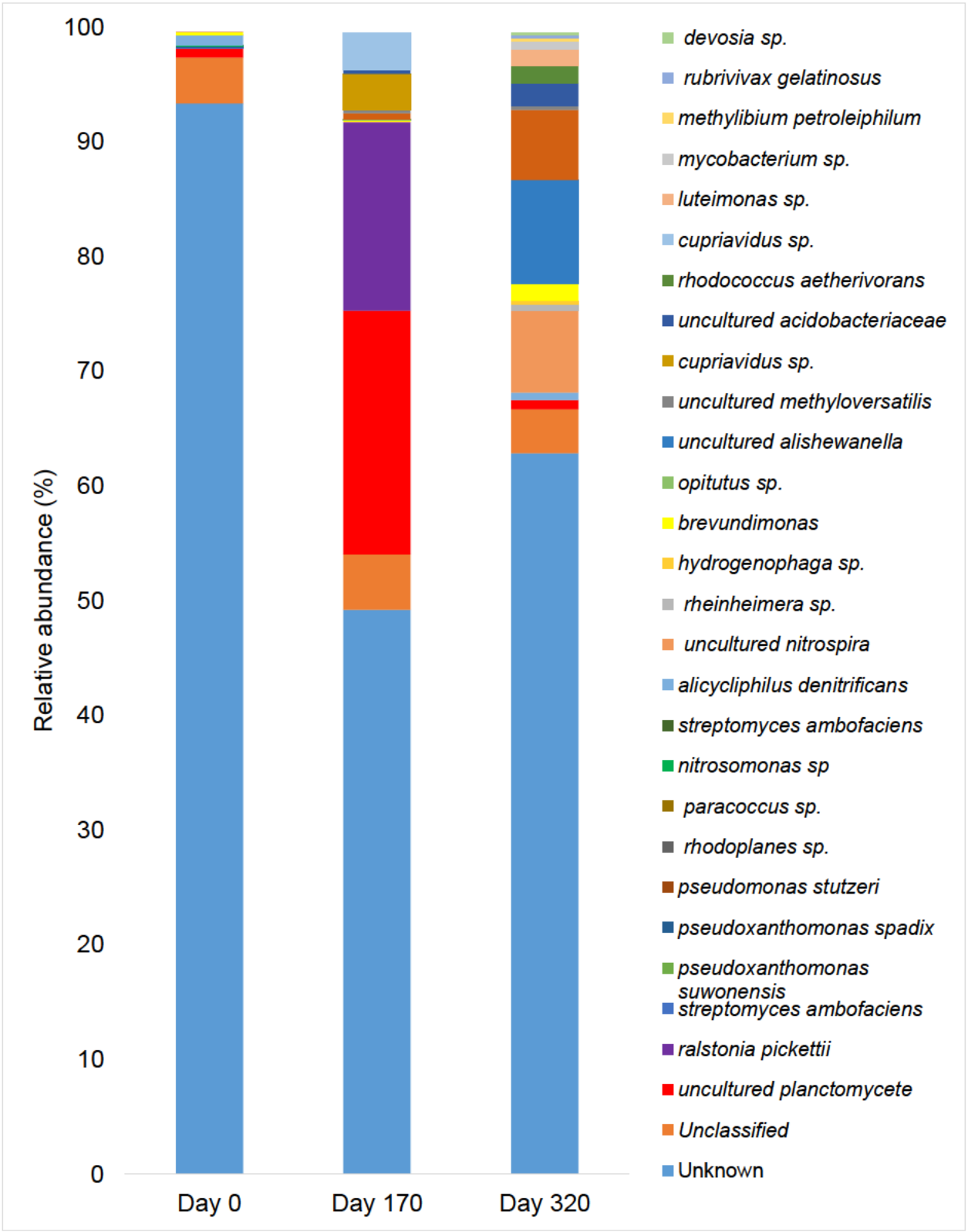

Figure 4.22: Relative abundance (%) of the microbial community structure species level from day 0, day 170 and day 320 present in Reactor 2 (2:1 ratio).

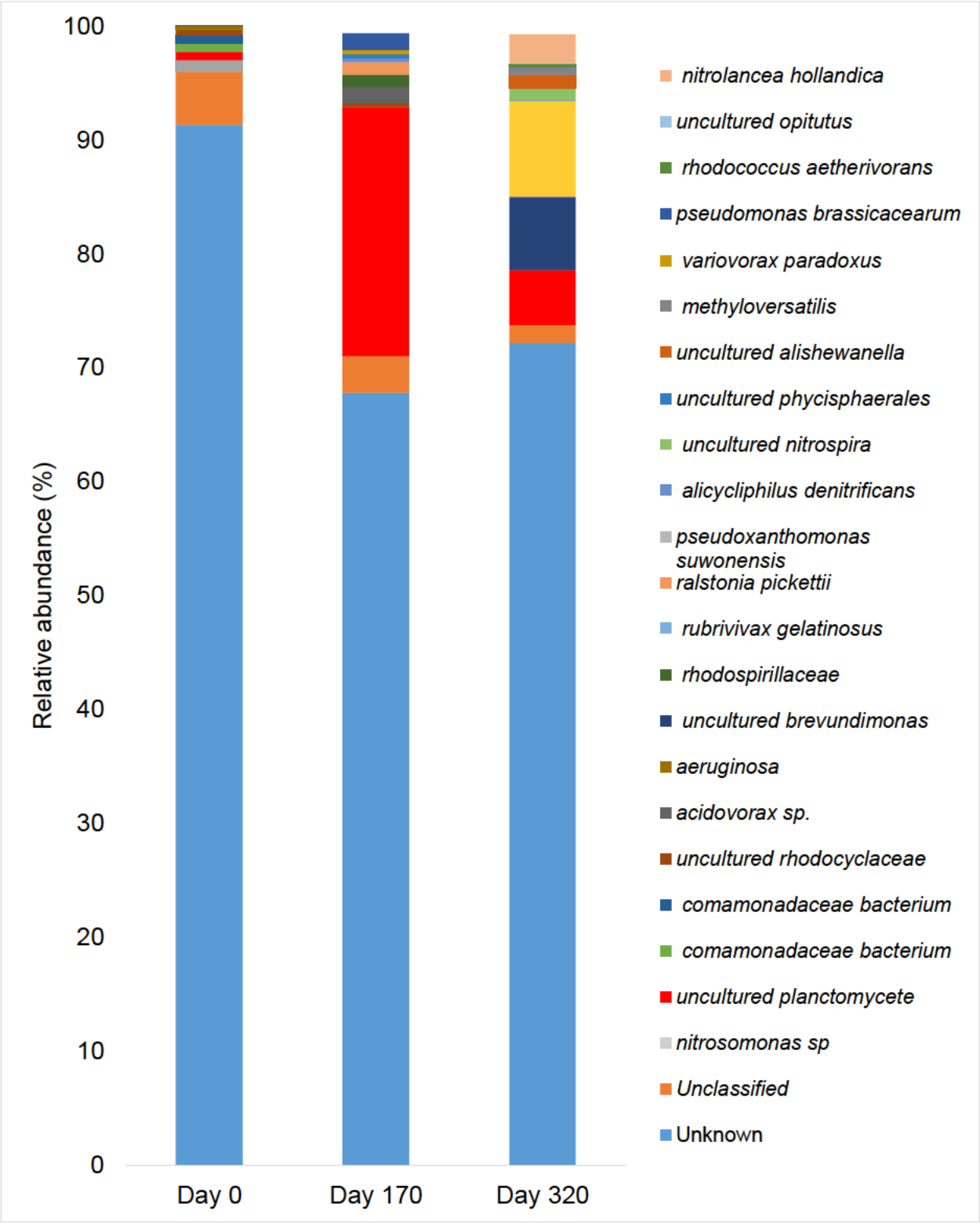

Figure 4.23: Relative abundance (%) of the microbial community structure species level from day 0, day 170 and day 320 present in Reactor 3 (1:2 ratio).

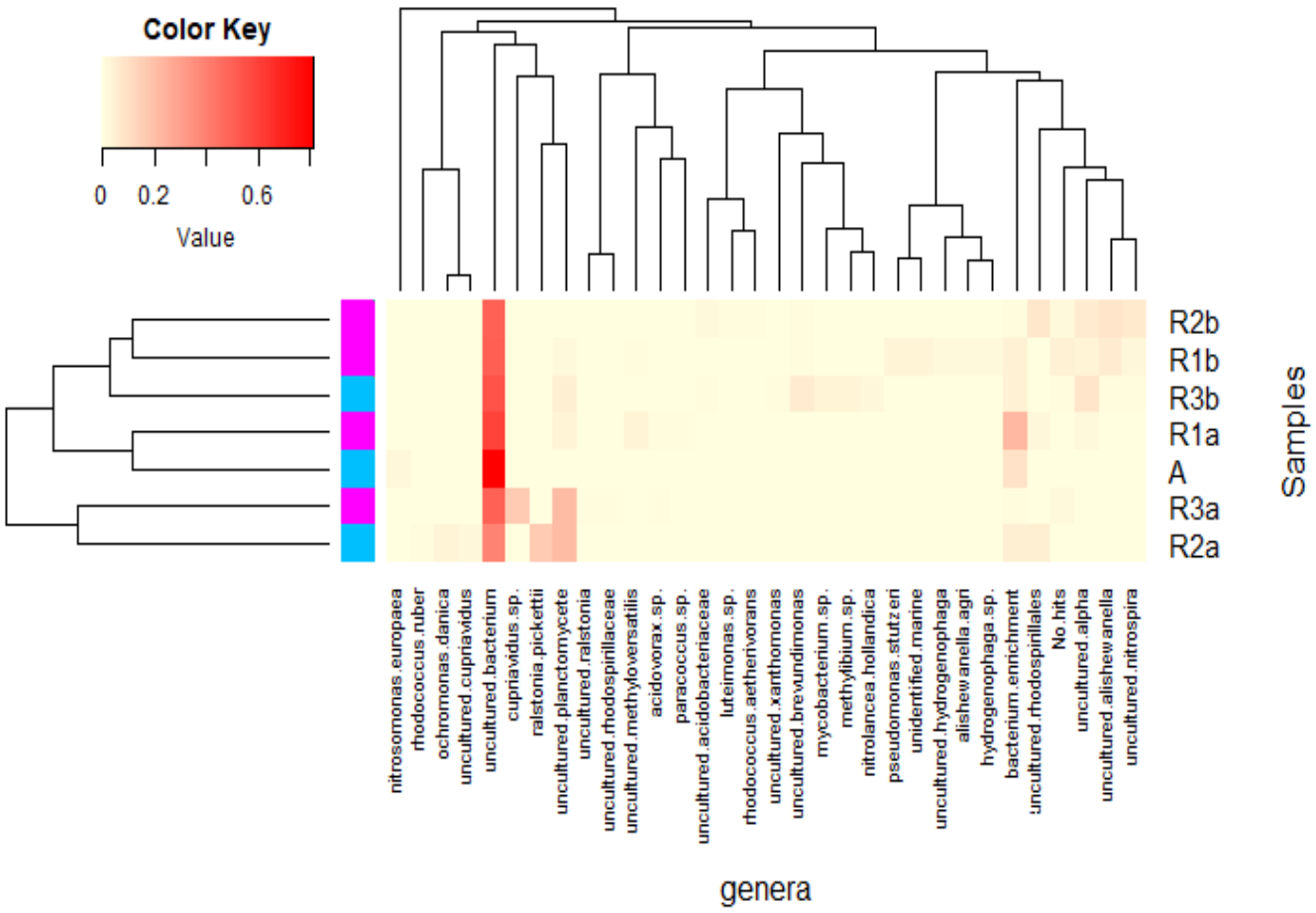

Figure 4.24: Heatmap of the most abundant bacterial genera in the reactors, cluster analysis was performed on the 32 most represented genera (abundance 1%) based on Bray-Curtis similarity index. A indicates the initial inoculum, R1a, R2a, R3a indicates Reactor 1, 2 and 3 respectively on the 170th day and R1b, R2b, R3b indicates Reactor 1, 2 and 3 respectively on 320th day.

4.4 Discussion

4.4.1 Microbial population dynamics within the reactors

The effect of different key substrate (NH_4^+-N: NO_2^--N) ratios on the microbial community structure (initial biomass containing anammox bacteria, AOB and NOB) within each rector was evaluated to understand complex microbial interaction that occurs inside the ASBRs. The 16S rRNA gene is commonly used to quantify microbial community structure and diversity using genomic DNA. Many studies use the abundance of 16S gene sequences to measure the relative abundance of the organisms containing those sequences. However, the gene copy numbers can vary greatly from each microorganism since many microorganism contain multiple copies of the gene in their genome. Thus, the disparity in the relative abundance of 16S gene sequences within an environmental sample can be attributed not only to variation in the relative abundance of different organisms but also to variation in genomic 16S copy numbers among those microorganisms (Kembel *et al*., 2012). The 16S rRNA and functional genes numbers in anammox bacteria are currently unknown (Tsushima *et al*., 2007). Furthermore, the gene copy numbers vary greatly in different anammox species. The variation in functional gene copy numbers (*hzo, hzs,* and *nirS*) observed in this study can be attributed to the variation in the gene copy numbers among the anammox bacteria. Wang *et al*. (2016), concluded that an anammox bacterium cell contains more than one copy of the functional genes and the 16S rRNA gene.

A positive correlation between the anammox functional genes and a steady effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations suggesting positive anammox activity was observed on the 170th day. However, a substantial reduction of these genes was observed on the 320th day could suggest that the anammox activity was reduced. Connan *et al*. (2016) observed substantial increase in anammox functional gene (*hzo*) in a reactors operated for approximately 120 days.

The above results show that even though anammox bacteria are present in all the reactors, the anammox population was not sufficient enough to compete with nitrifying and denitrifying bacteria. This concurs with the N performance since NO_2^-

-N (consumed)/ NH_4^+-N (removed), NO_3^--N (produced)/ NH_4^+-N (removed) ratios were not within the reported anammox stoichiometry. Other studies have reported similar results during anammox enrichment whereby the observed stoichiometric ratios were not in agreement with reported anammox stoichiometric ratio (Anjali and Sabumon, 2014, Laureni *et al*., 2015).

Additionally, FNA concentration above inhibitory concentration suggests that anammox activity was reduced by FNA concentration above the inhibitory level. Free nitrous acid concentration of 70 µg HNO_2^--N/L and a deterioration in anammox activity was observed during anammox enrichment in anammox gel-immobilized column reactor (Zhang *et al*., 2017). Also, Zhang *et al*. (2016) observe a rapid increase in FNA concentration reaching 70 µg HNO_2^--N/L accompanied by loss of anammox activity on 347th day of reactor operation.

Despite the fact that the reactors were anaerobic (DO concentration below 0.5 mg/L), AOB and NOB were present, suggesting that they can exist in both higher and lower DO concentrations. Lam *et al*. (2009) demonstrated that anammox bacteria can co-exist with AOB and simultaneously co-contributes to N removal. The AOB growth rate is approximately three times faster at low DO levels (1 mg/L) (Yan *et al*., 2010). At low DO concentration AOB could provide NO_2^--N to anammox bacteria through the oxidation of NH_4^+-N in the marine environment and wastewater treatment (Yan *et al*., 2010).

Furthermore, various inhibitory concentrations of DO and FNA observed in the reactors could have led to inhibited anammox activity. Zhang *et al*. (2016) also observed that a combination of long term (900 days) anammox enrichment, presence of other microbial groups, and presence of various inhibitors (DO, FA and FNA) resulted in unstable anammox performance. Overall, a decrease anammox functional genes on 320th day indicated that anammox bacteria were not favoured by long term enrichment in all three reactors. For optimal activity, anammox bacteria require stable and specific conditions (Tomaszewski *et al*., 2017a).

4.4.2 Illumina high-throughput sequencing

The microbial community structure dynamics of the three reactors in response to different substrate (NH_4^+-N: NO_2^--N) ratios were revealed by Illumina-based metabarcoding sequencing of 16S rRNA gene. Alpha diversity reflects the abundance and diversity of a microbial community (Wen *et al*., 2016). Chao1 and ACE indices used to estimate the richness of microbial structure show that the highest species richness was observed in Reactor 1, Reactor 3 and Reactor 2 on the 320th day and Reactor 2 on 170th day. The different ammonium: nitrite ratios in the reactors had a very strong effect on the microbial composition, this could have promoted the enrichment of other bacterial species on the 320th day of reactor operation compared to 170th day. The Shannon-Wiener index also indicated that the enrichment on the 320th day harboured the highest diversity of bacterial species in all the reactors and enrichment on 170th day harboured the lowest diversity in all the reactors. indices

The co-existence of bacteria belonging to the phylum *Proteobacteria* and *Planctomycetes*, observed in this study suggest that both groups were actively involved in N removal in all the reactors. Gonzalez-Martinez *et al*. (2016) reported similar observations, where *Proteobacteria* and *Planctomycetes* were found to be most abundant in a CANON reactor. Members of other phyla *Bacteroidetes*, *Actinobacteria* and *Euryarchaeota* were also found, although at varying abundances in the reactors. These phyla are widely reported in anaerobic reactors treating wastewater and are known to co-exist with *Planctomycetes* (Rosenkranz *et al*., 2013; Li *et al*., 2016). Other studies have also observed the presence of these phyla in anammox reactors although the reactors were operated under strict anammox conditions suggesting their importance for anammox enrichment and process stability (Costa *et al*., 2014). Members of phylum *Acidobacteria* have been reported as versatile heterotrophs capable of degrading complex carbohydrates with the additional ability to reduce nitrate (Ginige *et al*., 2005).

A microbial shift was observed with *Nitrospira* (NOB belong to this phylum) dominating in all the reactors on the 320th day, which correlates with an increase in NO_3^--N concentration within all the reactors. Thus, a successful nitrification process

was obtained on the 320th day due to the presence and activity of *Proteobacteria* which harbours two nitrifying bacterial groups. Joss *et al*. (2011) identified the activity and abundance of AOB and NOB (NOB; competing with anammox for NO_2^-) as a key to anammox reactor instability. Furthermore, deterioration in anammox population might be as a result of the long operational period of 320 days. Hoekstra *et al*. (2018) reported that long sludge retention time resulted in deterioration and increase of non-active and non-anammox cells in the reactor, thus decreasing the biomass-specific activity.

At class level, the different substrate ratios showed a significant impact on *Planctomycetia*, as indicated by an increase in their population at 170th day. However, prolonged exposure to varying substrate ratio drastically lowered the abundance of *Planctomycetia* in all three reactors. Further confirming instability and deterioration of the anammox process, which could be caused by the proliferation of AOB and NOB within the reactors.

Within the *Proteobacteria* phylum, the abundance of *Betaproteobacteria* was high for all the different ammonium: nitrite ratios. *Betaproteobacteria* (which harbours AOB) are responsible for the oxidation of NH_4^+ to NO_2^- (first step of nitrification process) in WWTPs (Zeng *et al*., 2014). A similar observation was made regarding other members of class *Proteobacteria* including *Alphaproteobacteria*. *Alphaproteobacteria* harbours known genera such as *Nitrobacter* and *Nitrococcus* (affiliated with NOBs) which are responsible for the oxidation of NO_2^- to NO_3^-; the second step of nitrification (Bae *et al*., 2012). On the 320th day, an overall increase in *Gammaproteobacteria* with known genera affiliated with AOB (*Nitrosococcus*) and NOB (*Nitrococcus*) was observed. Different authors have also reported these genera as most dominant in WWTPs or natural ecosystems (Schmidt *et al*., 2003, Awolusi *et al*., 2015).

Overall, the qPCR results indicated a similar trend when compared to Illumina sequencing data in Reactor 1. The difference between the qPCR and Illumina sequencing results could be caused by the limitations that exist in these assays. It has been assumed that there is a constant relationship exists between the cell number and the 16S rRNA gene copy number. However, Hao *et al*. (2009) reported

that 16S rRNA gene copies cannot be directly converted into cell counts, because anammox bacteria might have different 16S rRNA operon copy numbers from species to species, and the 16S rRNA operon of anammox bacteria are not presently known. Furthermore, qPCR cannot differentiate between viable and non-viable cells, since the genomic DNA can persist even after cell death (Tsushima *et al*., 2007). Therefore, qPCR technique can also overestimate active cell numbers within the reactor.

4.5 Conclusion

The microbial community structure within a biological wastewater treatment system is crucial to the operation of the system as a whole. Suitable microbial assemblage is required for successful and effective N removal in wastewater treatment.

The qPCR result demonstrated that the microbial community was predominated by AOB, NOB and anammox bacteria in all the reactors. Reactor 3 (1:2) showed a high abundance of anammox bacteria followed by Reactor 2 (2:1) and Reactor 1 (1:1.32) respectively on the 170th day, thereafter the anammox bacteria decreased throughout the study in all the reactors. This concurs with the NO_2^--N (consumed)/NH_4^+-N (removed) and NO_3^--N (produced)/NH_4^+-N (removed) ratios that were within the reported anammox stoichiometric ratio in Reactor 3 (containing high NO_2^--N). Whereas, the NO_2^--N (consumed)/NH_4^+-N (removed) and NO_3^--N (produced)/NH_4^+-N (removed) ratios in Reactor 1 (containing balanced NH_4^+-N: NO_2^--N) and Reactor 2 (containing high NH_4^+-N) were either higher or lower than the reported anammox stoichiometric ratio. Additionally, high FNA concentrations (above the reported inhibitory concentration) did not affect anammox bacteria, this was indicated by the increase in anammox functional gene on the 170th day of operation. On the contrary FNA inhibition was observed on long term operation of the reactor (320th day) resulting in anammox functional genes being lower in Reactor 3, Reactor 1 and Reactor 2 respectively.

The AOB population was favoured by fluctuating DO concentrations caused by oxygen leakage, resulting in a proliferation of AOB population observed in Reactor 1 (1:1.32) followed by to Reactor 3 (1:2) and Reactor 2 (2:1) on 170th and 320th day. The NOB population was high in Reactor 3 (1:2) followed by Reactor 1 (1:1.32) and Reactor 2 (2:1) respectively throughout the study period. This was not expected since FNA concentration were within the reported inhibitory concentration for NOB. The increase in NOB population on the 320th day revealed that NOB recovered from FNA inhibition after 170 days rather than being eliminated.

High-throughput sequencing indicated an overall impact of the different substrate ratios on the selection of microbial community structure across the reactors.

Proteobacteria phylum was the most dominant in all the reactors. A shift in population abundance was observed during the study period, with increased *Proteobacteria* population abundance in Reactor 1 (1:1.32) on the 320th day of operation compared to Reactor 2 (2:1) and Reactor 3 (1:2) respectively. Reactor 2 showed a high abundance of *Nitrospira* followed by Reactor 1 and Reactor 3 respectively on the 320th day of operation. An increase in phylum *Planctomycetes* (affiliated with anammox bacteria) population was observed up to 170 days of operation, thereafter a drastic decrease in their abundance was observed in all the reactors further confirming the qPCR results.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The effect of key substrate ratios on N removal performance and microbial structure of ASBRs was evaluated. Three ASBRs containing different ammonium: nitrite ratios namely; 1:1.32 (Reactor 1), 2:1 (Reactor 2) and 1:2 (Reactor 3) were operated for a period of 320 days. The result of this work revealed variations in N (NH_4^+-N and NO_2^--N removal and NO_3^--N production) performance in all the reactors throughout the study period. The highest NRE was achieved in Reactor 3 (1:2), Reactor 1 (1:1.32) and Reactor 2 (2:1) respectively. Additionally, Reactor 3 achieved high N_2 gas production followed by Reactor 2 and Reactor 1.

High NO_2^--N concentration in Reactor 3 had a positive influence on anammox activity, since the NO_2^--N (consumed)/NH_4^+-N (removed) and NO_3^--N (produced)/NH_4^+-N (removed) observed were closer to the reported anammox stoichiometry. This resulted in high abundance of *hzo* gene and *Planctomycetes* on the 170th day, a decrease (*hzo* gene and *Planctomycetes*) observed on 320th day can be attributed to the deterioration of anammox bacteria within the reactor. Additionally, high FNA above inhibitory concentration did not inhibit anammox bacteria on the 170th day in this reactor. However, on the 320th day high FNA concentration inhibited the anammox bacteria, this was demonstrated by a drastic decline in the *Planctomycetes* population.

As for Reactor 1 and Reactor 2, the ratios of NO_2^--N (consumed)/NH_4^+-N (removed) and NO_3^--N (produced)/NH_4^+-N (removed) observed were not within the reported anammox stoichiometric values of 1.32 and 0.26. The observed ratios correlated with the high-throughput sequencing result, which indicated a microbial shift from *Planctomycetes* (affiliated with anammox bacteria) enrichment towards *Proteobacteria* and *Nitrospira* enrichment. This suggests that the N removal in the studied reactors were as a result of the combined microbial (anammox bacteria and other heterotrophic bacteria) activity. In Reactor 1 and 2, the FNA concentrations were below the reported inhibitory concentration for anammox bacteria on the 170th day of reactor operation. On the 320th day, anammox bacteria were inhibited by

FNA concentration above the reported inhibitory concentration evident by a sharp decrease in the anammox population.

Furthermore, anammox bacteria and AOB were not inhibited by since FA concentrations were below the reported inhibitory concentration in all three reactors. On the contrary, NOB was not inhibited by the FA concentrations although the FA concentrations were above the reported inhibitory concentration for NOB. However, the FNA concentration did not affect AOB since the FNA concentrations were below the inhibitory concentration and the proliferation of AOB was observed in all three reactors throughout the study. Additionally, *Nitrospira* (NOB) population was inhibited by FA and FNA concentrations on the 170th day. However, *Nitrospira* population was able to recover from this inhibition since *Nitrospira* population increased on day 320 of reactor operation.

Overall, different substrate ratios (NH_4^+-N and NO_2^--N) increased the anammox bacteria in all the reactors on day 170 with Reactor 3 showing the highest anammox population followed by Reactor 2 and 1 respectively. However, on the 320th day, the abundance of anammox bacteria decreased in all the reactors. The proliferation of AOB alongside NOB on day 320 was observed in all the reactors, with Reactor 3 (1:2) showing a high abundance of AOB and NOB followed by Reactor 1 (1:1.32) and Reactor 2 (2:1). It is worth noting that Reactor 1 (1:1.32), Reactor 2 (2:1) and Reactor 3 (1:2) favoured a synchronous enrichment of *Proteobacteria* and *Planctomycetes* on the 170th day of operation, thus supporting microbial populations that compete for similar substrates (NH_4^+-N and NO_2^--N). However, long term operation, intermittent spike of DO and the presence of other toxic nitrogenous compounds (FA and FNA) inevitably inhibited the *Planctomycetes* population, resulting in the proliferation of *Nitrospira* population on day 320. On the 320th day, there was an overall decrease in *Planctomycetes* and an increase in *Proteobacteria* and *Nitrospira* in all the reactors as a result of long term operation of the reactors and formation of inhibitory compounds.

5.2 Recommendations

A long start-up period is a major challenge in the application of anammox process. Therefore future studies should focus on using inoculum containing dominant anammox bacteria, to enable fast start-up of the reactor. Furthermore, optimum operational control (DO, pH, FA, FNA and substrate) is crucial in anammox activity and stability. Future studies could also incorporate an online operational control system and continues feeding. This strategy could be helpful in minimizing FNA inhibition and suitable for anammox bacterial growth.

The presence of nitrifying, denitrifying, anammox bacteria and other microbial group in anammox reactors remains a challenge. More details should focus on the optimization of microbial community structure under influent fluctuation and different operational conditions using real wastewater.

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APPENDICES

APPENDIX A. Supplementary data for CHAPTER 3

1. Preparation of samples and chemical analysis with HACH kit

Nitrogen, Ammonia:

Salicylate method: 0.4 – 50 mg/L NH_3-N

i. Select the test. Insect the adapter or light or light shield if required.
ii. Prepared sample: Add 0.1 mL of sample to one AmVer™ Diluent Reagent Test 'N Tube for High Range Ammonia Nitrogen
iii. Blank Preparation: Add 0.1 mL of ammonia-free water to one AmVer™ Diluent Reagent Test 'N Tube for High Range Ammonia Nitrogen.
iv. Add the contents of Ammonia Salicylate Reagent Powder Pillow for 5 mL sample to each vial.
v. Add the contents of one Ammonia Cyanurate Reagent Powder Pillow to each vial.
vi. Cap the vials tightly and shake thoroughly to dissolve the powder.
vii. Start the instrument timer. A 20 minute reaction period will begin.
viii. Zero the instrument. The display will show: 0.0 mg/L NH_3-N.
ix. Read the results in NH_3-N.

Nitrate

Cadmium Reduction Method: 0.3 - 30.0 mg/L NO_3-N

i. Select the test. Insect the adapter or light or light shield if required.
ii. Fill the sample cell with 10 mL of sample.
iii. Prepared sample: Add the contents of one NitraVer 5 Nitrate reagent Powder Pillow. Insert a stopper into the cell.
iv. Start the instrument timer. A one minute reaction period will begin. Shake the cell vigorously until the timer expires.
v. When timer expires, start the timer again. A five-minute reaction will begin. An amber colour will develop if nitrate is present.

vi. Blank preparation: When the timer expires, fill a second sample cell with 10 mL of sample.

vii. Wipe and insert the blank into the cell holder.

viii. Zero the instrument. The display will show: 0.0 mg/L NO_3^--N.

ix. Within the two minutes the timer expires, wipe and insert the prepared sample into the cell holder.

x. Read the results in mg/L NO_3^--N.

Nitrite:

Ferrous sulfate method for powder pillows: 2 – 250 mg/L NO_2^-

i. Select the test. Insect the adapter or light or light shield if required.

ii. Fill the sample cell with 10 mL of sample.

iii. Prepared sample: Add the contents of one NitriVer® 2 Nitrite Reagent Powder Pillow.

iv. Insert the stopper and shake.

v. Start the instrument timer. A ten minute reaction period will begin. To prevent low results, leave the sample on a flat surface and do not disturb it during the reaction period.

vi. Blank preparation: Fill a second sample cell with 10 mL sample.

vii. Wipe and insert the blank into the cell holder.

viii. Zero the instrument. The display will show: 0.0 mg/L NO_2^-.

ix. After the timer expires, cap and gently invert the prepared sample twice. Avoid excessive mixing or low results will occur.

x. Wipe the prepared sample and insert it into the cell older

xi. Read results in mg/L NO_2-.

APPENDIX B. Supplementary data for CHAPTER 4

1. Fluorescence *in-situ* hybridization (FISH)

Reagents Required

Paraformaldehyde powder

NaOH (1 N)

HCl (Dilute)

Deionized H2O

1X PBS (0.145 M NaCl, 0.0027 M KCl, 0.0081 M Na2HPO4, 0.0015 M KH2PO4, pH 7.4)

Materials Required

Filter units

Glassware and stir bar (dedicated for formaldehyde solution)

Hot plate with magnetic stirrer

Thermometer

Ventilated hood

Gloves and eye protection

Procedure

i. For 1 L of 4% Formaldehyde, add 800 mL of 1X PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C. Take care that the solution does not boil.

ii. Add 40 g of paraformaldehyde powder to the heated PBS solution.

iii. The powder will not immediately dissolve into solution. Slowly raise the pH by adding 1 N NaOH dropwise from a pipette until the solution clears.

iv. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered.

v. Adjust the volume of the solution to 1 L with 1X PBS.

vi. Recheck the pH, and adjust it with small amounts of dilute HCl to approximately 6.9.

vii. The solution can be aliquoted and frozen or stored at 2-8 °C for up to one month.

Sample fixation

The biomass samples was metabolically inactive by paraformaldehyde fixation. The biomass samples were suspended in a 1:3 (v/v) ratio of 1 x phosphate buffered saline (PBS) solution: 4 % Paraformaldehyde, and frozen for overnight. The biomass samples were centrifuged at 10000 rpm for 5 minute. Thereafter, the biomass samples were washed twice with 1x PBS and re-suspended in a 1:1

solution of 1 x PBS: absolute ethanol. The sample was stored at -20 °C for further analysis.

Microscope Slides preparation

Teflon-coated slides were cleaned in alcohol (1% HCl in 70%Ethanol) and soaking in diluted Poly-L-Lysine solution (1:10) (Sigma-Aldrich, Germany) for 5 minutes at room temperature. The slides were air dried overnight at room temperature.

Cell pre-treatment using lysozyme

Enzymatic pre-treatment with lysozyme allows for cell permeability, whiles minimizing the loss of target molecules and cell integrity (Furukawa *et al*., 2006). Lysozyme opens up the peptidoglycan layer and allows for increased cell permeability and improved signal intensity (Moter and Göbel, 2000). To facilitate cell dispersion the fixed samples were treated with 10 µl of lysozyme (Sigma-Aldrich, Germany) 1mg/mL. The mixture was incubated at 37°C for 30 minutes, samples were diluted further by the addition of 100 µl of sterile water for even dispersion and vortexed at maximum speed for 5 min.

2. Detection of selected bacterial groups using polymerase chain reaction

The PCR was carried out with primer sets targeting AOB, NOB and anammox bacteria functional genes (*hzs*, *hzo* and *nirS*) (Table 4.2). Positive amplification for all primer sets was obtained during the study indicating the presence of the targeted bacteria (AOB and NOB) within all three reactors (Figure B1 and B2). For nitrifying bacteria, both AOB and NOB resulted in positive amplification at the expected size of the primers and were further confirmed by sequencing and analysis.

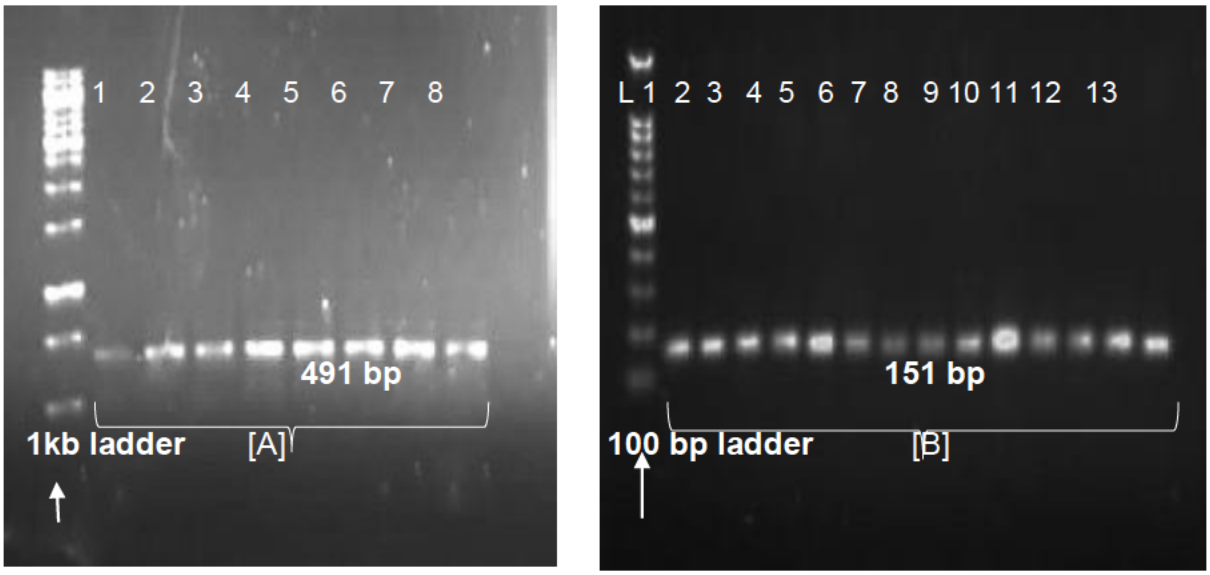


Figure B1: Agarose gel depicting specific PCR products. Plate [A] corresponds to AOB, lane L contains 1 kb ladder, and lane 1 – 8 contains positive amplification of amoA-1F/ amoA-2R primer (491 bp). Plate [B] corresponds to NOB, lane L contains 100 bp ladder, and lane 1 – 14 contains positive amplification of NSR 1113F/ NSR 1264R (151 bp).

Successful amplification of the anammox bacteria was obtained using functional gene (*hzs*, *hzo* and *nirS*) targeted primers with the expected size amplified products (Figure 4.3). Primer set hzsA_526F/ hzsA_1829R targeting the hydrazine synthase gene showed positive amplification with 526 bp product (Figure B2[A]). Primer sets hzocl1F1/ hzocl1R2 targeting hydrazine oxidoreductase gene showed positive amplification with 470 bp product (Figure B2[B]). Additionally, AnnirS379F/ nirS821R targeting nitrite oxidoreductase showed positive amplification bands on agarose gel of the expected size of 442 bp (Figure B2[C]).

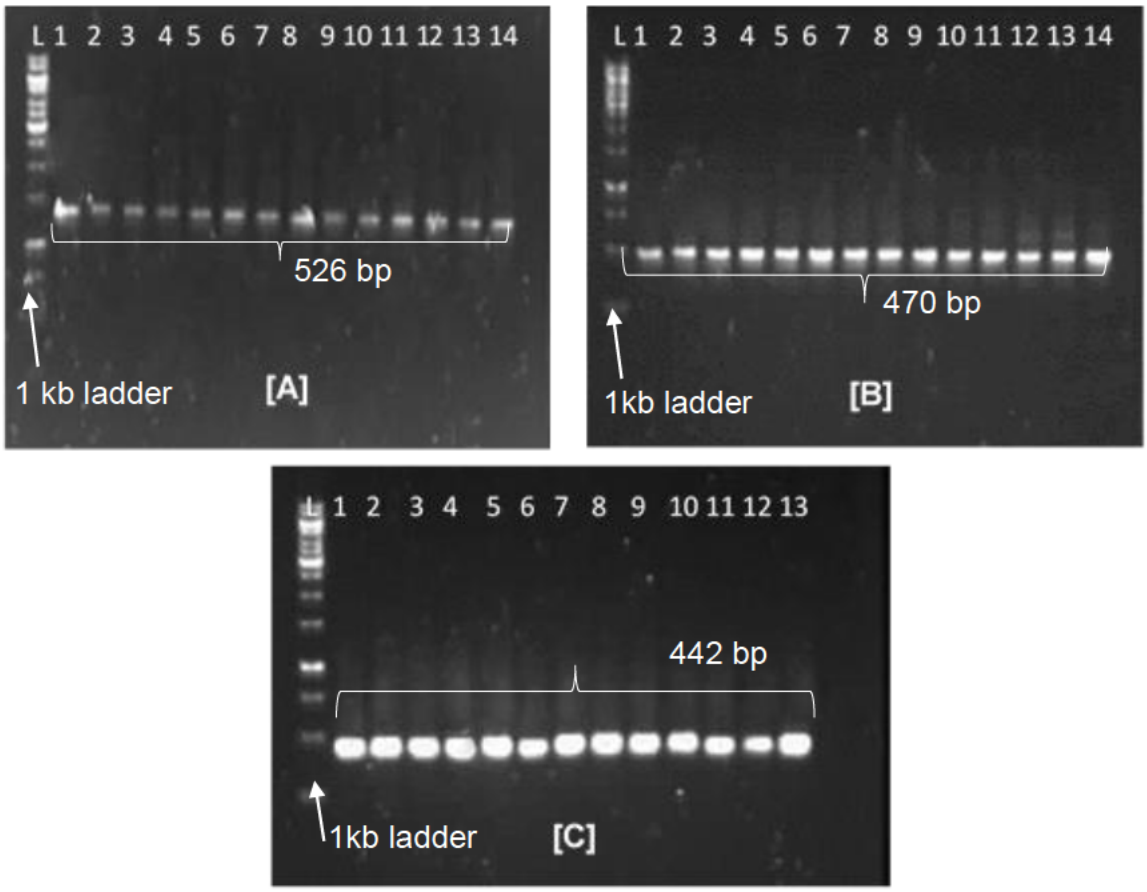
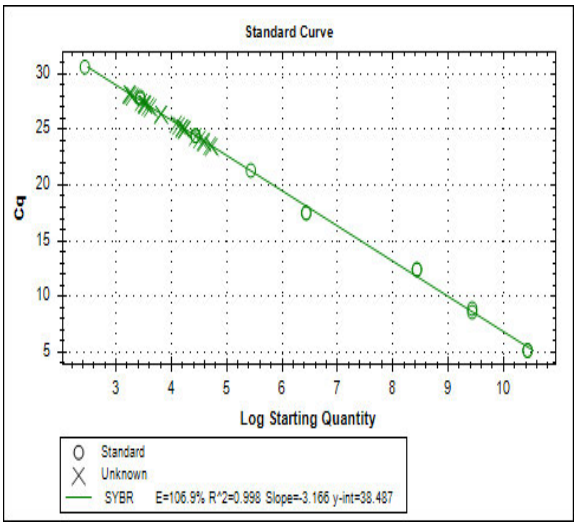


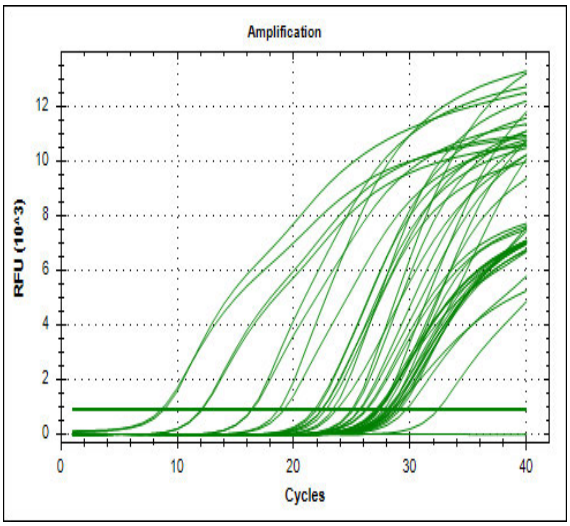
Figure B2: Agarose gel depicting PCR products from anammox functional gene primers. Plate [A] corresponds to *hzs* gene amplicons at 526 bp (lane 1 – 14) and lane L contains 1kb ladder. Plate [B] corresponds to *hzo* gene amplicons at 470 bp (lane 1 – 14) and lane L contains 1 kbp molecular weight marker. Plate [C] correspond s *nirS* gene amplicons at 442 bp (lane1 - 13 and lane L contains 1 kbp ladder.

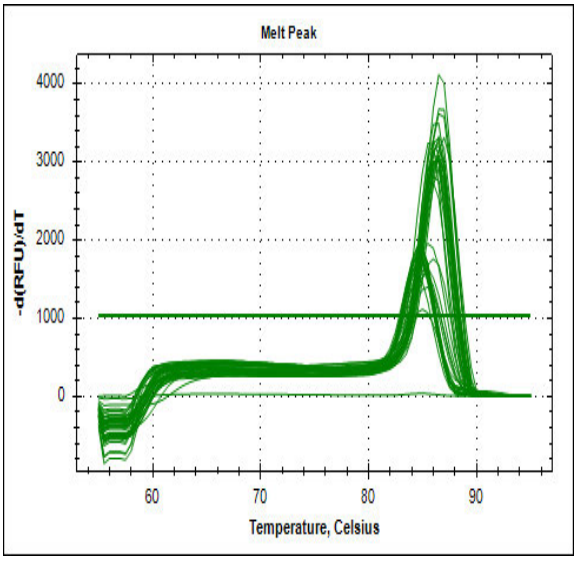
3. QPCR Quality control Parameter Data

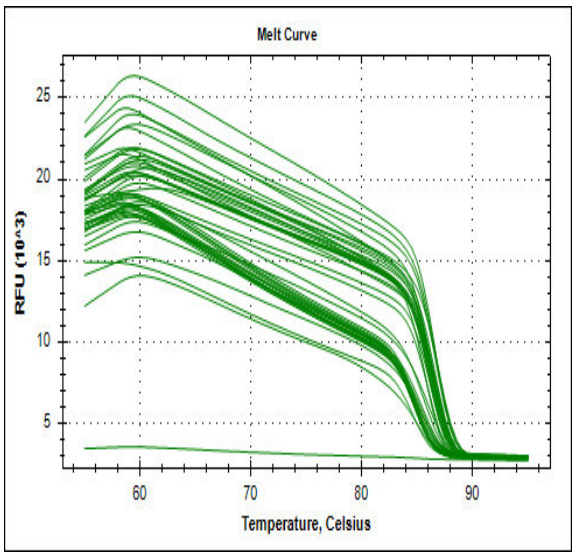
Parameters for the qPCR standard curve, obtained after optimisation is shown below. The qPCR efficiencies were between 89 and 106 % and the standard curves were linear over six order of magnitude (R2>0.99).

Hzo gene (anammox bacteria):

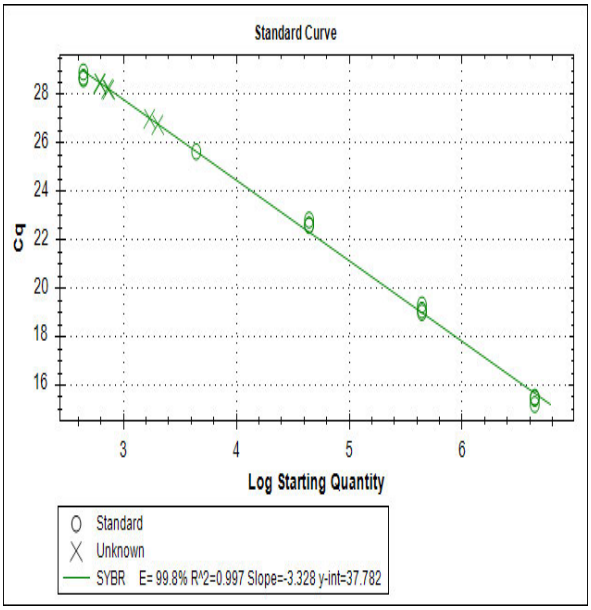


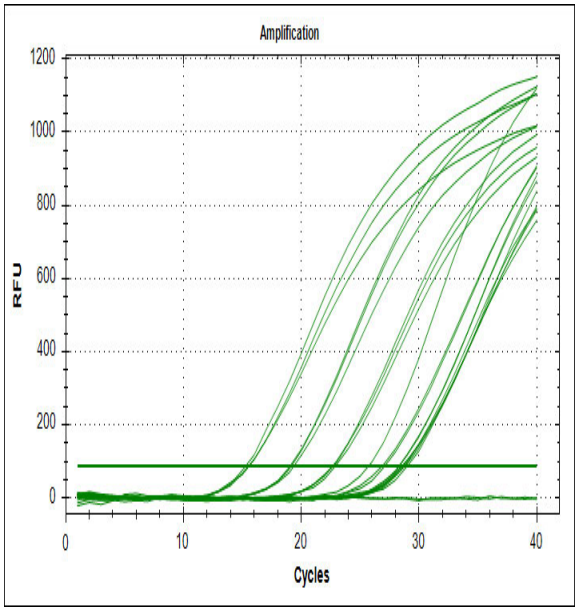


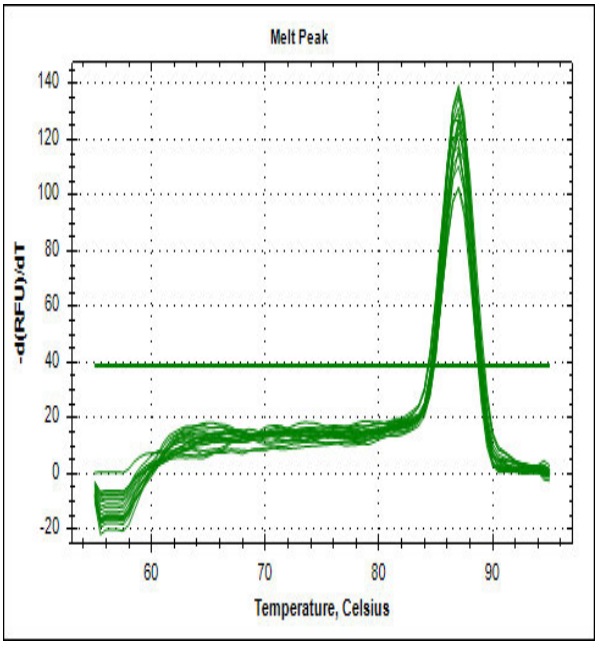


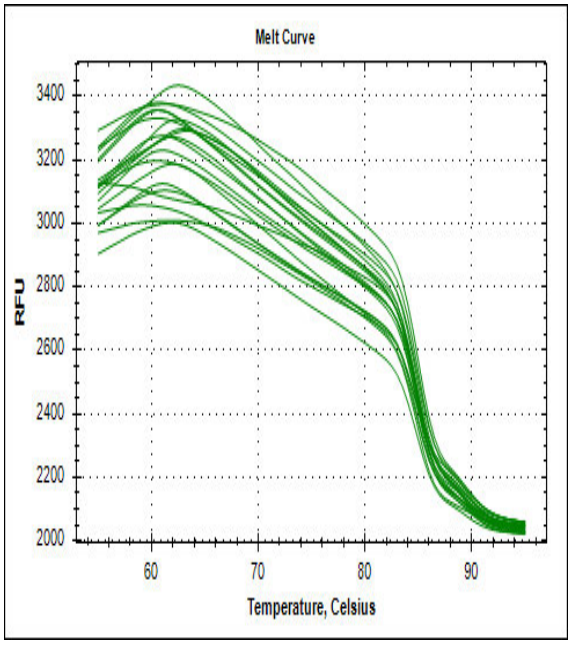


Ammonia oxidizing bacteria (AOB):

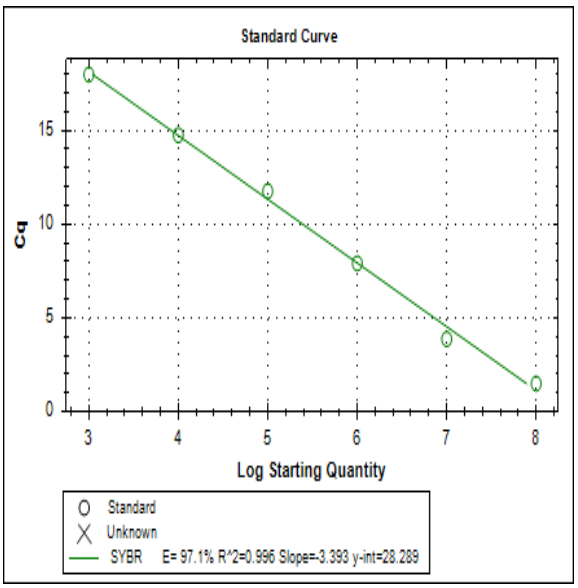


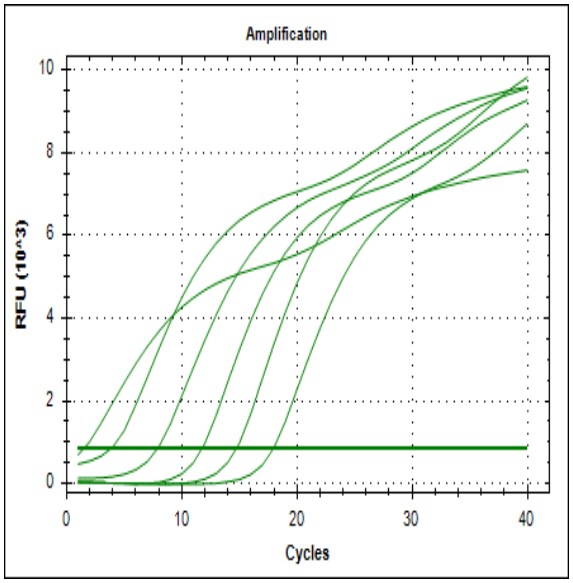


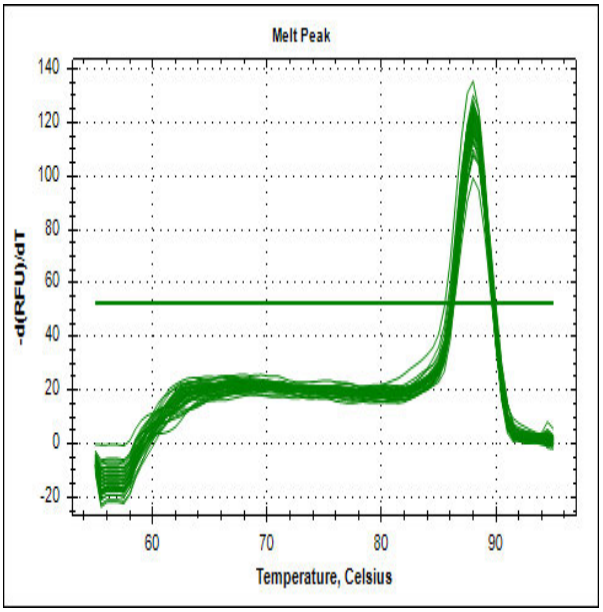


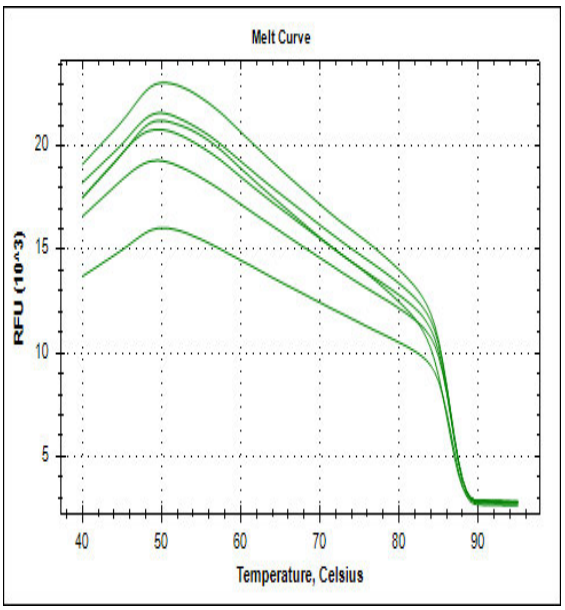


Nitrite oxidizing bacteria





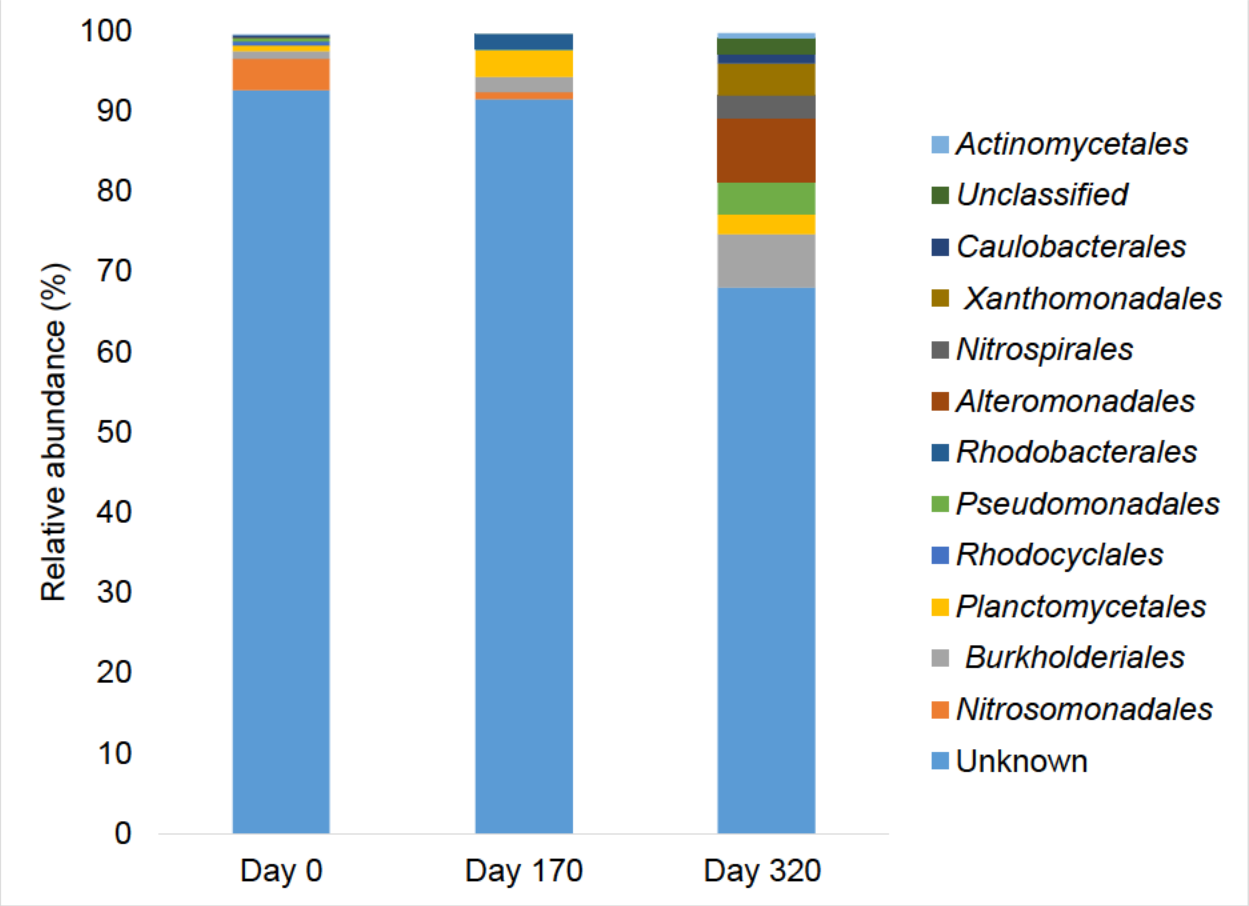

Melt Peak
-d(RFU)/dT
140
120
100
80
60
40
20
0
-20
60
70
80
90
Temperature, Celsius


Melt Curve
RFU (10^3)
20
15
10
5
40
50
60
70
80
90
Temperature, Celsius

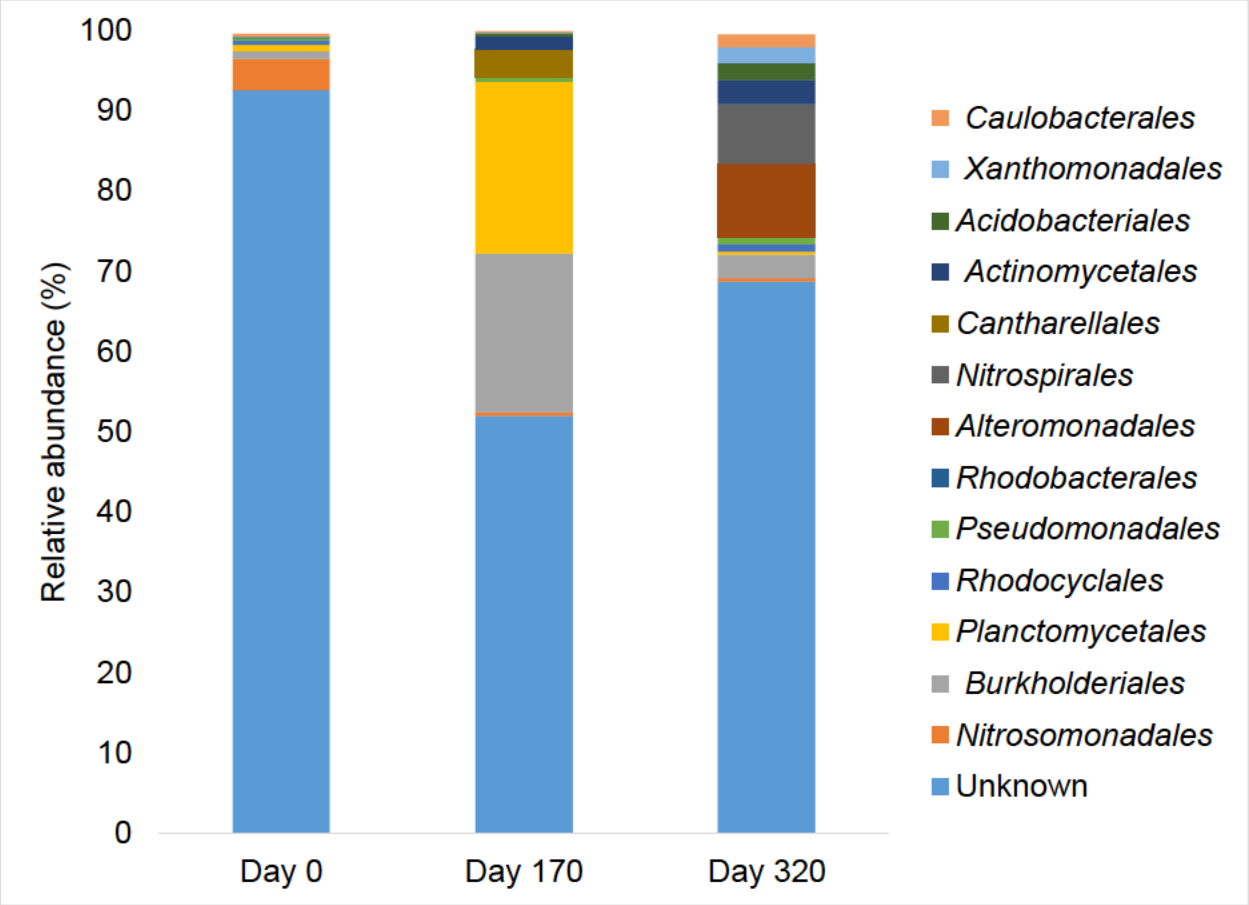
4. Illumina high through-put sequencing

Order Classification:

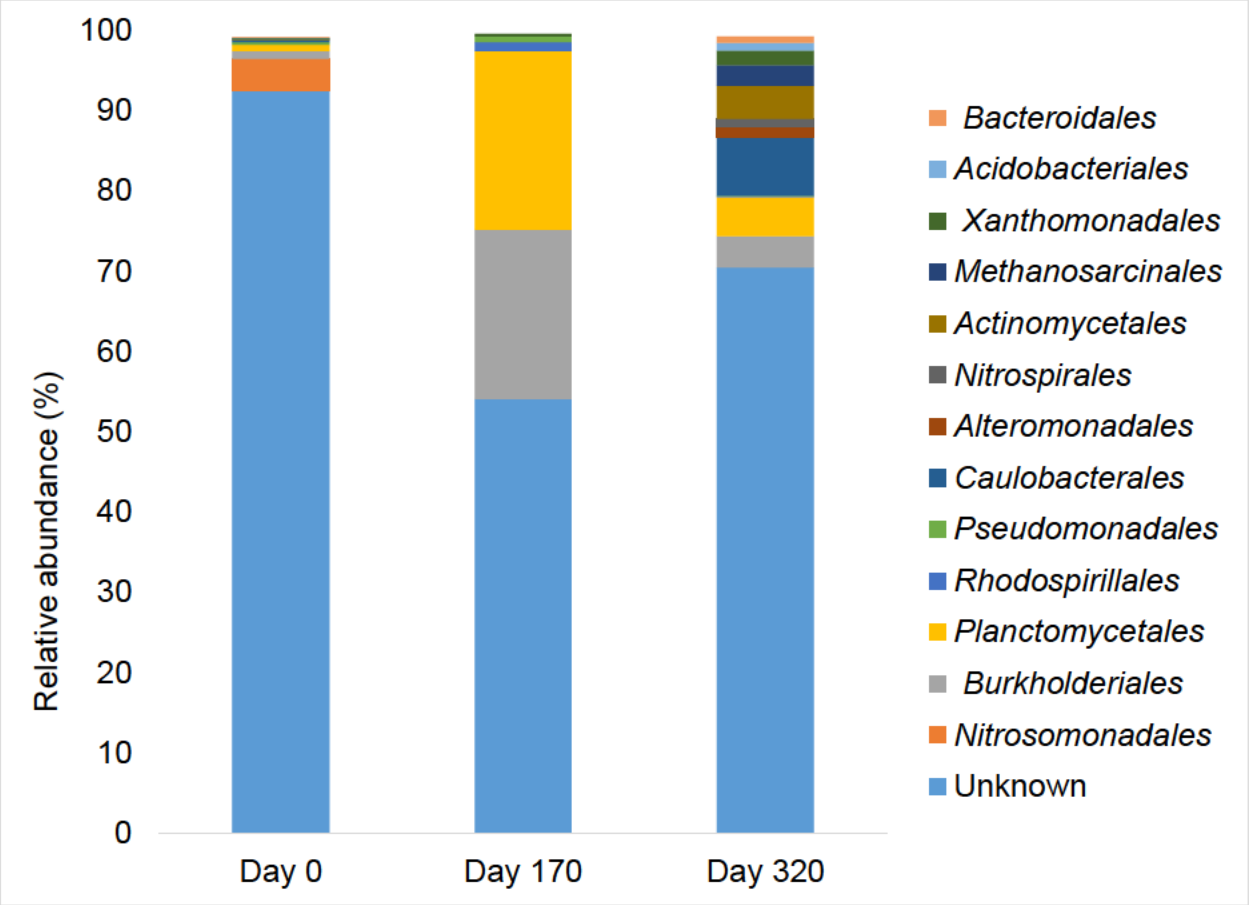
Reactor 1



Reactor 2


100
90
80
70
60
50
40
30
20
10
0
Relative abundance (%)
Day 0
Day 170
Day 320
Caulobacterales
Xanthomonadales
Acidobacteriales
Actinomycetales
Cantharellales
Nitrospirales
Alteromonadales
Rhodobacterales
Pseudomonadales
Rhodocyclales
Planctomycetales
Burkholderiales
Nitrosomonadales
Unknown

Reactor 3


100
90
80
70
60
50
40
30
20
10
0
Relative abundance (%)
Day 0
Day 170
Day 320
Bacteroidales
Acidobacteriales
Xanthomonadales
Methanosarcinales
Actinomycetales
Nitrospirales
Alteromonadales
Caulobacterales
Pseudomonadales
Rhodospirillales
Planctomycetales
Burkholderiales
Nitrosomonadales
Unknown

APPENDIX B. Publication

ENVIRONMENTAL TECHNOLOGY
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Taylor & Francis

Effect of ammonium to nitrite ratio on reactor performance and microbial population structure in anammox reactors

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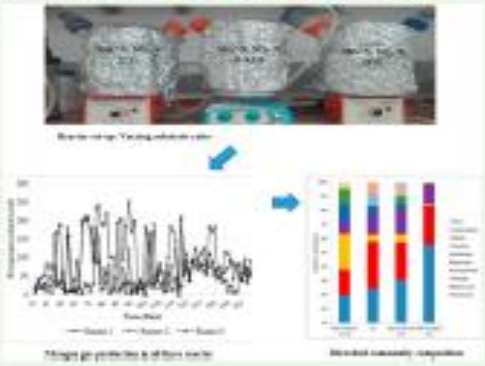
ABSTRACT

Anaerobic ammonium oxidation (anammox) presents an efficient alternative for conventional nitrogen removal process. In this study, the effect of varying Substrate (ammonium to nitrite) ratios on reactor performance and microbial community structures within three anaerobic sequencing batch reactors (ASBRs) was investigated. Three 1 L ASBRs (Reactors 1, 2 and 3) were operated under similar operational conditions. By varying the ammonium to nitrite ratios, a significant variation in nitrogen removal was observed after 170 days of operation: nitrogen removal efficiencies of 67.17 ± 7.29%, 57.13 ± 11.18% and 56.26 ± 17.05% in Reactors 3, 2 and 1 respectively were achieved. Similarly, using quantitative PCR, an overall variation in the population of anammox bacteria, ammonia oxidizing bacteria (AOB), *Nitrospira* and copy numbers of *nirS*, *hzo* and *hzs* genes were observed with varying degrees of expression. High throughput sequencing analysis further showed a shift in microbial community structure with an overall increase in population of *Planctomycetia* from 0.76% to (3%, 25% and 26%) and *Betaproteobacteria* from 5.38% to (19%, 21% and 43%) within Reactors 1, 2 and 3, respectively. In conclusion, different substrates ratio showed a significant influence on the overall nitrogen removal rate as well as the abundances of the different microbial groups.



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KEYWORDS
Wastewater; nitrogen removal; anammox; ammonia oxidizing bacteria; nitrite oxidizing bacteria

1. Introduction

Anaerobic ammonium oxidation (anammox) is a more environmentally sustainable and cost-effective technology alternative for nitrogen removal from ammonium-rich wastewater [1]. The anammox process is carried out by anammox bacteria, which oxidize ammonia under anoxic conditions, with nitrite as electron acceptor to produce dinitrogen gas [2]. Anammox bacteria belong to the order *Planctomycetales*. To date, six *Candidatus* genera of anammox bacteria have been defined including: *Brocadia*, *Kuenenia*, *Scalindua*, *Jettenia*, *Anammoxomicrobium* and *Anammoxoglobbus* [2]. Anammox bacteria have not yet been isolated as pure cultures because they coexist with other microbial species [2]. Previous studies have demonstrated the co-culture of anammox bacteria, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) inside the same reactor under oxygen limited conditions [3]. Under these conditions, the AOB can provide nitrite to the anammox bacteria, an interaction analogous to that in

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