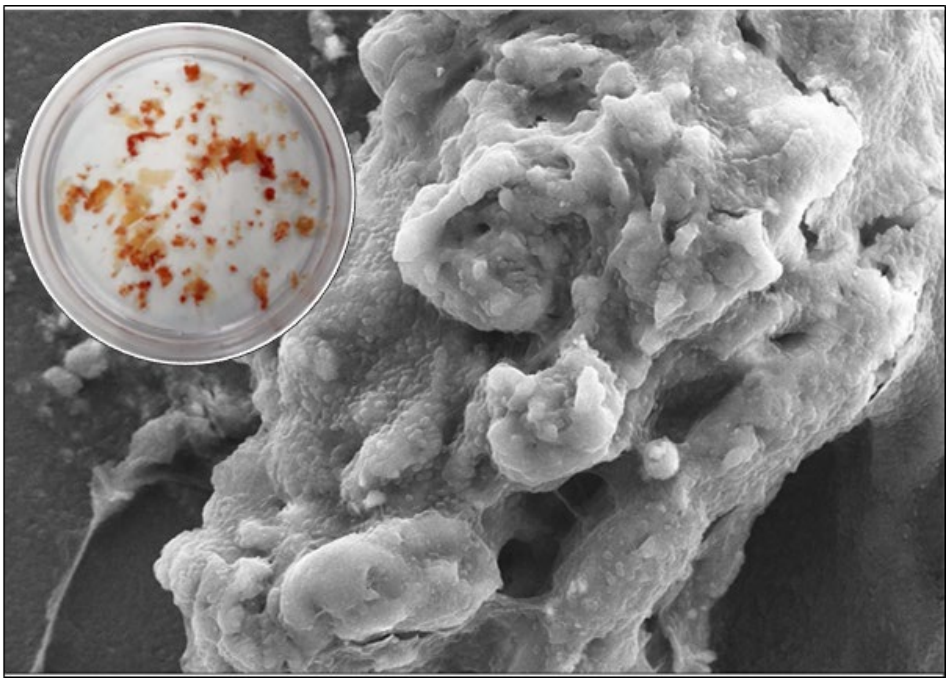
Development of a Two-Stage Nitritation-Anammox Process for Improved Ammonia Removal from Wastewater



Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Introduction

Partial nitritation-anammox (PN-A) process with the advantage of energy savings has been demonstrated as a sustainable and efficient alternative for nitrogen removal from wastewater without the organic carbon requirement. Several wastewater treatment plants around the globe have implemented the single-stage PN-A process, which combines both partial nitritation (PN) and anaerobic ammonium oxidation (ANAMMOX) reactions in a single unit. However, these single-stage systems are associated with a myriad of bottlenecks including nitrite-oxidizing bacteria (NOB) competition with ammonia-oxidizing bacteria (AOB) and anammox bacteria, for oxygen and nitrite respectively. In this study, a two-stage PN-A system for sustainable ammonia removal was implemented. The study was carried out in two phases: 1) enrichment and optimization of growth conditions for aerobic ammonia-oxidizing bacteria and anammox bacteria, and 2) coupling of the AOB and anammox bacteria activities in a two-stage PN-A lab-scale system for removing ammonia from synthetic wastewater.

The enrichment and optimization of growth conditions for AOB

The enrichment and optimisation of growth conditions for AOB was carried out in two identical 3 L sequencing batch reactors (SBR) operated under different DO concentrations (Reactor 1: 0.3-1.0 mg/L; Reactor 2: 1.3-2.0 mg/L) and pH ranges (Phase 1: pH 7.5-7.9; Phase 2: pH 8-8.5). A significant difference (t-test: <0.05) in the nitrite accumulation was observed in the two reactors with reactor 1 (Phase 1: 51%; Phase 2: 65%) showing better performance compared to the reactor 2 (Phase 1: 47%; Phase 2: 55%). There was also evidence of higher NOB suppression in reactor 1 compared to reactor 2. Therefore, a combination of DO range 0.3-1.0 mg/L and pH 8-8.5 proved to be more effective in achieving AOB enrichment and subsequent NOB suppression in this study. The FA concentration (1.87 and 3.18 mg/L) achieved in this study was above the inhibitory threshold (0.1-1.0 mg/L) of NOB, hence, it contributed to the suppression of NOB activity whilst the Free Nitrous Acid (FNA) effect was negligible.

Enrichment and optimization of growth conditions for anammox bacteria

Enrichment and optimization of growth conditions for anammox bacteria were carried out in two parts. First, local activated sludge sources (Kingsburgh, Shallcross, and Northern Work Wastewater Treatment Plants) were used as seed culture for the bioprospection and enrichment of indigenous anammox bacteria in different reactors. The initial screening of the samples

showed no indication of their presence in the local sludge. Upon enrichment of the seed sludge in different reactors for 60-100 days, one of the reactors have shown moderate anammox activity, which was further confirmed using molecular techniques. The results from this study indicate that anammox bacteria may be present in undetectable levels in the conventional wastewater treatment systems, which can be enriched by providing ideal growing conditions.

In addition to the enrichment of anammox bacteria from the local wastewater treatment plants, seed culture from an established anammox-mediated reactor (Columbia University, USA) was also mass cultivated in three different reactors (2 SBR and 1 UASB reactor). Among the different reactors, the UASB showed stable anammox activity within 190 days compared to the SBRs. The highest nitrogen removal efficiency of 81±14% was observed in the UASB reactor towards the last stage of operation (day 187-309) when the NO_2-/NH_4+ (1.12±0.28) and NO_3-/NH_4+ (0.17±0.12) ratios were close to the stoichiometric ratios expected of anammox process. Furthermore, shotgun sequencing of the UASB reactor sample confirmed the reactor progression towards the enrichment of the Planctomycetes, the phylum harbouring anammox bacteria. It revealed an increase in the anammox bacterial population in the UASB reactor with *Candidatus* Kuenenia dominating throughout. The sample collected on day 260 contained the highest concentration of anammox with a relative abundance of *Candidatus* Kuenenia at ca. 74%. On day 309, about 10% drop in the relative abundance of anammox bacteria was observed, whilst the relative abundance of *Nitrospira*- and *Nitrobacter*-affiliated NOB were below 1% on the same day. The Shannon and Simpson indices also corroborate the fact that the reactor progressed towards the enrichment of fewer population groups with over 30% reduction in these indices observed on the last day of this study.

The two-stage partial nitritation-anammox process

In the second phase of the study, a two-stage PN-A reactor was established and operated for approximately 200 days. In the PN reactor of the two-stage system, although the activity of NOB was successfully inhibited, their complete washout from the reactor could not be achieved. This is in agreement with earlier studies. Operation of the AOB reactor under DO concentration of approximately 1.5 mg/L led to better performance (in terms of nitrite accumulation) when compared to DO concentration below 1 mg/L. In addition, the incorporation of a mixer in the AOB-reactor and the maintenance of operational temperature at 34±1°C led to a significant improvement in the process performance (t-test: $p<0.05$). It was observed that the NO_2-/NH_4+ ratio in the influent to anammox reactor was a critical factor in

the operation of the two-stage PN-A system. The nitrogen removal rate in the anammox reactor increased from 0.16±0.13 kg-N/m^3-day by 55% when the NO_2-/NH_4+ ratio increased from 0.24±1.95 to 1.22±1.98. Based on qPCR investigation of the two-stage reactor, the anammox bacteria: AOB, and anammox bacteria: NOB ratios in the AMX-reactor were 5 and 3.2 respectively, which indicated enrichment of anammox bacteria in that reactor, whilst AOB: NOB ratio in the same reactor was 0.71. As expected, in the AOB-reactor, the AOB: NOB ratio was higher (1.8), whilst anammox: AOB was 0.013. This indicated that the condition within the AOB-reactor and AMX-reactor achieved the enrichment of anammox bacteria and AOB respectively.

Conclusions

A two-stage laboratory-scale PN-A lab-scale system for the treatment of high ammonia concentration in wastewater was developed. Overall, the findings from this study provided empirical data and experience for making an informed decision about the pilot-scale two-stage partial nitritation-anammox systems. With the current energy challenges in South Africa, retrofitting anammox-mediated systems within the current activated sludge systems could be a sustainable approach to achieve the PN-A system that offers the advantage of efficient nutrient removal coupled with achieving substantial energy savings.

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RESEARCH OUTPUTS

Journal Articles

1. Gasa, N.P., Nnadozie, C.F., Kosgey, K., Bux, F. and Kumari, S., 2019. Effect of ammonium to nitrite ratio on reactor performance and microbial population structure in anammox reactors. Environmental Technology, DOI: 10.1080/09593330.2019.1610076

2. K. Kosgey, K. Chandran, J. Gokal, S.L. Kiambi, F. Bux, S. Kumari. Critical analysis of biomass retention strategies in mainstream and sidestream anammox-mediated nitrogen removal systems. Environmental Science and Technology (Under review)

3. K. Kosgey, O.O. Awolusi, S.L. Kiambi, M. Allam, A. Ismail, F. Bux, K. Chandran, S. Kumari. Impact of reactor configuration on bacterial population dynamics and process performance in three anammox-mediated systems. Biotechnology and Bioengineering (Under review)

Conference presentations

1. K. Kosgey, S.L. Kiambi, F. Bux, K. Chandran, S. Kumari. Impact of Reactor Configuration on Process Performance and Microbial Population Dynamics in Three ANAMMOX-Mediated Systems Poland, Kazimierz Dolny, 9-11 September 2019.

2. N.P. Gasa, S Kumari, C.F. Nnadozie and F. Bux, (2018). Evaluation of the effect of ammonium: nitrite ratio on anammox reactor efficiency. Water Institute of Southern Africa (WISA) 2018 Biennial Conference and Exhibition. Cape Town International Conventional Centre, Cape Town, South Africa National. 26-28 June 2018.

3. K. Kosgey, S.L. Kiambi, F. Bux, K. Chandran and S. Kumari (2018). Effect of fluctuating operating conditions on physical characteristics of Anammox biomass. Water Institute of Southern Africa (WISA) 2018 Biennial Conference and Exhibition. Cape Town International Conventional Centre, Cape Town, South Africa National. 26-28 June 2018.

4. J. Gokal, S. Kumari, T.A. Stenström and F. Bux, (2018). Bioprospecting for indigenous Anammox bacteria in South African wastewater treatment plants. Water Institute of Southern Africa (WISA) 2018 Biennial Conference and Exhibition. Cape Town International Conventional Centre, Cape Town, South Africa National. 26-28 June 2018.

5. J. Gokal, S. Kumari, T.A. Stenström, and F. Bux, (2018). Community profiling of an adapted microbial consortia in an autotrophic N-removing bench scale reactor. IWA World Water Congress and Exhibition 2018. Tokyo Big Sight, Koto, Tokyo, Japan 16-21 September 2018.

6. K. Kosgey, S.L. Kiambi, F. Bux, K. Chandran, and S. Kumari, (2018). Potential application of magnetic fields for the retention of Anammox biomass in reactors. IWA World Water Congress and Exhibition 2018. Tokyo Big Sight, Koto, Tokyo, Japan 16-21 September 2018.

7. J. Gokal, S. Kumari, T.A. Stenstrom, and Bux, F. (2016). High rate Nitrogen removal through a stable Anammox-Nitrification process in a bench scale CSTR Oral presentation of the 19th South African Society of Microbiology (SASM) biennial congress, Coastlands, Umhlanga, Durban, South Africa, 17-20 January 2016.

8. J. Gokal, S. Kumari, T.A. Stenstrom, and F. Bux (2016). High rate Nitrogen removal through a stable Anammox-Nitrification process in a bench scale CSTR Oral presentation at the Water Institute of Southern Africa (WISA) Conference and Exhibition, Durban International Convention Centre, Durban 15-19 May 2016.

9. S. Kumari and F. Bux. 2017. Two-Stage Nitritation-Anammox: A promising sustainable wastewater treatment option, WRC Symposium, Technical session: Wastewater – tapping into this resource: Emerging Wastewater Treatment options, September 2017, Birchwood Hotel, Johannesburg

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LIST OF ABBREVIATIONS

amoA	Ammonia monooxygenase
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
CANON	Completely autotrophic nitrogen-removal over nitrite
CANR	Complete Autotrophic Nitrogen Removal
CO_2	Carbon dioxide
CSTR	Continuous stirred tank reactor
DNRA	Dissimilatory nitrite reduction to ammonia
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
FA	Free ammonia
FNA	Free nitrous acid
HAO	Hydroxylamine oxidoreductase
HRT	Hydraulic retention time
MBBR	Movable bed biofilm reactor
MLE	Modified Ludzack Ettinger process configuration
MLSS	Mixed Liquor Suspended Solids
N	Nitrogen
N_2	Nitrogen gas
NH_3	Ammonia
NH_4^+	Ammonium ion
NirS	Nitrite reductase functional gene

NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite Oxidizing Bacteria
NOR	Nitrite oxide reductase
OLAND	Oxygen limited autotrophic nitrification and denitrification
PCR	Polymerase chain reaction
pH	Potential of hydrogen ion
PN-A	Partial nitrification-Anammox
qPCR	Real-time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SAA	Specific anammox activity
SBR	Sequencing batch reactor Sodium
SHARON	Single reactor high activity ammonium removal over nitrite
SNAD	Simultaneous nitrification, anammox, denitrification
SNRR	Specific Nitrogen Removal Rate
SO_4^{2-}	Sulphates
SRT	Sludge Retention Time/ Solids Retention Time
TOC	Total organic carbon
UASB	Upflow anaerobic sludge bed
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Introduction

The conventional biological nitrogen removal process relies on the two-stage process (nitrification and denitrification), carried out by the synergistic action of nitrifying and denitrifying bacteria, cannot objectively, be considered as a sustainable process. It is highly energy consuming; produces excessive sludge and significant amounts of greenhouse gases and ozone-depleting N_2O (Hu et al., 2013). Thus, the removal of nitrogen from high ammonia constituent influent wastewater streams is a major challenge for treatment plants worldwide. Anaerobic ammonia oxidation (anammox) is a unique process carried out by anammox bacteria, which, under anaerobic conditions, convert ammonium directly to nitrogen gas using nitrite as electron acceptor (Van Niftrik and Jetten, 2012, Kartal et al., 2012). Thus, by compressing the conventional two-stage nitrification-denitrification pathways into a single metabolic pathway, an anammox process represents a cost-effective and environmental-friendly technology for ammonia removal from wastewater, which is currently being applied for biological nitrogen removal in different parts of the world including China, the Netherlands, and the USA. The anammox process may present a viable alternative to the conventional process due to its comparatively lower costs, energy, and waste footprints. Since the start-up of the first anammox WWTP in Rotterdam, Netherlands, anammox has emerged as an alternative to conventional nitrogen removal from ammonia-rich wastewater all over the world (Van der Star et al., 2007).

Anammox bacteria require nitrite for the effective removal of ammonia from wastewater. However, nitrite is more toxic than ammonium to these bacteria in a pH range of 6.7~8.3 (Strous et al., 1999b, Dapena-Mora et al., 2007). On the other hand, studies have also reported a substrate limitation effect on the kinetic behaviour anammox bacteria at low concentrations (<10 mg NO_2^-–N/L) (Bettazzi et al., 2010). Hence, a partial nitrification system producing the appropriate ratio of nitrite to ammonium nitrogen is a prerequisite for successful nitrogen removal. To meet this, ammonia is partly oxidized to nitrite by oxygen-limited aerobic ammonia oxidizers. The nitrite produced, together with a part of the remaining ammonia, is converted to dinitrogen gas by

anammox bacteria. It was recently shown that both types of bacteria can co-exist in one reactor, provided that the system was kept oxygen-limited (Sliekers et al., 2003). Thus, the existing pilot and full-scale studies couple the anammox process with the single reactor system for high ammonium removal over nitrite (SHARON) reactor, fluidized bed reactor (FBR), gas-lift reactor, upflow anaerobic sludge blanket (UASB) reactor, complete autotrophic nitrogen removal over nitrite (CANON) reactor, oxygen-limited autotrophic nitrification/denitrification (OLAND) reactor, single-stage nitrogen removal using anammox and partial nitritation (SNAP) or aerobic deammonification (Third et al., 2005, Furukawa et al., 2006, Bagchi et al., 2012) to provide the optimum environmental conditions for anammox activity. However, recent reports indicate that a single-stage nitritation process encounters difficulty maintaining stable partial nitrification (Cho et al., 2010, Terada et al., 2011). One of the main advantages of separating PN-A into 2 different reactors is the flexibility of being able to operate the anammox reactor under an anoxic condition to avoid NOB competition for nitrite (Pérez et al., 2014), which is unavoidable in a single-stage PN-A reactor (Cao et al., 2017). Furthermore, the first-stage PN reactor plays a positive role in maintaining the stability of the dominant bacteria in the anammox reactor (Liu et al., 2018). Moreover, it helps in the reduction of process instability because of influent organics, inhibitors, and toxic chemicals (Lackner et al., 2014, Li et al., 2017). Hence, the two-stage PN-N system can ensure the effective and full utilization of the anammox bacteria through the provision of adequate substrates and optimal environmental conditions.

1.2 Research Aim

The purpose of the study is essential to improve the performance of the conventional single-stage anammox process by developing a 2-stage Nitritation-Anammox process. Therefore, the major objectives of this project include the following:

1. Identification of suitable growth/operational conditions to selectively enrich Ammonia Oxidising Bacteria (AOB) and Anammox bacteria from a mixed consortium
2. Selective enrichment and optimization of growth conditions for AOB and Anammox bacteria in separate bioreactors
3. Integration of enriched AOB and Anammox reactors into a two-stage continuous Nitritation-Anammox process and evaluation of the potential for scale-up

4. Comparison of the performance of the integrated (two-stage) reactor to the conventional single-stage Anammox reactor

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nitrogenous compounds, often discharged in high concentrations from municipal and industrial wastewaters, can create serious problems when released untreated into the environment (Foglar and Briški, 2003, Terada et al., 2011). In receiving waters they can be potent drivers of eutrophication, induce water stagnation, odour problems, ammonia (NH_3) and nitrite (NO_2^-) toxicity, and cause a critical decrease in the dissolved oxygen (DO) concentration (Arbib et al., 2014, Cho et al., 2016). Globally, biological treatment has been the method of choice for reducing or removing these pollutants from wastewater (Punzi et al., 2015). Nitrogen (N) compounds are recycled within the biosphere by the metabolic actions of specific organisms capable of oxidizing and reducing these N- compounds to free dinitrogen gas (N_2) as per the Nitrogen Cycle on the next page (Figure 2-1).

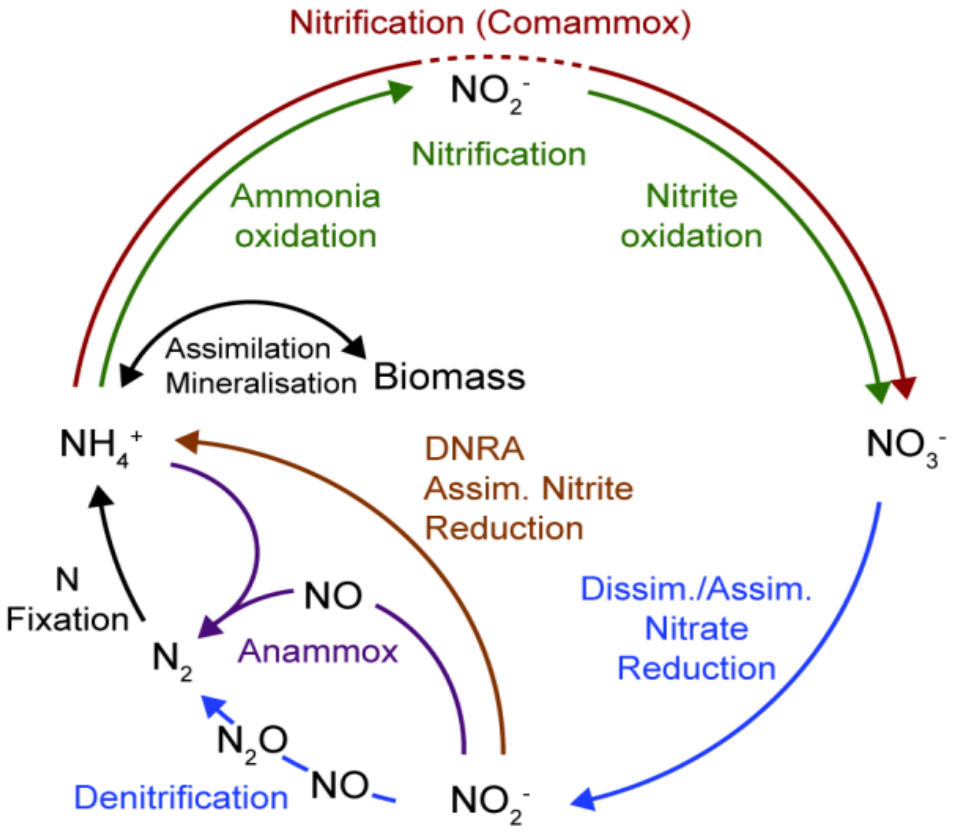


Figure 2-1: The Nitrogen Cycle (Daims et al., 2016)

The complete Nitrogen Cycle is divided into three different, yet interlinked processes – those of nitrification, denitrification, and anammox. Nitrification is a chemolithoautotrophic process by which ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) under strict aerobic conditions, and is conducted in two sequential oxidative stages: NH_4^+ to NO_2^- (ammonium oxidation), and NO_2^- to NO_3^- (nitrite oxidation) (Ahn, 2006). Each stage is performed by two different bacterial genera (ammonia-oxidizing bacteria and nitrite-oxidizing bacteria) (Yu et al., 2013, Daims et al., 2016) or by a single organism known as complete ammonia oxidation (comammox) bacteria (Lawson and Lucker, 2018). The ammonium or nitrite are used as an energy source during these reactions and molecular oxygen as an electron acceptor, while carbon dioxide (CO_2) is used as a carbon (C) source (Awolusi et al., 2015). Ammonia is first oxidized to NO_2^- by ammonia-oxidizing bacteria (AOB) (equation 1.1) and the NO_2^- produced as their metabolic by-product is then oxidized to NO_3^- by the nitrite-oxidizing bacteria (NOB) (equation 1.2) (Costa et al., 2006).

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+$$ Equation (1.1)

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^-$$ Equation (1.2)

These two processes require aeration which takes up the bulk of energy utilized in any wastewater treatment plant (WWTP) (Stefansdottir et al., 2018). Denitrification is a subsequent process where heterotrophic denitrifiers reduce oxidized nitrogen species to gaseous nitrogen under anaerobic conditions, using NO_2^- and/or NO_3^- instead of oxygen as electron acceptors and organic matter as a C and energy source (Breisha, 2010). Denitrification can be carried out by many heterotrophic microorganisms as part of their primary or secondary metabolic processes (equation 1.3).

$$2NO_3^- + H^+ + organic\ carbon \rightarrow N_2 + HCO_3^-$$ Equation (1.3)

The anammox process is a relatively new addition to the nitrogen cycle and represents a direct conversion from NH_4^+ to N_2 gas. Anammox bacteria carry out anaerobic oxidation of NH_4^+ directly to dinitrogen gas, using NO_2^- as an electron acceptor, and inorganic CO_2 as a C source, thus they represent a potent alternative to the conventional N removal pathways (Bae et al., 2010).

It was previously assumed that nitrification was the primary process for NH_4^+ removal, however, it was found that full nitrification accounts for less than 43% of NH_4^+ removal (Milner, 2008). Thus, the remaining NH_4^+ is removed by other mechanisms, possibly by anaerobic ammonia oxidation (Schmidt et al., 2003). It is currently estimated that up to 50% of the loss of bound N from the world's oceans is attributed to the anammox process (Arrigo, 2005, Shu et al., 2011).

Concern for energy consumption due to aeration during nitrification has become a major disadvantage for the nitrification process (McCarty, 2018). Hence, the search for alternative energy neutral or positive approach. Consequently, due to their significant contribution to N cycling, their metabolic efficiency and cost-effective implementation, the anammox bacteria represent an attractive alternative to the conventional processes at the industrial scale. Currently, anammox is gaining popularity as an energy-saving and efficient alternative to the traditional nitrification-denitrification approach to nitrogen removal (Zhang et al., 2017).

2.2 The nitrifying bacteria

The majority of nitrifiers in wastewater remains uncultivable, and thus, only very few strains of AOB (25 species) and NOB (8 species) have so far been identified and classified based on conventional cultivation techniques (Egli et al., 2003, Wojnowska-Baryla et al., 2010). The growth rate of autotrophic bacteria (nitrifiers) is five times slower than that of heterotrophic bacteria in WWTPs (Ozdemir et al., 2011). Thus, the nitrifiers form only 3-20% of the total bacteria in activated sludge (Gerardi, 2002, Xia et al., 2010, Yu et al., 2011, Cydzik-Kwiatkowska et al., 2012), which makes the isolation of nitrifiers difficult. However, the successful application of molecular techniques to the complex environmental samples has helped to unravel the complexity and diversity of these groups in nature (Gao and Tao, 2012).

The 16S rDNA sequences revealed that these two groups of nitrifying bacteria (AOB and NOB) are phylogenetically distinct (Daims and Wagner, 2010). All ammonia oxidizers can be classified in the β-subclass of *Proteobacteria* except *Nitrosococcus*, which belongs to a distinct branch of the γ-subclass. The NOB can be found within the α- and γ-subclasses of *Proteobacteria*, with the exceptions of *Nitrospira*, which has its distinct phylum (Duan et al., 2013) and *Nitrospina*, which belongs to the δ-subclass of *Proteobacteria* (Zeng et al., 2012). Due to their low specific growth rate and sensitivity to stress from environmental and operational factors, their population and physiological activities can limit the rate of biotransformation of nitrogen in many WWTPs.

2.3 Ammonium oxidizing bacteria enrichment for partial-nitrification

Turk and Mavinic (1986) earlier noted that ammonia removal in bioreactors treating wastewater via a partial-nitrification route as opposed to the conventional nitrification process would be more economically beneficial. Partial nitritation-anammox (PN-AMX) and partial denitrification-anammox (PD-AMX) could achieve a reduction of about 57% and 48% in oxygen demand for nitrogen removal whilst sludge production could be reduced by as much as 84% and 66% respectively (Zhang et al., 2019). Enriching for AOB which results in nitrite accumulation can be achieved by NOB inhibition or washout by harnessing the differences in their (AOB and NOB) physiological characteristics and responses to operating conditions (Ge et al., 2015b). Different factors or a combination of many have been proposed and employed in bioreactors treating different wastewater types to achieve enrichment. The primary strategies employed for AOB

enrichment and partial nitrification include temperature, pH, DO, sludge retention time (SRT), inhibitor and real-time aeration control (Peng and Zhu, 2006, Sinha and Annachhatre, 2007, Blackburne et al., 2008).

2.3.1 DO concentration

The NOB (1.2-1.5 mg/L) has a higher oxygen half-saturation constant compared to AOB (0.2-1.5 mg/L) (Ge et al., 2015a). This higher oxygen affinity of AOB is an important factor when enriching at low DO concentrations. The growth rate of AOB is 2.6 times more than that of NOB when DO concentration is between 0.5 and 1.0 mg/L (Chen et al., 2016). Stable nitrite accumulation was reported at DO concentration of 1-2 mg O_2 L^{-1} (Ruiz et al., 2006, Vázquez-Padín et al., 2010), whilst lower DO levels of 0.3-0.7 mg L^{-1} (Blackburne et al., 2008, Ma et al., 2009, Zeng et al., 2013) were also reported for nitrite accumulation. Guo et al. (2010b) and Tian et al. (2011) proposed a limited filamentous bulking process at low DO, for simultaneous removal of suspended solids and nitrogen, thus reducing the overall aeration consumption. Furthermore, Guo et al. (2013) reported about 85% nitrite accumulation in a limited filamentous bulking process at DO of 0.5-1.0 mg L^{-1} in a lab-scale anoxic/oxic system. Therefore, combining partial nitrification and limited filamentous bulking process at low DO concentration could be an energy-effective solution for wastewater treatment.

2.3.2 Real-time control

The real-time control for partial nitrification ensures the determination of aeration duration via direct and indirect online parameters. The direct online control strategy involves measuring the nitrogen species (nitrite, nitrate, and ammonia) with probes to obtain information on aeration within the reactor. Bartrolí et al. (2010) reported complete ammonia conversion to nitrite using an automatic feed-forward control system with ammonia and DO probes to maintain optimal DO concentration. Efficient direct on-line control approach depends on robustness of the nitrogenous probes to determine the end of nitrification. Presently affordability and reliability of the online nutrient probes is still a challenge Zanetti et al. (2012). On the other hand, the indirect real-time control strategy employs general water quality probes (i.e. pH, DO, redox potential, oxygen utilization rate and blower frequency), which are more cost-efficient and dependable (Yang et al.,

2007, Gu et al., 2012, Lee et al., 2013, Ge et al., 2015a). The ammonia variations during nitrification reflect on water quality parameters, therefore these indirect parameters could be used in monitoring aeration, whilst the online characteristic points on pH profiles can indicate the ammonium oxidation. Chen et al. (2016) recorded up to 94.12% nitrite accumulation rate with DO, pH, and temperature automatically controlled by a programmable logic controller.

2.3.3 Alternating anoxic and aerobic operation

Although all nitrifiers are known to be slow growers, NOB has a lower specific growth rate than the AOB (Daims and Wagner, 2010) and this can be exploited for nitritation within sequencing batch reactors (Turk and Mavinic, 1986, Blackburne et al., 2008). The ability of sequencing batch-type processes to operate multiple aerobic-anoxic modes can be exploited for partial-nitrification (Blackburne et al., 2008). Katsogiannis et al. (2002) and Katsogiannis et al. (2003) noted that operating short aerobic phases can enhance the suppression of NOB and nitrite accumulation. Shi et al. (2011) also reported a sustained nitrite accumulation rate of above 90% in a sequencing batch reactor treating synthetic wastewater when the temperature was kept above 20°C. Ge et al. (2014) by alternating anoxic/aerobic phases in a reactor treating municipal wastewater achieved about 82% nitrite accumulation. This mechanism is based on a proposed mathematical model (Bournazou et al., 2013) which assumes that NOB inhibition during alternating aerobic/anoxic operation was due to NOB enzyme deactivation under anoxic conditions and reactivation during the aerobic phase. Furthermore, AOB recovery from oxygen starvation is more rapid with resultant nitrite build-up. In summary, alternating anoxic/aerobic phases can be an effective route for achieving nitrogen removal via partial nitrification.

2.3.4 pH with free ammonia (FA) and free nitrous acid (FNA)

FA (free ammonia) and FNA (free nitrous acid) are known to inhibit the activities of AOB and NOB in bioreactors. Whilst several studies have reported the successful inhibition of nitrite oxidation using FA (Shi et al., 2010, Im et al., 2014), many others have noted adaptation of NOB to high FA concentration with a resultant limitation of its long-term suppression of NOB (Hawkins et al., 2010). The FA and FNA are determined by pH, temperature, ammonia or nitrite levels (Equations 2.1 and 2.2) (Anthonisen et al., 1976). The influence of pH on FA and FNA has been

noted, therefore pH can be regulated to achieve nitritation due to different inhibition on AOB and NOB (Peng and Zhu, 2006). In bioreactors, pH greater than 7.5 usually favours nitrite accumulation (Philips et al., 2002, Antileo et al., 2003). Different threshold FA inhibition concentrations have been reported by various authors. Anthonisen et al. (1976) reported that 10-150 mg N/L and 0.1-1.0 mg N/L of FA were necessary for AOB and NOB inhibition respectively, whereas Bae et al. (2002) reported 0.1-4.0 mg L^{-1} as an inhibitory range for NOB and Liang and Liu (2007) observed that AOB could acclimatize to high FA at 122-224 mg/L. FNA equally plays a key role in the NOB inhibition at low pH (<7.5) (Sinha and Annachhatre, 2007) and NOB has been reported to be more sensitive to FNA than AOB. According to Han et al. (2003) FA and FNA inhibitions effect on nitrite oxidation activity can be recovered once the inhibition factor is removed. Han et al. (2003) further suggest that other factors including SRT, substrate concentrations, etc. should be considered when FA or FNA is to be used as the control parameter for partial nitrification.

$$FA = \frac{17 S_{NH4\text{-}N} 10^{pH}}{14(e^{6344/(273+T)} + 10^{pH})} \qquad \text{Equation (2.1)}$$

$$FNA = \frac{47 S_{NO2\text{-}N}}{14(e^{-2300/(273+T)} 10^{pH} + 1)} \qquad \text{Equation (2.2)}$$

2.3.5 Substrate concentration

Optimizing the carbon: nitrogen (C: N) ratio is also essential for efficient nitrogen removal in waste treatment systems. Low C/N ratio is beneficial to nitrite accumulation, whereas a high ratio inhibits nitrifiers especially NOB while supporting the growth of heterotrophs (Okabe et al., 1996); Organic carbon loading rates higher than 2 kg TOC m^{-3} d^{-1} retarded nitritation while encouraging competition between the autotrophs and heterotrophs (Prá et al., 2012). Mosquera-Corral et al. (2005) also reported organic carbon loading higher than 0.3 g TOC/L (C/N = 0.3 g/g) resulted in competition between heterotrophs and autotrophs with a resultant detrimental effect on nitrite accumulation. In lab-scale moving bed biofilm reactors, (Zafarzadeh et al., 2011) observed that nitrite accumulation showed correlation with a decrease in C: N ratio and maximum nitrification noted at C: N ratios < 6. Naseer et al. (2013) reported a high nitrite accumulation rate of over 95%

when the C: N ratio was 2.33. Apart from organic loading rates, the influent carbon source could also impact on partial nitrification since it hinges on enzymes like every other biochemical reaction. The lowest enzyme activity was observed with malate and succinate, followed by acetate, whilst the highest activity was noted when butyrate and caproate were used as substrate (Richardson and Ferguson, 1992, Ge et al., 2015b).

2.3.6 Inhibitor

When toxic substances inhibit the nitrifier population, their cell growth and the oxidation of nitrogen species are affected. The addition of NOB inhibitors is a strategy for achieving partial nitrification. NOB inhibitors include volatile fatty acids, sulphide, hydroxylamine, heavy metals, chlorite, sodium chlorate, cyanate, halide, azide, hydrazine, salts, fulvic acids and organic chemicals (Peng and Zhu, 2006, Sinha and Annachhatre, 2007). According to Erguder et al. (2008), NOB was found to be more sensitive to sulphide than the AOB. At pulse doses (>40 mg/L) NOB was affected more than AOB. In an SBR operated under 2-day cyclic aerobic and anoxic conditions up to 75%, nitrite accumulation was obtained at an initial sulphide concentration of 45 mg/ L at a pH of 7.5 ± 0.2 (Erguder et al., 2008). Correlation between fulvic acid loadings and nitrite accumulation in a biofilm process was observed. There was no nitrite accumulation at fulvic acids loading <0.002 kg (TOC)/m^3 h, however, at loading rate 0.002±0.02 kg (TOC)/m^3 h nitrite built up was observed with nitrite concentration reaching 11.4 mg/L (Erguder et al., 2008). The efficiency of partial nitrification correlates with influent salinity and the application duration of salt (Ye et al., 2009, Aslan and Simsek, 2012). Ginestet et al. (1998) studied the toxicity effect of azide and observed that it impaired NOB more than AOB at a concentration of 0.3 µmol/L with a resultant 50% nitrite accumulation. Similarly, Philips et al. (2002) reported inhibitions of NOB with cyanate and hydrazine.

At the moment, most studies regarding AOB enrichment for partial nitritation have focussed on substrate concentration and temperature control. However, pH and DO concentration control have received less attention.

2.4 The anammox bacteria

Anammox bacteria are a specialized group of chemolithoautotrophs that are major contributors to the global nitrogen cycle (Hirsch et al., 2011). They form a distinct, deep branching phylogenetic group within the phylum Planctomycetes, under the unculturable genus *Candidatus* (Shu et al., 2011). At present, five different genera have been described, as outlined in Table 2-1 (Boumann et al., 2009, Ali et al., 2013, Awata et al., 2013). These include the genera *Brocadia*, Kuenenia, Scalindua, Anammoxoglobus and Jettenia (Strous et al., 1999a, Schmidt et al., 2003, Kartal et al., 2004, Tsushima et al., 2007, Jetten, 2008, Bagchi et al., 2012).

Table 2-1: Currently Elucidated Anammox Species

Genus	Species	Electron Acceptor	References
Brocadia	*Candidatus* Brocadia anammoxidans	NO^{2-}	(Strous et al., 1999a)
	Candidatus Brocadia fulgida	NO^{2-}	(Kartal et al., 2004)
	Candidatus Brocadia sinica	NO^{2-}	(Hu et al., 2010)
Kuenenia	*Candidatus* Kuenenia stuttgartiensis	NO^{2-}	(Schmid et al., 2000)
Scalindua	*Candidatus* Scalindua brodae	NO^{2-}	(Schmidt et al., 2003)
	Candidatus Scalindua wagneri	NO^{2-}	(Schmidt et al., 2003)
	Candidatus Scalindua sorokinii	NO^{2-}	(Kuypers et al., 2003)
	Candidatus Scalindua Arabica	NO^{2-}	(Woebken et al., 2007)
	Candidatus Scalindua sinooifield	NO^{2-}	(Li et al., 2010)
	Candidatus Scalindua zhenghei	NO^{2-}	(Hong et al., 2011)
	Candidatus Scalindua richardsii	NO^{2-}	(Fuchsman et al., 2012)
Jettenia	*Candidatus* Jettenia asiatica	NO^{2-}	(Tsushima et al., 2007)
Anammoxo-globus	*Candidatus* Anammoxoglobus propionicus	NO^{2-}	(Kartal et al., 2004)
	Candidatus Anammoxoglobus sulfate	SO_4^{2-}	(Liu et al., 2008)

Although they possess similar morphological and physiological characteristics, the divergence between the five different anammox genera is relatively large and the sequence similarity at the 16S rRNA gene level is often less than 85% (Jetten et al., 2003, Boumann et al., 2009). The high degree of genetic variance could explain the existence of many unique characteristics that set them apart from other bacterial clades (Kartal et al., 2004). These characteristics include:

- Highly diverse cell morphology and cell arrangement across genera (Shu et al., 2011).
- Intracellular compartmentalization in the form of an Anammoxasome
- Diverse cell wall constituents such as the distinct lack of peptidoglycan in the anammox cell wall and the especially remarkable ladderane lipid composition of the anammoxasome wall (Rattray, 2008, Shu et al., 2011).
- Extremely diverse, and relatively unique metabolic requirements and ecological niches (Shu et al., 2011). Anammox activity has been reported both at temperatures as low as -2.5°C in sea ice, and as high as 70°C in hydrothermal vents (Rattray, 2008).

2.5 Anammox Metabolism and Growth

The Anammox process and its potential application to wastewater treatment are currently in vogue. In comparison to the traditional nitrification-denitrification process, this autotrophic process consumes 100% less biodegradable organic carbon and at least 50% less oxygen, thus greatly lowering operating cost (Breisha, 2010). The stoichiometric anammox reaction (Figure 2-2), outlines the balanced reaction for the oxidation of NH_4^+ under anoxic conditions.

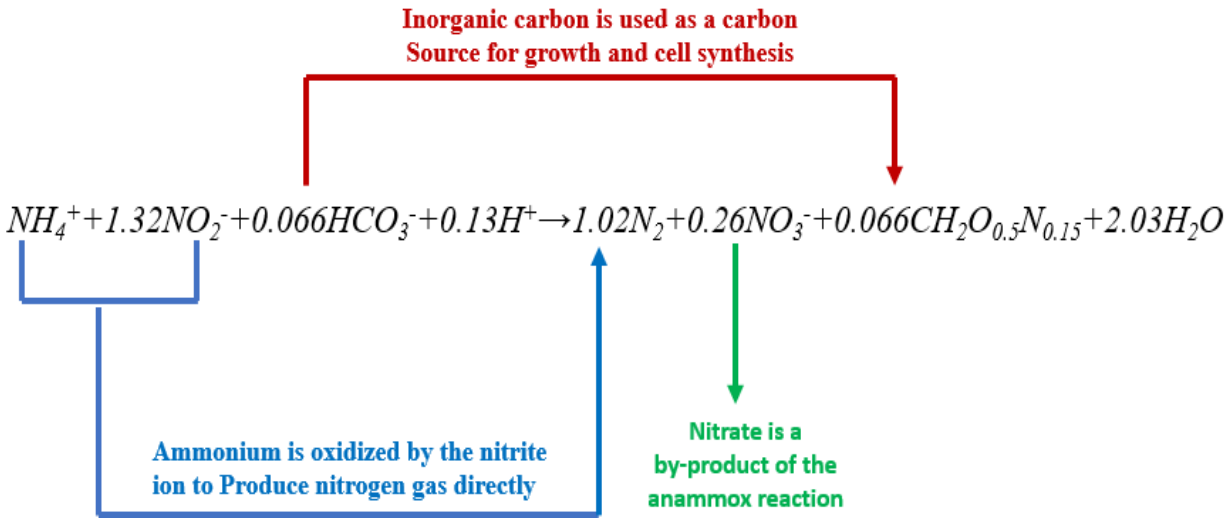


Figure 2-2: The balanced anammox reaction

As indicated from the reaction above (Figure 2-2), the anammox metabolic reaction requires an optimum ratio of 1mol NH_4^+:1.32 NO_2^-, no organic carbon and acts in the complete absence of oxygen. The inorganic carbon used for cell synthesis is also low, indicating that the anammox bacteria is an extremely slow-growing organism with a μmax = 0.065 d^{-1} and a doubling time (t1/2 = ln2/μmax) of 11 days (Ni and Zhang, 2013). Its slow growth rate is characteristic of the K strategist model of growth − where a specific population may have a high substrate affinity and a low maximum growth rate adapted to low substrate concentrations (Whang et al., 2009). Most significantly, detailed studies demonstrated a maximum specific ammonium oxidation rate of 55 nmol NH_4/min/mg protein with nitrite as an electron acceptor − a reaction which is 25-fold faster than chemolithotrophic ammonium oxidation with *Nitrosomonas* spp. (Keller et al., 2002).

2.6 Anammox Cultivation and Enrichment

Since anammox bacteria have not yet been isolated in pure culture, they require very specialized growth conditions to be cultivated. In many instances, previous studies have focused on the selective enrichment of anammox bacteria from a mixed environmental microbial community. By controlling the growth conditions of the environmental sample to favour the growth of anammox bacterial communities, a specialized N-removing consortium can be selected for (Gonzalez-Gil et al., 2014). This consortium would consist of the main nitrifying or denitrifying populations, as

well as the anammox populations which exist at a higher population density relative to the other populations (Egli et al., 2003, Burmølle et al., 2014, Gonzalez-Gil et al., 2014). Table 2-2 below outlines the optimum growth conditions for anammox bacteria, as per previous enrichment studies, however, it should be noted that many of these values are biomass dependent and would need to be tailored to the Specific anammox Activity (SAA) prevailing within the experimental reactors.

Table 2-2: Important Anammox Growth Parameters

Parameter	Optimum Range	Inhibitory Range	Caveat	Reference
Nitrite Conc.	Dictated by the SAA of the culture	11 mg/L HNO2	FNA is inhibitory	(Fernández et al., 2012, Puyol et al., 2014b)
Ammonia Load	Dictated by the SAA of the culture	>20 mg/L FA	FA in inhibitory	(Puyol et al., 2014a, Fernández et al., 2012)
Nitrate Conc.	-	50 mM	Needed for denitrification	(Suneethi et al., 2014)
Sulphide Load	-	>32 mg/L (pre-acclimation)	Substrate dependent	(Duan et al., 2013)
pH	7.2-7.6	6.8>pH>8.0	Mixed Cultures, Granules or continuous/ recycled systems	(Jin et al., 2012)
Temperature	30-40	T>40 or T<20	temperature affects FA and FNA conc.	(Lotti et al., 2015)
Organic Carbon	Not needed for the Anammox Process	>2 mM of most organic C compounds	Co-cultures of anammox with heterotrophic denitrifiers	(Güven et al., 2005, Jenni et al., 2014)
Inorganic Carbon	HCO_3^-: TN ratio of 1.2	<1.2 mg-C/L	Carbonic acid may decrease the pH and result in the formation of FA and FNA.	(Kimura et al., 2011, Jin et al., 2014)
HRT	-	-	Dictated by SAA of the culture	-

Parameter	Optimum Range	Inhibitory Range	Caveat	Reference
SRT	Biomass needs to be retained for as long as possible.	-	Enrichment periodic washout of faster-growing organisms	(Lotti et al., 2014)
Dissolved Oxygen	Anaerobic	<1% air saturation	Co-cultures of anammox and Nitrifiers	(Jin et al., 2012)

Considering that anammox bacteria cannot yet be cultivated in pure culture, obtaining suitable quantities of the inoculum makes its study and investigation difficult. This is especially true for large scale biotechnological applications, where the sensitivity and slow growth rates characteristic of the anammox species make such systems prone to failure (Ni and Zhang, 2013). This can be circumvented through the use of specially designed enrichment bioreactors that provide a rigorously controlled environment specifically for the selection, enrichment and long term maintenance of these fastidious organisms. Although the literature is extensive, the cultivation of anammox from conventional sludge is still cumbersome. Some fundamental problems that are common to many enrichment studies is the need to maintain anaerobic environments, to optimally control N-loading, and to somehow retain the slow-growing anammox biomass within the reactor while simultaneously washing out competing populations (Lotti et al., 2014, Pérez et al., 2014, Suneethi et al., 2014). Consequently, the use of a suitable reactor configuration and feed-regime that allows for these needs is essential.

The sequencing batch reactor (SBR) was the most common experimental set-up for anammox enrichment and has been successfully applied to many anammox enrichment studies. Despite this, the batch mode of operation offered by the basic SBR may not provide ideal conditions for long term anammox cultivation since Anammox bacteria are often inhibited by their substrates as well as suboptimal nitrogen loading (Carvajal-Arroyo et al., 2014a, Carvajal-Arroyo et al., 2014b). As such, reactors with a continuous mode of operation are currently preferred. Continuous reactor operation has been applied to other reactor types that are modified for maximal biomass retention, granule formation or attached growth systems (Bagchi et al., 2012, Lotti et al., 2014). Table 2-3 below outlines the different reactor types used in previous enrichment studies and the benefits of each for successful anammox cultivation.

Table 2-3: Preferred Anammox Reactor Types

Reactor Type	Advantages	Disadvantages	References
SBR	• Useful for the screening of environmental samples • Relatively simple to set up and operate.	• Not suitable for scale-up or mass cultivation • May get biomass loss during decanting phase	(Hu et al., 2005, Jin et al., 2008, Bagchi et al., 2012)
UASB	• Promotes the formation of granules • Efficient mixing and mass transfer • Good biomass retention	• Shear forces can become difficult to control on an undefined culture • Equipment and setup costs are high and relatively complex.	(Abma et al., 2007, Ma et al., 2013)
CSTR	• Efficient mass transfer • Continuous operation	• Equipment and setup costs are high and relatively complex.	(Suneethi et al., 2014)
MBBR	• Utilizes carrier particles − thus enabling longer SRT • Can be operated in a variety of modes and configurations	• Limited literature is available on the most suitable type of carrier material. • Mass transfer and bulk mixing can become inefficient depending on the particles used.	(Ekström, 2010, Regmi et al., 2016)
FBR	• No wasting of sludge − extremely high SRT • Stratification of target populations	• Difficulty sampling • Low control over mass transfer and nutrient diffusion through the bed	(Gao and Tao, 2012)
MBR	• Optimum biomass retention through attached growth/ biofilm formation. • Ideal for co-culture systems where bacterial niches are allowed to form naturally.	• Membrane fouling • Difficulty in sampling at low culture densities (i.e. at the initial stages of enrichment)	(Van Der Star et al., 2008, Bagchi et al., 2012, Huang et al., 2016)

2.7 Factors affecting the success of enrichment

The anammox bacterial species are highly sensitive to many biotic and abiotic factors within their microenvironment. These factors are often multifaceted, either enhancing growth or inhibiting growth as a function of concentration. Incongruously, even the growth substrates (ammonia and nitrite) that are essential to the anammox metabolism can become inhibitory above a specific concentration threshold, at which point they will be deleterious to anammox bacterial growth (Hu et al., 2012). Substrate concentration, pH, temperature, dissolved oxygen, organic matter, salinity, sulphide, and biomass retention have been shown to significantly impact the success of enrichment: either by directly or indirectly affecting the growth of anammox bacteria.

Unfortunately, experimentally derived values for these factors often differ significantly from each other, particularly about the effects of minimum inhibitory concentrations (IC_{50}). This trend is observed in many batch anammox experiments due to the use of non-standardized experimental design, the difference in anammox sludge types, uncharacterized relative microbial community composition, or unquantified degree of enrichment; thus it is difficult to accurately determine inhibitory or stimulating concentrations from literature alone (Jaroszynski et al., 2012). Nonetheless, it does serve as an approximate guideline for further anammox enrichment attempts. These inhibitory factors are discussed below in detail.

2.7.1 Substrate Concentrations, pH and Temperature

As per the metabolic pathways discussed above (Figure 2-3), NH_4^+ as the primary substrate is oxidized by NO_2^- to generate N_2 gas, and thus form the total N complement required for cellular metabolism. According to the anammox stoichiometric ratio, the N-source in the influent feed matrix should be 1 part NH_4^+: 1.32 parts NO_2^-, however, this ratio is the hypothetical optimum based on metabolic calculations for pure culture, and may not directly apply to enrichment studies, especially those initialized with a mixed microbial population containing very low concentrations of anammox bacteria (Van der Star et al., 2007).

A gradual increase of total N, according to the activity of the constituent biomass, allows for the microbial population to adjust to the new conditions within the reactor system, preventing overloading. Furthermore, anammox bacteria are particularly prone to inhibition effects from both NH_4^+ and NO_2^-, particularly in the form of Free Ammonia (FA) and Free Nitrous Acid (FNA) respectively. The FA is a function of pH and can diffuse into the cell through the cell

membrane, changing the cytoplasmic pH and neutralizing the membrane potential (Jaroszynski et al., 2012). Jaroszynski et al. (2012) further determined that FA had directly affected specific anammox activity (SAA) when the FA exceeded an inhibitory threshold of 2 mg N/L. In direct contrast, Puyol et al. (2014a) stated that it was the high pH and not FA that caused anammox inhibition, implying that operating the reactors at a pH of below 7.6 will not allow for the formation of inhibitory FA concentrations. This discrepancy in results could again be due to the differences in microbial diversity and growth modes between these studies, as non-standardized anammox sludge from vastly different types of reactors were used for the comparison.

Similarly, Puyol et al. (2014b) also stated that FNA is less inhibitory than the ionized NO_2^- species, in direct contrast to results shown by Ma et al. (2010) and Fernández et al. (2012). Regardless, NO_2^- is toxic to a wide variety of microorganisms by inhibiting the production of adenosine triphosphate (ATP) through the destabilization of proton gradients (Carvajal-Arroyo et al., 2014a). At high pH, FNA formation is minimal, and often lower than the reported inhibitory values of <0.2 mg/L (Chai et al., 2015).

Thus, to prevent the accumulation of the inhibitory FA and FNA, and to maintain the optimum metabolic performance of the constituent microbial populations, the concentrations of Ammonia and Nitrite substrates, as well as the pH and temperature must be controlled within an optimal range.

2.7.2 Dissolved Oxygen

Being an obligate anaerobe, dissolved oxygen is one of the most critical control factors for the proliferation of anammox bacterial species. It was initially reported that anammox bacteria are reversibly inhibited at very low DO concentrations of 0.5%, while a higher oxygen concentration (>18%) leads to irreversible inhibition (Egli et al., 2003, Jin et al., 2012). Although the anammox species itself is highly susceptible to oxygen inhibition, when grown in a co-culture with aerobes the anammox process exhibits some form of recalcitrance − even under largely aerobic conditions (Zekker et al., 2014). Co-culture studies have shown anammox recovery even after exposure to fully aerobic conditions (8 mg-O_2/L) (Hu et al., 2013). This unusual tolerance of anammox bacteria to relatively high concentrations of DO as an anaerobe is hypothesized to be through the spatial distribution of the anammox bacteria within the floc or granule (Ding et al., 2013, Zekker et al., 2014). Growth of an anammox co-culture with

aerobic autotrophs is currently being investigated in reactor systems like the CANON, SNAD and OLAND systems, where DO is a growing requirement for the coexisting populations. Nevertheless, stringent control over the DO within the system is essential to prevent of overgrowth of the aerobes and out the competition of the anammox population, and recent studies have indicated maintaining a constant DO concentration of 0.5-1 mg-O2/L will effectively stabilize the delicate balance between the AOB, NOB, and anammox bacterial populations (Zekker et al., 2014, Hu et al., 2013).

2.7.3 Carbon compounds

Anammox bacteria are chemolithoautotrophic, and utilize inorganic Carbon as a C source, often in the form of the bicarbonate ion (HCO_3^-). As per their metabolic needs, the inorganic Carbon is required in minimal concentrations relative to those of NH_4^+ and NO_2^- (Inorganic Carbon: TN = 1.20) (Jin et al., 2014). The HCO_3^- contained in the media may also assist in buffering the media through the formation of carbonic acid (H_2CO_3), and preventing the formation of FA and FNA.

Although anammox bacteria are autotrophs, low concentrations of organic carbon have been shown to enhance anammox activity (Güven et al., 2005, Dapena-Mora et al., 2004, Jin et al., 2012). Some types of anammox bacteria have also been shown to consume organic compounds, such as formate, acetate, and propionate to sustain their metabolism, however, they are strongly inhibited by similarly low concentrations of methanol (Kartal et al., 2007, Güven et al., 2005). Conversely, high concentrations of organic matter have been found to inhibit anammox activity (Jin et al., 2012). This mechanism of organic matter inhibition has yet to be verified, although two possible mechanisms have been hypothesized for the inhibition effects:

- The first is the phenomenon of out-competition. Due to the slow growth rates of anammox bacteria about competing heterotrophs, the addition of organic substrates would result in the heterotrophs out-growing − and by extension, outcompeting-the Anammox bacteria within the same system (Chamchoi and Nitisoravut, 2007, Lackner et al., 2014, Güven et al., 2005).
- The second hypothesis is that in the presence of high organic loads, the anammox bacteria may switch to an alternate metabolic pathway designed to metabolize the organic matter instead of the ammonia and nitrite (Güven et al., 2005). As the anammox populations are still the dominant species in the system, this type of metabolic pathway

conversion inhibition is usually reversible once conditions that favour the primary anammox pathway return (Kartal et al., 2007, Kartal et al., 2011, Güven et al., 2005).

Toxic organic matter is often regarded as strongly antimicrobial since it often results in cell death or irreversible inhibition upon exposure to very low concentrations. These toxic organic compounds include alcohols, phenolics, aldehydes and antibiotics (Jin et al., 2012).

- Alcohol and aldehydes at low concentrations (3-4 mmol/L methanol) have been found to inhibit anammox bacteria in marine sediments, however, the inhibitory concentrations within artificial systems are greatly varied (Jensen et al., 2007). The observed differences can be attributed to anammox species involved and the prevailing experimental conditions (Jin et al., 2012).

- Phenolic compounds are often found in industrial wastewaters and are often strong inhibitors of microbial activity, showing an IC_{50} of 678.2 mg/L in anammox batch tests. However, when grown in the presence of low concentrations of phenol (12.5 mg/L) the anammox activity is initially depressed, however, the anammox bacteria were able to adapt and recover nitrogen removal capability (Jin et al., 2012, Yang et al., 2013a).

- Some antibiotics have been shown to inhibit anammox growth, but the actual mechanism of inhibition and the minimum inhibitory concentrations have yet to be elucidated. The available studies on antibiotic effects are very limited and those that do exist have only focused on chloramphenicol, beta-lactams, and tetracycline (Jin et al., 2012). Although the effects of these antibiotics are largely inhibitory, some enrichment studies have utilized cell wall targeting antibiotics to provide a selectively competitive advantage to the anammox bacteria as they lack a peptidoglycan cell wall (Bagchi et al., 2012).

2.7.4 Iron (Fe_2^+) and other metals

Elemental iron is an important micronutrient for all living organisms and is a key component of numerous biological processes including photosynthesis, respiration, the tricarboxylic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Bi et al., 2014). Similarly, anammox bacterial metabolism utilizes a core of heme-based proteins (i.e.: hydrazine synthase (HZS); hydrazine dehydrogenase (HDH); hydrazine oxidase (HZO)) that requires chelating

ferrous iron to form their active regions (Harhangi et al., 2012, Kartal et al., 2011). Bi et al. (2014) found that an appropriate increase of Fe_2^+ from 0.03 mM to 0.12 mM increased heme-c synthesis, HDH activity and accelerated the start-up of an anammox enrichment reactor. These findings were corroborated by Liu and Ni (2015), who further noticed a decrease in anammox growth rates at high Fe_2^+ concentrations (>0.18 mM), while (Huang et al., 2014) found similar results for manganese (Mn_2^+) ions.

Copper (Cu_2^+) is also an important constituent of some enzymes in anammox bacteria, such as nitrite reductase, however, the inhibitory concentration (IC50) of Cu_2^+ was calculated to 12.9 mg/L, with concentrations as low as 5 mg/L causing almost complete anammox inhibition (Yang et al., 2013b). In contrast, the IC50 calculated by Zhang et al. (2015) was 32.5 mg/L, and that the anammox consortia could resist inhibition by 5 mg/L Cu_2^+. This discrepancy could be due to the different reactor configurations, the sludge type, and the relative microbial community compositions used in the study. The effects of other heavy metals on anammox bacterial growth and activity is still under investigation.

2.7.5 Salinity

As with the other inhibitory compounds; inhibitory effects depend on the type of salt, salt concentration, reactor design and population characteristics (Jin et al., 2012). The IC50 of Na_2SO_4 (at 11.36 g/L), NaCl (at 13.46 g/L) and KCl (at 14.9 g/L) were elucidated by Kartal et al. (2006), however, with long-term operation at high salts concentrations, the anammox bacteria can acclimatize to increased salt concentrations (Jin et al., 2011, Kartal et al., 2006). After adaptation, these anammox bacteria resisted salt stress, and Jin et al. (2011) reported that the salinity inhibition level of the adapted anammox bacterial populations was reduced from 67.5% to 43.1% by this acclimation.

2.7.6 Sulphides

Sulphate reduction to sulphide commonly occurs in anaerobic digestion systems, with this sulphide being in the form of highly toxic, corrosive and malodourous H_2S (Chen et al., 2008, Mahmood et al., 2007, Beristain-Cardoso et al., 2009). Reactor systems designed to enrich for the anammox bacteria under anaerobic conditions are also susceptible to H_2S production, which is problematic as H2S is strongly protein denaturing (Dapena-Mora et al., 2007). According to Jin et al. (2013), the mean IC_{50} for the anammox bacteria biomass was calculated to be

264 mg/L, however, the actual inhibitory concentration varies greatly between conditions and the species tested in each study. A sulphide concentration of 1-2 mM caused the specific anammox activity to decrease by 60%, while anammox activity was completely lost at a sulphide concentration of 5 mM (Dapena-Mora et al., 2007).

In stark contrast to the previous inhibitory data, Van de Graaf et al. (1997) reported that sulphide concentrations of up to 5 mM increased the Anammox activity. One likely explanation for these contrasting results could be attributed to the different anammox populations used in the respective studies. The anammox culture detected by Mulder et al. (1995) originated from a denitrifying fluidized bed reactor that used sulphide and organic acids as the major electron donors, while Dapena-Mora et al. (2007) used an enriched sludge from a municipal WWTP treating domestic wastewater. The dominant anammox species in the population originally observed by Mulder et al. (1995) was *Anammoxoglobus sulfate* which is more acclimatised to high sulphur conditions and uses SO_4^{2-} as an electron source.

2.7.7 Biomass Retention

Due to the K-strategist model of growth applied by the anammox bacteria, they are often outcompeted by faster growing, rate-specialist organisms. Selective biomass retention, that allows for the selective washout of faster-growing competing organisms, while still maximizing anammox bacterial retention, is amongst the most important parameters for both applying the anammox process at full scale, as well as during the enrichment phase (Jubany et al., 2008, Laureni et al., 2015, Tao et al., 2012). Furthermore, a growing body of evidence conjectures that anammox bacteria grow best in the presence of other bacteria through interspecies signalling responses, however, the relative populations of each within the biomass fraction need to be stringently controlled to prevent out-competition of the target anammox population (Chong et al., 2012, Geets et al., 2006, Gao et al., 2014a, Ding et al., 2013).

Reactor systems with high biomass retention, such as an immobilization or granulation process are ideal, and these types of systems can provide almost infinite biomass retention, create suitable growth niches for synergistic bacteria, and protect the anammox bacteria from inhibition effects (Ahn, 2006). To date, many different reactor designs have been used to enrich for anammox bacteria and will be further discussed below.

2.8 The Nitritation-Anammox Process

In the last two decades, several biological nitrogen removal technologies based on anaerobic ammonium oxidation (anammox) have been developed to treat ammonium rich wastewaters with low carbon to nitrogen ratio. In the anammox reaction; ammonia is oxidized to dinitrogen gas in the absence of oxygen, using nitrite as an oxidizing agent and electron donor, while carbon dioxide is used as a carbon source for cell growth. Consequently, the anammox process displays many advantages over the conventional biological nitrogen removal processes, particularly about the energy and associated cost saving (Gao et al., 2014a). It has been approximated that for the treatment of domestic and low C/N industrial wastewater, the need for organic carbon decreases by 100%, aeration requirements by 60% and sludge production by 90% (Lackner et al., 2014, Mulder, 2003). To exploit the potential of the anammox process at full scale, several new biological nitrogen removal processes have been developed around the anammox process. These designs are now commercially available and maximize the advantages of the anammox process as a way to shortcut conventional nitrification-denitrification while minimizing potential anammox inhibition. As far back as 2015, there are over 100 full-scale nitritation-anammox mediated systems installed across the globe as documented in detail by Bowden et al. (2015).

A common trait shared by many of these processes is that they involve partial nitrification (PN) or nitritation (oxidation of ammonia to nitrite) followed by anoxic oxidation of the remaining ammonia (by the anammox bacteria) in the presence of the generated nitrite as the electron acceptor (Bagchi et al., 2012, Keluskar et al., 2013). These PN/A processes utilize a particular combination of Ammonia oxidizing bacteria (AOB) and anammox bacteria to achieve autotrophic removal of nitrogen (Ding et al., 2013, Philips et al., 2002, Sliekers et al., 2003). It is both convenient and economical to achieve 50% partial nitrification by AOB (up to a condition wherein half of the ammonia present is converted to nitrite), followed by the anammox process to ensure total nitrogen removal (Breisha, 2010) (Figure 2-3). Complete autotrophic nitrogen removal (CANR) from the influent wastewater stream is an innovative process that can increase loading rate by about 5 times compared to the conventional nitrification-denitrification processes and is, therefore, a much-desired alternative process.

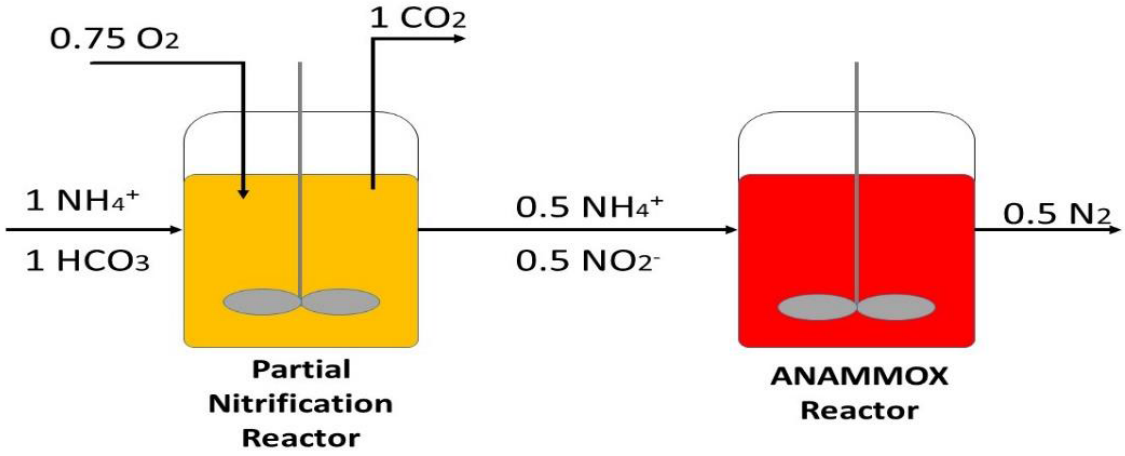


Figure 2-3: A schematic of the two-stage PN/A process

The application of the autotrophic nitrogen removal process may be applied to a single system, where both reaction stages occur simultaneously within the same reactor system or applied to a two-stage configuration, where the stages are spatially separated. Both single-stage and two-stage systems have been investigated to varying degrees of success, particularly in reactor systems like the CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) (Chang et al., 2013)), SNAD (Simultaneous partial Nitrification, Anammox, and Denitrification) (Langone et al., 2014), SHARON (Single reactor High activity Ammonium Removal Over Nitrite) (Shalini and Joseph, 2012) and OLAND (Oxygen Limited Autotrophic Nitrification Denitrification) (De Clippeleir et al., 2011) systems. These systems represent complex multi-species ecosystems that synergistically degrade influent nutrient loads. In the case of CANR (Complete Autotrophic Nitrogen Removal) (Mauricio-Iglesias et al., 2015), as in many other bioreactors, the coexistence of several kinds of microbial groups makes the control of such a system particularly challenging. Many of these microbial groups have similar growth conditions or require conditions that may be inhibitory to other bacterial groups. Additionally, operational conditions, reactor configurations and the microbial community within all have a direct effect on the efficiency of the process, making it difficult to accurately predict the performance of an anammox system.

2.8.1 The 2-stage Nitritation-Anammox Process

Partial nitritation-anammox remains one of the most important innovations in biological wastewater treatment in recent times which can be implemented in either single- or two-stage reactors. Presently, maintaining an efficient NOB washout strategy whilst retaining anammox and AOB in the same system is one of the most challenging goals in running a single-stage reactor (Stefansdottir et al., 2018). This phenomenon has led to exploring the possibility of a two-stage anammox reactor (PN-A reactor) whereby nitritation and anammox reactions are achieved in two separate reactors that are coupled (Piculell et al., 2016). In the 2-stage PN-A reactors, the nitritation and anammox reactions may be optimized individually.

According to (Dosta et al., 2015) the 2-stage PN-A process reduces the risk of heterotrophic competition with anammox bacteria and their inhibition by dissolved oxygen. (Liu et al., 2018) noted that the first-stage i.e. the partial nitritation, plays a positive role in conferring stability on the anammox community in the coupled second stage. Earlier on, studies have shown that the presence of biodegradable organic matter in wastewater could impact negatively the anammox process (Chamchoi et al., 2008, Kumar and Lin, 2010). However, in the two-stage PN-A reactors, the first stage (PN) could serve in removing this biodegradable organic matter from wastewater and thus enhance the stability in subsequent anammox reactor (De Graaff et al., 2011). Also, achieving high-rate mainstream ammonia removal through the two-stage PN-A process has been reported to be more feasible than via the single-stage process (Isanta et al., 2015, Reino et al., 2018).

Nitrosomonas europaea and one species within *Candidatus* Brocadia have been reported as the dominant functional bacteria in the partial nitritation and anammox reactors, respectively (Liu et al., 2018). Operation of PN reactor rich with ammonium in excess favours the selective enrichment of r-strategist AOB species such as *Nitrosomonas europaea* (Ahn et al., 2008, Reino et al., 2016). Earlier studies by Dosta et al. (2015) also identified members of the candidate genus Brocadia, identified as the key players in anammox activity and immigrant bacteria from the PN reactor to the following anammox reactor had no negative effect on the anammox function (Liu et al., 2018). Li et al. (2014) reported that AOB in the PN-SBR was mainly affiliated to *Nitrosomonas* sp. IWT514, and *Nitrosomonas eutropha*, whilst the anaerobic ammonium oxidizing bacteria in the anammox reactor were mainly affiliated to *Kuenenia stuttgartiensis*.

According to Jaroszynski and Oleszkiewicz (2011) a single-stage PN-anammox reactor system is simple in configuration, but the complex interactions that exist among AOB, NOB and anammox bacteria can be a great limitation. Moreover, the single-stage system needs stringent DO and pH control. However, the complex configuration in the two-stage reactor system may allow simpler system operation with improved performance and stability. Currently, the knowledge biochemical processes in a two-stage PN-anammox system are still incomplete and thus, require more studies. To this end, this study aimed to develop a stable 2-stage PN-A system for the removal of nitrogen from wastewater.

CHAPTER 3

OPTIMIZATION OF REACTOR CONDITIONS FOR ENRICHING AMMONIA OXIDIZING BACTERIA

3.1 Introduction

Biological wastewater treatment harnesses the ability of the microorganisms to metabolize the pollutants in wastewater for synthesizing their cells' building blocks. These bioreactors are configured and operated in a way to select and enrich the specialized microbial consortium that is required for nutrient removal (Lopez-Vazques, 2009). The combination of partial nitritation and anaerobic ammonium oxidation has received a lot of attention recently due to the advantages it has as compared to other methods (Daverey et al., 2013, Blackburne et al., 2008). To date, no member of the anammox bacterial group has been cultured as a bacterial isolate. They are often found in the presence of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), as well as denitrifying bacteria. These organisms represent the nitrifying and denitrifying groups respectively, which act synergistically as an N removing microbial consortia. In most instances, this synergism is based on competition for N-substrates, where the metabolic by-products of one group forms primary substrates of another (Figure 3-1). Effective control of specific environmental factors can enhance the growth of specific groups while selectively inhibiting the growth of others. The most common enrichment strategies, as well as the full-scale commercial application of anammox has involved the concurrent enrichment of both anammox bacteria and AOB. The AOB group is responsible for the first stage of nitrification, which is the conversion of ammonia to nitrite. Both ammonia and nitrite are primary substrates for the anammox reaction.

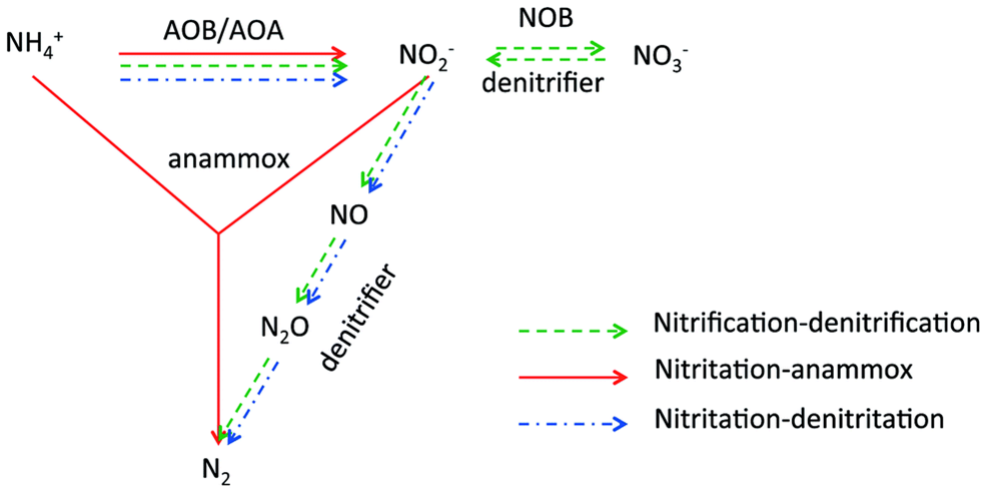


Figure 3-1: The Nitrogen removal pathways (Gao et al., 2014b)

Conventionally, the partial nitritation and anaerobic ammonium oxidation are conducted in a single reactor, however, efficient suppression of NOB growth during long-term operation is difficult. The NOB thus competes with anammox bacteria for nitrite and decreases the overall nitrogen removal efficiencies (Li et al., 2013). Therefore, two sequencing batch reactor system has been proposed whereby the effluent from partial nitritation reactor is used as the feed for the second reactor in which anaerobic ammonium oxidation would occur (Lackner et al., 2014). However, certain operational conditions need to be monitored for this simultaneous process of partial nitritation and anaerobic ammonium oxidation (anammox) to be successful (Li et al., 2011a). These operational conditions include dissolved oxygen concentration, temperature, HRT, substrate concentration and pH. The low dissolved oxygen concentration, temperature, hydraulic retention time and pH have been successfully used for partial nitritation, enriching AOB and oxidizing ammonium to nitrite while inhibiting NOB (Ciudad et al., 2007, Guo et al., 2010a, Van Hulle et al., 2007).

This chapter focused on the start-up of multiple laboratory-scale reactors for selective enrichment of AOB and Anammox bacteria respectively, and the selective enrichment of these species over the other organisms within the system. Physicochemical analysis of microbial biomass, chemical speciation for different forms of C and N in the growth media, and off-gas analyses were performed to close the mass balances of each reactor system. This was corroborated with quantitative PCR analysis to determine target population growth in each reactor. The enrichment of these organisms was enabled in three ways:

1. Enrichment of AOB from a conventional activated sludge/MLE system that is known to have an efficient nitrification system

2. Enrichment of anammox bacteria from activated sludge obtained from local WWTP

3. Mass cultivation of anammox bacteria from a previously characterized seed source obtained from an anammox enrichment reactor

3.2 Materials and Methods

3.2.1 The enrichment of AOB using SBR reactors

The major focus of this objective was to facilitate partial nitritation by enrichment of AOB while inhibiting NOB activity. The study was conducted in two identical cylindrical 3 L laboratory-scale sequencing batch reactors (SBR) with a working volume of 2.5 L. Magnetic stirrers at 100 rpm were used to ensure proper mixing. The reactors were placed inside the same incubator to maintain the same constant temperature of 35℃ for the entire experiment. Aeration was provided by the small air pump which was connected to a rotameter to control the flow rate. This pump was also connected to a timer to control the aeration period throughout the cycle.

3.2.2 Feed

An initial sample was sampled from the Kingsburgh wastewater treatment plant. Temperature, DO, and pH measurements of the initial sample were carried out on-site using the YSI 556 MPS (Multiprobe System) and before it was transported to the laboratory on the ice where the SBR reactors (for AOB enrichment) were seeded with an initial volume of 1 L. The initial NH_3-N, NO_2-N, NO_3-N concentrations of the seed sample were also measured on day 0.

3.2.3 Synthetic wastewater media

The synthetic media was prepared and fed to the SBR reactors throughout the experimental period (Table 3-1). The NH_3-N concentration was varied according to the rate at which ammonium is used upper cycle. The trace elements solution was also added to the media in minute concentrations as outlined by Table 3-2. The NH_3-N concentration fed was between the range of 20-100 mg/L as shown in Table 3-3. Ammonium sulphate was used as the ammonium nitrogen source.

Table 3-1: Synthetic wastewater composition

Chemical	Concentration (g/l)
$(NH_4)_2SO_4$	As per Table 2-4
$NaHCO_3$	0.42
KH_2PO_4	0.064
K_2HPO_4	0.064
$CaCl_2.2H_2O$	0.18
$MgSO_4.\ 7H_2O$	0.059
$FeSO_4$	0.009
Trace Elements	2 ml

Table 3-2: Trace elements composition

Chemical	Concentration (mg/l)
EDTA	0.15
$ZnSO_4.7H_2O$	0.43
$CoCl_2.6H_2O$	0.24
$MnCl_2.4H_2O$	0.99
$CuSO_4.5H_2O$	0.25
$NaMoO_4$	0.22
H_3BO_4	0.014
$NaSeO_4.10H_2O$	0.050

Table 3-3: Ammonium nitrogen concentration in the feed

Day	Average concentration (mg/l)
1-24	20.51
24-32	25.62
33-44	30.50
45-56	35.26
57-68	40.41
69-80	45.23

Day	Average concentration (mg/l)
81-90	50.28
91-98	55.45
99-110	60.40
111-122	65.37
123-134	70.45
135-144	75.46
145-158	80.4
159-168	85.36
169-178	90.28
179-185	95.56

3.2.4 Operational Parameters

The reactors were fed and drawn manually with a volume of 1 L of synthetic media. The HRT of the reactors was between 1.6-2.5 days depending on the ammonium nitrogen utilization rate. The airflow rate was kept between 0.5-0.9 litres/min depending on the oxygen uptake rate. Calculations of FA and FNA were according to method earlier described by Gokal (2017) and (Jiang et al., 2019). The two reactors operated at different DO concentration ranges at the same pH range as outlined in Table 3-4. A pH controller including a dosing pump and pH probe demonstrated in the figure was employed in controlling the pH during the experiment. The DO concentration was monitored by DO probe meter. The DO concentration was maintained within working ranges by sparging with 95% Arg, 5% CO_2.

Table 3-4: The operational conditions for the experimental runs in the SBR reactors for AOB enrichment

	AOB-SBR REACTOR 1	AOB-SBR REACTOR 2
Run 1	pH (7.5-7.9), DO (0.3-1) mg/L	pH (7.5-7.9), DO (1.3-2.0) mg/L
Run 2	pH (8-8.5), DO (0.3-1) mg/L	pH (8-8.5), DO (1.3-2.0) mg/L

3.2.5 Sample Collection

About 50 ml sample was drawn from the reactors, centrifuged and filtered using a 0.45µl syringe filters. The samples were analysed immediately for NH_4^+-N, NO_2^--N, and NO_3-N in DR 6000 to monitor the N-removal performance of each reactor.

3.2.6 Analytical Methods

Chemical analysis of the reactor effluent during the decanting phase of each respective reactor system was carried out to determine the nutrient utilization by the constituent microbial communities. The concentrations of nitrogen in the form of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) in the influent and effluent of the bioreactor were measured daily during the start-up phase and three times a week afterward for the rest of the experiment. Standard water analysis techniques (APHA et al., 1992) were performed with DR/6000 spectrophotometer and Hach kits (Hach CO., Loveland, CO, USA) for the nitrogen species.

Outlet gas was collected in Tedlar gas bags and measured using gas chromatography (GC), using the Agilent 7820 GC-TCD (Agilent, USA). Pure Nitrogen baseline was used as a standard to calibrate the GC, and Argon was used as a carrier gas. The sample was manually injected into the GC.

3.2.7 Calculations

The HRT was calculated as follows:

$$HRT\ (day) = \frac{reactor\ volume\ (L)}{\left(feeding\ rate\ (\frac{L}{day})\right)} \qquad \text{Equation (2.3)}$$

The concentration of FA and FNA were calculated according to (Anthonisen et al., 1976)

Free Ammonia (FA)

$$FA\ (\frac{mg}{L}) = \frac{17}{14} \times \frac{Total\ Ammonia\ Nitrogen \times 10^{pH}}{\frac{k_b}{k_w} + 10^{pH}} \qquad \text{Equation (2.4)}$$

$$\frac{k_b}{k_w} = Exp^{(\frac{6344}{273+T})} \qquad \text{Equation (2.5)}$$

Free Nitrous Acid (FNA)

$$FNA\ (\frac{mg}{L}) = \frac{46}{14} \times \frac{Total\ Nitrite\ Nitrogen}{k_a + 10^{pH}} \qquad \text{Equation (2.6)}$$

$$k_a = Exp^{(\frac{-2300}{273+T})} \qquad \text{Equation (2.7)}$$

The concentration of free ammonia (FA) and free nitrous acid (FNA) were calculated using equations (2.3)-(2.7) respectively. These equations were derived, as a function of pH, temperature and the sum of unionized and ionized forms, TAN and TNN, respectively from acid-base equilibrium (Anthonisen et al., 1976, Langone, 2013).

The ammonium oxidation efficiency (AOE) was estimated according to the following equation:

$$AOE\ (\%) = \left(\frac{NH_4{-}N_{\ influent} - NH_4{-}N_{\ effluent}}{NH_4{-}N_{\ influent}}\right) \times 100\% \qquad \text{Equation (2.8)}$$

Whereby NH_4-N $_{influent}$ is the nitrogen concentration in the liquid after the instant fill phase and NH_4-N $_{effluent}$ is the concentration of the ammonium nitrogen in the effluent at the end of the SBR cycle. The nitrite accumulation efficiency was estimated according to the following equation:

$$NAE\ (\%) = \left(\frac{NO_2{-}N_{\ effluent}}{NO_2{-}N_{\ effluent} + NO_3{-}N_{\ effluent}}\right) \times 100\% \qquad \text{Equation (2.9)}$$

Whereby NO_2-N $_{influent}$ is the nitrite nitrogen concentration in the effluent at the end of the cycle and NO_3-N $_{effluent}$ is the concentration of the nitrate-nitrogen in the effluent at the end of the SBR cycle.

The nitrogen loading rate (NLR), nitrogen removal rates (NRR) and nitrogen removal efficiency (NRE) were determined according to the following equation:

$$NLR\ (kgNm^{-3}day^{-1}) = \frac{Influent\ N\ concentration}{HRT\ (days)} \qquad \text{Equation (3.0)}$$

$$NLR\ (kgNm^{-3}day^{-1}) = \frac{[Influent\ N\ concentration - effluent\ N\ concentration]}{HRT\ (days)} \qquad \text{Equation (3.1)}$$

$$NRE\ (\%) = \frac{Influent\ N\ concentration - Effluent\ N\ concentration}{Influent\ N\ concentration}$$ Equation (3.2)

Where N concentration = (NO_2^--N) + (NH_4^+-N) + (NO_3^--N)

3.2.8 DNA Extraction

A 10 ml sample was taken from the settled biomass in the reactors at the end of the cycle in the SBRs. This sample was further centrifuged to obtain biomass of about 0.3 g, which was used in the extraction. Genomic DNA was extracted from the biomass samples using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany). The manufacturers' instructions were followed in the extraction process. The DNA samples were stored at -20° until further analyses.

3.2.9 Identification and quantification of key bacterial populations

The polymerase chain reaction (PCR) and shotgun sequencing were used in identifying and confirming the presence of key bacterial groups in the reactors (ammonia-oxidizing bacteria, *Nitrobacter* spp., *Nitrospira* spp.). Appropriate primer sets (Table 2-8) were employed for the PCR according to the method described by Gokal (2017) and Graham et al. (2007).

3.3 Results and Discussion

3.3.1 Comparison of SBR reactors 1 and 2 for AOB enrichment

The laboratory-scale sequencing batch reactors for AOB enrichment were operated for 185 days with increasing ammonia-loading rate as per Table 2-4. In the start-up phase (1-24 days), the DO concentration was controlled to be in the range of 0.3-1.0 mg/l for reactor 1 and 1.3-2.0 mg/l for reactor 2 (Figure 3-2). Figures 3-3 and 3-4 represent the measured nitrogen concentrations in the form of ammonium, nitrite, and nitrate in the effluent for reactors 1 and 2, respectively as well as the influent ammonium nitrogen concentration up to 175 days of operation.

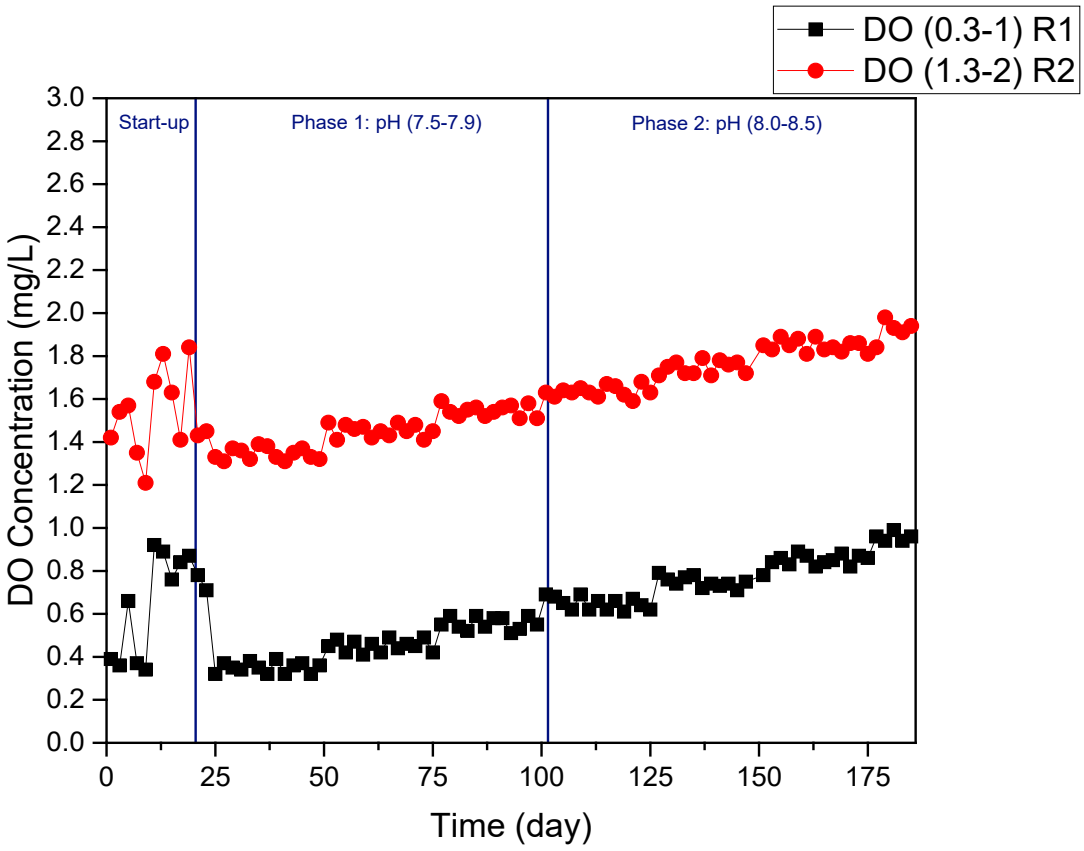


Figure 3-2: The DO concentration (mg/L) in the AOB reactors (R1 and R2) during the study period

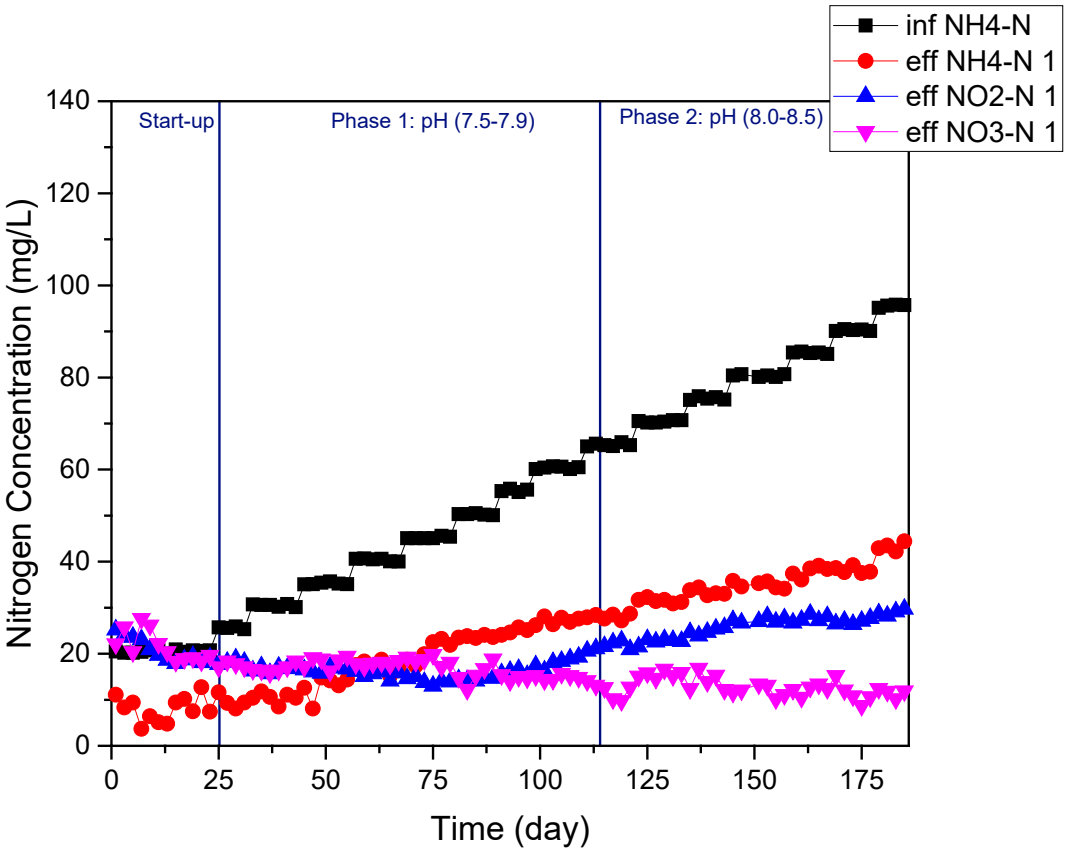


Figure 3-3: The influent and effluent nitrogen concentrations (NH_4, NO_2^-, NO_3^-) in reactor 1 (*R1)*

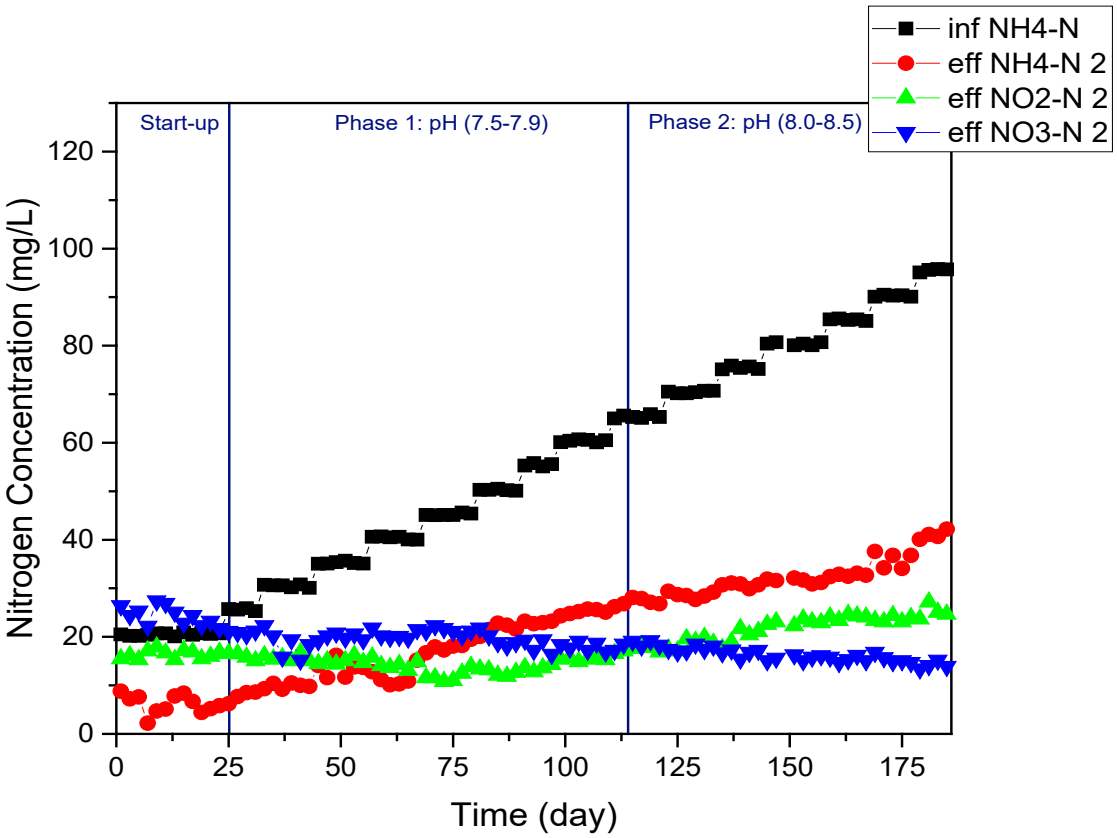


Figure 3-4: The influent and effluent nitrogen concentrations (NH_4, NO_2^-, NO_3^-) in reactor 2

An average ammonium nitrogen concentration of 20.53 mg/L was fed to both reactors 1 and 2 during the start-up period. During the start-up phase, the DO concentration was controlled and was between 0.3-1 mg/L for reactor 1 and 1.3-2 mg/L for reactor 2 (Figure 3-2). In reactor 1, during the first 10 days, the ammonium concentration in the effluent was low with an average of 2.78 mg/L (Figure 3-3). As shown in Figure 3-4, from day 1-10, the ammonium concentration in the effluent was equally low in reactor 2. These low concentrations of ammonium nitrogen may due to the long cycle time of 24 hours that both the reactors were exposed to in the first 10 days. The nitrate-nitrogen concentrations were highest in both reactors 1 and 2 in the initial period (Figure 3-3 and 3-4). This was expected since the enrichment stage conditions were not controlled and hence oxidation of nitrite to nitrate (nitratation) had occurred due to the activity of NOB. It was observed that the nitrite and nitrate concentrations in the initial stage of both reactors 1 and 2 were slightly higher than expected. This could have been as a result of the high nitrogen concentrations of the initial feed samples. This finding thus corroborates with earlier studies by Ruiz et al. (2003) and Yun and Kim (2003) that reported high nitrite and ammonia accumulation from reactors treating simulated industrial

wastewater with high ammonia concentration. Furthermore, pioneer works by Anthonisen et al. (1976) show that nitrite oxidation activity was selectively inhibited in the presence of free ammonia concentration ranging from 0.1-1.0 mg/L whereas inhibition threshold for AOB ranged from 10-150 mg/L. In this present study, the observed FA ranged between 1.87 and 3.18 mg/L as shown in Figure 3-5. This indicates that the reactors ran at the inhibitory FA threshold hence, it contributed to the nitrite build-up and suppression on NOB. On the other hand, the calculated FNA concentration in this study ranged 4.0×10^{-4}-3.1×10^{-3} mg/L (Figure 3-6) which was less than the reported inhibitory concentration of 0.02 mg /L. Hence, the effect of FNA concentration was negligible on NOB in this study. This result is similar to an earlier study by Zhang et al. (2018) who noted that only FA impacted the NOB and not FNA when treating high strength ammonia wastewater.

The DO concentration was increased by an average of 0.1 mg DO/L every 25 days on average for both reactors as displayed in Figure 3-2. This was done to promote the AOB growth while suppressing the NOB growth. In phase 1 of reactor 1 (Figure 3-3), the nitrite-nitrogen started to drop from day 24 to day 77 and gradually increased afterward until the end of the phase. The nitrate-nitrogen concentration in phase I was comparable to the nitrite-nitrogen concentration during the first 50 days, but between the 50^{th} and 80^{th} day, the nitrate-nitrogen concentrations were higher than the nitrite-nitrogen concentrations (Figure 3-3). In phase II, a constant increase in the effluent nitrite-nitrogen was observed.

In reactor 2 (Figure 3-4), the ammonium nitrogen in the effluent which had the lowest concentrations started to increase gradually from day 24 until it reached an average concentration of 11.5 mg/L, which was similar to nitrite-nitrogen on day 68. The nitrite nitrogen, on the other hand, remained lower than nitrate-nitrogen in this phase. However, towards the end of the phase (around 110^{th} day), the nitrite-nitrogen concentrations were comparable to the nitrate-nitrogen concentrations in the effluent. In phase II, the constant increase in the effluent nitrite-nitrogen concentrations was observed from day 113 onwards. On the contrary, a constant decrease in effluent nitrate nitrogen was observed in the reactor, similar to reactor 1 (Figures 3-3 and 3-4).

Therefore, in both reactors 1 and 2, the trend was the same. The nitrate-nitrogen concentration was highest in both reactors 1 and 2 during phase 1. However, it was always at a slightly higher concentration in reactor 2 as compared to reactor 1. There was also a significant difference in the accumulation of nitrite (t-test: <0.05) in both reactors with a higher percentage in reactor 1

(Figure 3-7). It also showed that nitrite oxidation was higher in reactor 2 as compared to reactor 1 which indicates the higher activity of NOB in reactor 2 and turn lower inhibition of NOB compared to reactor 1. In both reactors 1 and 2, the ammonium oxidation was higher than the nitrite accumulation. Since the ammonium oxidation into nitrite and nitrite oxidation into nitrate was based on AOB and NOB activities, respectively, the nitrite accumulation profiles (Figure 3-7) indicated the selective inhibitory impact of FA on NOB in this study. This observation was expected as Zhang et al. (2018) had noted that traditionally, FA is the first choice for nitrite build-up and NOB washout and the calculated FA in this study (Figure 2-8) was above the reported inhibitory levels reported by different authors (Anthonisen et al., 1976, Abeling and Seyfried, 1992).

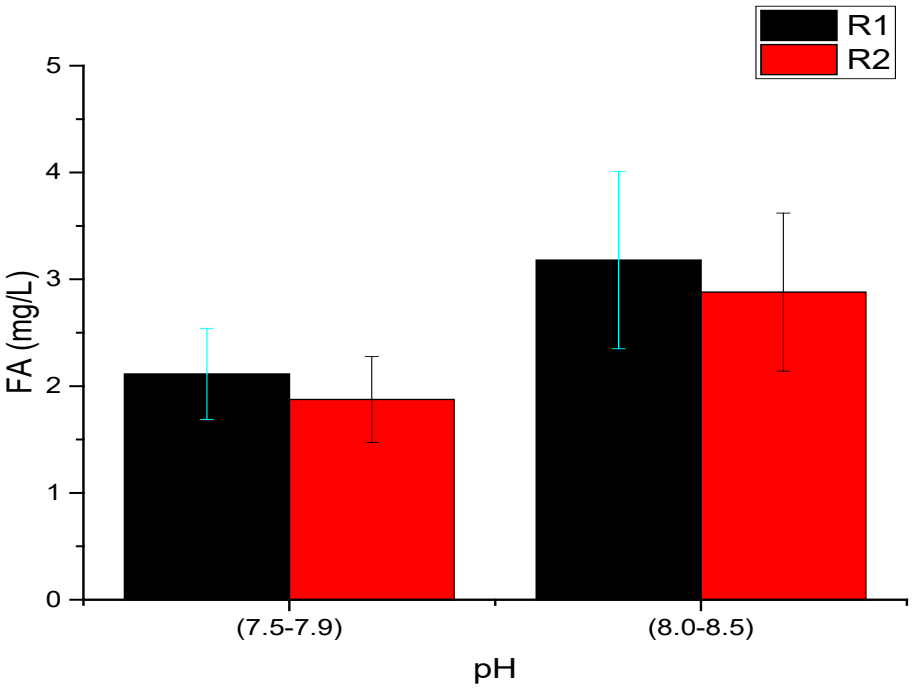


Figure 3-5: The concentration of free ammonia (FA) in reactor 1 and 2

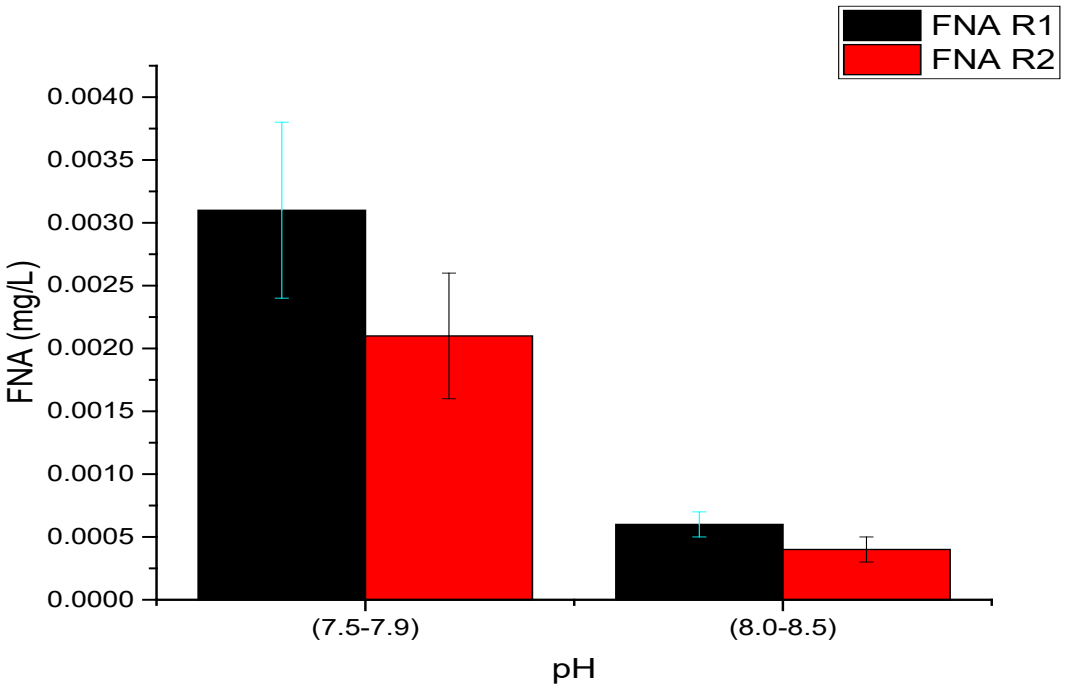


Figure 3-6: The concentration of free nitrous acid (FNA) in reactor 1 and 2

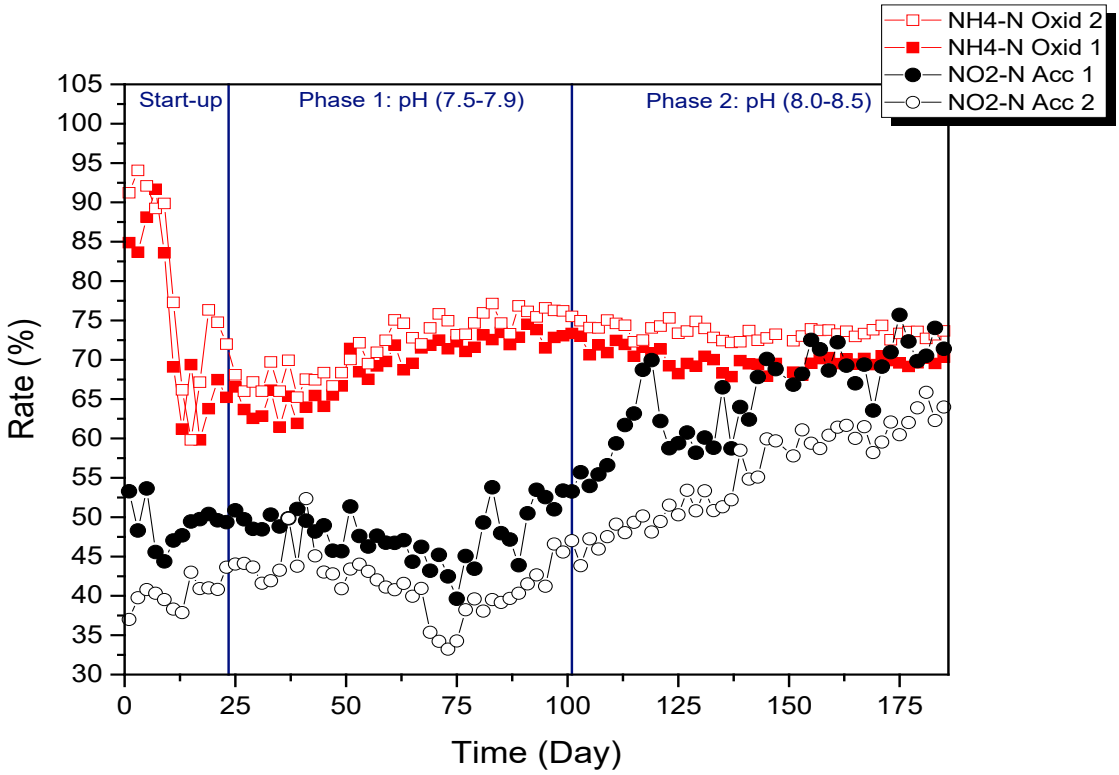


Figure 3-7: Oxidation and accumulation rates of ammonium and nitrite nitrogen in reactor 1 and 2

3.4 Conclusions

Two identical 3 L SBR reactors working at different DO concentrations (Reactor 1: 0.3-1.0 mg/L; Reactor 2: 1.3-2.0 mg/L) were used for AOB enrichment operated at two different phases (Phase 1: pH 7.5-7.9; Phase 2: pH 8-8.5).

- FA concentration achieved in this study was above the inhibitory threshold, hence, it contributed to the suppression of NOB whilst the FNA effect was negligible.
- During the AOB enrichment experiment, nitrite accumulation in both reactors was significantly different (t-test: <0.05) with reactor 1 showing better performance.
- There was evidence of higher nitrite oxidation observed in reactor 2 which signifies a higher proliferation of NOB. Hence, a combination of DO range 0.3-1.0 mg/L and pH 8-8.5 proved to be more effective in achieving a higher NO_2^- accumulation.

CHAPTER 4

OPTIMIZATION OF REACTOR CONDITIONS FOR ENRICHING ANAMMOX BACTERIA

4.1 Introduction

Anammox bacteria are sensitive to operational conditions such as temperature, pH and oxygen (Strous et al., 1997). Besides, these bacteria have slow growth rates that limit their ability to out-compete the faster-growing NOB and heterotrophic bacteria which present competition for nitrite (Strous et al., 1997, Zhang et al., 2017). Therefore, the optimization of operational parameters before mass-cultivation of anammox bacteria is important to avoid these competing organisms from taking over the system at the detriment of anammox.

The operational temperature, oxygen concentrations and loading rates are the key parameters that were chosen for optimization since literature survey revealed that these parameters influence not only bacterial community structures in the reactors but also the duration of reactor start-up period (Park et al., 2010, Hendrickx et al., 2014, Park et al., 2015, Yang et al., 2018). Despite previous investigations of the influence of these factors on the performance of anammox-mediated systems, the impact of reactor configuration on the growth of anammox bacteria is not well articulated. Also, few studies have characterized bacterial communities using high-resolution techniques such as shotgun sequencing in anammox-mediated systems. Therefore, in this objective, the operating conditions of anammox bacteria were optimized in different reactor configurations before their mass cultivation in SBRs (sequencing batch reactors) and UASB (up-flow anaerobic sludge blanket reactor) under the optimized operational conditions.

The slow growth rate of anammox bacteria necessitates the application of efficient biomass retention strategies (Lackner et al., 2014). This could include the promotion of biomass granulation in the reactors, the sequencing of reactor operations and biofilm development on carrier materials (Lackner et al., 2014). However, each of these strategies directly or indirectly influences the choice of a reactor configuration, the process control strategies and the wastewater treatment capacity (Christensson et al., 2013, Lackner et al., 2014, Val del Río et al., 2016).

Previous comparative studies of processes mediated by anammox have revealed that the start-up of anammox systems could vary considerably with reactor configuration (Wang et al., 2012) [ref]. Besides, a literature survey revealed disparities in nitrogen removal rates with reactor configurations as well as at different operational temperatures (Gilbert et al., 2015, Lackner et al., 2014, Lackner and Horn, 2013). Therefore, further optimization of the anammox process in different reactor configurations is still necessary for its widespread application.

4.2 Materials and Methods

4.2.1. Media Composition

The synthetic anammox growth media was prepared and fed to the reactors throughout the experimental period. Anammox growth media was prepared according to Van de Graaf et al. (1995) and is outlined in Table 4-1. The NO_3-N was only added during the first 14 days of enrichment to promote denitrification, thus facilitating excess COD removal. The NO_2^--N and the NH_4^+-N were added individually to the reactors to maintain a suitable C: N ratio.

Table 4-1: Feed composition for anammox bacterial enrichment

Synthetic wastewater		Traces nutrient solution	
Compound	**Concentration (mg/L)**	**Compound**	**Concentration (mg/L)**
$MgSO_4.7H_2O$	300	EDTA	0.015
$CaCl_2.2H_2O$	180	$ZnSO_4.7H_2O$	0.00043
$NaHCO_3$	1000	$CoCl_2.H_2O$	0.00024
K_2HPO_4	13.6	$MnCl_2.4H_2O$	0.00099
KH_2PO_4	13.6	H_3BO_4	0.000014
NaCl*	15	$CuSO_4.5H_2O$	0.00025
$FeSO_4$	0.005	$NaMoO_4.2H_2O$	0.00022
EDTA**	0.005	$NiCl_2.6H_2O$	0.00019
		$NaSeO_4.10H_2O$	0.00021

*provided for the first 333 days only, *Ethylenediaminetetraacetic acid

4.2.2 Reactor setup for enriching anammox from local sludge

A series of enrichment reactors (Figures 4-1 and 4-2) was established for the enrichment of anammox bacteria, and all were seeded with 30% (v/v) of the respectively activated sludge

mixed liquor (Table 4-2). All reactors were fed using the Van de Graaf's media (Table 4-1). The Northern anaerobic Gaslift utilized continuous sparging of Argon gas (2 L/min) (Figure 4-1A). The Northern 10 L SBR consisted of a glass bottle containing a magnetic stirrer bar (10 mm x 80 mm) and using a magnetic stirrer plate to maintain constant agitation at 100 rpm. The UASB reactor was constructed of Perspex according to the design below (Figure 4-1B). Upflow was achieved with a Waterfall 700 L/h water pump. In all instances, the 3 L SBR used was the Corning Disposable Spinner Flask (Corning, USA), and the MBBR utilized this same spinner flask with the addition of plastic filter media (Figure 4-1C). All reactors were operated at ambient temperature, with no active pH or DO control.

The various reactor configurations, i.e. the SBR, MBBR, UASB, and Gaslift reactors respectively, have previously proven to be effective for anammox enrichment (Jin et al., 2008, Dapena-Mora et al., 2004, Thuan et al., 2004, Tao et al., 2012, Lackner et al., 2014). Each reactor was operated for approximately 100 days under equivalent operation (Table 4-2).

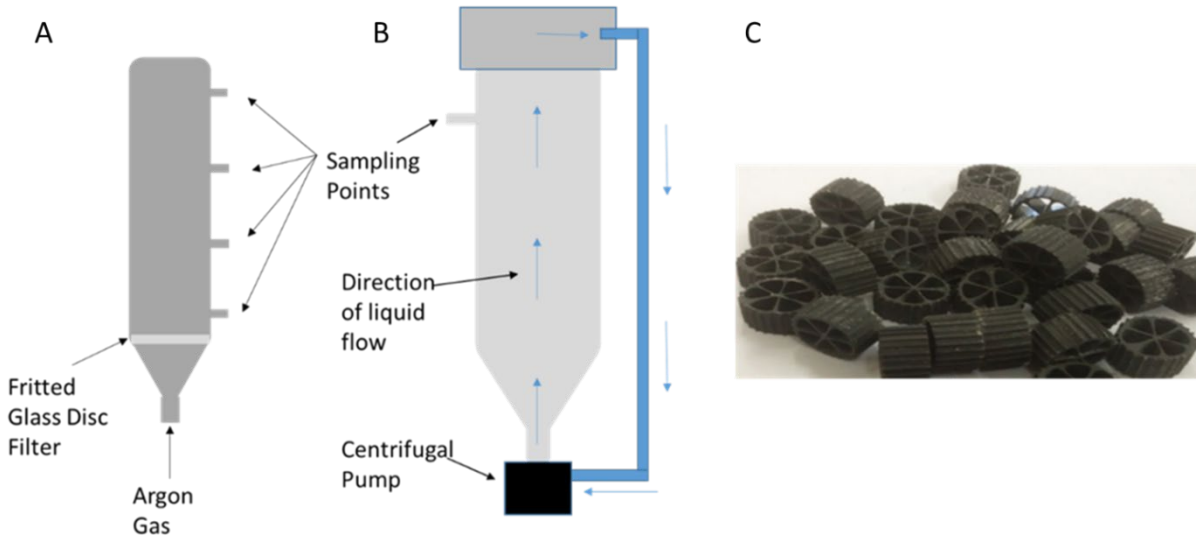


Figure 4-1: Reactor schematic diagram for A) the gas-lift reactor; B) the UASB reactor; and C) the carrier beads used for the MBBR

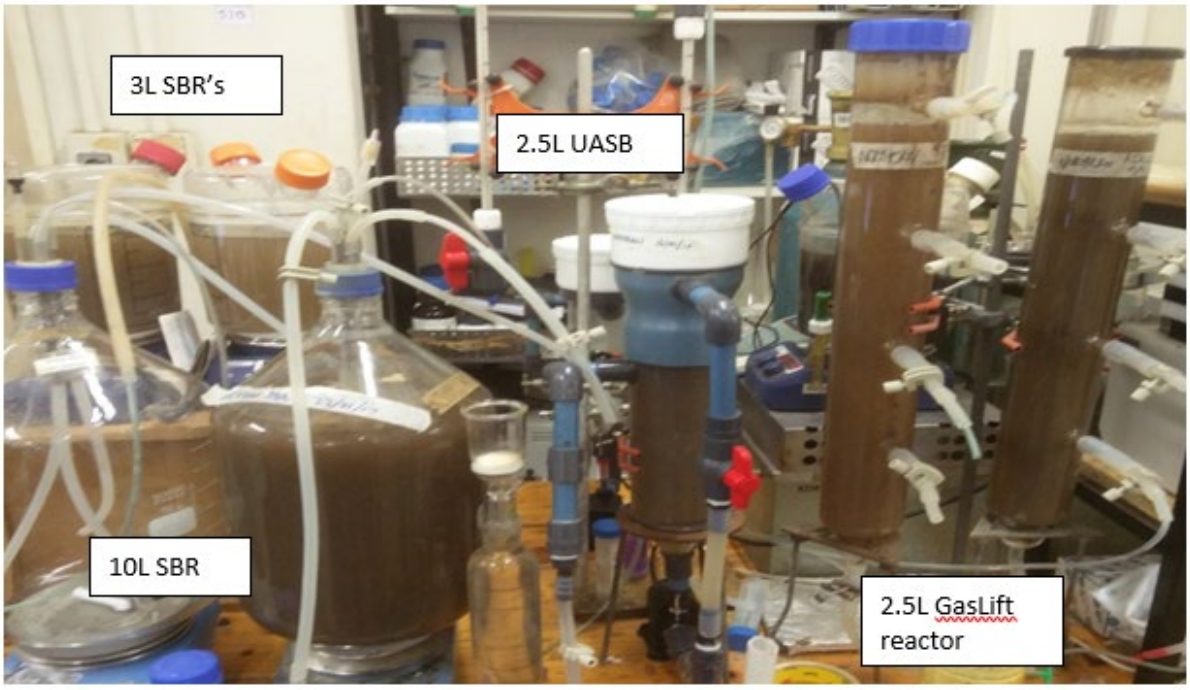


Figure 4-2: Pictorial image of the different reactor set up for the enrichment of anammox bacteria from the local sludge samples

Table 4-2: Summary of operational conditions for reactors

Sample	Reactor Type	Reactor Volume	Period of operation (d)	HRT	Average DO (mg/L)	Average temperature (°C)
Kingsburgh Aeration	SBR	3 L	76	3 days	0.70 ± 0.27	28.25 ± 1.98
Kingsburgh Anoxic	SBR	3 L	92	3 days	0.78 ± 0.30	30.24 ± 2.81
Shallcross	MBBR	3 L	99	3 days	0.75 ± 0.35	28.69 ± 1.66
Northern	SBR	10 L	110	2.5 days	1.18 ± 0.6	26.77 ± 1.35
	Gaslift	2.5 L	53	2.5 days	0.75 ± 0.33	27.21 ± 1.27
	UASB	2.5 L	80	3 days	0.75 ± 0.73	29.55 ± 1.72

4.2.3 Mass Cultivation of anammox from enriched seed culture

4.2.3.1 Reactor setup

Two sequencing batch reactors (SBRs) and an up-flow anaerobic sludge blanket reactor (UASB) were used for the mass cultivation of anammox bacteria from a known seed culture. The SBRs were mechanically agitated, and each had a 5 L and 7 L working volume (Figure 4-1). The UASB also had a 5 L working volume. Both SBRs were inoculated with equal portions of culture collected from the effluent of moving bed biofilm reactor (MBBR) mediated by anammox, while the UASB (Figure 4-1) was also inoculated with a different culture collected in the effluent of a mainstream MBBR, which was in turn fed with effluent from a sidestream MBBR.

4.2.3.2 Operational conditions

Both SBRs were operated under the same conditions of pH (6.7-8.0), temperature (36 ± 1°C) and dissolved oxygen (DO) (<0.5 mg/L), with the same hydraulic retention time and influent to effluent recycle ratio. The SBRs (Figure 4-3) and the UASB (Figure 4-4) were all operated using the synthetic feed described in Table 4-1. Sodium nitrite and ammonium sulphate were used to make the required concentrations of ammonium nitrogen (NH_4^+-N) and nitrite nitrogen (NO_2^--N). Influent NH_4^+-N and NO_2^--N concentrations for the SBRs were increased every three months from 50 mg N/L by 10 mg-N/L. DO was controlled in the influent to the SBRs and UASB by sparging with 95% Argon gas and 5% Carbon dioxide and the pH was maintained by dosing the influent with either 1 M HCl or 1 M NaOH. UASB was operated in different phases under slightly different conditions tabulated in Table 4-3. The influent NH_4^+-N and NO_2-N to the UASB ranged from 50±1 mg N/L and 36±11 mg N/L to 112±23 mg N/L and 145±40 mg N/L, respectively. The DO in the influent to UASB was maintained below 0.5 mg/L during the study period, except in phases II and III (Table 4-3). Furthermore, UASB was aerated in phase III at a rate of 40 ml/min. Hydrazine was added in the feed to UASB on the 175th day (phase V) to a final concentration of 0.33 mg/L. Continuous feeding was maintained in UASB throughout the operation period.

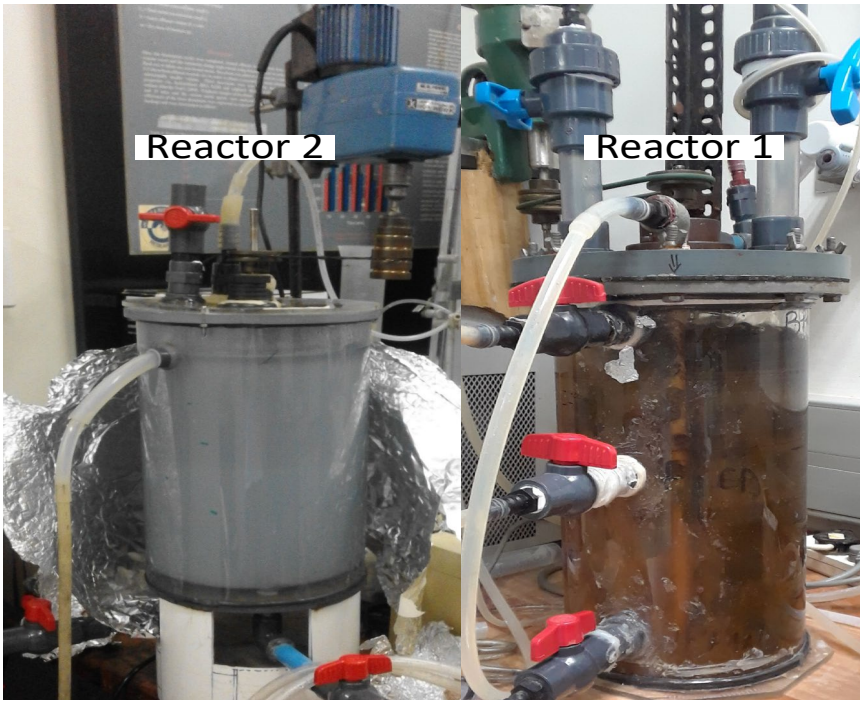


Figure 4-3: Pictorial image of the two SBRs used for anammox cultivation from a seed inoculum

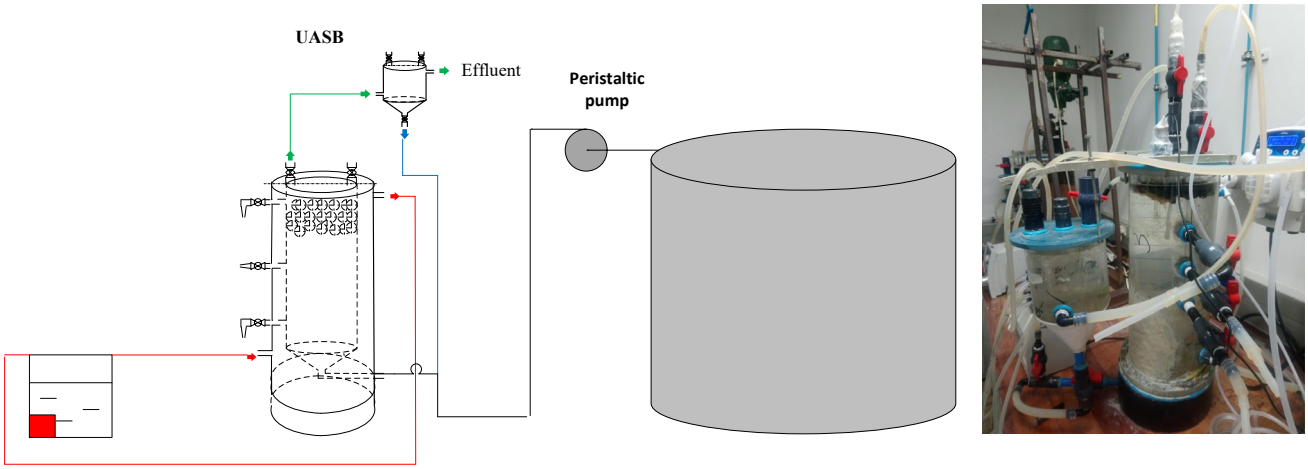


Figure 4-4: Schematic and pictorial diagrams of the UASB reactor for anammox cultivation

Table 4-3: UASB operational conditions in different phases

Phase	Period (days)	Aeration	Influent			
			DO (mg-O_2/L)	NH_4^+ (mg-N/L)	NO_2^- (mg-N/L)	Hydrazine (mg/L)
I	0-45	NO	0.5 ±0.1	50±1	56±4	0
II	46-79	NO	8.5±0.3	67±10	36±11	0
III	80-86	YES (40 ml/min)	8.5±0.3	80±1	45±1	0
IV	87-174	NO	0.3 ±0.2	74±11	51±10	0
V	175-186	NO	0.3 ±0.2	55±11	46±8	330
VI	187-250	NO	0.3 ±0.2	75±21	81±0.5	0
VII	251-309	NO	0.3 ±0.2	112±23	145±40	0

4.2.4 DNA Extraction

In UASB, 50 ml mixed liquors were collected on days 1, 125, 192, 260 and 309 and centrifuged to obtain about pellets from which DNA was extracted, while in the SBRs, 50 ml mixed liquors were collected on the last days of study. Genomic DNA was extracted from the biomass as described earlier in section 3.3.8.

4.2.5 Microbial community structure analysis

The polymerase chain reaction, 16S rRNA gene metabarcoding (SBRs) and shotgun sequencing (UASB) were used in studying the bacterial community structure of the reactors. Appropriate primer sets (Table 4-4) were employed for the PCR according to the method described by Gokal (2017) and Graham et al. (2007).

Partial 16S rRNA bacterial gene sequences (hypervariable regions V3-V4) were amplified using the Truseq tailed 341F/ 785R primer set. The PCR was done based on the method reported by Klindworth et al. (2013). The PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The quantity and quality of the DNA were assessed using the NanoDrop spectrophotometer and Qubit fluorometer analysis. The purified amplicons were sequenced at Inqaba Biotechnology Industries, South Africa on the Illumina MiSeq platform.

For shotgun sequencing of the UASB samples, multiplexed paired-end (2×300 bp) libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). The gDNA was sequenced on an Illumina MiSeq platform at the Sequencing Core Facility, National Institute for Communicable Diseases (Johannesburg, South Africa). The obtained paired-end reads were analysed with the use of sequence-based ultra-rapid pathogen identification (SURPI), a computational pipeline to rapidly classify next-generation sequencing reads according to their origin (Naccache et al., 2014). The raw sequencing data were further analysed using CLC microbial genomics module (Qiagen, Denmark) to uncover and compare the taxonomic and functional composition of microbial communities in each sample.

Table 4-4: The list of primers used for the PCR identification of anammox

Primer	Sequence (5'-3')	Gene target	Reference
PLA46 F	GGATTAGGCATGCAAGTC	anammox	(Van der Star et al., 2007)
Amx667 R	ACCAGAAGTTCCACTCTC		

4.3 Results and Discussion

4.3.1 Enrichment of ANAMMOX from activated sludge

The NH_4^+-N removal values did not show any stability for all the reactors sampled (Figure 4-5). These values are routinely higher than the influent ammonia concentrations for all reactor systems, however, this is typical of an anammox enrichment reactor at start-up (Wang et al., 2011, Trigo et al., 2006). The extra NH_4^+-N generated could be due to autolysis of bacterial cells within the system due to the low DO and dominance of denitrifiers at this time (Ding et al., 2017). This is corroborated by Figure 4-6, which indicates that the NO_3-N produced by all the 4 reactor systems displays the same trend of sharply increasing, and rapidly decreasing to negligible amounts for the first ~20 days of operation. After 20 days of operation, both the NH_4^+-N and NO_3-N levels fluctuate greatly, which could be due to an excessive NLR when the overall metabolism of the system is suppressed due to DO ingress, pH imbalance or temperature changes.

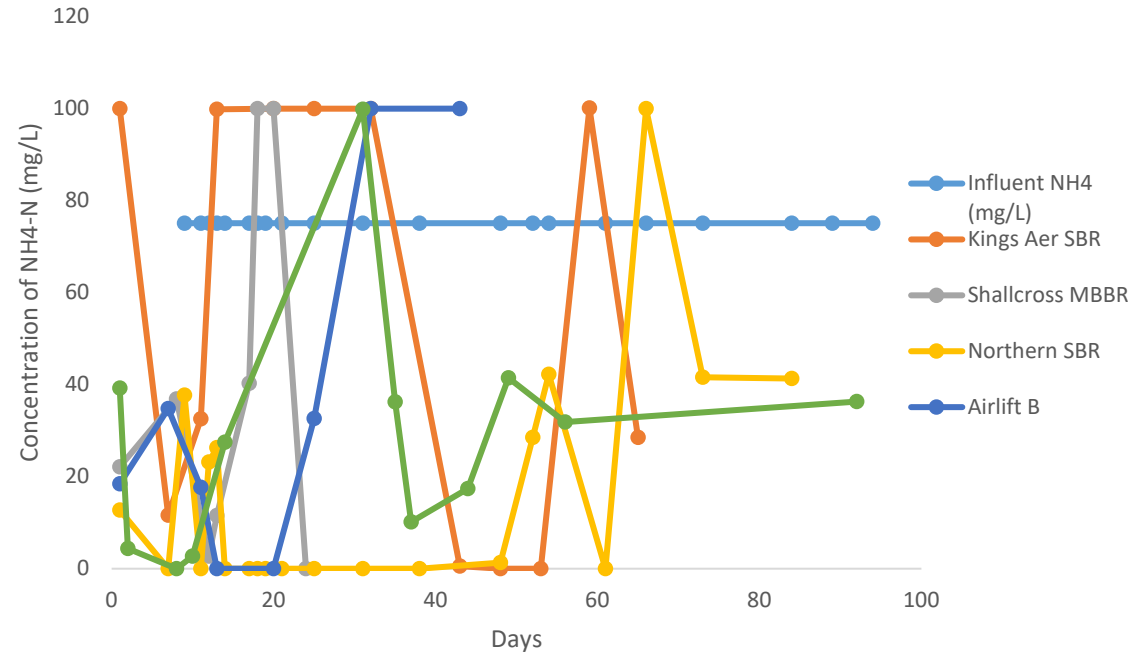


Figure 4-5: Variation in effluent NH_4^+-N concentration for each enrichment reactor

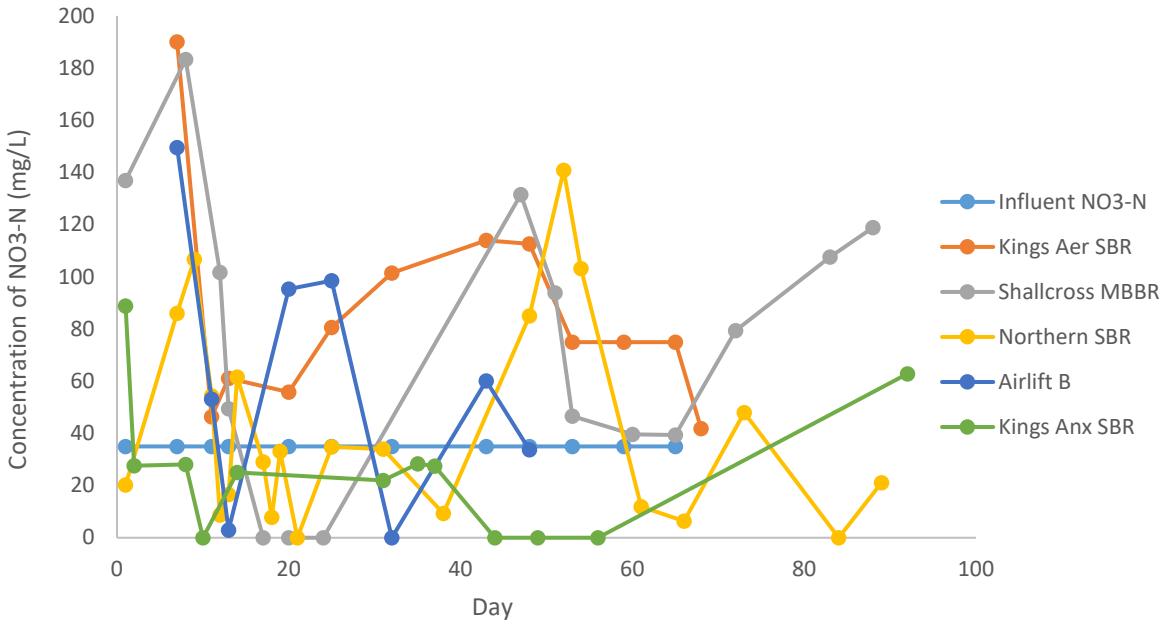


Figure 4-6: Change in effluent NO_3-N concentration for each enrichment reactor

Interestingly, NO_2^--N values did not display a complete removal within the system (Figure 4-7). There was the utilisation of NO_2^--N in all reactors over time, however, these values correlate more closely with the production of NO_3^--N during the same periods (Figure 4-6),

indicating NOB activity. Since the conversion of NO_2^--N to NO_3^--N is only partial, it indicates that the low DO within the system is effectively limiting the NOB population activity (Figuerola and Erijman, 2010).

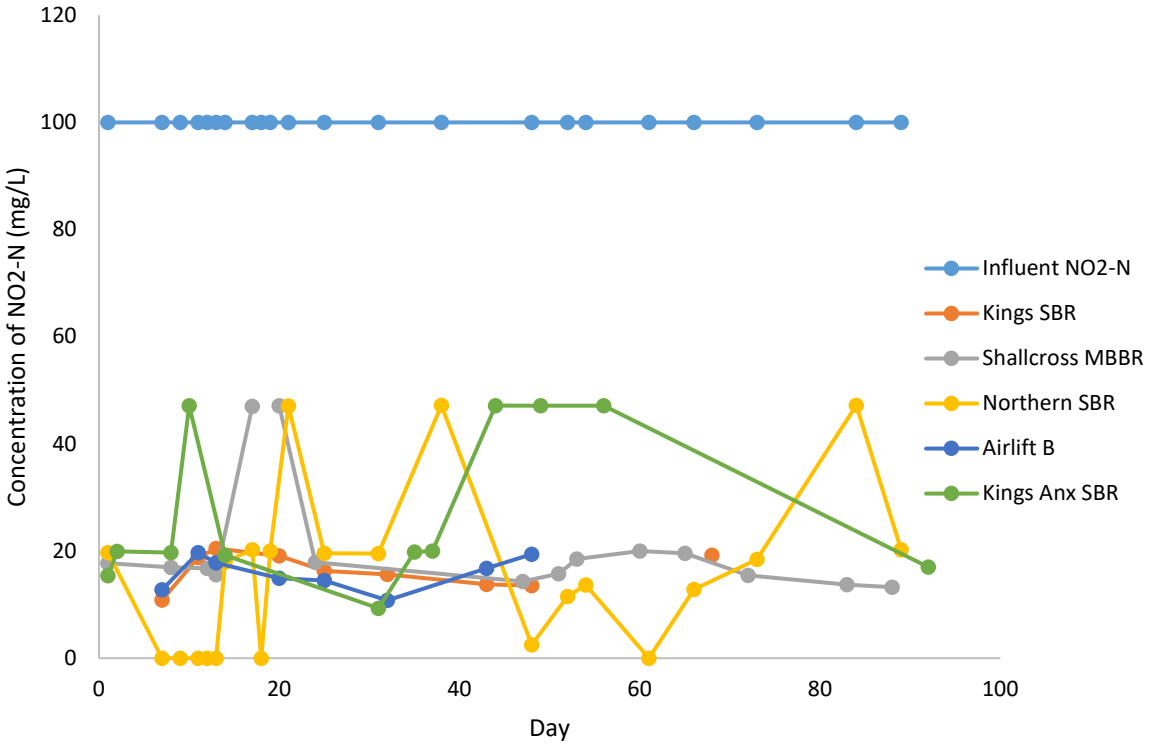


Figure 4-7: Change in effluent NO_2^--N concentration for each enrichment reactor

The pH values of all the reactors displayed a consistent increase above the average influent pH of 7.10 ± 0.10 (Figure 4-8). The largest pH increase occurred within the Airlift B and UASB reactors. This pH increase could be attributed to primarily the action of nitrifiers, which cause an increase in pH due to the nature of the nitrification metabolic pathway. Conversely, the anammox reaction and the denitrification pathway tends to decrease pH within the system. A sharp decrease in pH is only observed in the Kings Aeration SBR, with the pH decreasing from a high of 8.5 on Day 48 to 4.1 on Day 65. At day 65 the pH was manually adjusted back to 7.2, however, the system lost N-removal activity at this point due to the sharp pH decrease into the acidic range.

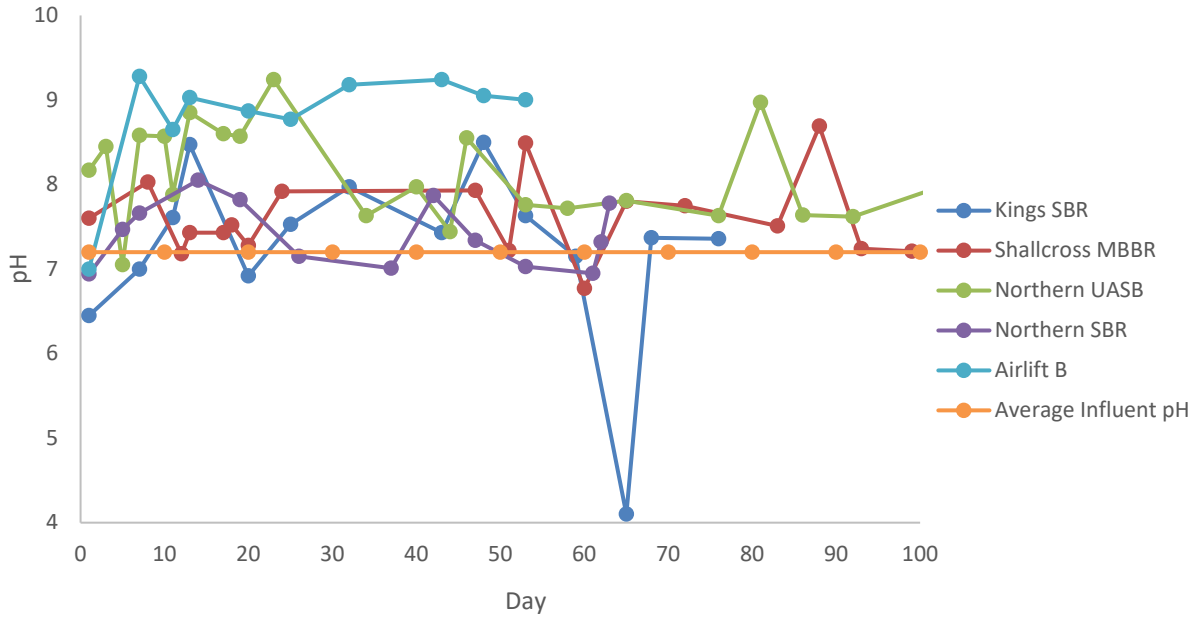


Figure 4-8: Variation in pH of the different enrichment reactors during the enrichment period

The chemical results for all the reactors did not display the characteristic NH_4^+–N, NO_2^--N utilization and NO_3-N production that are typical of enriched anammox cultures (Figure 4-5-4-7). Previous enrichment of anammox bacteria from activated sludge systems has been reported with a wide range of start-up times, however many of these positive enrichment results have been based on the reactor systems achieving the calculated stoichiometric ratios of NH_4^+: NO_2^- = 1:1.32 and NO_3^-: NH_4^+ = 0.26. In a mixed microbial consortium with multiple species utilizing the same substrates, the most metabolically dominant species would initially prevail (Kartal et al., 2013). The slower-growing organisms would still develop within the system, however, they would not significantly contribute to the overall removal of substrates on the macro-scale. Using chemical substrate utilization analysis alone may not detect trace amounts of anammox bacteria within the system. Molecular analysis using nested PCR will vastly enhance the detection limits of the screening and will be able to detect trace amounts of anammox bacteria that have not yet been detected chemically. Figure 4-9 displays a single positive result in screening for anammox bacteria using nested PCR.

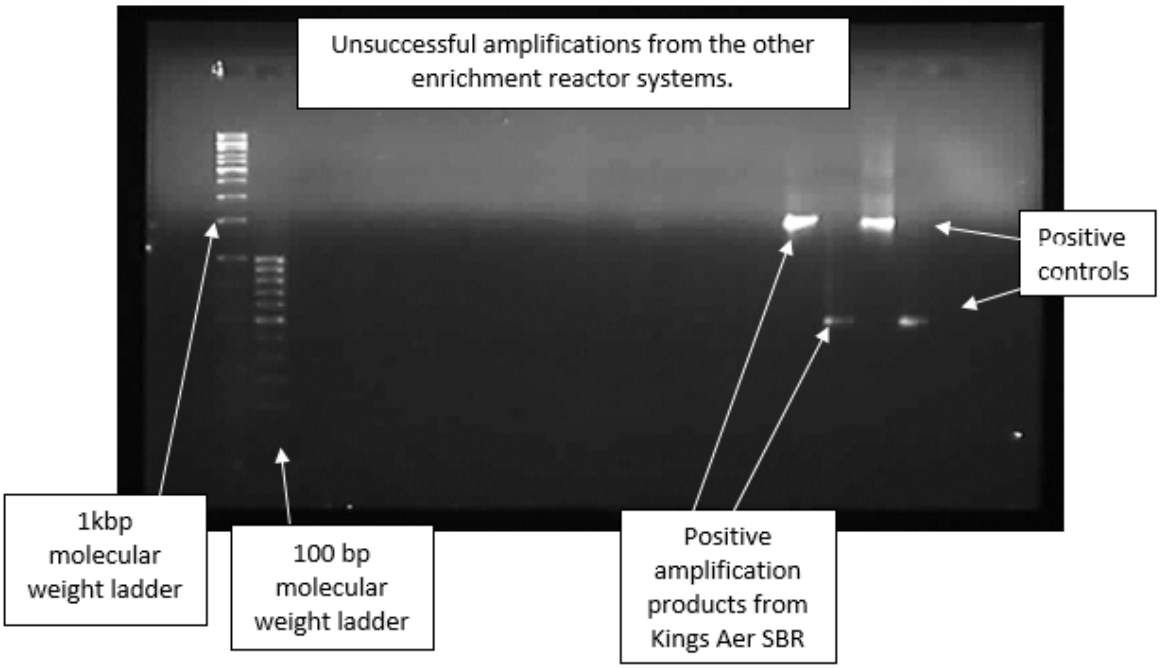


Figure 4-9: Screening of reactor samples using nested PCR with the Planctomycetes and anammox specific primer sets.

Out of all the enrichment samples tested, only the Kingsburgh Aeration 3 L SBR sample indicated a positive result for the presence of anammox bacteria. Despite this reactor displaying very erratic N removal, molecular analysis at Day 65 tested positive for the presence of anammox bacteria. Surprisingly, the sample obtained from the anoxic zone of the same WWTP was negative for the presence of anammox even at Day 85, despite being enriched under the same conditions. The low DO of the aeration tank at the time of sampling could have created sufficient nitrite for anammox bacteria to proliferate within the aeration tank. The limited nitrite produced by this low DO may also be utilized by other microbes before reaching the anoxic tank, thus causing starvation of the anammox bacteria within this tank. The Shallcross WWTP had a relatively high DO, which may explain the absence of anammox bacteria after enrichment, however, the Northern WWTP anoxic sludge, which contained a similarly low DO to the Kingsburgh sample was also negative for anammox bacteria after enrichment in 3 different reactor systems for up to 110 days. These results imply that while a low DO in the source inoculum plays a large role in whether anammox bacteria are present in detectable quantities, there are other factors at the source inoculum which may affect anammox enrichment at the bench scale.

4.3.2 Mass Cultivation of Anammox Bacteria from an Enriched Seed Culture

Cultivation in SBRs

The SBR-1 and SBR-2 were both operated for 290 days under anaerobic conditions using a similar feed. Reactor operational data showed gradual removal of ammonium and nitrite in the form of nitrogen gas and nitrate (Figure 4-10 and Figure 4-11). Both reactors showed the same trend in nitrite and ammonia depletion and an increment of nitrate. SBR-2 showed signs of stability from about 175th day of operation with gas production, while SBR-1 only stabilized from about the 200th day of operation (Figures 4-10 and 4-11). Nitrate production in the two reactors was high within the first 175 days. This could indicate that during this period, ammonia-oxidizing bacteria (AOB), nitrite oxidizing bacteria and denitrifying bacteria were the dominant bacterial populations in the reactors and nitrogen (ammonium and nitrite) removal was mainly through the conventional nitrification-denitrification route. However, since organic carbon concentration was not added in the effluent, nitrate removal might have been limited, hence its accumulation was inevitable. As a result, no gas was collected in this phase of enrichment although both reactors showed ammonia removal. After the 175th day, nitrate accumulation dropped, signalling a possible shift in the microbial population to anammox bacteria dominated, marking the onset of nitrogen gas production.

The gas collected from the two reactors was analysed by gas chromatography, against pure a nitrogen standard. Pure nitrogen gas has a retention time of 11.903 minutes at the described instrument parameters, and single peaks arising at the same retention times under identical conditions can be regarded as the same compound. Gas samples obtained from reactors 1 and 2 displayed a retention time of 11.911 and 11.907 respectively, confirming that the gas collected from the two reactors is nitrogen (Table 4-5). The slight disparities in the retention times could be attributed to the manual injection of samples into the GC. However, the biomass in both reactors was dark in colour, which might indicate the accumulation of Sulphur compounds as well as Sulphur-consuming organisms in the reactors. It is noteworthy that biomass enriched with anammox bacteria are red, a reflection of haem-bounded iron in their cells (Ferousi et al., 2017). This thus could be associated with indefinite retention of biomass in both reactors. In comparison, full-scale anammox-mediated systems report solids retention times (SRTs) of between 45 and 60 days. Therefore, continuously operated anammox-mediated UASB formed the next phase of the project (next section).

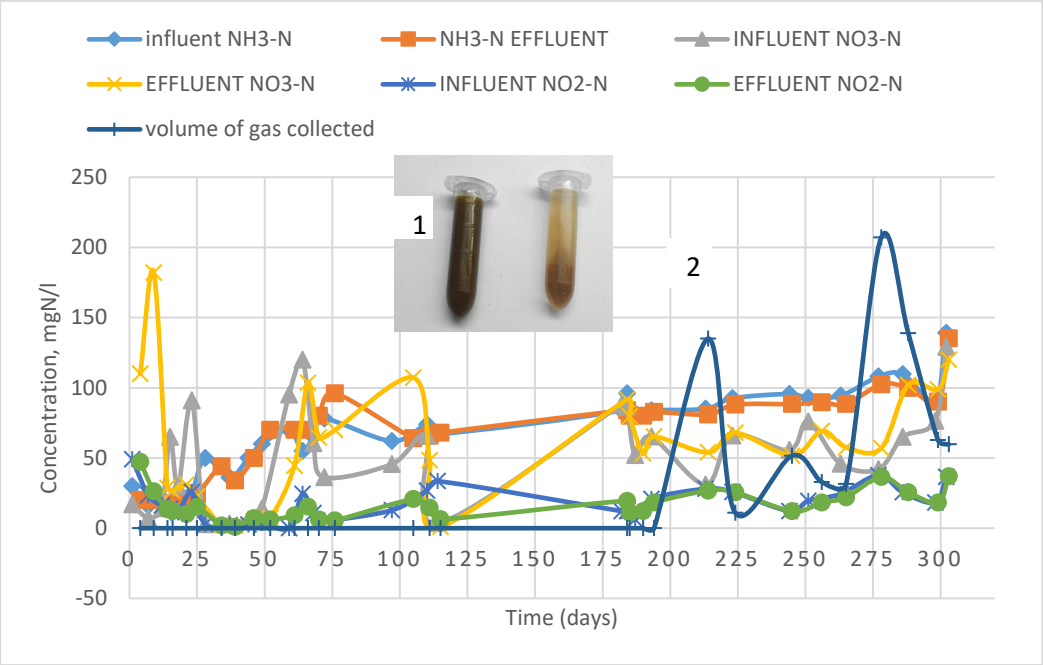


Figure 4-10: Performance of the anammox process in terms of nitrogen species conversions (SBR-1)

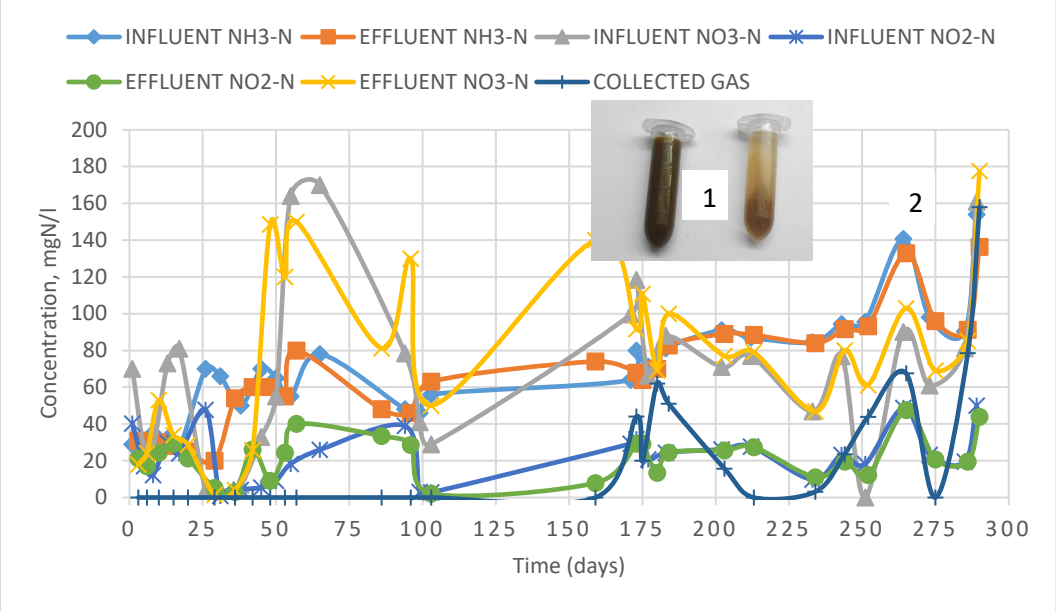


Figure 4-11: Performance of the anammox process in terms of nitrogen species conversions (SBR-2)

Signal 1: TCD1 B, Back Signal A

Peak #	RetTime [min]	Type	Width [min]	Area [25 µV*s]	Height [25 µV]	Area %
1	11.903	BB	0.2495	428.08615	20.67935	1.000e2
Totals :				428.08615	20.67935	

Signal 1: TCD1 B, Back Signal B

Peak #	RetTime [min]	Type	Width [min]	Area [25 µV*s]	Height [25 µV]	Area %
1	11.911	BB	0.2450	437.86130	21.11008	1.000e2
Totals :				437.86130	21.11008	

Signal 1: TCD1 B, Back Signal C

Peak #	RetTime [min]	Type	Width [min]	Area [25 µV*s]	Height [25 µV]	Area %
1	11.907	BB	0.2480	435.64429	21.17991	1.000e2
Totals :				435.64429	21.17991	

Table 4-5: GC output for A) Nitrogen Standard; B) Gas collected from SBR-1; C) Gas collected from SBR-2

Cultivation in UASB

The UASB displayed a different kind of performance, in stark contrast to the performance of SBRs. The nitrogen species concentrations in the effluent and ratios in the UASB reactors are shown in Figures 4-12 and 4-13. Although the SBRs and UASB were operated using a similar feed, UASB showed better performance in terms of nitrogen removal at the end of the operation (Figures 4-14).

However, in phase I, low nitrogen removal was observed, only 22±13%, an indication that the anammox bacteria had not adapted to the operating conditions. As a result, the ratios of nitrite consumed to ammonium consumed (NO_2^-/NH_4^+), and nitrate produced to ammonium consumed (NO_3^-/NH_4^+) ranged from below -10 to above 10, in stark contrast to the stoichiometric ratios expected of anammox process (Figure 4-13). During this phase also, incidences of higher effluent ammonium than influent ammonium were observed, an indication of possible lysis of dead bacterial cells.

In phase II, a drop in effluent ammonium, nitrite and nitrate concentrations were observed in the first ca. 15 days, followed by an increase, which continued until the end of the phase (Figure 4-12). The NO_2^-/NH_4^+ and NO_3^-/NH_4^+ ratios during this phase only fluctuated over a smaller region than in phase I (Figure 4-13). However, the NRE and NRR (20±24% and 0.005±0.008

kg-N/m^3/day, respectively) in this phase were slightly less than in phase I (22±13% and 0.006±0.003 kg-N/m^3/day), probably because of high influent DO in this phase.

Although phase III was short, the aeration of the reactor led to an improvement of process performance (0.02±0.01 kg-N/m^3/day and 28±12%) (Figures 4-14). Similarly, there was no noticeable change in the effluent ammonium, nitrite and nitrate concentrations from the reactor as well (Figure 4-12).

In phase IV, the NO_2^-/NH_4^+ ratio remained below the stoichiometric NO_2^-/NH_4^+ ratio expected of an anammox process (Figure 4-13). However, NRE and NRR increased to 47±20% and 0.07±0.04 kg-N/m^3/day, respectively. The addition of hydrazine on the 175th day (phase V) destabilized the process, leading to a drop in NRR and NRE to 0.004±0.03 and 0.99±25%, respectively. The NO_2^-/NH_4^+ and NO_3^-/NH_4^+ ratios also fluctuated over a wide region during this phase, similar to phase I. This thus indicated that the bacteria were inhibited by the added hydrazine. Indeed, 0.33g/L concentration of hydrazine in the influent was 4 times higher than the recommended concentration (Carvajal-Arroyo et al., 2013).

From phase VI, the NO_2^-/NH_4^+ and NO_3^-/NH_4^+ ratios were close to the stoichiometric ratios expected of anammox process, an indication that anammox bacteria were dominating from this phase (Figure 4-13). Similarly, a drop in the effluent ammonium, nitrite and nitrate concentrations were observed (Figure 4-12). The NRR and NRE in this phase were 0.27±0.16 kg-N/m^3/day and 81±14%, respectively, which were close to the literature reported values (Kotay et al., 2013). A further increase in the NLRs in phase VII was followed by a deterioration in process performance, possibly because of substrate inhibition (Figure 4-14). However, the NO_2^-/NH_4^+ and NO_3^-/NH_4^+ ratios in phase V remained close to the stoichiometric ratios expected of the anammox process (Figure 4-13). At the end of the study, the biomass was bright red (Figure 4-14), an indication of the high enrichment of anammox bacteria.

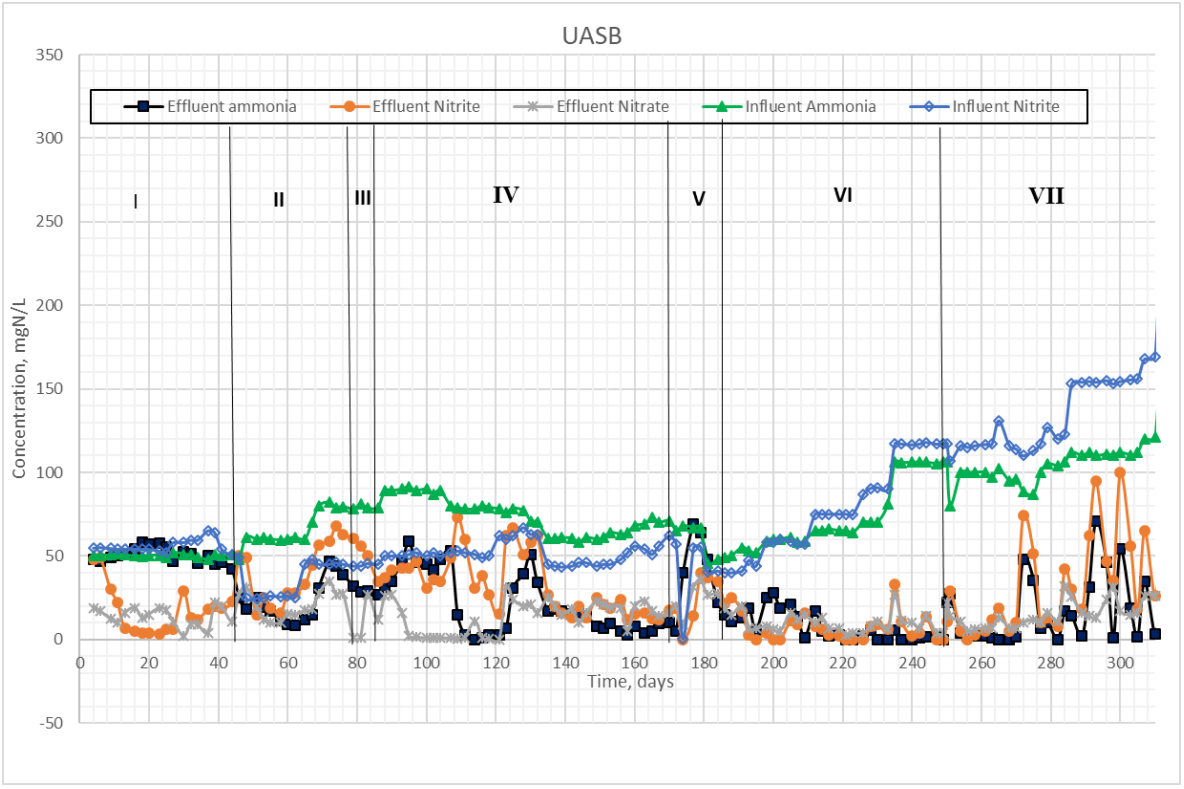


Figure 4-12: Effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations in UASB reactor

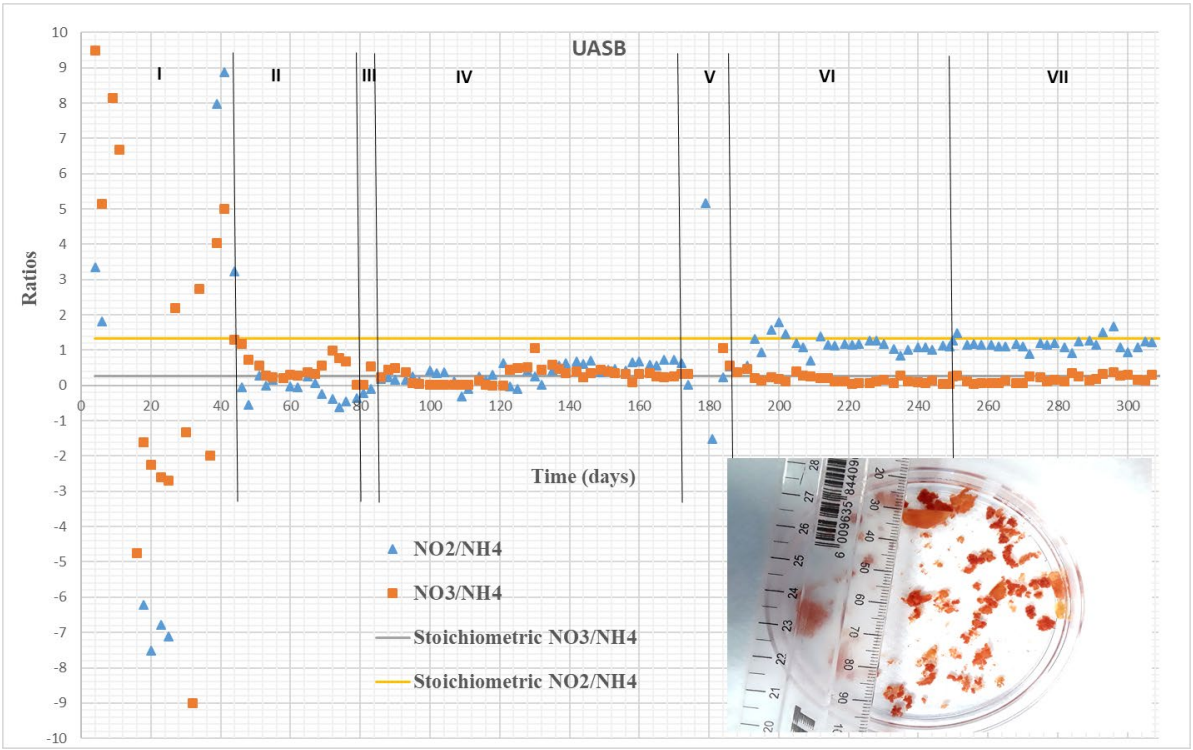


Figure 4-13: NO_2^--N/NH_4^+-N and NO_3^--N/NH_4^+-N ratios in UASB reactor

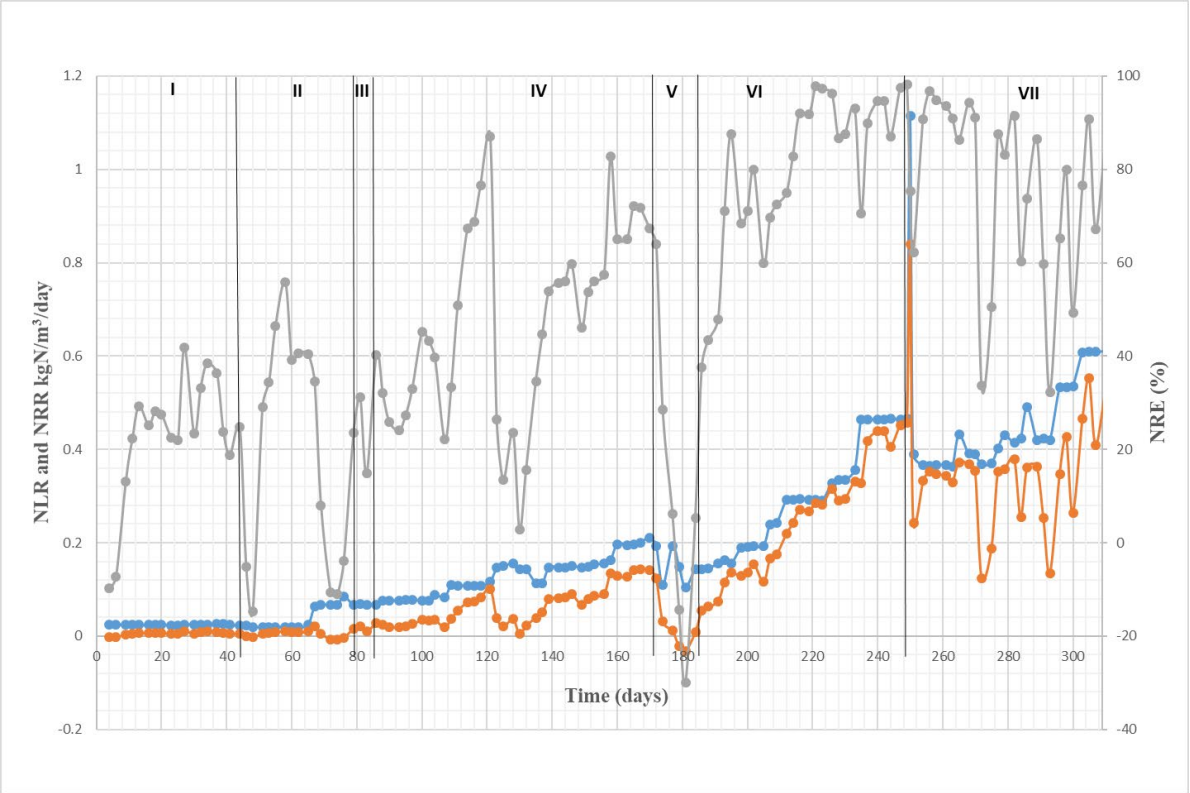


Figure 4-14: The profiles of nitrogen loading rate (NLR), nitrogen removal rate (NRR) and nitrogen removal efficiency of the UASB reactor

4.3.3 The total microbial community diversity in UASB as revealed by amplicon sequencing

The total bacterial community diversity in the reactor over the study period was investigated using Illumina (Miseq) sequencing platform. Based on Chao 1 and ACE indices the reactor progressed towards enriching a particular group of organisms as these indices were lowest from day 260 to 309 in the reactor (Table 4-6). Moreover, based on Pielou's Evenness (J) the reactor's evenness (Table 4-6) was also lowest during this period which indicated the prevailing conditions favoured fewer population groups compared to earlier periods. Variation was observed in the species richness of the reactor's microbial community. The Shannon and Simpson indices equally corroborate the fact that the reactor progressed towards the enrichment of fewer population groups as the lowest species richness was observed in days 260 and 309 samples. In total, 32 bacterial phyla apart from the unclassified and unidentified bacteria sequences were identified during this study (Figure 4-15), with Proteobacteria (phylum containing nitrifiers) and Planctomycetes (phylum containing anammox bacteria) being the

most abundant. The reactor progressed towards the enrichment of the Planctomycetes, the phylum containing anammox bacteria (Table 4-6).

Table 4-6: Analysis of microbial diversity in the reactor over the sampling period

	S.obs	S.chao1	S.ACE	Shannon	Simpson	Pielou Evenness (J)
Inoculum	3506	4649.150	4666.748	3.952174	0.8079099	0.09898496
UASB125	3551	4928.696	4870.179	4.506347	0.9073554	0.1109955
UASB192	3445	4551.230	4537.018	4.151466	0.8841733	0.1085623
UASB260	2755	3876.061	3705.665	2.088718	0.4529908	0.05718997
UASB309	3065	4176.555	4106.805	2.659774	0.5659765	0.07050491

Shotgun sequencing showed a decrease in the relative abundance of anammox bacteria from 45% in the inoculum to ca. 5% in phase IV (day 125) (Figure 4-16). However, a gradual increase in the anammox bacterial population was observed in the course of study between day 125 and 260, with *Candidatus Kuenenia* dominating throughout. On the last day of the study, the relative abundance of anammox bacteria was ca. 66%. Thus, the sample collected on day 260 which showed a relative abundance of *Candidatus Kuenenia* at ca. 74% contained the highest concentration of anammox (Figure 4-16).

On the contrary, the relative abundance of *Nitrospira*-affiliated NOB decreased in the course of study, reaching below 1% on the last day of study, while *Nitrobacter*-affiliated NOB remained below 1.5% during the entire study period (Figure 4-16). Similarly, Nitrosomonas-affiliated AOB, which dominated over all the other AOB during most of the study period, decreased after 125th day to below 1% at the end of the study period. This could thus indicate that the granulation of biomass in the reactor (Figure 4-13), could have favoured anammox bacteria while suppressing other nitrogen consuming organisms. Also, the maintenance of anaerobic conditions from phase IV could have favoured anammox bacteria over other nitrogen consuming bacteria.

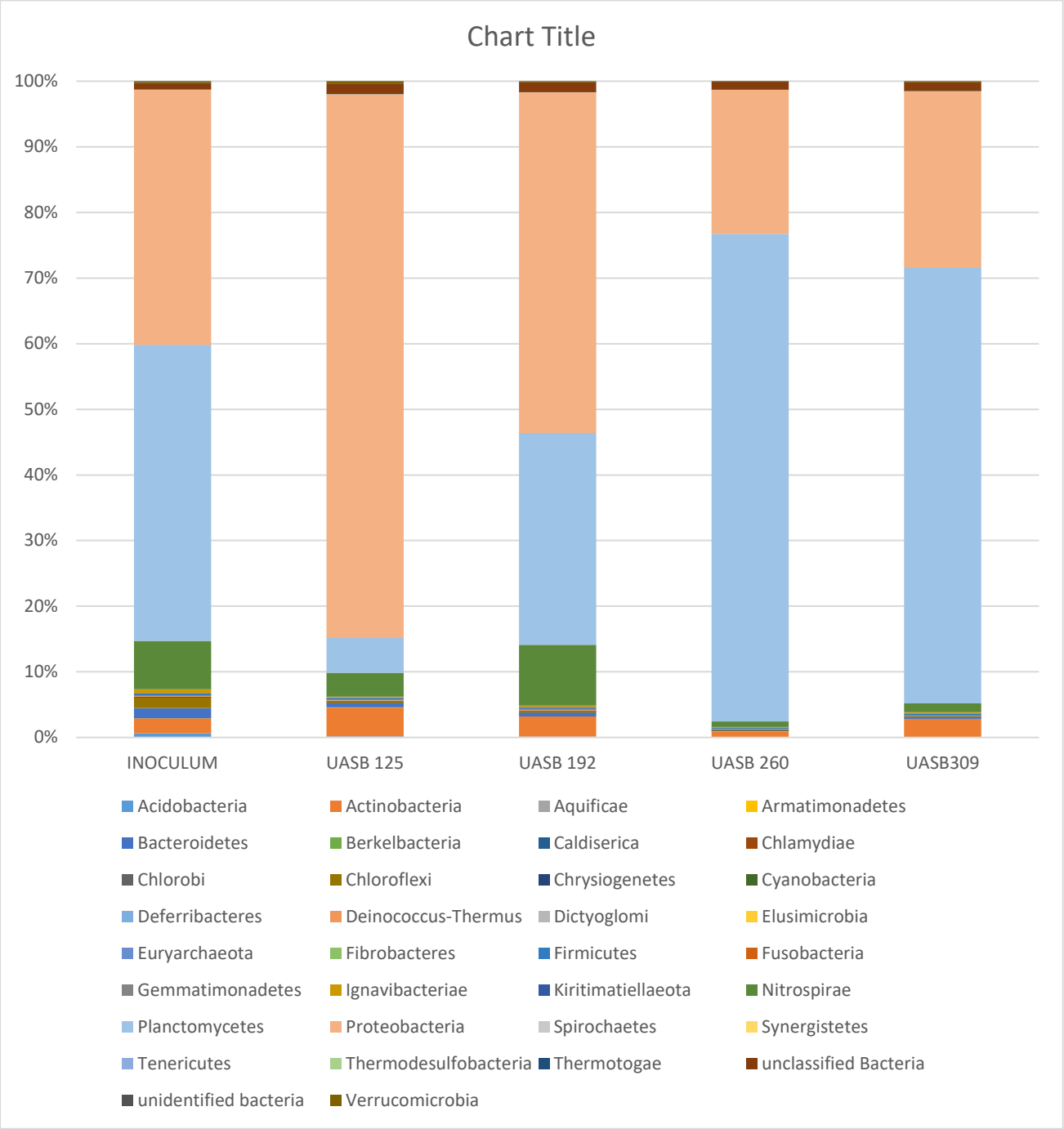


Figure 4-15: Taxonomic distribution of different bacterial phylogenetic groups at different sampling periods in the reactor at the Phylum level. The percentages of the phylogenetically classified sequences are plotted on the y-axis

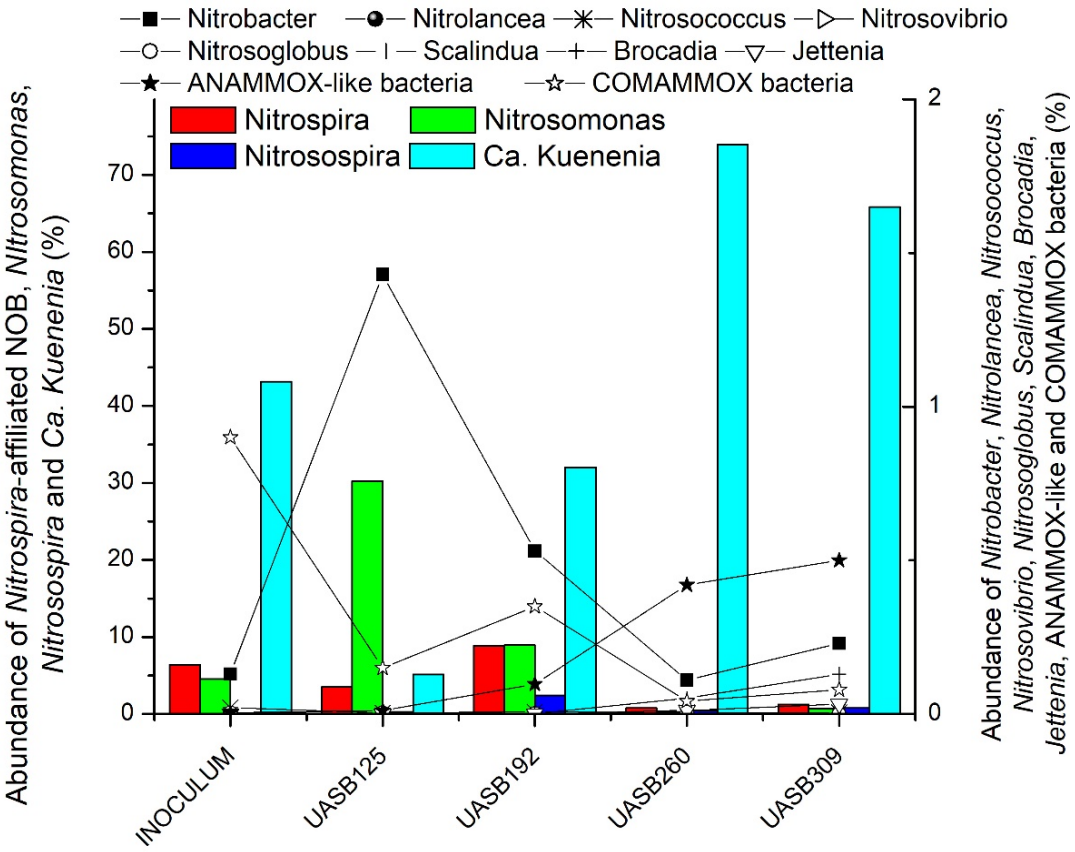


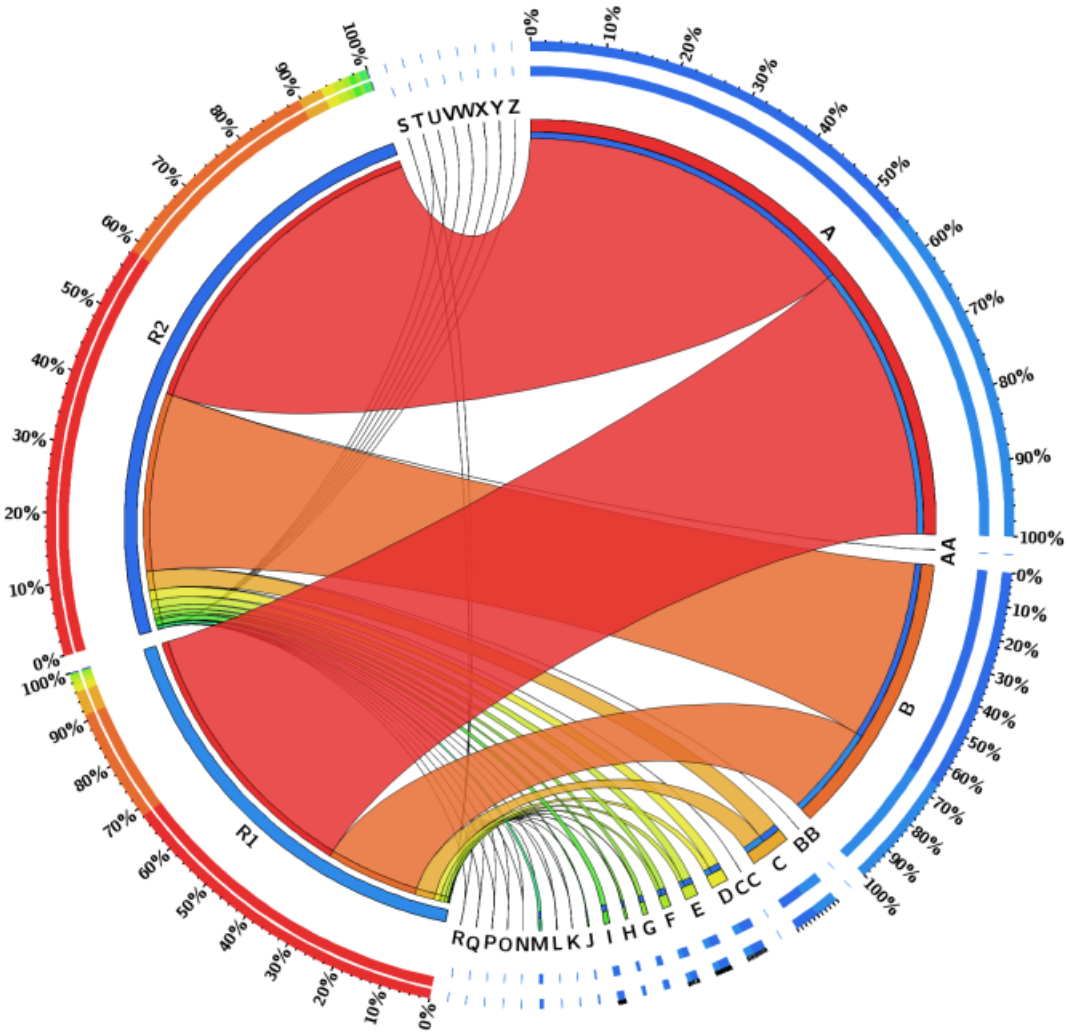
Figure 4-16: Autotrophic bacterial growth in UASB based on shotgun sequencing

4.3.4 Phylogenetic characterization of bacterial communities in the SBRs using amplicon sequencing

Bacterial 16S rRNA gene amplicon paired-end sequencing results of the SBRs corroborated the chemical data analyses which revealed low activities of anammox bacteria. As shown in Figure 4-17, the presence of *Planctomycetes* phylum to which anammox bacteria belong, was below 1% on the last day of operation of the reactors. On the contrary, bacteria belonging to *Proteobacteria* phylum dominated in the SBRs, while those belonging to *Chloroflexi* phylum were the third most abundant (Figure 4-17). As expected of anammox-mediated systems, bacteria belonging to many other phyla to which other nitrogen removing bacteria belong such as *Nitrospirae* phylum were found in both reactors (Figure 4-17).

All the known AOB, *Nitrobacter*-affiliated NOB and many heterotrophic bacteria belong to *Proteobacteria* phylum (Kersters et al., 2006). Their dominance in these reactors indicates that the operational conditions in the SBRs favoured their growth more than it favoured anammox

bacteria. This is probably because the variation in the reactor operations during the sequencing of the reactor operations affected anammox bacterial growth, leading to out-competition.



Key for interpretation					
Proteobacteria	A	TM6	K	AC1	U
Chloroflexi	B	WPS_2	L	GN02	V
Chlorobi	C	Acidobacteria	M	Nitrospirae	W
Bacteroidetes	D	Cyanobacteria	N	OP11	X
Actinobacteria	E	Firmicutes	O	OP8	Y
Gemmatimonadetes	F	Armatimonadetes	P	Tenericutes	Z
OD1	G	GAL15	Q	TM7	AA
Verrucomicrobia	H	SBR1093	R	WS2	BB
Thermi	I	NKB19	S	WS6	CC
Planctomycetes	J	Chlamydiae	T		

Figure 4-17: Bacterial community structures in the SBRs as determined through pyrosequencing

4.3.4 Conclusions

Anammox bacterial enrichment was carried out from local sources (Kingsburgh, Shallcross and Northern works) and known anammox seed culture (using 2 SBR and 1 UASB reactor)

- There was evidence of anammox presence in the local source (Kingsburg aeration tank) as evidenced by molecular identification however, their cultivation into an enriched state still requires further optimization.
- The low enrichment of anammox bacteria in the SBRs led to low levels of nitrogen removal.
- The UASB reactor configuration supported the enrichment of anammox within approximately 190 days whereas the SBR configuration did not support their enrichment within the same period.
- The highest nitrogen removal efficiencies (81±14%) was observed in the UASB reactor during stage VI when the NO_2^-/NH_4^+ and NO_3^-/NH_4^+ ratios were close to the stoichiometric ratios expected of typical anammox process.

CHAPTER 5

THE TWO-STAGE PARTIAL NITRIFICATION-ANAMMOX

5.1 Introduction

Presently, maintaining an efficient NOB washout strategy whilst retaining anammox and AOB in the system is seen as the most challenging one in the single-stage reactor (Stefansdottir et al., 2018). This phenomenon has led to exploring the possibility of a two-stage anammox reactor (PN-A reactor) whereby nitritation and anammox reactions are achieved in two separate reactors that are coupled (Piculell et al., 2016). The two-stage anammox reactors configuration allows individual optimization of the processes with the advantage of reducing the risk of heterotrophic bacteria competition and dissolved oxygen toxicity (Dosta et al., 2015). The partial nitritation-anammox (PN-A) technology is currently being employed in side stream wastewater treatment systems and in industrial wastewater streams, which are known for high temperatures and high ammonium concentrations (Hoekstra, 2017, Lackner et al., 2014). Nitrate build-up which signifies the proliferation of NOB was also observed in the reactor when there was a reduction in temperature to below 20°C (Stefansdottir et al., 2018).

An in-depth understanding of the bacterial community structure of bioreactors could enhance process performance and control. Various molecular techniques have been employed in analysing nitrogen removal reactors. These techniques rely on oligonucleotide primers or probes targeting 16S rRNA or functional genes of these nitrogen removal community. These include denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), quantitative real-time PCR and next-generation sequencing (NGS) (Cho et al., 2014, Ye and Zhang, 2013, Yu et al., 2011).

According to different studies, members of Nitrosomonas spp. are usually dominant in reactors carrying out partial nitritation (Dosta et al., 2015, Ahn et al., 2011), whilst Chlorobi, Chloroflexi, and Bacteroidetes have been reported to coexist with Planctomycetes the phylum harbouring anammox bacteria (Qiao et al., 2008, Cho et al., 2010). In a study by Dosta et al. (2015) using NGS (pyrosequencing), it was noted that in a partial nitritation reactor, despite the number of phyla and richness observed, the microbial community was dominated by the members of phylum Proteobacteria (89.7 ± 1.6%) and Nitrosomonas was the most abundant genera. The abundance of AOB community in partial nitrifying reactors could be due to several factors including their higher tolerance for O_2 and NO_2^-, and higher growth rates at high

ammonia concentrations (Dosta et al., 2015). On the other hand, NGS analysis of the anammox reactor revealed a completely microbial composition compared to the partial nitritation reactor. The phylum Proteobacteria were not significantly represented whilst Chlorobi, Chloroflexi, and Bacteroidetes were coexisting with Planctomycetes, and within the Planctomycetes, members of the candidate genus *Brocadia* were the most abundant (Dosta et al., 2015).

Most of the known nitritation-anammox processes are single-stage reactors, where the process is controlled in a single reactor by carefully controlling the operational conditions. AOB and anammox are two metabolically diverse groups of bacteria and require specific operational conditions for their optimal activity. In single-stage reactors, maintenance of the system stability, therefore, is a challenge and often leads to system breakdown. The purpose of the study is essential to improve the performance of the conventional single-stage anammox process by developing a 2 stage nitritation-anammox process for enhanced process control. The findings of this study have the potential for the development of comparatively low energy usage technology for wastewater treatment which is becoming essential due to current energy crises in South Africa.

5.2 Materials and methods

5.2.1 Reactor setup

The two-stage PN-A system consisted of one reactor for aerobic oxidizing bacteria (AOB-reactor) and another for anaerobic ammonium oxidizing bacteria (AMX-reactor). The AOB-reactor and AMX-reactor were operated under aerobic and anaerobic conditions, respectively. AOB-reactor was installed on the first stage while the AMX-reactor was installed on the second stage (Figures 5-1 and 5-2). A settler was installed between the AOB- and AMX-reactors for retention of biomass washed out of the AOB-reactor. The AOB-reactor was operated in two different modes, first as UASB reactor (Figure 5-1), and then as continuously stirred tank reactor (CSTR) (Figure 5-2). This was achieved by installing a mixer in the reactor in the second part of the study. AMX-reactor was operated as a UASB during the entire study period. A 3 L working volume was maintained in each reactor during the study period.

In the first period of the study (both the AOB-reactor and AMX-reactor as UASB), the reactors were operated at room temperature (21-25°C) (Table 3-1). During this period, the AOB-reactor was aerated at 40 ml/min while anaerobic conditions were maintained in an AMX-reactor (Table 3-1). Feeding and effluent removal from both reactors was implemented with a four-

channel peristaltic pump (Gilson, UK). The pH in both reactors was controlled with automatic pH controllers (Etatron, Italy) (≥7.7 in AOB-reactor and 7.0-7.5 in AMX-reactor). The hydraulic retention time (HRT) was kept constant at 0.31 days throughout the first period (AOB-reactor was operated as UASB), while the influent NH4+ concentrations to the AOB reactor was 95.17±11.20 mg-N/L during the same period.

In the second period of the study, the AOB reactor was re-inoculated with biomass from the anaerobic tank of a full-scale wastewater treatment plant (Kingsburg wastewater treatment plant, Durban). Following the re-inoculation of the AOB reactor, the temperature in both the AOB-reactor and AMX-reactor was maintained at approximately 34±1°C (Table 5-1). Also, a mixer was incorporated in the AOB-reactor, and the reactor was mixed at approximately 80 revolutions per minute throughout the study. The AOB-reactor was aerated at 40 ml/min during the first 77 days in the second part, while the aeration rate was maintained at 140 ml/min from the 78th day of the study. The HRT was kept at 0.46 days in phase I of the second period, and at 0.25 days in phase II of the second period (Table 5-1). The influent NH_4^+ concentrations to the AOB reactor were 186.21±35.09 mg-N/L and 200 mg N/L in phases I and II, respectively. In both parts I and II, the reactors were operated using Van de Graaf et al. (1995) synthetic feed as outlined in Table 4-1. The AOB reactor was aerated during both periods of study (Table 5-1).

Table 5-1: Operational conditions of the two-stage reactors

Period of study		Influent NH_4^+ concentration to AOB reactor	NLR kg-N/m^3-day	Operational temperature (°C)	Aeration rate (ml/min)	HRT (days)
First period		95.17±11.20	0.16±0.08	Ambient (21-25)	40	0.31
Second period	Phase I (1-77 days)	186.21±35.09	0.34±0.29	34±1	40	0.46
	Phase II (78-130 days)	200±0	0.55±0.16	34±1	140	0.25

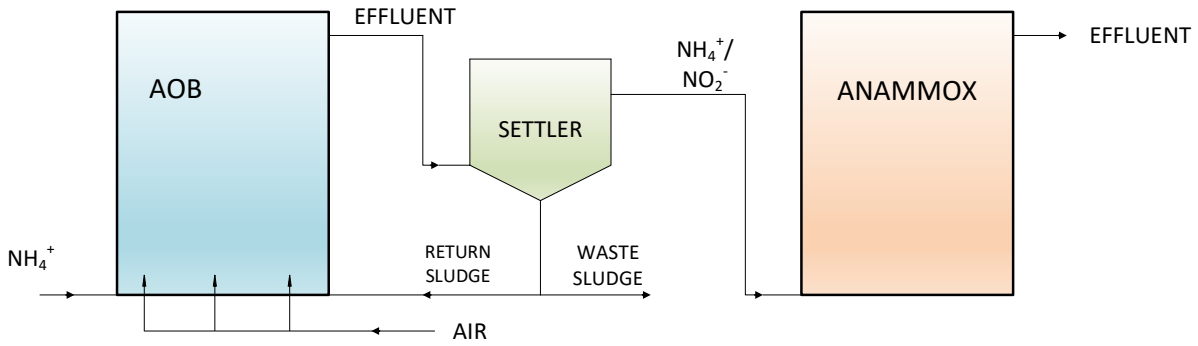


Figure 5-1: Schematic diagram of the AOB-reactor as UASB (first period)

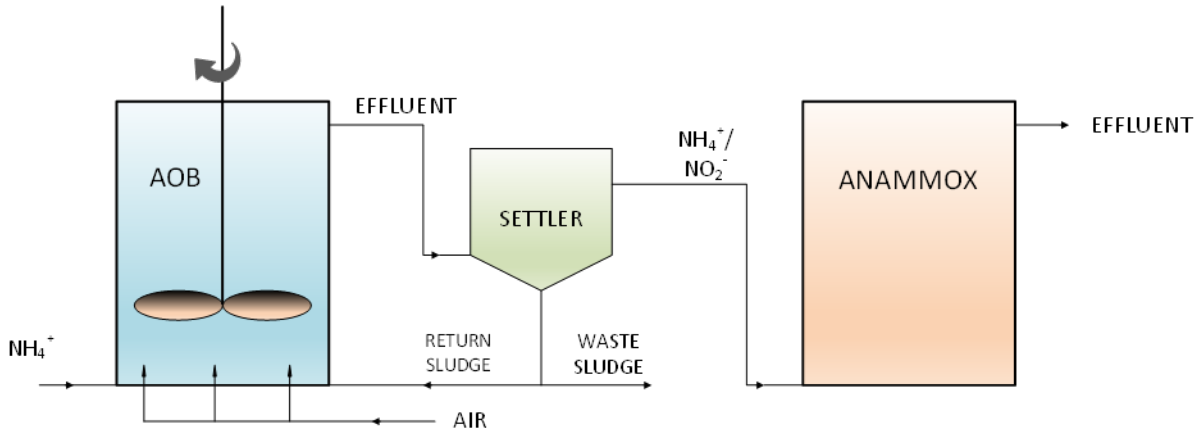


Figure 5-2: Schematic diagram of the AOB-reactor as CSTR (Second period)

5.2.2 Analytical method

The concentrations of ammonium, nitrite, and nitrate were analysed in the effluent from each reactor twice a week using the colorimetric method (HACH, USA). Samples were first filtered through 0.45µm filters before analysis. Standard water analyses were performed for the nitrogen species as described in section 3.3.6.

5.2.3 DNA extraction

Biomass was collected from the two partial nitritation reactors (AOB enrichment) for DNA extraction. For the 2-stage PN-anammox reactors, biomass samples were collected in phase I from each reactor on the 34th day of operation of reactors, whilst in phase II, biomass collection and extraction was done on days 1, 36, 70 and 130. Genomic DNA was extracted from all the biomass, quality and quantity of the extracted DNA were ascertained as described in section 3.3.8.

5.2.4 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) of the total bacteria, AOB, NOB (*Nitrospira and Nitrobacter*) and anammox bacteria were carried out using the appropriate primer sets (Table 5-2) according to the modified method described by Steinberg and Regan (2009). The Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) was employed for the qPCR reactions and the optimized thermocycling conditions are shown in the appendix (Table S1). The qPCR reaction mixture was made up of 4 µL of PowerUP SYBR Green Master Mix (Applied Biosystems, USA), 0.5 µL of each primer (final concentration of 0.4 µM), 2 µL of template DNA (final concentration of 1 ng), and molecular grade water to a final volume of 10 µL. For each experimental setup, appropriate negative controls containing no genomic DNA were subjected to the same amplification condition. The specificity of each qPCR assay was confirmed by using both melting curve analysis and electrophoresis in 1.2% (w/v) agarose gel for the presence of the expected gene product sizes. In each assay, a plot of the threshold cycle (C_q) against the logarithmic starting quantity value of every 16S rRNA gene fragment (target DNA) was made and the standard curve with linear range having regression analysis correlation coefficient (R^2) value that is greater than 0.98 and efficiency within the 90 to 110% range (Awolusi et al., 2018, Yapsakli et al., 2011).

Table 5-2: The list of primers used for the polymerase chain reaction in this study

Primer	Sequence (5'-3')	Gene target	Reference
AmoA-1F	GGGGTTTCTACTGGTGGT	Ammonia monooxygenase	(Rotthauwe et al., 1997)
AmoA-2R	CCCCTCKGSAAAGCCTTCTTC		
Nitro 1198f	ACCCCTAGCAAATCTCAAAAAACCG	*Nitrobacter*	(Graham et al., 2007)
Nitro 1423r	CTTCACCCCAGTCGCTGACC		
NSR1113F	CCTGCTTTCAGTTGCTACCG	*Nitrospira*	(Dionisi et al., 2002)
NSR1264R	GTTTGCAGCGCTTTGTACCG		
AnnirS379F	TCTATCGTTGCATCGCATTT	anammox *nirS* genes	(Li et al., 2011b)
AnnirS821R	GGATGGGTCTTGATAAACA		
P338F	ACTCCTACGGGAGGCAGCAG	Total Bacteria	(Gokal, 2017)
P518R	ATTACCGCGGCTGCTGG		

5.3 Results and discussion

5.3.1 Performance of the reactors

When both reactors (AOB and anammox reactor) were operated as UASB, the effluent NO_2^-, NO_3^- and NH_4^+ concentrations from the AOB reactor was 41.0±25.9 mg-N/L, 3.62±5.83 mg-N/L and 2.51±2.19 mg-N/L, respectively (Figure 5-3). The effluent concentrations from the anammox reactor during the same period were 19.23±21.46 mg-N/L, 1.93±5.30 mg-N/L and 2.92±3.54 mg-N/L, respectively (Figure 5-3). During this period, the DO in the AOB reactor was below 0.8 mg/L, while the DO in the anammox reactor was below 0.5 mg/L. The effluent analysis revealed that NH_4^+ removal (49.82±27.35%) occurred in the AOB reactor despite the low effluent NO_2^- concentrations. This thus indicates that denitrification in the first stage could have occurred, possibly due to the presence of heterotrophic bacteria or anammox bacteria in this reactor that might have been favoured by the low DO. Several researchers have previously suggested that denitrifiers in autotrophic systems could use Fe^{2+} and organic carbon from dead bacterial cells and soluble microbial products to reduce NO_2^- or NO_3^- to nitrogen gas (Kindaichi et al., 2007, Agrawal et al., 2017, Kiskira et al., 2017). Also, despite the low NO_2^-/NH_4^+ ratio in the effluent from the AOB-reactor, removal of nitrogen (NO_2^- and NH_4^+) was observed in the anammox reactor (Figure 5-3). However, the nitrogen removal rate (NRR) was only 0.084±0.069 kg-N/m^3-day, despite the nitrogen removal efficiency (NRE) being 53.71±13.14% (Figure 5-4). Furthermore, the performance of the entire system fluctuated during the entire period, with incidences where higher effluent nitrogen (NO_2^-, NO_3^- and NH_4^+ concentrations) being observed occasionally. This could have been driven by endogenous decay because of fluctuating operating conditions, possibly because there was no mixing in the reactors. The operation of the reactors under room temperature in this period could have also affected system performance as both AOB and anammox have previously been reported to favour higher temperatures 30°C-38°C (Dapena-Mora et al., 2004, Hellinga et al., 1999). The NRR of the system were also lower than those reported from full scale systems (Van der Star et al., 2007). It was thus concluded that the lack of mixing, room temperatures, and low feeding rates led to the observed low nitrogen removal during this phase of the study. Therefore, the AOB reactor was modified with the installation of a mixer, and both the AOB and anammox reactors were heated to 34±1°C. The further operation, however, was split into two phases: in phase I, the DO was 0.53±0.16 mg/L, in phase II, the DO was 1.48±0.84 mg/L in the AOB reactor.

After the AOB-reactor operational mode was switched from UASB to CSTR followed by re-inoculation, low nitrogen removal was observed in the early periods, with incidences of higher effluent nitrogen than the influent being during the first 20 days, leading to negative NRE values. In similarity to the anammox systems in the previous section (4.3), this could have been caused by the endogenous decay of the bacteria since they were yet to adapt to the new reactor conditions. Besides, during phase I, the effluent NH_4^+ concentrations were on the increasing trend, an indication of low nitrification rates (Figure 5-3). Similarly, the effluent NH_4^+ concentrations from the anammox reactor increased synchronously with the effluent NH_4^+ concentrations from the AOB reactor, a clear indication of the lack of enough electron acceptors for the proper functioning of the anammox system. Indeed, in phase I, the effluent NO_2^-/NH_4^+ ratio from AOB-reactor in phase was 0.24±1.95, which was lower than the stoichiometric 1.32 required for anammox process. This was despite the maintenance of approximately 2.5 mg/L mixed liquor suspended solids (MLSS) in both reactors during the second study period, besides maintaining the operational temperature at 34±1°C. Therefore, after 77 days of operation of the AOB-reactor as CSTR at 40 ml/min aeration rate, the aeration rate was increased to 140 ml/min in phase II in an attempt to enhance the activities of AOB, and consequently the performance of the two-stage system.

Following the increase of the aeration rate in the second phase of operation of AOB-reactor as CSTR (days 78-130), gradual increases in the effluent NO_2^- concentrations from the AOB-reactor were observed, leading to a decrease in the effluent NH_4^+ concentrations. The effluent NO_2^- concentrations from the AOB reactor increased to 37±23 mg-N/L in phase II, from 16±14 mg-N/L in phase I. During this period, the NO_2^-/NH_4^+ in the effluent from the AOB-reactor was 1.22±1.98, which was an improvement from what was observed in phase I. Similarly, an improvement in both NRR and NRE was observed in the AMX-reactor, from -966.87±2610.06% and 0.16±0.13 kg-N/m^3-day in phase I to 51.63±22.20% and 0.29±0.14 kg-N/m^3-day, respectively, in phase II (Figure 5-6). This thus indicates that maintaining the operational DO at approximately 1.5 mg/L in phase II (days 78-130) played a critical role in the improvement of the performance of the partial nitrification/anammox system. Regmi et al. (2014) also reported that maintaining a DO at approximately 1.5 mg/L enhances the partial nitrification-anammox system. The observed variation in both NRE and NRR in AMX-reactor in phases I (days 1-77) and II (days 78-130) were significantly different (t-test: $p<0.05$). Also, in both the first period and the second, the overall NREs of the two-stage system was higher than the NRE of the AMX-reactor (Figures 5-4 and 5-6), an indication that some of the nitrogen

was being removed in the AOB reactor, possibly through the activities of the anammox bacteria and heterotrophic bacteria. Indeed, characterization of the bacterial communities through qPCR revealed the presence of anammox bacteria in both the AOB-reactor and the AMX-reactor (section 5.3.2).

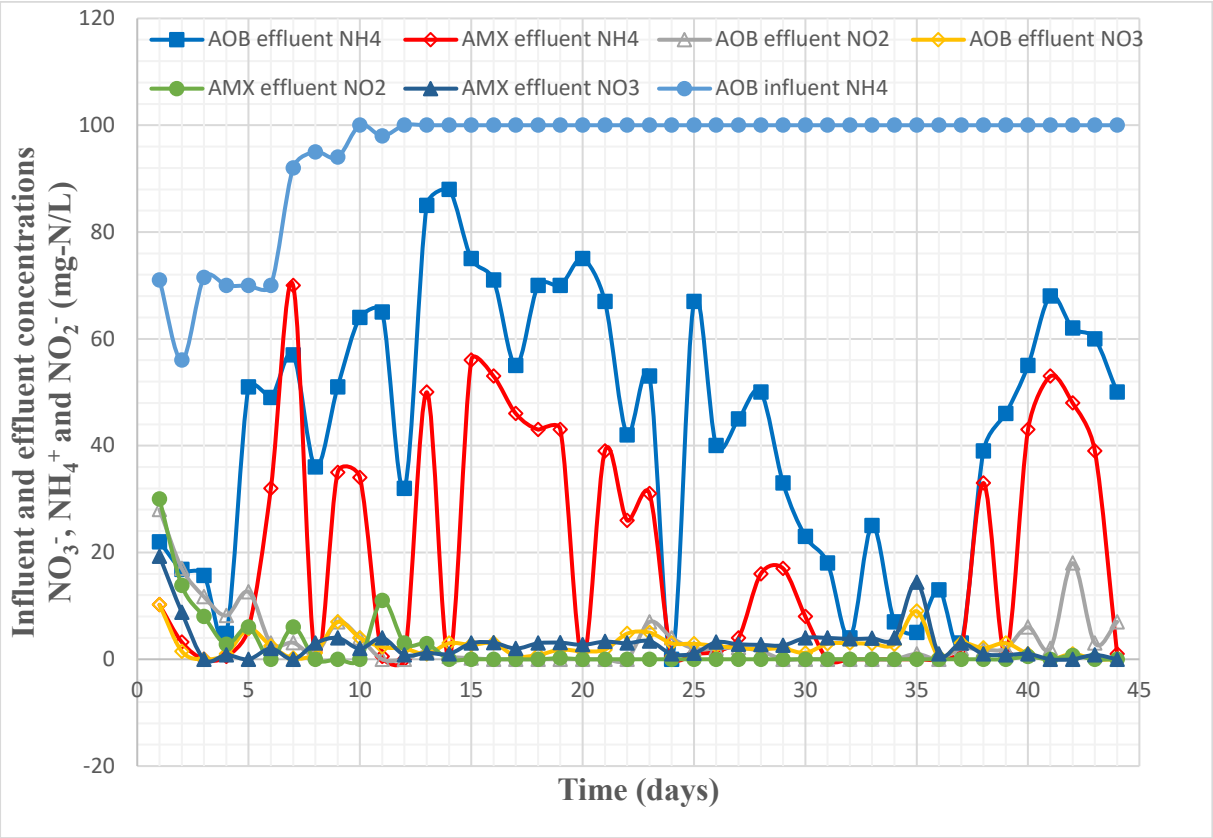


Figure 5-3: Influent and effluent concentrations of NO_2^-, NO_3^- and NH_4^+ from AOB reactor and AMX-reactor in the first period of study (when AOB reactor was operated as UASB)

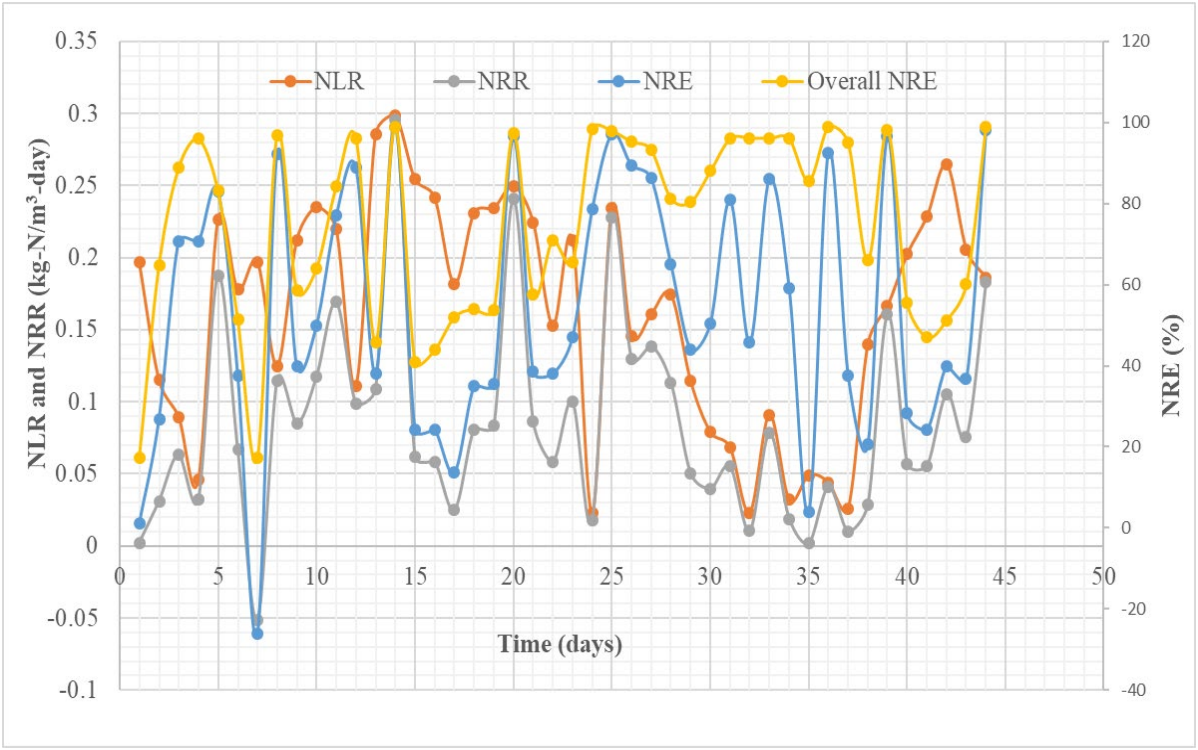


Figure 5-4: NLR, NRR, and NRE in the AMX-reactor in the first period of study (when AOB reactor was operated as UASB)

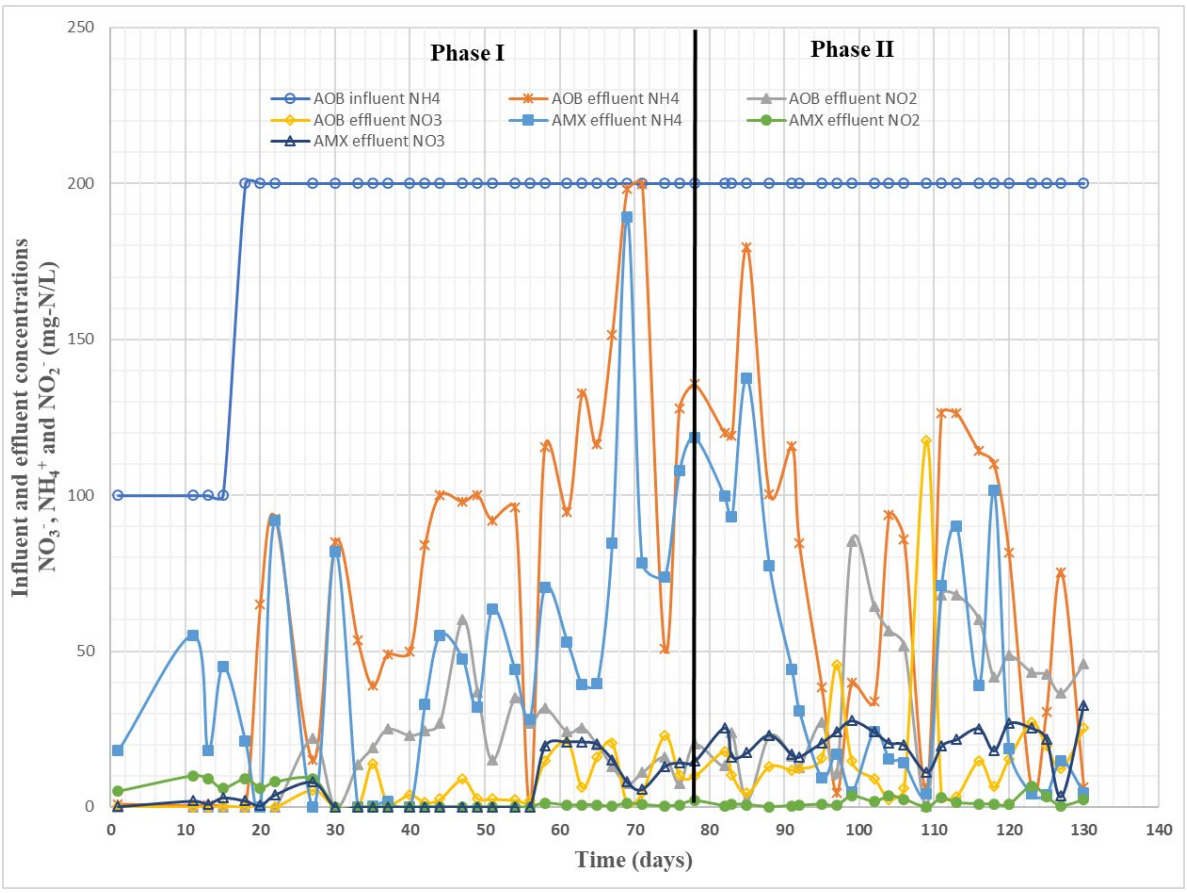


Figure 5-5: Influent and effluent concentrations of NO_2^-, NO_3^- and NH_4^+ from AOB and AMX-reactors in the second period of study (when AOB reactor was operated as CSTR)

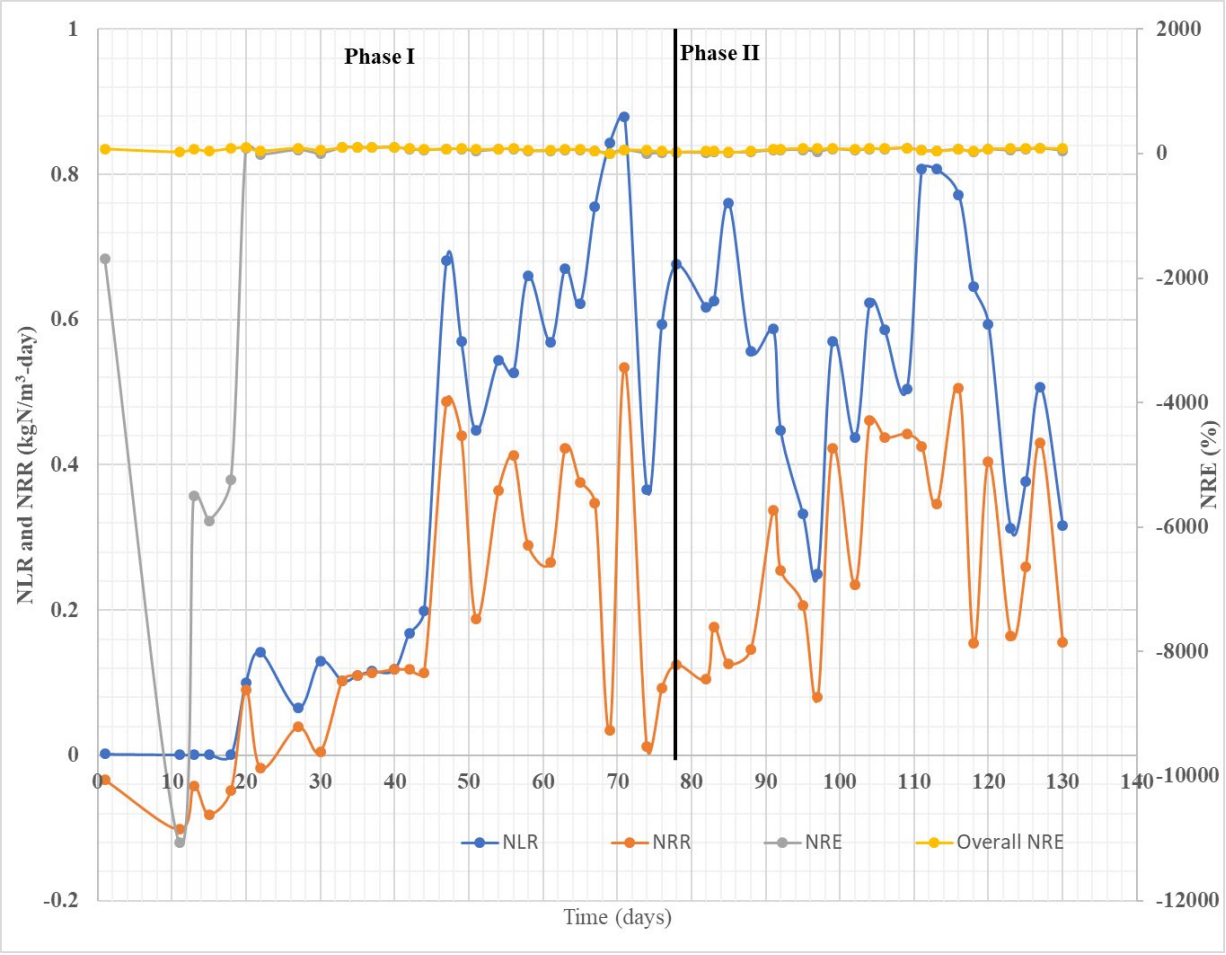


Figure 5-6: NLR, NRR and NRE in the AMX-reactor in the second period of study during phases I and II (when AOB reactor was operated as CSTR).

5.3.2 Characterization of bacterial populations

The quantification of AOB, NOB and anammox bacteria was done using qPCR. When the AOB-reactor was operated as UASB, the AOB, anammox bacteria, *Nitrospira* sp. and total bacteria were quantified (Table 5-3). When the AOB was re-inoculated and operated as CSTR, quantification of AOB, anammox bacteria, *Nitrobacter*-affiliated NOB, *Nitrospira*-affiliated NOB were done four times in the course of study (Figure 5-7). At the end of the study, reddish granules were observed in both AOB- and AMX-reactors which indicate the presence of anammox bacteria (Figure 5-8).

Although both AOB and anammox reactors were inoculated with biomass from an existing anammox reactor when both reactors were operated as UASB, the abundance of AOB, NOB and anammox bacteria were conspicuously different after 44 days of reactor operation (Table 5-3). As expected, the lower DO concentration in the anammox reactor favoured anammox bacterial growth in the anammox reactor, while the growth of AOB was favoured in the aerated reactor AOB reactor (Table 5-3). Based on qPCR investigation of the two-stage reactor, the anammox bacteria: AOB, and anammox bacteria: NOB ratios in the AMX-reactor were 5 and 3.2 respectively which indicated enrichment of anammox bacteria in that reactor, whilst AOB: NOB ratio in the same reactor was 0.71. As expected, in the AOB-reactor, the AOB: NOB ratio was higher (1.8), whilst anammox: AOB was 0.013. This indicated that the two reactors were suitable for enrichment of anammox (AMX-reactor) and AOB (AOB-reactor) notwithstanding the limited growth of NOB. A similar observation has been made by Miao et al. (2018), noting that although the activity of NOB can be inhibited, their complete washed out from the reactor under the long SRT (50 days) is difficult. This long SRT was similar to the operation in this present study hence the inability to completely suppress NOB.

However, on changing the mode of operation of the AOB reactor to CSTR from UASB as well as re-inoculating the reactor with biomass from a full-scale plant (Kingsburg wastewater treatment plant), a different bacterial composition was observed. Although *Nitrobacter* spp. remained low in both AOB and anammox reactors during the entire study period (≤7800 copy numbers), *Nitrospira* spp. and AOB varied synchronously in both AOB and anammox reactors. Anammox bacteria in the AOB reactor gradually increased approximately 21900 times during the first 70 days of the study (phase I), probably because the average bulk DO concentration during this period was below 0.6 mg/L, which is conducive for anammox bacteria (Strous et al., 1997).

Table 5-3: Abundance of AOB, Nitrospira, anammox bacteria during the operation of AOB-reactor as UASB

	Anammox bacteria	AOB	NOB (*Nitrospira*)	Total bacteria
AOB-reactor	6.41E+02±1.98E+02	5.01E+04±4.67E+04	2.78E+04±1.34E+04	1.39E+06±7.05E+05
AMX-reactor	1.30E+05±1.55E+05	2.81E+04±6.37E+03	3.98E+04±9.50E+03	7.36E+05±1.52E±05

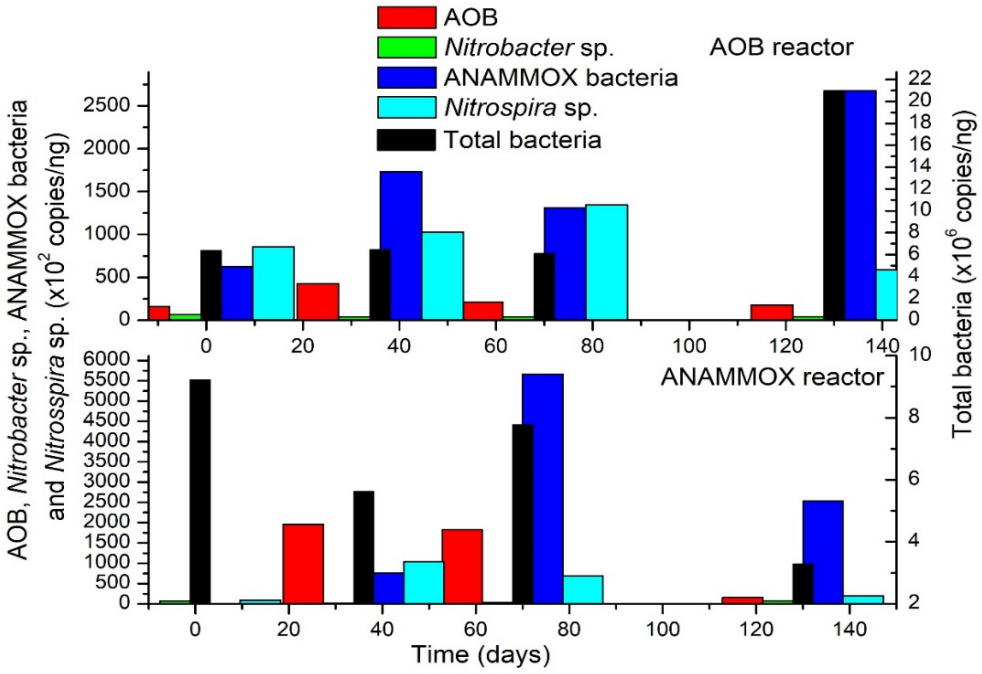


Figure 5-7: Copy numbers of AOB, anammox bacteria, Nitrospira spp., Nitrobacter spp. and total bacteria in both AOB- and AMX-reactors

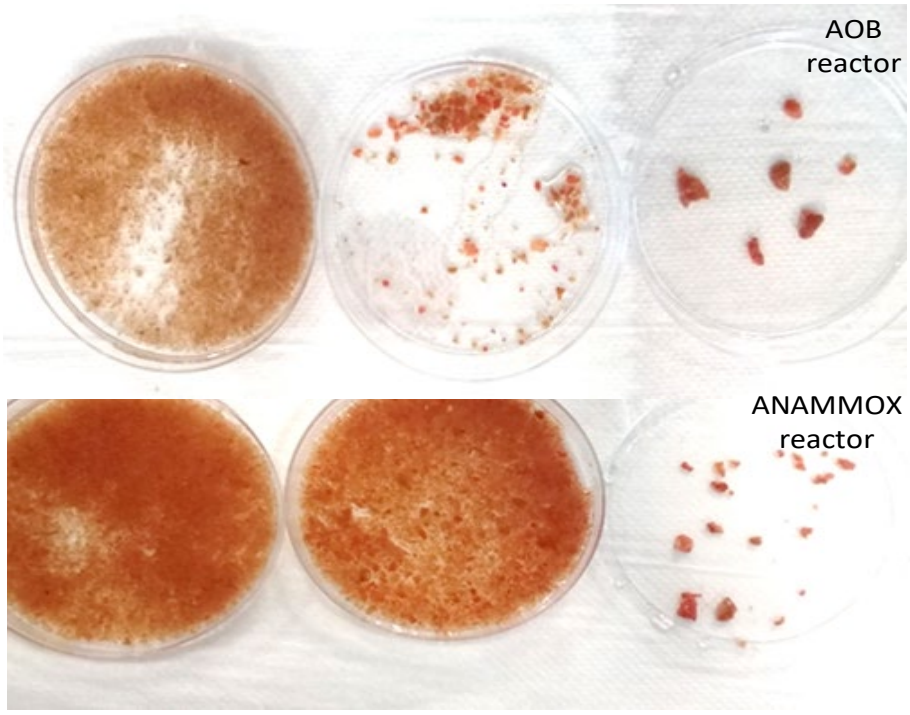


Figure 5-8: Granulation in both AOB and anammox reactors

5.3.3 Comparison of two-stage to single-stage partial nitrification/anammox system

The two-stage system studied here displayed the importance of maintaining DO at approximately 1.5 mg/L in the bulk liquid with the AOB reactor in the first stage, as opposed to less than 1 mg/L. However, in a single-stage partial nitrification-anammox system, maintaining DO concentrations above 0.5 mg/L would be detrimental to anammox bacteria as this would lead to inhibition. This was possible because, in a two-stage setup, AOB-conducive conditions were maintained in the first stage, while the conditions conducive to anammox bacteria were maintained in the second stage. Therefore, the compartmentalization of bacterial activities in a two-stage setup could make the operation of the two-stage systems a bit less complex, compared to single-stage systems that would require strict regulation of DO to avoid DO-inhibition of anammox bacteria, in turn leading to an increase in the instrumentation cost of single-stage systems. This observation is in line with that of earlier authors (Jaroszynski and Oleszkiewicz, 2011) who stated that a single-stage reactor system could be simple in terms of configuration, however, the complex interaction between AOB, NOB and anammox bacteria can be a challenge (Table 5-4). Moreover, it requires very stringent oxygen and pH control in

contrast with the two-stage system, which has a more complex configuration but allows simpler system design and operation which can lead to higher efficiency and reliability. The two-stage PN-A system is robust and capable of reduced recovery time following a possible system upsets. Moreover, the first-stage PN in the 2-stage design can also play a positive role in stabilizing the dominant microbial population in the second-stage (anammox reactor). However, an imbalance in the effluent's stoichiometric ratios of NH_4^+/NO_2^- from the PN stage will destabilize the anammox community in the second-stage since. Therefore, in this regard, the two-stage configuration is disadvantaged compared to the single-stage configuration as the NO_2^-/NH_4^+ ratios need to be strictly regulated (Table 5-4). In addition, it is necessary to install two reactors (each with its control units) in a two-stage configuration, which could raise the capital and operational costs above that of a single-stage configuration (Table 5-4) (Szatkowska et al., 2007).

Table 5-4: Comparison of single- and two-stage anammox-mediated systems

One stage	Two-stage	References
❖ Low capital and operational costs (one reactor) ❖ No need to maintain NO_2^-/NH_4^+ ratios above 1 ❖ Control of the C/N ratio in the influent is difficult ❖ Simultaneous regulation of conditions for AOB and anammox bacteria (process control is difficult)	❖ High capital and operational costs (two reactors) ❖ Necessary to maintain NO_2^-/NH_4^+ ratios above 1 ❖ Ease of C/N ratio control in the influent ❖ Independent regulation of conditions for AOB and anammox bacteria (process control is easy)	(Szatkowska et al., 2007, Van der Star et al., 2007, Rosenwinkel and Cornelius, 2005)

The operation of two-stage systems could also make the use of different reactor configurations in the first and second stages possible, leading to an improvement of process performance as it was done in this study. The CSTR employed in the first stage for partial nitrification made it possible to homogenize the operating conditions within the reactor, particularly oxygen, enabling the growth of AOB, while the UASB could have favoured the growth of anammox bacteria in the second stage as it retains the faster settling granules while washing out the slow settling flocs.

However, the application of two-stage PN-A systems at the full-scale level is limited compared to single-stage systems (Lackner et al., 2014). This could be stemming from the simplicity of reactor configuration in single-stage setups compared to two-stage setups. The stratification of bacterial communities in the biofilms and granules from anammox-mediated systems has made the single-stage systems attractive, as this could enable complementary activities by the AOB and anammox bacteria. For instance, AOB generates NO_2^- for anammox bacteria and in the process controlling DO concentration in the reactors, while the anammox bacteria remove the NO_2^- in the process preventing its accumulation to inhibitory levels for the AOB. Furthermore, operating granular reactors and biofilm based-systems enable the maintenance of high biomass concentration in the reactors (Lackner et al., 2014), making the two-stage systems non-attractive to single-stage systems. According to Jaroszynski and Oleszkiewicz (2011), and Lackner et al. (2014) the single-stage reactor system is more attractive than the two-stage from the commercial point of view since the single-stage is based on the proven reputation of SBR and MBBR technologies.

5.4 Conclusions

A two-stage partial nitrification-anammox system consisting of two reactors one for aerobic oxidizing bacteria (AOB-reactor) and a second one for anaerobic ammonium oxidizing bacteria (AMX-reactor) were operated under aerobic and anaerobic conditions, respectively. In the first part of the study, both the AOB-reactor and AMX-reactor were operated as UASB at ambient temperature, and in the second part of the study, the AOB-reactor was operated as CSTR and the temperature in both reactors was maintained at 34±1°C. The major findings of these investigations are as stated below:

- Although the activity of NOB can be inhibited, their complete washed out from the reactor could not be achieved in agreement with literature
- Operation of AOB reactor under DO concentration of approximately 1.5 mg/L led to better performance than at DO concentration below 1 mg/L
- Incorporation of mixing unit in the AOB-reactor and the maintenance of operational temperature at 34±1°C led to a significant improvement in process performance (t-test: $p<0.05$)
- The NO_2^-/NH_4^+ ratio is critical in the operation of two-stage partial nitrification/anammox systems as the NRR increased from 0.16±0.13 kg-N/m^3-day by 55% when the ratio increased from only 0.24±1.95 to 1.22±1.98

- NOB growth in partial nitrification/anammox system can be effectively regulated by controlling the DO in the reactors
- Based on qPCR investigation of the two-stage reactor, the anammox bacteria enriched in the AMX-reactor whilst AOB was enriched in the AOB-reactor.

6.1 GENERAL CONCLUSIONS

Developing an efficient, stable and cost-effective bioprocess technology for removing nitrogenous compounds from wastewater is of great interest globally and especially in South Africa considering the current protracted energy crisis. The PN-A system offers the advantages of efficient and cost-effective nitrogen removal without the need for organic carbon. This study aimed to develop a stable 2-stage PN-A system for the removal of nitrogen from wastewater.

Two identical 3 L SBRs were used for the optimisation of operational conditions (DO and pH) for AOB. The nitrite accumulation in the two SBRs (Reactor 1: 0.3-1.0 mg/L; Reactor 2: 1.3-2.0 mg/L) were significantly different (t-test: <0.05). DO levels of 0.3-1.0 mg/L and pH range of 8-8.5 supported the highest NO_2^- accumulation, indicating a better AOB activity and NOB inhibition. The FA concentration (1.87 and 3.18 mg/L) achieved during the AOB enrichment was above the inhibitory threshold (0.1-1.0 mg/L), hence, it contributed to the suppression of NOB whilst the FNA effect was negligible. Overall, there was evidence of higher NOB suppression in SBR reactor 1 during the AOB enrichment since its nitrate accumulation was lower (Phase 1: 18 mg/L and Phase 2: 13 mg/L) compared to SBR reactor 2 (Phase 1: 21 mg/L; Phase 2: 17 mg/L).

The UASB and SBR reactor systems were used for the mass cultivation of anammox bacteria obtained from a previously enriched anammox reactor. The highest nitrogen removal efficiencies (81±14%) during anammox mass cultivation was observed in the UASB reactor in phase VI. The NO_2^-/NH_4^+ (1.12±0.28) and NO_3^-/NH_4^+ (0.17±0.12) ratios close to the stoichiometric 1.32 and 0.26 ratios, respectively, expected of anammox process was observed from around the 190th day of operation of UASB. Shotgun sequencing of the UASB reactor sample revealed the dominance of the Planctomycetes which harbours anammox bacteria, with *Candidatus* Kuenenia being the dominant species. The Shannon and Simpson indices also corroborate the fact that the reactor progressed towards lower species diversity indicating successful enrichment of anammox bacteria within the UASB reactor.

A two-stage PN-A system consisting of one reactor for aerobic oxidizing bacteria (AOB-reactor) and another for anaerobic ammonium oxidizing bacteria (AMX-reactor) was successfully established. In the two-stage partial nitrification-anammox reactor, the operation of the AOB reactor under DO concentration of approximately 1.5 mg/L led to better performance than at DO concentration below 1 mg/L. Incorporation of a mixer in the AOB-

reactor (the two-stage system) and the maintenance of operational temperature at 34±1°C led to a significant improvement in process performance (t-test: $p<0.05$) in the two-stage process. The NO_2^-/NH_4^+ ratio in the effluent of the PN stage (AOB reactor) was found to be a critical factor in the operation of the subsequent anammox reactor of the two-stage PN-A system. Nitrogen removal rate increased from 0.16±0.13 kg-N/m^3-day by 55% when the ratio was increased from 0.24±1.95 to 1.22±1.98. It is important to maintain the temperature during the start-up period at >30°C and then gradually reduce it to ambient temperature after the system has stabilized. Maintaining the PN-A two-stage system at dissolved oxygen concentrations approximately 1.5 mg/L in the first stage to enable effective functioning of AOB is important. Based on qPCR investigation of the two-stage reactor, the anammox bacteria: AOB, and anammox bacteria: NOB ratios in the AMX-reactor were 5 and 3.2 respectively which indicated enrichment of anammox bacteria in that reactor, whilst AOB: NOB ratio in the same reactor was 0.71. As expected, in the AOB-reactor, the AOB: NOB ratio was higher (1.8), whilst anammox: AOB was 0.013. This indicated that the condition within the AOB-reactor and AMX-reactor achieved the enrichment of anammox bacteria and AOB respectively.

RECOMMENDATIONS

1. Single-stage PN-A systems are recommended to two-stage systems as they present cheaper alternatives in terms of the capital and operational costs than two-stage systems, although DO inhibition of anammox bacteria in two-stage systems configurations could be negligible as only the first reactor is aerated.

2. PN-A systems based on biofilms and granules have high biomass concentrations and hence, their development could be ideal for wastewaters containing high nitrogen concentrations such as digestate and source-separated urine.

3. With the current energy challenges in South Africa, retrofitting anammox-mediated systems within the current activated sludge systems can be a good way to achieve the PN-A system which offers the advantage of efficient nutrient removal coupled with achieving substantial energy savings.

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APPENDIX

Table S1: The optimized primer conditions

Real-time PCR step	Quantitative real-time PCR conditions using the primers				
	AOB	*Nitrospira* spp.	*Nitrobacter* spp.	Anammox	Total Bacteria
	amoA-1F/amoA-2R	NSR1113F/NSR1264R	Nitro1198f/Nitro1423r	AnnirS379F/AnnirS821R	P338F/P518R
1. Initial activation	3:30 min at 95°C	3:30 min at 95°C	3:30 min at 95°C	3:30 min at 95°C	3:30 min at 95°C
2. Denaturation	0:30 min at 95°C	0:30 min at 95°C	0:30 min at 95°C	0:30 min at 95°C	0:30 min at 95°C
3. Annealing	0:30 min at 54°C	0:30 min at 65°C	0:30 min at 52°C	0:30 min at 55°C	0:30 min at 58°C
4. Extension	0:30 min at 72°C	0:30 min at 72°C	0:30 min at 72°C	0:30 min at 72°C	0:30 min at 72°C
5. Read fluorescence	Read	Read	Read	Read	Read
6. Go to step 2 for	40 times	40 times	40 times	40 times	40 times
7. Melt curve	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s
8. Read fluorescence	Read	Read	Read	Read	Read