DETERMINING THE EFFICIENCY OF THE ANAMMOX PROCESS FOR THE TREATMENT OF HIGH- AMMONIA INFLUENT WASTEWATER

Submitted in fulfilment of the degree of Master of Applied Science: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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Declaration

Determining the efficiency of the ANAMMOX process for the treatment of high- ammonia influent wastewater

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

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Reference Declaration in Respect of a Master's Dissertation

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Dedication

To my mother.

For her patience, her love, her understanding, her support.

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Abstract

Domestic wastewater contains a high nutrient load, primarily in the form of Carbon (C), Nitrogen (N), and Phosphorous (P) compounds. If left untreated, these nutrients can cause eutrophication in receiving environments. Biological wastewater treatment utilizes a suspension of microorganisms that metabolize this excess nutrient load. Nitrogen removal in these systems are due to the synergistic processes of nitrification and denitrification, each of which requires its own set of operating parameters and controlling microbial groups. An alternative N-removal pathway termed the anammox process allows for total N-removal in a single step under anoxic conditions. This process, mediated by the anammox bacterial group, requires no organic carbon, produces negligible greenhouse gases and requires almost 50 % less energy than the conventional process, making it a promising new technology for efficient and cost-effective N-removal. In this study, a sequencing batch reactor (SBR) was established for the autotrophic removal of N-rich wastewater through an anammox-centric bacterial consortia. The key microbial members of this consortia were characterized and quantified over time using molecular methods and next generation sequencing to determine if the operational conditions had any effect on the seed inoculum population composition. Additionally, local South African wastewater treatment plants were screened for the presence of anammox bacteria through 16S rRNA amplification and enrichment in different reactor types.

A 3 L bench scale SBR was inoculated with active biomass (~ 5 % (v/v)) sourced from a parent anammox enrichment reactor, and maintained at a temperature of 35 °C ± 1 °C. The reactor was fed with a synthetic wastewater medium containing no organic C, minimal dissolved oxygen (< 0.5 mg/L), and N in the form of ammonium and nitrite in the ratio of 1:1.3. The reactor was operated for a period of 366 days and the effluent ammonium, nitrite and nitrate were measured during this period. The hydraulic retention time was controlled at 4.55 days from Day 1 to Day 250, and thereafter shortened to 1.52 days from Day 251 to Day 360 due to an increased nitrogen removal rate (NRR). During Phase I of operation (Day 1 to Day 150), the reactor performance gradually increased up to an NRR of ~160 mg N/day. During Phase II (Day 151 to Day 250), the overall reactor performance decreased with the NRR decreasing to ~90 mg N/day, while Phase III (Day 251 to Day 366) displayed a gradual recovery of NRR back to the reactor optimum of ~160 mg N/day. The accumulation of nitrate in the effluent during the latter parts of Phase II and Phase III, coupled with oxygen ingress (~2.1 mg/L) in the same period, indicated that

it was not the anammox pathway that was dominating N-removal within the reactor, but more likely the second half of the nitrification pathway mediated by the nitrite oxidizing bacteria (NOB).

This was further confirmed through molecular analysis, which indicated that the bacterial population had shifted significantly over the course of reactor operation. Quantitative PCR methods displayed a decrease in all the key N-removing population groups from Day 1 to Day 140, and a marginal increase in anammox and aerobic ammonia oxidizing bacteria from Day 140 – Day 260. From Day 300 onwards, NOB had started dominating the system, simultaneously suppressing the growth of other N-removing bacterial groups. Despite this, the NRR peaked during this period, indicating an alternative mechanism for ammonia removal within the reactor system. A total population analysis using NGS was also performed, which corroborated the QPCR results and displayed a population shift away from anammox bacteria towards predominantly NOB and members of the phylum *Chloroflexi*. The proliferation of aerobic NOB and *Chloroflexi*, and the suppression of anammox bacteria, indicated that DO ingress was indeed the primary cause of the population shift within the reactor. Despite this population shift, N-removal within the reactor remained high. New pathways have recently emerged which implicate these two groups as potential N oxidizers, with specific NOB groups showing the ability for oxidation of ammonia through the comammox process, and members of the Phylum *Chloroflexi* being capable of nitrite reduction. This could imply that an alternate pathway was responsible for the majority of N-removal within the system, in addition to the anammox and conventional nitrification pathways.

Additionally, in an attempt to detect a local anammox reservoir, eleven wastewater systems from around South Africa were screened for the presence of anammox bacteria. Through direct and nested PCR-based screening, anammox bacteria was not detectable in any of the activated sludge samples tested. Based on the operating conditions of the source wastewater systems, a subset of three sludge samples were selected for further enrichment. After 60-110 days of enrichment in multiple reactor configurations, only one reactor sample tested positive for the presence of anammox bacteria. Although this result indicates that anammox bacteria might not be ubiquitous within every biological wastewater system, it is more likely that anammox bacteria might only be present at undetectable levels, and that an extended enrichment prior to screening is necessary for a true representation of anammox bacterial prevalence in an environmental sample.

Preface

Research Outputs:

- 1 book chapter:

Gokal, J.; Awolusi, O. O.; Enitan, A. M.; Kumari, S.; and Bux, F. 2017. Chapter 4: Molecular characterization and quantification of microbial communities in wastewater treatment systems. In **Shukla, P.** *Microbial Biotechnology: an Interdisciplinary Approach*. CRC Press. Florida. ISBN: 13:978-1-4987-5677-8

- 1 Water Research Commission Technical Report:

Sheena Kumari, Jashan Gokal, Oluyemi Awolusi, Kriveshin Pillay, Nashia Deepnarain and Faizal Bux. 2016. The Activated Sludge BIOS- A real time tool to evaluate Activated Sludge Process Performance. *WRC Technical Report K5/2376:*

- 2 Conference Presentations:

-SASM 2016- High rate nitrogen removal through a stable anammox-nitrification process in a bench scale SBR at low dissolved oxygen concentrations

Jashan Gokal, S. Kumari, T. A. Stenström and F. Bux

-WISA 2016- Maintaining a stable anammox-nitrification consortium with the potential for high- rate ammonia removal

Jashan Gokal, S. Kumari, T. A. Stenström and F. Bux

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List of Abbreviations

ANAMMOX	Anaerobic ammonium oxidation
AOB	Ammonia oxidizing bacteria
AS	Activated Sludge
BABE	Bio-augmentation batch enhanced
BNR	Biological nutrient removal
bp	Nucleic acid base pairs
C	Carbon
CANON	Completely autotrophic nitrogen-removal over nitrite
CAS	Conventional activated sludge process configuration
CO_2	Carbon dioxide
CSTR	Continuous stirred tank reactor
Cu^{2+}	Cupric ion
ddH_20	Double-distilled deionized water
DAPI	4′, 6′-diamidino-2-phenylindol
DEAMOX	Denitrifying Ammonia Oxidation reactor configuration
DEMON	Deammonification reactor configuration
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
ED	Electrodialysis
EDTA	Ethylenediaminetetraacetic acid
EGSB	Expanded granular sludge bed
FA	Free ammonia
FAM	6-carboxyfluorescein
FBR	Fixed bed reactor
Fe^{2+}	Ferrous ion
FISH	Fluorescence *in situ* hybridization
FNA	Free nitrous acid
FRET	Fluorescence Resonance Energy Transfer
H_2S	Hydrogen Sulphide

HAO	Hydroxylamine oxidoreductase functional gene
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ion
HRT	Hydraulic retention time
HDH	Hydrazine dehydrogenase functional gene
HZS	Hydrazine synthase functional gene
IC_{50}	Minimum inhibitory concentration
IE	Ion exchange
MBR	Membrane bioreactor
MBBR	Movable bed biofilm reactor
MLE	Modified Ludzack Ettinger process configuration
MLSS	Mixed liquor suspended solids
MSA	Multiple sequence alignment
MSG	Monosodium glutamate
Mn^{2+}	Manganese ion
N	Nitrogen
N_2	Nitrogen gas
N_2O	Nitrous oxide
NH_3	Ammonia
NH_4^+	Ammonium ion
NirS	Nitrite reductase functional gene
NLR	Nitrogen loading rate
NO	Nitric oxide
NO_x	Generic nitrogen oxides
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOB	Nitrite oxidizing bacteria
NRR	Nitrogen removal rate
O_2	Oxygen gas
OLAND	Oxygen limited autotrophic nitrification and denitrification
OTU	Operational Taxonomic Units
PBS	Phosphate buffered saline

PC	Phosphatidylcholine
P/C/I	Phenol/Chloroform/Isoamyl Alcohol mix
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
pH	Potential of hydrogen ion
PN/A	Partial nitrification/Anammox
PO_4^-	Orthophosphate ion
PVP	Polyvinyl pyrrolidine
Q	Quinone
QS	Quorum sensing
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RO	Reverse osmosis
SAA	Specific anammox activity
SAM	S-adenosyl methionine
SBR	Sequencing batch reactor
SDS	Sodium dodecyl sulphate
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
$t1/2$	Doubling time of a bacterial species
TE	Tris/ Ethylenediaminetetraacetic acid buffer
TSS	Total suspended solids
UCT	University of Cape Town process configuration
μmax	Specific growth rate of a bacterial species
UASB	Upflow anaerobic sludge bed
VBNC	Viable but non-culturable
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

Chapter 1: Introduction

1.1. General introduction

Fresh water is a basic requirement of the metabolic activities of every living organism on the planet, and is a fundamental prerequisite to life itself. Despite this, accessible fresh water resources are rapidly dwindling due to unrestrained anthropogenic activities. As a cornerstone of the industrialized world, fresh water is used in a variety of industrial processes, which in turn produce large quantities of contaminated wastewater effluent. This industrial wastewater can contain a vast array of hazardous chemicals, heavy metals or recalcitrant organics, which can only be removed from this stream with specialized treatment systems. Wastewater may also arise from household waste, agricultural activities or non-toxic industries, and this wastewater is characterized largely by high nutrient loads, primarily consisting of Carbon (C), nitrogen (N) and phosphorous (P). These compounds are regularly utilised as growth substrates by various microorganisms, and it is this phenomenon that is exploited for the biological remediation of wastewater.

Biological wastewater treatment plants are engineered systems that provide a complex ecological niche for microbial growth. The biotic and abiotic factors prevailing at a particular plant serves to shape a resident microbial community comprising of a wide variety of specialist organisms. These microorganisms act synergistically to metabolize excessive nutrient loads from the influent waste stream.

Included among this complex microbial community are those specialist organisms that are capable of transforming potentially harmful ammonium (NH_4^+) into inert dinitrogen gas (N_2). This degradation pathway is through the coupled processes of aerobic autotrophic nitrification of ammonia (NH_3) to nitrate (NO_3), followed by anoxic heterotrophic denitrification from NO_3 to N_2 gas. Conventional ammonia removal from wastewater, accomplished through the combination of the spatially and metabolically separated processes of nitrification and denitrification, is an energy intensive process that generates a high amount of waste sludge (Bagchi et al., 2012). Furthermore, when the available carbon-to-nitrogen (C/N) ratio is low, as it is in many ammonium rich wastewaters, a readily biodegradable organic matter source must be added to achieve complete denitrification (Ni & Zhang, 2013). The increased oxygen demand for aerobic nitrification, the extra organic substrate addition for denitrification, and the surplus

sludge generated all increase the treatment cost of nitrogen containing wastewater (Ni & Zhang, 2013).

Until the latter half of the 20th century, ammonium was only considered metabolically active under oxic conditions, and thus the sole pathway for ammonia removal lay through the route of aerobic nitrification (Jetten et al., 2009). Although this conventional nitrogen removal pathway has been widely utilised in BNR processes, its numerous disadvantages have prompted the development of alternate nitrogen removal pathways that achieve the same goal in a far more efficient, cost effective manner.

The anaerobic ammonium- oxidation (anammox) process has emerged as one of the most efficient, sustainable and cost effective alternatives to the conventional nitrification/denitrification process. The "anammox" bacterial clade- as an abbreviation of "ANaerobic AMMonium OXidation"- is uniquely able to convert ammonia to dinitrogen gas under anaerobic conditions using nitrite as the electron acceptor (Strous et al., 1999b). The unique ability of a single bacterial group to hydrolyse ammonia directly to dinitrogen gas in a single step implies vast energy, time and cost savings over the conventional dual processes of nitrification and denitrification. Thus, by exchanging the conventional two step nitrogen removal pathway for the efficiency of the one step anammox process, a far more elegant ammonia removal process may be realised. Within the context of limited space, limited budget, and minimal energy expenditure, the implications of a self-contained nitrification-denitrification process through anammox becomes a clear advantage over the conventional means, particularly within developing countries, and especially within the current South African climate.

1.2. Rationale of the present study

Autotrophic N removal methods centred on the anammox process have been successfully implemented internationally as a cost effective and efficient N-removal technology. Implementation of a similar process for the treatment of low C/N wastewaters in South Africa would enable significant cost and energy savings, and an improvement over the currently utilised conventional nitrogen removal processes. Barriers to large scale deployment of this new technology are the lack of access to suitable anammox seed inoculum, and the difficulty of local enrichment. Since anammox bacteria have not yet been isolated in pure culture, current anammox-based studies involve the selective enrichment of anammox bacteria either from natural environments or existing anaerobic

reactors treating low C/N wastewaters. Comparisons between the conclusions drawn from these studies can often be ambiguous due to fundamental differences between these studies. Some of these differences include the level of anammox enrichment, the anammox bacterial species present, the operational conditions of the reactor used to enrich for anammox bacteria itself, and perhaps most importantly the characterization and contribution of the other organisms present within that same system (Jin et al., 2012b). Consequently, enrichment of anammox bacteria from both conventional wastewater sludge, as well as partially enriched anammox co-cultured sludge, still remains a significant challenge. More recent studies have reported that anammox bacteria can synergistically co-exist with other microorganisms, and this co-existence can play an important role in treating N-rich wastewater (Guo et al., 2016; Reeve et al., 2016; Langone et al., 2014; Liang et al., 2014; van den Berg et al., 2017). Further study into the anammox process, and its relationship to other synergistic microbial groups, could provide valuable insight into the development of an anammox-centric wastewater treatment system for South Africa.

1.3. Scope of study

The overall aim of this study was to develop a laboratory scale bioreactor for stable autotrophic N removal mediated by an anammox-nitrifier microbial consortium. The long term efficiency of this reactor system was assessed based on nitrogen removal performance, and compared with the constituent microbial population that mediates this N removal, in order to determine whether the relative population sizes of certain key populations have an impact on the N-removal activity of the system. Additionally, anammox bacterial enrichment was attempted from the activated sludge of indigenous wastewater treatment plants to identify a local reservoir for anammox bacteria. Previous anammox enrichment attempts in South Africa have yielded limited results and determining a new, accessible source for potential anammox seed biomass can rapidly advance local anammox based research.

1.4. Aim

To enrich for a stable anammox-nitrifying consortia within a laboratory scale biological reactor system for the removal of high influent N.

1.5. Objectives

i. Design and operation of a laboratory scale sequencing batch reactor (SBR) for the enrichment of a high rate N-removing anammox-nitrifier bacterial consortia.

ii. Determination of reactor efficiency by chemical analysis of influent and effluent samples.

iii. Identification and quantification of the major constituent N-removing microbial species, using PCR, QPCR and 16S rRNA community analysis.

iv. Screening and initiation of laboratory scale reactors for the enrichment of indigenous anammox bacteria from South African wastewater treatment plants.

Chapter 2: Literature Review

2.1. Biological wastewater treatment

The natural environment inherently possesses the capacity to clean contaminated water through the combined action of physical, chemical and biological processes. Unfortunately, the effluent wastewaters arising from anthropogenic activities are often polluted far beyond the remediation capacity of natural processes alone and need to be treated (Smith et al., 1998). In the metropolitan context, wastewater treatment refers to the use of specifically engineered systems that purify effluent wastewater prior to release into the natural environment. These engineered processes are designed to mimic the physical, chemical and biological principles used by the natural environment, but at a vastly accelerated rate.

Bioremediation of organic pollutants within a natural environment is primarily accomplished through the activity of its constituent microbiota, as a function of their metabolic processes. This phenomenon was discovered by Ardern and Lockett in 1914, where they noticed that microbial biomass retained from a previous wastewater treatment process remained active, and could be used as a starting inoculum to treat fresh, raw wastewater (Ardern & Lockett, 1914; Ardern, 1914). This biomass was termed "activated sludge" since it contained actively metabolizing microorganisms, and its development into the "Activated Sludge (AS) Process" represented a revolutionary enhancement to the biological wastewater treatment technology of its time (Sheik et al., 2014; Orhon, 2014).

Over a century later, the AS process is still the most widely utilized biological treatment method in the world (Keller et al., 2002; Gernaey et al., 2004; Bitton, 2005). It utilises a suspension of microorganisms that aerobically degrade the excess organic materials present within untreated wastewater by metabolically converting those nutrients into new biomass. Since its discovery, the AS-based biological wastewater treatment processes have been extensively modified to treat wastewater effluent from many diverse sources (Gernaey et al., 2004; Sheik et al., 2014).

Biological Nutrient Removal (BNR) is the most common modification to the conventional AS process. There are many different BNR plant configurations, each varying with regard

to influent flow rates to the reactors, recycle regime between reactors, and arrangement of differently sized reactors (Metcalf & Eddy, 2003; Ayanda & Akinsoji, 2011). All BNR plants are designed for the concurrent removal of organic matter, nitrogen and phosphorous from raw influent wastewater, however the particular configuration used can impact on the overall efficiency of the entire process. The best BNR plant configuration for any particular application largely depends on the influent wastewater characteristics and stringency of final effluent standards.

2.1.1. Nitrogen in wastewater

Organic matter containing Carbon (C), Nitrogen (N) and Phosphorous (P) are primary nutrients that can cause impairment within a receiving environment. The reactive N-species are particularly strong drivers of eutrophication, and promote excessive growth of phytoplankton and toxic algae if released untreated into receiving environments (Cai et al., 2013). Additionally, the uncontrolled release of ammonia can also result in a decrease in dissolved oxygen within the water column, toxicity to aquatic life and the eventual death of fish and shellfish in the water body (Terada et al., 2011; Ahn, 2006). More alarmingly, exposure to nitrates and nitrites can pose serious human health risks such as cancer and methaemoglobinaemia in new-born infants (Breisha, 2010).

Nitrogen enters the sewage stream primarily as urea, where it dissociates into ammonia (NH_3) through a process of urea hydrolysis (Eq. 1) (Awolusi et al., 2014). The ammonia formed during this process fluctuates between its base state, to the more acidified, more reactive ammonium (NH_4^+) molecule through the pH dependent process of ammonification (Eq. 2).

$$CO(NH_2)_2 + H_2O \longrightarrow 2NH_3 + CO_2 \quad (1)$$

$$NH_3 + H_2O \overset{\Delta pH\ ,\ \Delta T}{\Longleftrightarrow} NH_4^+ + OH^- \quad (2)$$

The NH_4^+ molecule remains in solution where it is oxidized by bacterial catalysts in the presence of oxygen to produce the oxidation products NO_2^- and NO_3, as well as the NOx gases, which are necessary intermediates in the conversion of ammonia to dinitrogen gas (Eq. 3) (Ghafari et al., 2008).

$$NH_4^+ \longrightarrow NO_3^- \longleftrightarrow NO_2^- \longrightarrow NO \longleftrightarrow N_2O \longrightarrow N_2 \quad (3)$$

Although the primary N input to the influent wastewater is in the form of NH_4^+, all of these N species can exist within the wastewater treatment system concurrently (Paetkau & Cicek, 2011). In order to successfully safeguard human health and protect receiving environments, robust and effective removal of NH_4^+–N, NO_3–N and NO_2^-–N from the effluent wastewater stream needs to occur before being released into the environment for reuse.

2.2. Biological nitrogen removal

There are two main approaches for the removal of reactive nitrogen species from wastewater streams; physicochemical and biological. The most common conventional physicochemical treatment processes are reverse osmosis (RO), ion exchange (IE), electrodialysis (ED) and activated carbon adsorption (Ghafari et al., 2008; Gupta et al., 2015; Capodaglio et al., 2015). These abiotic methods are often prohibitively costly, and may not work effectively in all types of wastewaters (Ghafari et al., 2008; Khin & Annachhatre, 2004). Consequently, many developing countries favour the biological approach to wastewater treatment, due to its cost effectiveness, efficiency, sustainability and flexibility (Ghafari et al., 2008; Ge et al., 2014; Gupta et al., 2015; Nourmohammadi et al., 2013). Biological N removal occurs much the same way as it does in nitrogen cycling within the biosphere, using microbial metabolism as the catalyst for bioconversion of N-species (Figure 1).

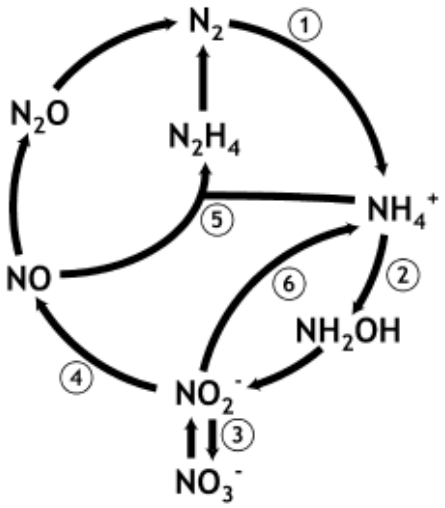

Figure 1: The nitrogen cycle. (1) Dinitrogen gas fixation; (2) aerobic ammonium oxidation; (3) aerobic nitrite oxidation; (4) denitrification; (5) anaerobic ammonium oxidation; and (6) dissimilatory nitrate and nitrite reduction to ammonium (Jetten, 2008).

Nitrogen removal in any biologically mediated process can be attributed to two interlinked and co-dependant processes- those of nitrification and denitrification (Costa et al., 2006). These processes occur synergistically, each mediated by specific and diverse microbial groups, in both natural and engineered ecosystems (Terada et al., 2011).

Nitrification is the bacterially mediated process whereby ammonia is oxidised to nitrate under aerobic conditions, and is conducted in two sequential oxidative stages by different bacterial genera (Ahn, 2006; Mess et al., 2012). The bacteria mediating this stage are considered to be "chemolithoautotrophic", since they utilise ammonia or nitrite as a primary energy source, molecular oxygen as an electron acceptor, and carbon dioxide as a carbon source (Ahn, 2006; Breisha, 2010). These two groups of microorganisms are broadly characterised as the ammonia oxidising bacteria (AOB) and the nitrite oxidising bacteria (NOB) respectively, and are primarily responsible for the conversion of ammonia to nitrite (ammonia oxidation, Eq. 4) and nitrite to nitrate (nitrite oxidation, Eq. 5 and Eq. 6)

$$NH_4^+ + 1.5\,O_2 \longrightarrow NO_2^- + 2H^+ + H_2O \quad (4)$$

$$NO_2^- + 0.5\,O_2 \longrightarrow NO_3^- \quad (5)$$

$$NH_4^+ + 2\,O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \quad (6)$$

The most prolific AOB groups identified within the wastewater system include *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus*. Although these groups are genetically diverse, they are all members of the ß-Proteobacterial clade (Breisha, 2010). The most commonly reported NOB includes; *Nitrobacter, Nitrospira, Nitrospina,* and *Nitrococcus,* These microorganisms fall within the α-Proteobacterial subdivision, with *Nitrobacter* generally being considered the model organism for nitrite oxidation (Breisha, 2010; Metcalf & Eddy, 2003).

In the subsequent process of denitrification; oxidised NO_2^- and NO_3 are reduced to gaseous nitrogen oxides (nitric oxide (NO) and nitrous oxide (N_2O)), and free nitrogen gas (N_2) by denitrifying bacteria under anoxic conditions (Terada et al., 2011).

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \quad (7)$$

In contrast to the chemolithoautotrophs of the nitrification step, denitrification is mediated by chemolithoheterotrophic microorganisms, which use nitrite and/or nitrate instead of oxygen as electron acceptors, with organic matter as the carbon and energy source (Banihani et al., 2012). Denitrifiers are often ubiquitous in nature and are particularly common among the Gram negative bacteria; specifically among *Pseudomonas, Alcaligenes, Paracoccus,* and *Thiobacillus* spp., although some Gram positive bacteria and a few archaea have shown denitrifying capacity as well (Breisha, 2010; Patureau et al., 1998; Metcalf & Eddy, 2003).

2.2.1. Factors affecting the efficiency of nitrogen removal in wastewater systems

Since it is a biologically mediated process, biological nitrogen removal proceeds relatively slowly when compared to other physicochemical methods (Khin & Annachhatre, 2004). As with any biologically mediated process, the bioconversion of influent ammonia to dinitrogen gas is dependent on:

- The presence and quantity of the necessary bacterial populations; and
- Their potential metabolic activity under the prevailing conditions.

As the processes of nitrification and denitrification are carried out by two functionally different microbial groups, the requirements for each process are fundamentally different. Thus, although these processes represent the two complementary halves of a single bioconversion pathway, each requires its own controlled set of operating parameters (Metcalf & Eddy, 2003).

The dissolved oxygen (DO) concentration has an enormous influence on the success of the nitrogen removal process. Since nitrification involves the oxidation of N-species, nitrification processes requires a high DO concentration, whereas a high DO has a negative influence on biological denitrification as this is often an anaerobic process. According to Metcalf and Eddy (2003), the amount of DO required for complete oxidation of ammonia is as high as 4.57 g O_2/g N. Accordingly, nitrifying bacteria requires a high degree of aeration in order to achieve an aerobic microenvironment that promotes their metabolism. In contrast, the denitrification process requires strictly anoxic conditions (van Rijn et al., 2006). In the presence of oxygen, denitrifying bacteria choose to preferentially utilise oxygen as a terminal electron acceptor instead of nitrate or nitrite

(Wang et al., 2007). In fact, the presence of oxygen actively represses the enzymes involved in the $NO_x \rightarrow N_2$ pathway, and it is only in the absence of oxygen that nitrogen oxides will be used as electron acceptors (Mess et al., 2012). In order to achieve these operational parameters, the processes of nitrification and denitrification are commonly physically separated, which often result in increased complexity of the system.

The influent C/N ratio is another important control measure for biological nitrogen removal (Akunna et al., 1992). Nitrification is a chemolithoautotrophic process and utilises inorganic CO_2 instead of organic carbon. In contrast, heterotrophic denitrification, by its very nature requires an organic carbon source, and the type of carbon source can have a significant effect on the denitrification rates (Bernat & Wojnowska-Baryła, 2007; Dhamole et al., 2007). A poor C/N ratio leads to improper denitrification; while a high C/N ratio may cause accumulation of nitrite or production of nitrous oxides (NO_x) (Patureau et al., 2000).

Critically, this C/N ratio directly influences the growth competition between fast growing heterotrophic and slower growing autotrophic microorganisms (Keller et al., 2002). An overgrowth of heterotrophic microorganisms in the system results in out-competition of the more specialised autotrophic nitrifier population. In this scenario, the activity of the nitrifier microbes would be suppressed, resulting in an accumulation of NH_4^+ in the system, and insufficient NO_3 being produced in the nitrification stage. The denitrifiers, which utilise NO_3 as an electron donor, would not have access to this essential metabolite and thus be unable to complete the denitrification process. Additionally, the accumulation of NH_4^+ in the system could reach toxic that could send the system into shock, thus crashing the entire treatment process.

2.3. Anaerobic ammonium oxidation

2.3.1. The anammox process

In 1977, based on thermodynamic calculations of the nitrogen cycle, Broda predicted that an autotrophic microorganism that could oxidise NH_4^+ under anaerobic conditions was a theoretical possibility (Kartal et al., 2013; Broda, 1977). This anaerobic oxidation of NH_4^+ was observed almost two decades later in a denitrifying reactor, and the process was soon confirmed to be biological in nature, thus proving Broda's original hypothesis (van de Graaf et al., 1995; Jetten et al., 1998; Kartal et al., 2011; Strous et al., 1999b). Termed

the anammox process by Mulder et al. (1995), this metabolic pathway and the organism that mediates it has redefined the traditionally accepted model for nitrogen cycling in the biosphere, and allowed for a more accurate understanding of global nitrogen cycling processes (Dang et al., 2015).

In the conventional nitrification/denitrification pathway, NH_4^+ is degraded to N_2 gas via three different microbially mediated reactions: the 2-stage aerobic nitrification reactions that oxidises NH_4^+ to NO_3 in the presence of oxygen; and the denitrification reaction that reduces this NO_3 to N_2 under anaerobic conditions. The anammox process, which represents an alternative pathway, allows for NH_4^+ to be oxidised directly to N_2 in a single step, thus circumventing the conventional process (Figure 2).

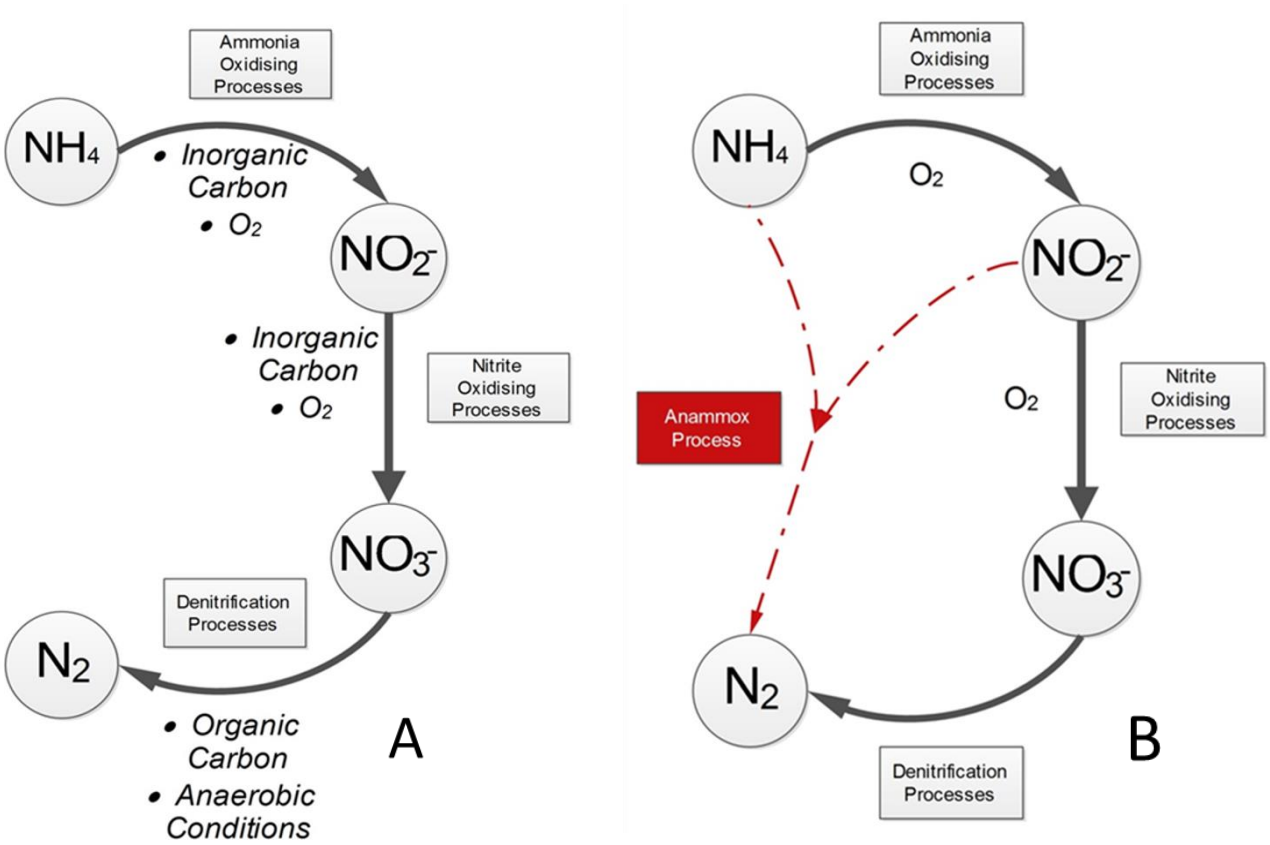

Figure 2: Comparison of: A) the conventional nitrification/denitrification process with B) the anammox process

Once the existence of the anammox process, and the bacteria that mediates it, was confirmed by Mulder et al. (1995) and van de Graaf et al. (1995), ecologists quickly started examining the biosphere for evidence of the anammox pathway in natural biogeochemical

cycling of nitrogen. To the astonishment of researchers, the anammox process was found to play a far more significant role than previously anticipated:

- In the cold marine sediments off the coasts of Greenland, nitrogen gas production primarily originates from anammox and denitrification processes, where the contribution of anammox is approximately 1–35 % of total nitrogen gas produced (Rysgaard et al., 2004).
- In the anoxic waters around the Golfo Dulce (Costa Rica), the anammox process accounts for 19–35 % of the total nitrogen gas in the water column, and this can increase to as much as 58 % in some marine zones (Dalsgaard & Thamdrup, 2002).
- The Black Sea is the world's largest anoxic basin, and it has been found that up to 40 % of the total nitrogen gas in this water column is generated through the anammox process (Jetten et al., 2003; Kuypers et al., 2003).

In fact, marine anammox bacteria have been found to be responsible for up to 67 % of the dinitrogen gas production within continental shelf sediments, and are thus more important for loss of fixed nitrogen than pure denitrification processes alone (Jetten et al., 2003; Dalsgaard & Thamdrup, 2002). This is due to the complex ecological processes of the marine system, where these interlinked processes of nitrification, denitrification and anammox are seen working in concert for N cycling: 1) ammonium is partially oxidized to nitrate in the aerobic surface layer; 2) this nitrate is transported to the anoxic zones by ocean currents, and it is then reduced to nitrite by denitrifying bacteria; and 3) anammox bacteria in the anoxic and anaerobic zones utilise the produced nitrites to oxidise remaining ammonia to dinitrogen gas. The combination of ammonium, nitrite and the deep sea anoxic environment creates a suitable habitat for anammox bacteria, and it is consequently estimated that up to 50 % of the loss of bound N from the world's oceans globally is attributed to the anammox process (Arrigo, 2005; Qinglong, 2011; Ding et al., 2013).

Just as conventional nitrification and denitrification are microbially mediated processes, the anammox process occurs through the biological action of specific anammox bacterial groups. They enable the anammox process as a function of their basic metabolic processes: carbon dioxide is used as a carbon source to produce biomass, while nitrite functions as both an electron acceptor for ammonium oxidation and also an electron donor for the reduction of carbon dioxide (Figure 3).

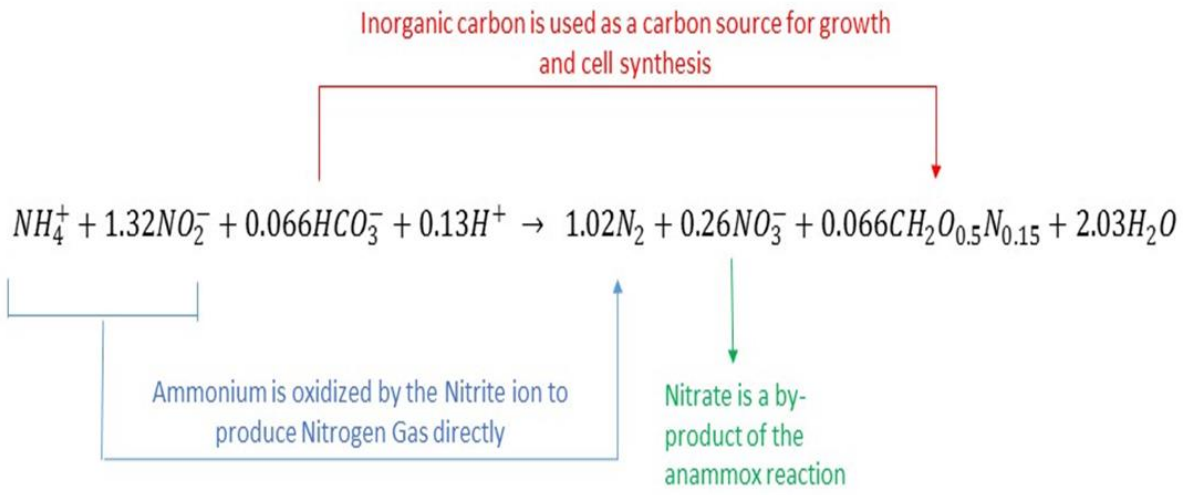

Figure 3: The balanced stoichiometric anammox equation (adapted from van der Star *et al.* (2007))

As indicated from the balanced anammox reaction, the anammox process requires an optimum ratio of 1mol NH_4^+:1.32 NO_2^-, no organic carbon and acts in the complete absence of oxygen. Most significantly, a maximum specific ammonium oxidation rate of 55 nmol NH_4/min/mg protein with nitrite as electron acceptor was observed for enriched anammox cultures- a reaction which is 25-fold faster than conventional chemolithotrophic ammonium oxidation with *Nitrosomonas* spp. (Keller et al., 2002).

The importance of the anammox process for nitrogen cycling in the biosphere has emphasized its prospective use for wastewater remediation, due to high efficiency and greatly reduced operating costs (Breisha, 2010). As a result, the anammox process appears to be a sustainable biotechnology for wholly autotrophic ammonia removal, however the slow growth rate and sensitivity of the anammox bacteria that mediate this process represents a major bottleneck to be overcome before successful widespread full-scale application (Jin et al., 2012a; Ahn, 2006).

2.3.2. Anammox bacteria

The anammox bacterial clade is a member of the order Planctomycetales, which falls within the phylum Planctomycetes- a relatively large group within the Bacterial domain (Kartal et al., 2004). Although extremely varied and ubiquitous within the biosphere, the members of this phylum exhibit many oddities that make them unique among other

bacteria, and are regarded as being genetically closest to the root of the bacterial domain (Brochier & Philippe, 2002).

The phylum Planctomycetes have been divided into eight culturable genera: *Pirellula, Gemmata, Planctomyces, Isosphaera, Blastopirellula, Rhodopirellula, Schlesneria* and *Singulisphaera*, and five unculturable *Candidatus* genera, to which the anammox bacteria belong (Qinglong, 2011). Anammox bacteria form a deeply branched phylogenetic group within the Planctomycetes, a separate order; "*Candidatus* Brocadiales", and family; "*Candidatus* Brocadiaceae". This family consists exclusively of anammox bacteria and encompasses all known genera capable of anaerobic ammonium oxidation (Van Niftrik, 2013). To date, five different genera have been described, as outlined in Table 1 (Awata et al., 2013; Boumann et al., 2009; Ali et al., 2013). These include the genera *Brocadia, Kuenenia, Scalindua, Anammoxoglobus* and *Jettenia* (Tsushima et al., 2007a; Schmidt et al., 2003; Jetten, 2008; Kartal et al., 2004; Strous et al., 1999b; Bagchi et al., 2012).

Table 1: Currently elucidated anammox species

Genus	**Species**	**References**
Brocadia	*Candidatus* Brocadia anammoxidans	Strous et al. (1999)
	Candidatus Brocadia fulgida	Kartal et al. (2007)
	Candidatus Brocadia sinica	Hu et al. (2012)
Kuenenia	*Candidatus* Kuenenia stuttgartiensis	Schmid et al. (2000)
Scalindua	*Candidatus* Scalindua brodae	Schmidt et al. (2003)
	Candidatus Scalindua wagneri	Schmidt et al. (2003)
	Candidatus Scalindua sorokinii	Kuypers et al. (2003)
	Candidatus Scalindua arabica	Woebken et al. (2008)
	Candidatus Scalindua sinooifield	Li et al. (2010)
	Candidatus Scalindua zhenghei	Hong et al. (2011)
	Candidatus Scalindua richardsii	Fuchsman et al. (2012)
Jettenia	*Candidatus* Jettenia asiatica	Tsushima et al. (2007)
Anammoxoglobus	*Candidatus* Anammoxoglobus propionicus	Kartal et al. (2007)
	Candidatus Anammoxoglobus sulphate	Liu et al. (2008)

The different anammox species also show some characteristic traits between them. *Candidatus* Scalindua spp. are marine anammox species adapted for high salinity and lower temperature conditions, with corresponding lower growth rates when compared to other known anammox species (Awata et al., 2013; Humbert et al., 2010). These *Ca.* Scalindua spp. are thus not the most suitable for commercial wastewater treatment. *Candidatus* Anammoxoglobus sulphate is an unusual anammox bacterial species in that it uses sulphate (SO_4^{2-}) instead of NO^{2-} as the electron donor for the oxidation of NH_4^+,

and thus shows strong promise for the treatment of sulphur containing wastewaters (Liu et al., 2008). Of the currently elucidated species, the *Ca.* Brocadia and *Ca.* Kuenenia have shown the most potential for nitrogen removal from wastewater as they have been previously enriched from wastewater plants, have relatively high growth rates and show high N-removal activity (Lotti et al., 2012b; Bagchi et al., 2012).

2.3.2.1. Physiological characteristics of anammox bacteria

The Planctomycetes as a whole are an unusual group within the bacterial domain, and have many features that set them apart from other bacterial clades (Kartal et al., 2004). The unique characteristics of this group include:

- Highly diverse cell morphology and cell arrangement across genera (Qinglong, 2011).
- Intracellular compartmentalisation (Van Niftrik, 2013)
- Diverse cell wall constituents (Fuerst, 2004)
- Highly diverse, and relatively unique metabolic requirements and ecological niches (Qinglong, 2011).

The anammox species in particular also display an unusual cell structure, especially when compared to other bacterial groups (Figure 4) (Van Niftrik, 2013). In particular, the cell wall composition, the internal membrane structure and the internal compartment, the anammoxosome, is unusual even when compared to other members within the same phylum.

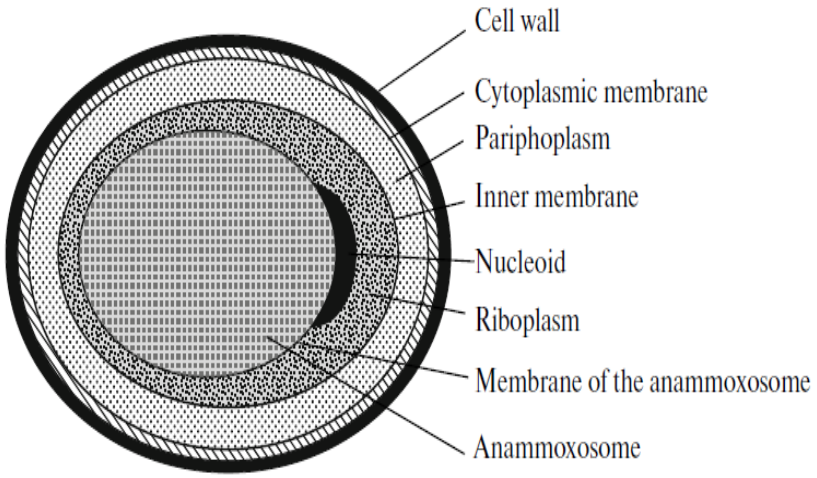

Figure 4: The anammox bacterial cell plan showing compartmentalisation within the anammox cell (Nozhevnikova et al., 2012)

The cell wall

The anammox bacterial cell wall was originally thought to not contain peptidoglycan like other members of the Planctomycetes phylum (Jetten et al., 2003; Van Niftrik, 2013). In stark contrast to bacterial members of other phyla, where the cell wall is largely composed of peptidoglycan, the anammox cell wall was assumed to be proteinaceous in nature (Jetten et al., 2003). Recent findings have since revealed that anammox bacteria do indeed have a thin peptidoglycan layer in their cell walls, but they also contain a predominant proteinaceous layer (van Teeseling et al., 2015). The structure and function of this protein layer is as yet unknown (van Teeseling et al., 2015).

The cell membrane

Where most bacterial species have a single cell membrane, the anammox bacteria possess multiple membranes that enclose and protect the cell. These have been divided into the cytoplasmic membrane, the paryphoplasm and the intracytoplasmic membrane. Further genetic evidence from *K. stuttgartiensis* has indicated the presence of a vacuolar-type H^+-translocating inorganic pyrophosphatase that might function as a transmembrane proton pump, however this protein shows closer structural homology to those within the archaeal domain rather than to closely related members of the bacterial domain (Russ et al., 2012).

The anammoxosome

The anammoxosome is the largest compartment within the anammox cell, comprising of up to 60 % of the total cell volume (Van Niftrik, 2013). It is the location for the anammox reaction (Equations 8-11), and is thus central to the metabolism of the cell (Van Niftrik et al., 2004; van Teeseling et al., 2013; Kartal et al., 2011). The anammoxosome membrane is extensively folded, which increases the surface area for the anammox reaction to take place by possibly enhancing the contact points for metabolic enzymes to act (Figure 5) (Van Niftrik, 2013).

$$NO_2^- + 2H^+ + 2e^- \rightarrow NO + H_2O \qquad (E_0' = +0.38\,V) \qquad (8)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \qquad (E_0' = +0.06\,V) \qquad (9)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \qquad (E_0' = -0.75\,V) \qquad (10)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (\Delta G^{0'} = -357\,kJ.mol^{-1}) \qquad (11)$$

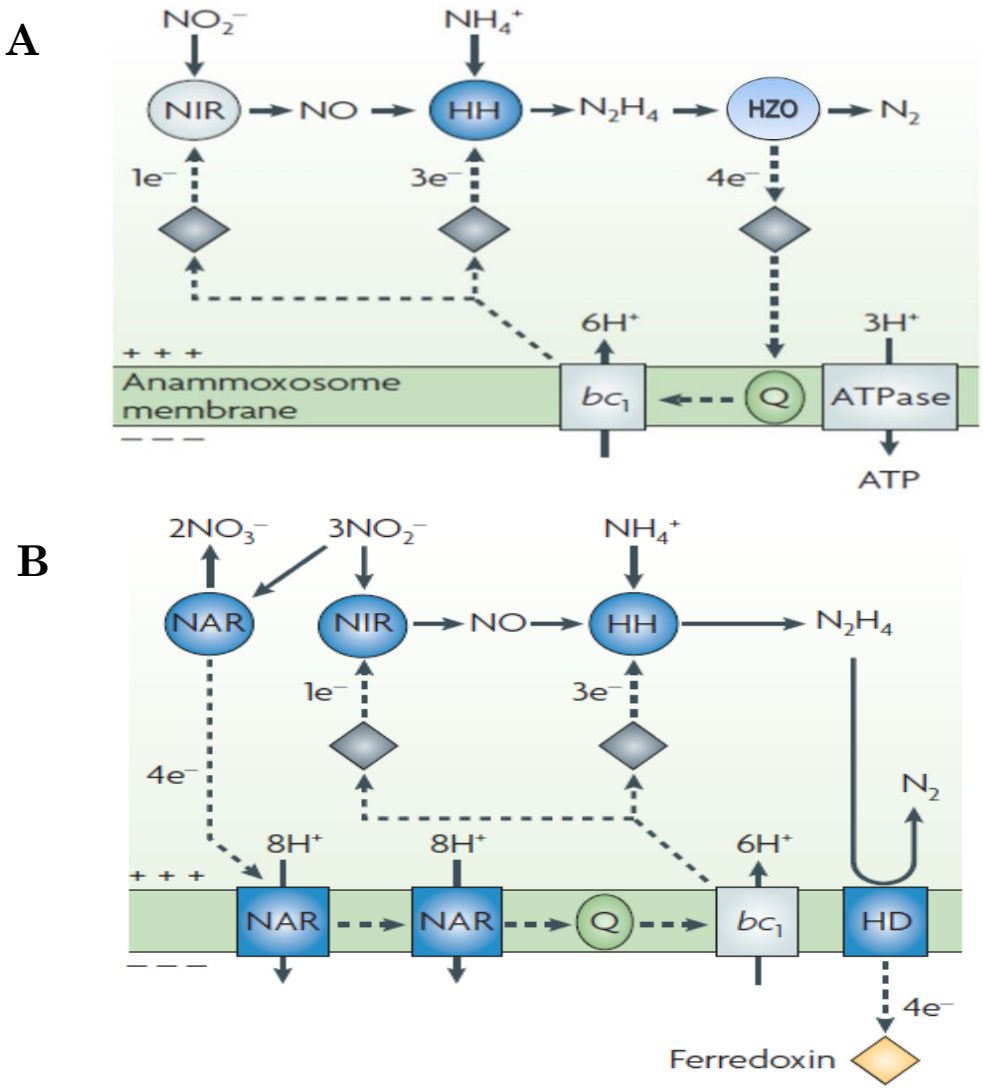

Figure 5: Hypothetical catabolism and electron transport through the anammoxosome (Kartal et al., 2011); a) Pathway of ammonium oxidation using nitrite as the electron acceptor to produce the intermediate hydrazine; b) Generation of Ferredoxin as a terminal electron acceptor in the acetyl-CoA pathway.

In Figure 5a, the generation of a proton motive force (PMF) over the anammoxosomal membrane allows for NO_2^- to be reduced to nitric oxide. This nitic oxide then combines with ammonium to produce hydrazine, along with the uptake of three low-energy electrons (Kartal et al., 2011). The oxidation of this hydrazine to nitrogen yields four high-energy electrons, which flows with the gradient through the quinone (Q) pool and the H^+–translocating cytochrome *bc*1 complex, thereby generating a second positive PMF (Kartal et al., 2011). This PMF energizes the proton-translocating ATPase for the production of ATP in the riboplasm. In Figure 5b the PMF-driven reversed electron

transport combines central catabolism with NO_3^- reductase to generate ferredoxin for carbon dioxide reduction in the acetyl-CoA pathway. Hydrazine and nitrite oxidation to nitrate can donate high-energy electrons to ferredoxin, which complete the anammox reaction (Kartal et al., 2011).

The anammoxosome has also been found to contain tubule like structures and electron dense particles (Fuerst, 2005; Van Niftrik et al., 2008). The tubular structures are hypothesized to be cytoskeletal supports involved in anammoxosome stability and division, or metabolically active protein configurations involved in the anammox metabolism; while the electron dense particles resemble bacterioferritins, iron-storage particles that serve as a reservoir for the heme-containing proteins that participate in the anammox reaction (Shimamura et al., 2008; Van Niftrik et al., 2008, 2004; Strous et al., 2006). Although the application of the anammox process has currently been in vogue, a thorough understanding of the anammoxosome structure and function still remains to be elucidated.

Ladderane lipids

Among the more unusual attributes of the anammox cell are the ladderane lipids of the anammoxosome membrane. The core ladderane lipids within the anammoxosome membrane consist of C18 and C20 fatty acids methyl esters and contain either 3 or 5 linearly concatenated cyclobutane rings. These rings are either bound to a glycerol backbone with an ester bond, or as alkyl chains with an ether bond (Figure 6) (Sinninghe Damsté et al., 2005). Furthermore, these intact polar lipids containing the core ladderane structures may have different types of polar head groups, including phosphatidylglycerol (PG), phosphatidylethanolamine (PE) or phosphatidylcholine (PC)or (Rattray et al., 2010; Boumann et al., 2009).

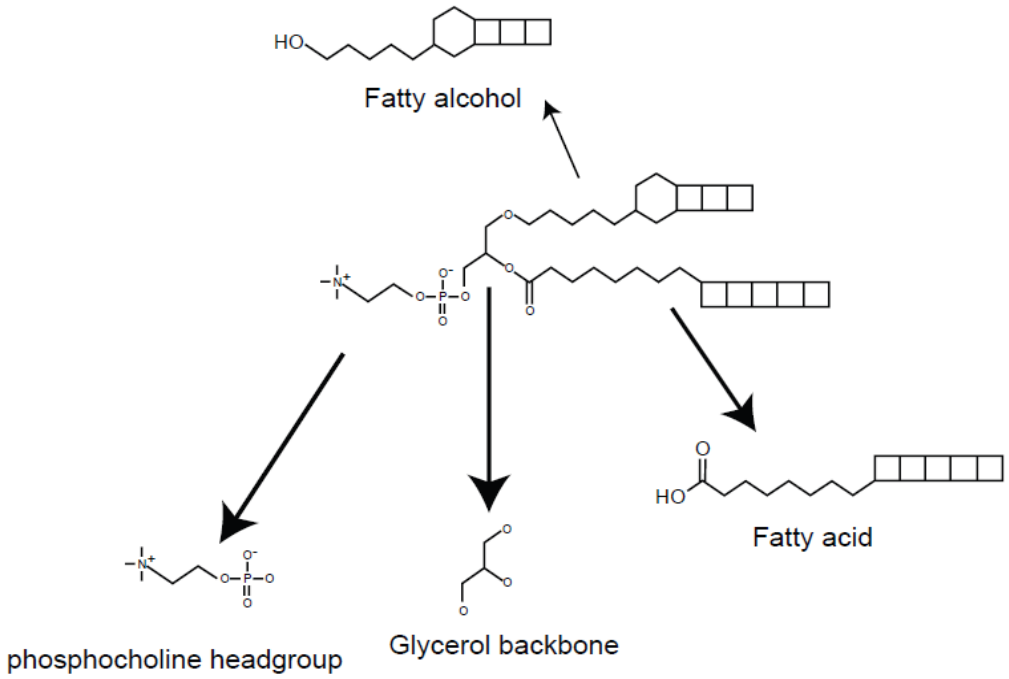

Figure 6: Schematic of ladderane lipids and its structural components (Rattray, 2008)

Ladderane lipids are unique to anammox bacteria, however their functional significance remains unknown. Since they represent an impermeable, dense membrane in comparison to conventional membranes (up to 1.6 kg/dm^3 vs 1.0 kg/dm^3), the evolution of ladderane lipids could be an adaptation to the cytotoxic effects of the anammox metabolism reaction intermediates hydrazine and hydroxylamine (Rattray et al., 2008; Jetten et al., 2009; Sinninghe Damsté et al., 2005).

2.3.2.2. Phylogenetic characteristics of anammox bacteria

Although they possess similar morphological and physiological characteristics, divergence between the five different anammox genera is relatively large and the sequence similarity at 16S rRNA gene level is often less than 85 % (Figure 7) (Boumann et al., 2009; Jetten et al., 2003, 2009). This large divergence still remains a mystery. Planctomycetes as a phylum, and particularly anammox bacteria, grow slowly, hence the phylogenetic distance cannot be due to rapid evolution. Conversely, a single event of "quantum evolution", usually characterised by a phylogenetic tree with a long stem and short branches, is not observed with anammox bacteria (Jetten et al., 2003). The two prevailing hypotheses explaining this genetic distance imply that:

- Since the Planctomycetales constitute an old lineage, individual genera genetically diverged quite early from each other, and thus had had more time to evolve (Brochier & Philippe, 2002); or that
- The genetic architecture of the Planctomycete ribosome may have allowed for a larger degree of genetic freedom, thus allowing a greater genetic drift between individual species and genera at the 16S rRNA level, without compromising ribosome function (Jetten et al., 2003).

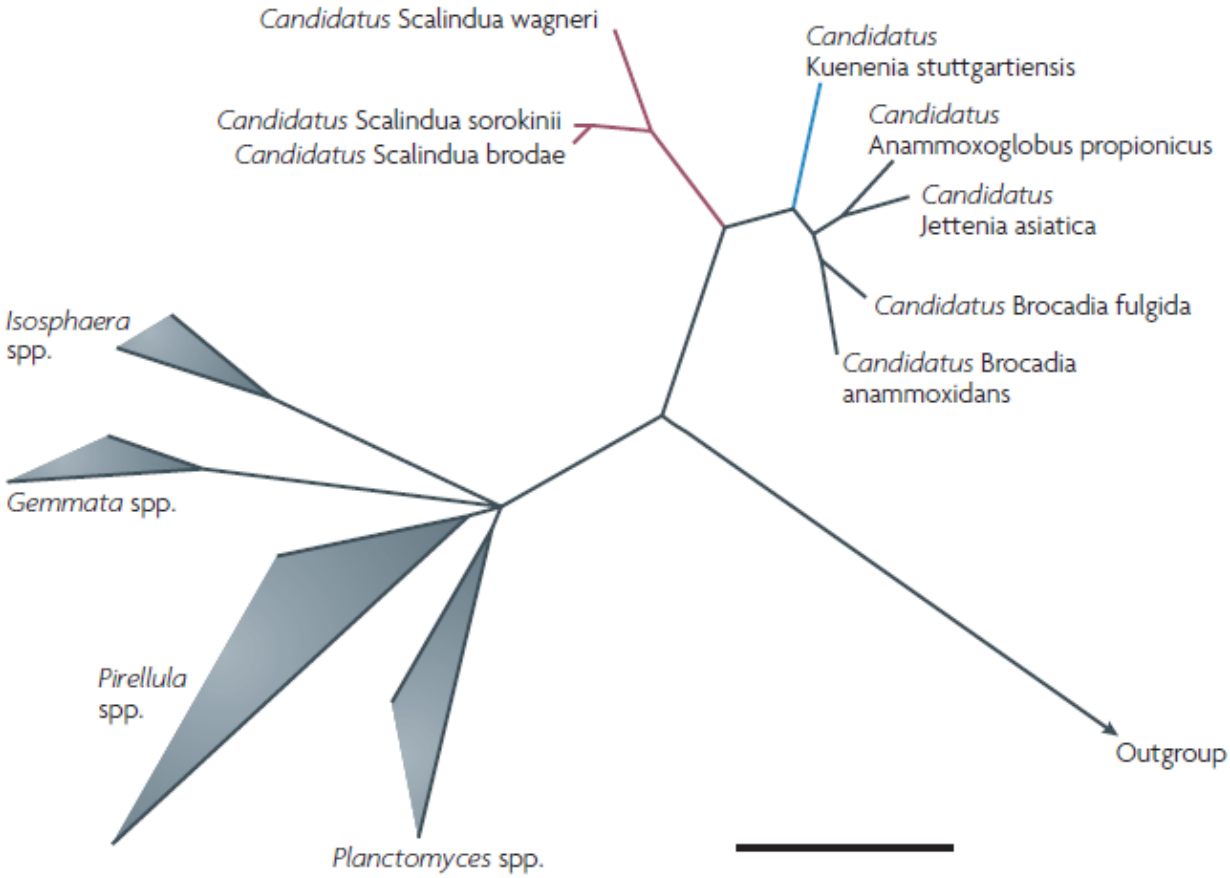

Figure 7: The 16s rRNA-gene-based phylogenetic tree of anammox bacteria. The scale bar represents 10 % sequence divergence (Kuenen, 2008).

The first metagenome of the enriched anammox culture: *Candidatus* Kuenenia stuttgartiensis was released in 2006, and elucidated the complete mechanism for the anammox metabolism (Strous et al., 2006). It included the enzymes that facilitated the anammox reactions (Equations 8-11 above):

a) the reduction of nitrite to nitric oxide (NO) by cd1 nitrite reductase (NirS);

b) condensation of ammonium and NO into hydrazine by hydrazine synthase (HZS); and
c) oxidation of hydrazine to dinitrogen gas by hydroxylamine oxidoreductase (HAO)-like hydrazine dehydrogenase (HDH) (Kartal et al., 2011).

The metagenomic sequences of three other anammox species were subsequently reported: a marine species: *Candidatus* Scalindua profunda; and two wastewater species: *Candidatus* Brocadia fulgida and *Candidatus* Jettenia asiatica (van de Vossenberg et al., 2013; Gori et al., 2011; Hu et al., 2012). Surprisingly, all of them displayed major differences to the *Ca.* K. stuttgartiensis metagenome.

Although the metagenomic approaches provide valuable information on gene content, these technologies do not adequately describe the activities of the microbial community, or how these activities vary with respect to space, time, environmental factors or other biotic and abiotic interactions (Bagchi et al., 2016). Nonetheless, the high degree of genetic variance could explain diversity of anammox bacteria with regard to the utilization of alternative carbon sources, alternating metabolic pathways and enzymes, as well as the adaptability of anammox bacteria to vastly different, often extreme, environmental niches.

2.3.2.3. Ecology and interspecies interactions

"A species' niche is determined by the traits that allow an organism to gather resources, evade enemies and any other factor that influences its relative birth and death rates" (Chase & Myers, 2011). In the case of the anammox bacteria, its unique metabolism is often the singular determinant to its ecological niche. Although isolation has proven difficult, the anammox bacteria have been detected in a number of different terrestrial (soil, wetland); aquatic (lake, estuary, river, sea); and engineered (oil reservoir, monosodium glutamate (MSG) wastewater and pharmaceutical waste, landfill leachate) ecosystems (Ali et al., 2013; Ni & Zhang, 2013). Being strictly anaerobic microorganisms, yet needing nitrites or sulphates as an electron acceptor, restricts the anammox to a very narrow growth niche at the oxic/anoxic interface of the given environment.

Oxic/anoxic interfaces are abundant in nature, particularly within biofilms and flocs. Biofilms are the predominant arrangement of microorganisms in the natural ecosystems, and is a mode of living which promotes close spatial and metabolic interactions between different species of microorganisms (Nozhevnikova et al., 2015). Anammox bacteria have

a tendency to form biofilms, and thus may naturally form stable, commensal relationships with other ammonia oxidising bacteria (Schmidt et al., 2002). Bacteria in biofilms are more protected against physiological or metabolic stress than their planktonic counterparts, and allows for these organisms to gain a fitness advantage particularly in stressful conditions (Burmølle et al., 2014). As an example: in oxic environments, AOB would oxidize ammonium to nitrite by utilising the oxygen within the microenvironment, thereby protecting anammox bacteria from the inhibitory effects of oxygen exposure, while anammox bacteria would convert the produced nitrite and the remaining ammonium to dinitrogen gas.

The bacterial members of a multispecies biofilm are either cooperative or competitive, depending on the molecular mechanisms of interaction (Burmølle et al., 2014). It has been found that the synergism and competition between anammox bacteria and their companion bacteria are regulated by ecological factors such as substrate, dissolved oxygen (DO) and organic matter (Ding et al., 2013). Anammox bacteria, along with AOB, denitrifiers, and in some cases NOB, interact based on co-metabolism of similar substrates (i.e.: NH_4^+, NO_2 and NO_3). When a substrate is limiting, a cooperative relationship can turn competitive, where the organisms with the greatest affinity for the limiting substrate outcompetes the other organism. Anammox bacteria may have adapted to this model of cooperative-competitive growth as it is an extremely slow growing organism with a specific growth rate (μmax) of 0.065 d^{-1}, and a doubling time ($t1/2 = \ln 2/\mu$max) of 11 days (Ni & 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).

Further evidence of anammox bacteria as a commensal, community-centric organism is the molecular evidence of genes encoding homologues of the S-adenosyl methionine (SAM) quorum sensing (QS) molecules (Ding et al., 2013; Strous et al., 2006). QS is often associated with bacterial communication in multispecies biofilms, and has been demonstrated to play important roles in development of biofilm formation across species boundaries (Burmølle et al., 2014). The presence of a QS system within anammox may explain its close relationship with companion bacterial species, as well as its current inability to be cultivated in pure culture, however not enough is known about quorum sensing signalling in anammox to exploit this for biotechnological application.

Although the eco-physiology of the anammox bacteria has yet to be elucidated in its entirety, the metabolism of anammox bacteria; (i.e.: the autotrophic oxidation of ammonium in the absence of oxygen), shows strong promise for the cost-effective, biological removal of high N loads from effluent wastewaters.

2.3.3. Anammox application for wastewater treatment

Biological nutrient removal processes are now integrating the anammox process as a novel and far more efficient approach to treating high ammonia containing wastewaters (Kartal et al., 2013; Strous et al., 2002; Schmidt et al., 2003). Nitrogen-rich wastewater, such as anaerobic sludge digestion liquid, landfill leachate, monosodium glutamate wastewater, nitrogen fertilizer production wastewater, coking wastewater and other industrial wastewaters are often rich in ammonium but low in COD (Jin et al., 2012a; Strous et al., 1997; Lotti et al., 2012a). This makes it difficult to treat in a conventional biological nitrogen removal system without incurring excess costs.

The anammox process shows many advantages over the conventional biological nitrogen removal processes, particularly with regard to the energy and associated cost saving (Gao et al., 2014b). In fact, despite the obvious advantages of having complete nitrogen removal in a single tank, it has further 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% (Mulder, 2003; Lackner et al., 2014).

2.3.3.1. Advantages over the conventional biological nitrogen removal process

Minimal organic Carbon input

The final denitrification step of the conventional nitrification/denitrification process, in which NO_3 is converted to N_2 gas, is mediated by heterotrophic denitrifiers. These organisms use organic carbon as a carbon source. This carbon source often needs to be manually dosed into the system since the influent may not contain adequate amount of readily biodegradable carbon sources to achieve the required level of nitrate reduction (Lu et al., 2014). Due to the chemolithoautotrophic nature of the anammox process no external organic carbon sources are required (van de Graaf et al., 1995; Hao et al., 2009; Ni & Zhang, 2013). This is especially promising for the treatment of those influent wastewaters with a low C/N ratio (Jaroszynski & Oleszkiewicz, 2011).

Low sludge production

Due to its slow growth rate, the biomass yield of anammox process is very low in relation to its ammonia conversion efficiency, and as a consequence very little sludge is produced (Strous et al., 1997). The low sludge production can significantly lower operating costs, especially in areas where sludge disposal is difficult, expensive or irregular.

Higher NH_4^+ conversion efficiencies

A further advantage of this low sludge production and high efficiency, especially with regard to biofilm systems, is that reactors can be run with higher biomass concentrations and volumetric loading rates within a smaller physical area (Abma et al., 2007). This effectively decreases the volume of anammox reactors, decreasing the spatial footprint needed for N-removal, reducing land usage.

Lower greenhouse gas production

While Biological Nitrogen Removal does successfully remove the aqueous N-load from wastewater, the aerobic nitrification and heterotrophic denitrification stages have been shown to be responsible for releasing significant proportions of N_2O and NO gas into the atmosphere (Chandran, 2011). The greenhouse equivalence of N_2O is approximately 300 times that of CO_2, and it is thus a potent contributor to global warming and depletion of the ozone layer (Ravishankara et al., 2009; Dameris, 2010). The anammox process does not utilise oxygen for the treatment of N-containing wastewaters, thus neither N_2O nor NO gases are formed as intermediates of the anammox reaction.

Minimal aeration required

Lastly, but perhaps most importantly, provision of elemental oxygen (O_2) through energy intensive aeration is not needed (Carvajal-Arroyo et al., 2014b; Hao et al., 2009). Furthermore, as hypothesized by Strous et al. (1997), if the anammox process is combined with a preceding aerobic nitrification step, only a portion of the ammonium load needs to be nitrified to nitrite, and a coupled anammox process combines any remaining ammonium with the nitrite produced in the preceding step to yield dinitrogen gas and complete the process. These combined processes offers further advantages such as less oxygen and alkalinity demand, with a significantly lower nitrate production that leads to negligible production of undesirable by-products like N_2O and H_2S (Ahn, 2006). These

combined processes are often operated in a single reactor unit, allowing for reduced aeration (1 kWh/kg N) and external organic load requirements; saving up to 90% of the operation costs (Wang et al., 2010).

2.3.3.2. Challenges of initiating the anammox process at full scale

Although application of the anammox process is a much anticipated new frontier in wastewater treatment, it still faces some bottlenecks to widespread implementation. Ironically, the very characteristics that make the anammox bacteria so unique and so useful within the wastewater system are the self-same characteristics that hinder its practical application. The major obstacle for the application of the anammox process at full scale is the lack of sufficient seed biomass (Tao et al., 2013). The anammox bacteria that mediate the anammox process are extremely slow growing organisms, and the difference in growth rates between nitrifiers and anammox bacteria is significant, with μmax at least ten times higher for the aerobic AOB and NOB (Gustavsson, 2010). Consequently, start-up of the anammox process can thus take several months and the enrichment of sufficient seed biomass to inoculate a large scale system requires an extensive cultivation period that is often labour-intensive, prone to out-competition and demanding a large time- and monetary investment (Ni & Zhang, 2013; Van Dongen et al., 2001).

Furthermore, once enriched, acclimatization of the anammox bacteria to real wastewater streams poses its own set of challenges. While domestic wastewater has been successfully treated at pilot scale, the characteristics of industrial wastewaters vary based on source, sampling point and even time of day (Breisha, 2010). The complexity of industrial effluent can make the anammox process difficult to initiate, particularly vulnerable to suppression, and can complicate the recovery from inhibition (Jin et al., 2012a; Bagchi et al., 2010). A lack of operator knowledge on how to adapt the anammox process to their particular wastewater streams has also resulted in its failure in many pilot scale attempts and has led to scepticism on the real world applicability of this technology.

Nevertheless, the advantages of the anammox process over the conventional biological nitrogen removal processes outweighs its challenges. While full scale implementation of the anammox process is still limited, it is not impossible. New technologies, reactor designs and operational configurations have been implemented in various forms at the pilot scale to mitigate these challenges and promote scale-up, and the successful anammox reactor at the Rotterdam WWTP (Netherlands) is proof of this.

2.3.3.3. Types of anammox processes and configurations

In order to both mitigate some of the aforementioned challenges and exploit the potential of the anammox process at full scale, several new biological nitrogen removal processes have been developed around the anammox process (Table 2). These designs are now commercially available, and maximise on the advantages of the anammox process as a way to shortcut conventional nitrification-denitrification, while minimizing potential anammox inhibition.

A common trait shared by many of these processes is that they involve partial nitrification 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 (Keluskar et al., 2013; Bagchi et al., 2012). These types of processes can be collectively referred to as a partial nitrification/anammox (PN/A) process, of which the CANON, SHARON and OLAND processes are typical examples of. These PN/A processes utilise a particular combination of AOB and anammox bacteria to achieve autotrophic removal of nitrogen (Ding et al., 2013; Phillips et al., 2002; Sliekers et al., 2003).

In these processes, AOB first convert ammonium into nitrite, and then anammox bacteria convert ammonium and nitrite into dinitrogen gas (Yan et al., 2012; Langone et al., 2014; Keluskar et al., 2013). 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). Interestingly, as anammox bacteria cannot directly utilise urea, AOB can also convert urea into usable ammonium (Ding et al., 2011; Phillips et al., 2002). This relationship is a substrate based synergism, as the anammox bacteria obtain both ammonium and nitrite from AOB when wastewaters contain urea.

Table 2: Comparison of current anammox process configurations

Process name	Abbreviation	Advantages	Disadvantages	References
Single-reactor system for high activity ammonia removal over nitrite, coupled with the anammox process	SHARON/ Anammox	• High N-loading and conversion rates • Significant saving in energy and operating costs.	• Technically complex to implement and operate. • Limited to wastewaters with a C/N ratio of ≤0.15	van Dongen et al. (2001); Sri Shalini and Joseph (2012)
Completely autotrophic nitrogen removal over nitrite	CANON	• Oxygen limiting conditions reduces aeration costs • Allows for complete N removal in a single tank	• Requires stringent aeration control. • Prolonged exposure to ammonia-limiting conditions results in the development of NOB populations.	Third et al. (2005; Bagchi et al. (2012)
Simultaneous nitrification, anammox, denitrification	SNAD	• Simultaneous removal of ammonium and organics in a single reactor	• Difficult regulatory control for maintain the population equilibrium for AOB, anammox and denitrifiers	Chen et al. (2009)
Oxygen limited autotrophic nitrification and denitrification	OLAND	• Similar to CANON process • Utilises oxygen limited conditions to culture both AOB and anammox	• Achieves lower NRR than similar CANON processes due to less stringency of control	Bagchi et al. (2012)
DEamMONification	DEMON	• Currently employed at full scale	• Achieves lower NRR than the CANON process	Wett (2007)
DEnitrifying AMmonia OXidation	DEAMOX	• Allows denitrification of nitrate using sulphide as an electron donor	• Application is restricted to sulfur-containing wastewaters. • Low NRR • Competition between anammox and heterotrophic denitrifiers	Kalyuzhnyi et al. (2006)
Bio-augmentation batch enhanced	BABE	• Easily retrofitted onto existing AS plants • Currently operating at full scale for nitrification	• Not yet applied to anammox processes outside of lab scale	Bagchi et al. (2012)

As the PN/A systems are designed around completely autotrophic bacteria, they cannot effectively treat influent wastewaters with a high organic load. The recently developed SNAD process enables simultaneous removal of nitrogen and biodegradable organics in a single system, through the synergistic action of an autotrophic AOB population, the anammox population, as well as a heterotrophic microbial fraction (Wang et al., 2016; Chen et al., 2009). Furthermore, bio-augmentation of existing BNR plants using the BABE process has proven effective for nitrification at cold temperatures (Munz et al., 2012). While bio-augmentation of existing anammox-based technologies have not been implemented outside of laboratory scale studies, it has shown potential for mitigating the weaknesses of other anammox-based processes (Zhang et al., 2014; Bagchi et al., 2012; Gao et al., 2014b; Ma et al., 2016; Sabine Marie et al., 2015; van Loosdrecht et al., 2016).

Optimal control over such systems remains a potent challenge; especially when the preceding partial nitritation process needs to produce an anammox influent according to the defined optimum ammonia to nitrite ratio (1:1.32) for optimum activity (van de Graaf et al., 1997; Joss et al., 2011; Fux et al., 2002; Xing et al., 2013a). The advanced control measures that can be used to maintain an appropriate influent nutrient ratio include manipulating the temperature and the hydraulic retention time (HRT), controlling competitor microbial species by inhibition of nitrite oxidizing bacteria by increasing free ammonium, or strictly manipulating the dissolved oxygen concentration (Monballiu et al., 2013; Lackner et al., 2014).

2.3.3.4. The Rotterdam plant as a case study

The first full scale anammox wastewater treatment system (Figure 8) was established in 2002, and has been operating at the Sluisjesdijk sludge treatment plant in Rotterdam (The Netherlands) to remove nitrogen from concentrated sludge reject water (Abma et al., 2007; Van Niftrik, 2013). This reactor, which has a design load was 500 kg-N/d, is operated in the SHARON-anammox configuration and is partially fed with nitritated sludge from a neighbouring nitritation process (van der Star et al., 2007; Van Dongen et al., 2001).

The innovative design features of this reactor played a large role in the success of the anammox start-up. The innovative 3 phase separators allowed for maximal retention of the biomass, while still allowing for a sufficient hydraulic flow between chambers. Furthermore, mixing influent with both an additional recirculation flow from the effluent of the reactor, as well as with the off-gas produced by the reactor allowed for an adequate upflow velocity that promoted granule formation. Finally, online monitoring and control

systems at multiple points of the reactor allowed operators to rapidly respond to problems detected during reactor operation (van der Star et al., 2007).

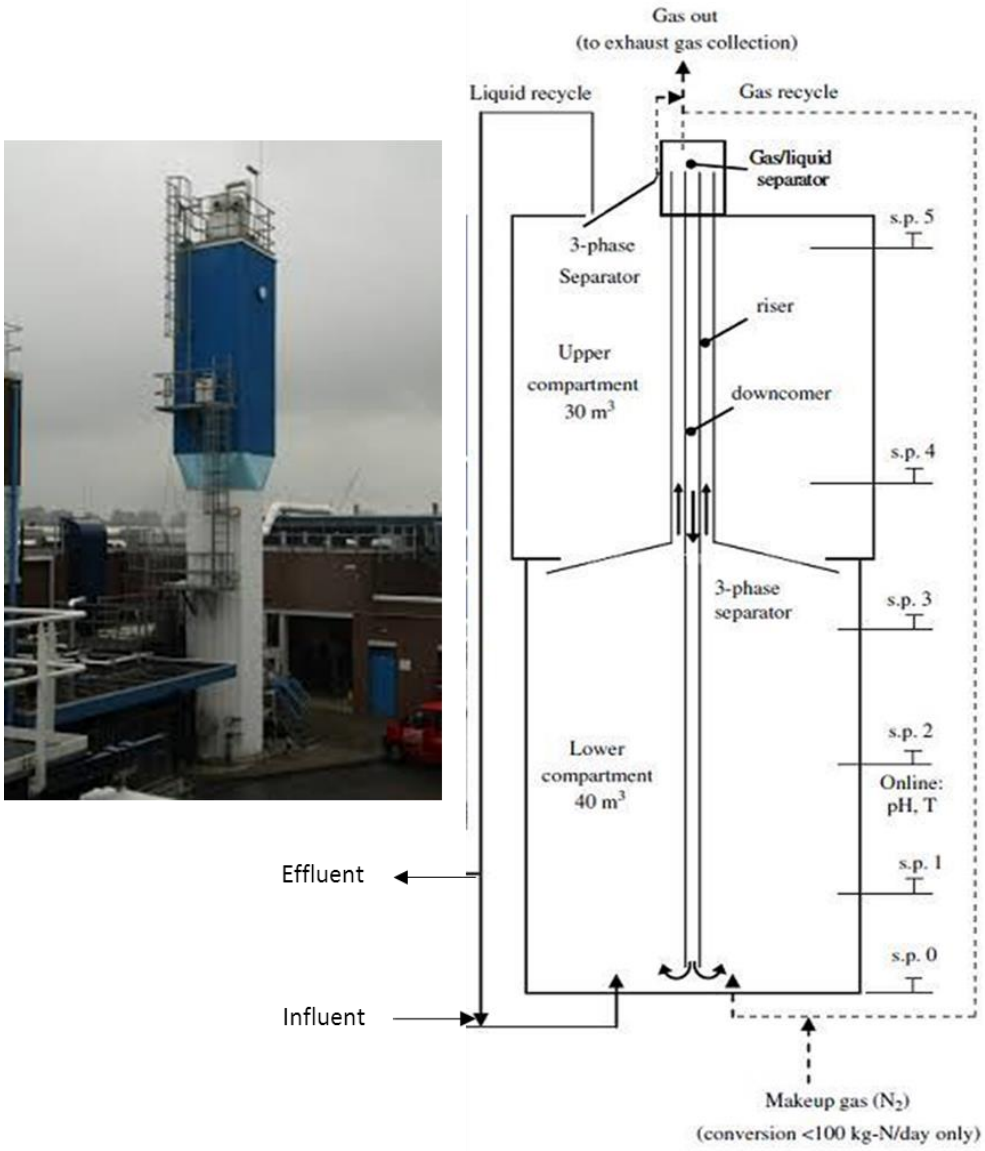

Figure 8: The full scale anammox UASB reactor in Rotterdam (Netherlands). The influent moves from the lower compartment to the less mixed upper compartment, allowing for biomass retention and effluent polishing. The gas generated by the reactor is fed back into the lower compartment to further promote mixing (van der Star et al., 2007)

Nevertheless, even with an optimal reactor system, precise operational control and knowledgeable operators, anammox activity was only seen 800 days after start-up, and the reactor only achieved its design N-loading rate of 7.1 kg-N/m^3/d on Day 1359 (van der Star et al., 2007). Additionally, the start-up period itself was not without difficulty. As observed by van der Star et al. (2007), some of the problems experienced included:

- Decreased N-removal due to incidental nitrite toxicity
- Loss of active anammox biomass due to sudden changes in the hydraulic regime
- Breakthrough of toxic methanol from nitritation
- Freezing of pipes leading to blockage of the influent stream
- Mechanical failure of pumps and compressor resulted in inefficient bulk liquid mass transfer
- Discharge of wastewater from mobile chemical toilets directly in the sludge line inhibited the key bacterial populations.

Despite the challenges experienced during this full-scale attempt, the Rotterdam reactor is still operational, and is now functioning significantly above its calculated design capacity and predicted efficiency (van der Star et al., 2007). Indeed, by learning from this first attempt, the anammox process has been incorporated into over 100 WWTW worldwide (Lackner et al., 2014). In fact, the application of the anammox process has been positively implemented for the treatment of landfill leachate, as well as sewage, food processing, yeast, tannery, monosodium glutamate, and pharmaceutical wastewaters respectively (Tang et al., 2014; Hu et al., 2010; Lotti et al., 2012a; Ali et al., 2013; Van Niftrik, 2013). One of the most significant findings of this study showed that biomass produced by existing anammox reactors can be used for the inoculation of other reactors, and that a larger inoculum of enriched active anammox biomass will decrease the start-up times of other anammox reactors (van der Star et al., 2007). Thus, the bottleneck for the widespread development of full scale anammox based systems remains the availability of large amounts of enriched anammox biomass.

2.3.4. Anammox Enrichment

Bacterial enrichment is a microbial culturing technique used to selectively propagate specific organisms from a mixed population, while inhibiting growth of other organisms within the same system. This method is often used when the classical microbiological approach based on the isolation of pure cultures cannot be achieved. For the anammox bacterial group, which are fastidious, slow growing organisms that have not yet been isolated in pure culture, highly concentrated enrichment cultures have been the only way to effectively study these organisms (Tsushima et al., 2007a; Ni & Zhang, 2013).

Although the literature is extensive, the cultivation of anammox from conventional sludge is still cumbersome, due to the excessively slow growth rates, long start-up times,

sensitivity to inhibition and tendency towards being outcompeted (Ni & Zhang, 2013). Anammox bacteria tend towards the K-strategy of growth (organisms that reproduce slowly but have a higher substrate affinity) instead of the R-strategy (rapid growth rate but utilising the growth substrates less efficiently) typical of competitor organisms (Awolusi et al., 2014; Cébron & Garnier, 2005; Kallistova et al., 2016). Anammox enrichment from conventional wastewater sludge is still complex, since batch enrichment methods are notorious for encouraging the growth of rate-specialist organisms (R-strategists) at the expense of more energetically-efficient organisms (K-strategists). The use of specially designed enrichment bioreactors can provide a rigorously controlled environment specifically for the selection, enrichment and long term maintenance of these fastidious organisms.

Consequently the use of a suitable reactor configuration and feed-regime that allows for these needs is essential. These reactors and operational conditions can be modified broadly in order to alleviate some of the fundamental problems common to many anammox enrichment studies: the need to maintain anaerobic or low DO environments; to optimally control N-loading; to minimise substrate inhibitory effects; and to somehow retain the slow-growing anammox biomass within the reactor while simultaneously washing out competing populations (Suneethi et al., 2014; Pérez et al., 2014; Lotti et al., 2014). Considering that obtaining suitable quantities of the enriched anammox culture is a prerequisite to successful large scale biotechnological application, the importance of successful anammox enrichment techniques should not be underestimated.

2.3.4.1. 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 (Jin et al., 2012a).

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 (Table 3). Unfortunately, experimentally derived values for these factors often differ significantly from each other, particularly with regard to 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, 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.

Substrate Concentrations, pH and Temperature

As per the metabolic pathways discussed above (Figure 3), NH_4^+ as the primary substrate is oxidised 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 a pure culture, and may not directly apply to enrichment studies, especially those initialised 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 is able to 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. (2014) 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. (2013) 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 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, in order to prevent the accumulation of the inhibitory FA and FNA, and to maintain 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.

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., 2012a). 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). In fact, 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 growth requirement for the coexisting populations. Nevertheless, stringent control over the DO within the system is essential to prevent of over growth of the aerobes and out competition of the anammox population, and recent studies have indicated maintaining a constant DO concentration of 0.5-1 mg-O_2/L will effectively stabilise the delicate balance between the AOB, NOB and anammox bacterial populations (Zekker et al., 2014; Hu et al., 2013).

Table 3: Summary of the most important parameters for anammox enrichment

Parameter	Optimum Range	Inhibitory Range	Exception	Reference
Nitrite Concentration	Dictated by the SAA of the culture	11mg/L HNO2	FNA is inhibitory and is a function of pH and Nitrite concentration	Fernández et al. (2012); Puyol et al. (2013)
Ammonia Load	Dictated by the SAA of the culture	>20mg/L FA	FA is inhibitory and is a function of pH and ammonia concentration	Fernández et al. (2012); Puyol et al. (2014)
Nitrate Conc.	-	50mM	Needed for denitrification in the case of an SNAD system	Suneethi et al. (2014)
Sulphide Load	-	>32mg/L (pre-acclimation)	Substrate dependent or anammox species dependent	Jin 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	>2mM of most organic C compounds	Co-cultures of anammox with heterotrophic denitrifiers	Jenni et al. (2014); Guven et al. (2005b)
Inorganic Carbon	HCO_3^-:TN ratio of 1.2	<1.2mg-C/L	Carbonic acid may decrease the pH and result in the formation of FA and FNA.	Jin et al. (2014); Kimura et al. (2011)
Hydraulic Retention Time	-	-	Dictated by SAA of the culture	-
Sludge Retention Time	Biomass needs to be retained for as long as possible.	-	Enrichment requires 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)

Carbon compounds

Anammox bacteria are chemolithoautotrophic, and utilise 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., 2004b; Jin et al., 2012a). In fact, 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; Guven et al., 2005). Conversely, high concentrations of organic matter have been found to inhibit anammox activity (Jin et al., 2012a). 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 in relation to competing heterotrophs, addition of organic substrates would result in the heterotrophs out-growing- and by extension, outcompeting-the Anammox bacteria within the same system (Chamchoi & 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, 2011; Guven 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., 2012a).

- 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., 2012a).
- 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., 2012a; Yang et al., 2013b).
- 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, b-lactams and tetracycline (Jin et al., 2012a). Although the effects of these antibiotics are largely inhibitory, some enrichment studies have utilised cell wall targeting antibiotics to provide a selectively competitive advantage to the anammox bacteria as they lack a peptidoglycan cell wall (Bagchi et al., 2010).

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 utilises 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 (IC_{50}) of Cu^{2+} was calculated to 12.9 mg/L, with concentrations as low as 5mg/L causing almost complete anammox inhibition (Yang et al., 2013a). In contrast, the IC_{50} calculated by Zhang et al. (2015) was 32.5 mg/L, and that the anammox consortia could resist inhibition by 5mg/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.

Salinity

As with the other inhibitory compounds; inhibitory effects depend on the type of salt, the salt concentration, reactor design and population characteristics (Jin et al., 2012a). The IC_{50} 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 have been found to be able to 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.

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 H_2S 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. It is possible that 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.

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 maximising 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 grows 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 is 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.

2.3.4.2. Bioreactor designs for anammox enrichment

As described above, successful anammox bacterial enrichment requires very precise control over a number of physical, chemical and biological factors. The ideal enrichment reactor should maximise on those factors that promote anammox bacterial growth, while simultaneously minimizing the effect of the factors inhibit it. Thus, the most important considerations of an anammox bioreactor include:

- A system that allows for the creation of an anaerobic, oxygen limited environment;

- The ability to maintain a high hydraulic retention time without toxicity accumulation, thus allowing for an extended exposure time of the anammox cells to fresh nutrients and advantageous growth conditions;
- Efficient biomass retention such that the slow growing anammox bacterial cells are retained within the reactor system, while faster growing competitor organism are washed out;
- Suitable pH and temperature regulation for the promotion of anammox metabolism and growth. Effectively maintaining pH can also retard the formation of severely inhibitory concentrations of FA and FNA within the system;
- Sufficient agitation to promote mass flow within the reactor and promote granulation without excessive shear forces which could damage the biological material.

Anammox enrichment studies have been attempted using many different types of reactor systems, each with their own advantages and disadvantages (Table 4). With careful operational control over the aforementioned factors, the anammox process was successfully implemented in all these reactor systems, albeit to varying degrees of efficiency.

The sequencing batch reactor (SBR) was the most common experimental set-up for anammox enrichment, and has been successfully applied to many anammox enrichments studies (Dapena-Mora et al., 2004b; Galí et al., 2007; Wang et al., 2012; Vega De Lille et al., 2015). Despite this, the batch mode of operation offered by the basic SBR may not provide ideal conditions for long term anammox cultivation due to the fact that Anammox bacteria are often inhibited by their own substrates as well as suboptimal nitrogen loading (Puyol et al., 2013; Carvajal-Arroyo et al., 2014b). As such, reactors with a continuous mode of operation are currently preferred. The CSTR builds on the advantages offered by the SBR, but can offer a much longer hydraulic retention time and a continuous source of fresh substrate, with the simultaneous removal of toxic metabolic by-products. While the CSTR offers a longer hydraulic retention time, and avoids cell starvation due to optimal control of substrate flow rates, it still suffers from inefficient biomass retention. The use of membrane based bioreactor systems (MBRs) had the exact opposite problem to the CSTRs- biomass retention was excellent, however bulk mixing and substrate mass transfer through the biofilm were suboptimal. A hybrid solution, in the form of MBBRs were developed that maximized on the advantages of both the MBR and CSTR systems.

Table 4: Comparison of the most common reactor types used for anammox enrichment

Reactor Type	Abbrev-iation	Advantages	Disadvantages	References
Sequencing Batch Reactor	SBR	• Useful for the screening of environmental samples • Simple to set up and operate.	• Not suitable for scale-up or mass cultivation • Biomass loss during decanting	Bagchi et al. (2012); Hu et al. (2005); Jin et al. (2008)
Continuous Stirred Tank Reactor	CSTR	• Efficient mass transfer • Continuous feeding regime prevents nutrient shock loading.	• Equipment and setup costs are high and relatively complex. • Poor biomass retention	Suneethi et al. (2014)
Fixed Bed Reactor	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 et al. (2012)
Membrane Bioreactor	MBR	• Optimum biomass retention through attached growth • Allows for multi-species biofilms to form	• Membrane fouling • Difficulty in sampling at low culture densities (i.e. at the initial stages of enrichment)	Bagchi et al. (2012); Huang et al. (2016); Van Der Star et al. (2008)
Moving Bed Biofilm Reactor	MBBR	• Utilizes carrier particles- thus enabling longer SRT • Can be operated in a variety of modes and configurations	• Limited literature on the most suitable type of carrier material. • Bulk mixing can become inefficient depending on the particles used.	Regmi et al. (2016); Christensson et al. (2013)
Upflow Anaerobic Sludge Blanket	UASB	• Promotes formation of granules • Efficient mixing and mass transfer • Good biomass retention	• Shear forces can become difficult to control on an undefined culture	Abma et al. (2007); Ma et al. (2013)
Air-lift/ Gas-lift	Air-lift/ Gas-lift	• Easy to operate stably • Shear forces induce rapid granulation.	• Foaming can often occur. • High pressure pumps required for different sludge concentrations.	Khin & Annachhatre (2004); Hendrickx et al. (2012)
Expanded Granular Sludge Bed	EGSB	• Efficient sludge-wastewater contact • Allows for higher loading rates. • Most conducive to the formation of granules	• High upward velocities prevent complete removal of suspended solids and colloidal matter. • May result in washout of biomass	Seghezzo et al. (1998); Chen et al. (2010)

The MBBR systems utilised carrier particles to serve as attachment sites for bacterial biofilm formation (which allowed for more efficient biomass retention), and these particles were dispersed within the bulk liquid (thus allowing for efficient mass transfer). MBBR systems still remain a popular choice for anammox enrichment studies, especially within co-culture processes, however the MBBR systems are not ideally suited for large scale due to the difficulty in working with the carrier particles.

The UASB, Airlift and EGSB all incorporate the advantages of the MBBR through the natural formation of anammox granules. These reactors exploit the natural tendency of anammox bacteria to "clump" together, and use flow rates and shear forces to direct the sludge to form compact granules (Figure 9). Although the exact mechanism for granulation remains unknown, they are hypothesized to be mediated by the turbulent up-flow rates, suboptimal nutrient loading and biochemical signalling. These granules form naturally, without the need for carrier particles, and represent a unique frontier in wastewater treatment processes.

Figure 9: Examples of highly enriched anammox granules from long-term UASB reactors (Ni et al., 2010; Tang et al., 2011)

Despite the many reactor configurations developed and applied to anammox bacterial enrichment, very little work has compared the effect of these different reactor systems on

both anammox and on the synergistic microbial populations co-existing within the same system. As these populations will all be subject to the same environmental stresses, determining the optimum operational parameters that establishes a balance between these competing populations, and mitigates the excessive growth of any one population group, will be critical to the development of a stable, cost-effective, anammox-centric wastewater treatment system.

Chapter 3: Initialization and operation of a high rate N-removing Nitrification-Anammox reactor system

3.1. Introduction

Biological wastewater treatment systems represent complex environmental niches, where physical, chemical and biological phenomena act in concert to shape a constituent microbial community. The microbes that thrive within these environments are often specialist organisms that are able to metabolize the high nutrient load from the influent wastewater stream. Organic and inorganic nitrogen compounds are primary nutrients which are often found in excess within domestic and industrial effluents. In WWTPs, these nitrogen compounds are metabolically converted by specialist N-removing microbial groups comprised predominantly of the nitrifier, denitrifier and anammox bacterial groups respectively. These specialist N-removing microbial groups form a complex synergism wherein the metabolic by-products of one microbial group forms the primary growth substrates for another (Arrigo, 2005; Kox & Jetten, 2015).

The intimate synergism between these key N-removing groups makes *ex situ* isolation of any single member challenging, however they can collectively be cultured as a synergistic consortia under appropriate enrichment conditions. Laboratory scale biological reactors have been widely used to selectively enrich for slow growing organisms and specific microbial groups from a mixed microbial consortia. Stringent regulation of the enrichment reactor conditions can promote the proliferation of a single target group while suppressing the growth of competitors.

This chapter focuses on the initialization and operation of a bench scale bioreactor for the autotrophic removal of NH_4^+, with the reactor conditions set to specifically promote the growth of a nitrifier-anammox population. The reactor was operated for a period of 365 days and the nitrogen removal rates were measured. Since the metabolism of the nitrifying, denitrifying and anammox groups are N-centric, tracking the N fluxes within the microbial community as a whole can indirectly determine the metabolic efficiency of the entire constituent bacterial consortia for autotrophic N-removal.

3.2. Methodology

3.2.1. Characterisation of the seed inoculum

3.2.1.1. Source of seed inoculum

An enriched nitrifier-anammox sample was obtained from an existing CANON-anammox reactor operated at the University of Colombia (USA). The parent reactor was a 20 L laboratory scale SBR that was maintained at a constant temperature of 35 ºC and a pH range of 7.5-7.9 (Park et al., 2010). This reactor was fed with partially nitrified anaerobic digestion centrate containing an average of 490 ± 194 mg NH_4^+–N/L and 518 ± 222 mg NO_2^-–N/L (Park et al., 2010). The system was operated as a sequential nitritation-anammox reactor, with the DO maintained below 1 mg/L. The population distribution, type of microbial growth (i.e. biofilm, planktonic or granular), and activity rates of each individual groups within the reactor remains unknown.

3.2.1.2. Biomass determination

The total solids and total volatile solids were measured in duplicate according to the standard methods with slight modifications (APHA, 2012).

For the TSS measurement, 10 mL of the stock sample was added to a cleaned, pre-weighed ceramic crucible. These crucibles were heated at 120 °C for 5 h after which they were placed in a desiccator containing silica gel to cool. The crucibles were then weighed on a Mettler-Toledo ME204 analytical balance (Mettler-Toledo International Inc., USA) to determine the dry biomass.

To calculate the volatile component of the biomass, these crucibles were subsequently incinerated at 550 °C for 15 min. The crucibles were cooled in a desiccator containing silica gel and weighed on an analytical balance.

The difference in grams between the empty crucible and post heating at 120 °C was recorded as TSS, while the difference between the crucible and post heating at 550 °C was recorded as VSS.

$$TSS = Mass_{(Crucible+Biomass)} - Mass_{(Crucible)} \quad (12)$$

$$VSS = Mass_{(Crucible+Biomass)} - Mass_{(Crucible\ after\ incineration\ at\ 550°C)} \quad (13)$$

3.2.1.3. Light microscopy

The sludge sample was diluted (1:50) and a Gram Stain, Neisser Stain and Wet Mount were performed. These slides were analysed at 1000x under oil immersion using the Zeiss Axiolab Light Microscope equipped with the Zeiss Archroplan 1000x objective lens (Carl Zeiss, Germany).

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

FISH was performed in three phases: Fixation, Probe Hybridization and Microscopic Analysis, as per the protocol outlined by Amann (1995),

Sample fixation

The seed sample was rendered metabolically inactive by paraformaldehyde fixation. The cells were suspended in a 1:3 (v/v) ratio of 1 x Phosphate Buffered Saline (PBS) solution: 8 % Paraformaldehyde, and frozen for 3 hours. Thereafter, the cells were washed twice with 1x PBS, resuspended in a 1:1 solution of 1 x PBS: Absolute Ethanol, and thereafter stored at -20 °C until required.

Probe hybridization

The fixed samples were diluted five-fold with ddH_2O. Flocs were disrupted by agitation using a FinePCR FineVortex One-Touch Vortexer Mixer (FinePCR, South Korea) at maximum speed for 5 min, coupled with sonication (2 W for 2 min), with a Microson XL-2000 ultrasonic liquid processor (Qsonica, USA). Subsequently, 10 µL of the sample was applied to each of the wells on a 10 well Teflon coated microscope slide and dried at 48 ºC for 10 min. Excess intracellular moisture was removed through a successive dehydration series (50 %, 80 % and 100 % Ethanol at 3 min each). A hybridization buffer (containing a final concentration of 0.9 M NaCl, 0.02 M Tris-HCl, 0.02 % sodium dodecylsulfate (SDS), and 30 % formamide (v/v)) was prepared for each respective probe (Table 5) and added to each well containing the sample.

Table 5: List of FISH probes

Probe name	Sequence (5'→ 3')	Target	Fluorophore	Reference
EUB338	GCTGCCTCCCGTAG GAGT	Most Bacteria	5' 6-FAM	Amann et al. (1990)
EUB338II	GCAGCCACCCGTAG GTGT	Planctomycetales	5' 6-FAM	Daims et al. (1999)
EUB338III	GCTGCCACCCGTAG GTGT	Verrucomicrobiales	5' 6-FAM	Daims et al. (1999)
AMX368	CCTTTCGGGCATTG CGAA	All anammox bacteria	5' 6-FAM	Kartal et al. (2007)

Each set of fluorescent probes were added to the hybridization buffer on each well to a final concentration of 5 ng/μL, and the slides were incubated at 48 ºC overnight. Following incubation, the slides were rinsed in a wash buffer (0.02 M Tris/HCL, 0.01 % SDS, 0.1 M NaCl, 0.005 M EDTA) for 45 min at 48 ºC. After washing, the slides were rinsed with ddH_2O, dried and counter-stained with the DNA stain 4′, 6′-diamidino-2-phenylindol (DAPI) at a working concentration of 0.25 μg/mL. Slides were sealed with VectaShield Mounting Medium (Vectorlabs, USA) and a cover slip, and stored in the dark at -20 ºC.

Microscopic analysis

Slides were examined using an Axiolab Apotome (Carl Zeiss, Germany) microscope containing the FLUOR fluorochrome filter set. Image analyses were examined with the Zeiss Axio Vision (version 4.6) imaging software.

3.2.2. Media Composition

The composition of the anammox growth media and trace elements solution is outlined in Table 6 and Table 7 respectively, as originally described by van de Graaf et al. (1997), and modified by the addition of sodium nitrate ($NaNO_3$) as per the findings of Uyanik et al. (2011). As per the recommendations of Uyanik et al. (2011), the $NaNO_3$ component was only added at the start for the first 10 days of reactor operation to promote denitrification activity, however no additional organic C was added. The NH_4^+–N (added as $(NH_4)_2SO_4$) and the NO_2^-–N (added as $NaNO_2$) concentrations were varied through the course of the enrichment based on the N utilization rates of the reactor (measured as described in 3.2.4.1), while still keeping to a relative NH_4^+:NO_2^- ratio of ~1:1.5, (Bagchi et al., 2010; Chamchoi & Nitisoravut, 2007; Bae et al., 2010).

Table 6: Media for anammox bacterial enrichment (van de Graaf et al., 1997)

Media Component	Concentration (mg/L)
Sodium Nitrate ($NaNO_3$)	50 (as NO_3−N)
Sodium Nitrite ($NaNO_2$)	Variable (from 30mg NO_2^-−N/L to 160 mg NO_2^-−N/L)
Ammonium Sulfate ($(NH_4)_2SO_4$)	Variable (from 15mg NH_4^+−N/L to 100 mg NH_4^+−N/L)
Sodium Hydrogen Carbonate ($NaHCO_3$)	1250
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	0.025
Calcium Chloride Dihydride ($CaCl_2.2H_2O$)	0.3
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.2
Ferric Sulphate ($FeSO_4$)	0.00625
Ethylene Diamine Tri-Acetic Acid (EDTA)	0.00625

A mixture of trace elements was further added to the van de Graaf medium to promote growth of the anammox bacteria (Table 7). Trace Element Solution 1 comprised of an iron solution while Trace Element Solution 2 contained a mixture of other physiologically relevant metals. Both solutions were made bioavailable through chelation with EDTA, and were each added to the van de Graafs media at a concentration of 1.25 mL/L (Bae et al., 2010).

Table 7: Trace element composition for anammox bacterial enrichment (Bae et al., 2010)

Trace Element Solution 1	Concentration (g/L)
Ferric Sulphate ($FeSO_4$)	5
Ethylene Diamine Tri-Acetic Acid (EDTA)	5
Trace Element Solution 2	**Concentration (mg/L)**
Ethylene Diamine Triacetic Acid (EDTA)	15
Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	0.43
Cobaltous Chloride Hexahydrate ($CoCl_2.6H_2O$)	0.24
Manganese Chloride Tetrahydrate ($MnCl_2.4H_2O$)	0.99
Cupric Sulfate Pentahydrate ($CuSO_4.5H_2O$)	0.25
Sodium Molybdate Dihydride ($NaMoO_4.2H_2O$)	0.22
Nickel Chloride Dihydride ($NiCl_2.2H_2O$)	0.19
Sodium Selenate Decahydrate ($NaSeO_4.10H_2O$)	0.21
Boric acid (H_3BO_4)	0.014
Sodium Tungstate Dihydride ($NaWO_4.2H_2O$)	0.050

The media was sparged with Argon and CO_2 gas consecutively to remove any DO and to maintain inorganic C within the media. The pH of the media was adjusted to pH 7.2 -7.5 using a 2 M HCl and a 5 M NaOH solution respectively.

3.2.3. Reactor Setup and Initialisation

A sequencing batch reactor was used for the mass cultivation of the seed inoculum. The bioreactor consisted of a sealed glass fermenter (3 L working volume) (New Brunswick Scientific, USA) seated on a hotplate/magnetic stirrer (Fried Electric, Israel) (Figure 10). Agitation within the reactor was enabled with a 50 mm x 10 mm magnetic stirrer bar, and a constant stirring speed of 80 rpm was maintained. Reactor temperature was maintained at 34 °C ± 2 °C using the hotplate and an internal heat exchange connected to a circulating water bath (Labcon, South Africa).

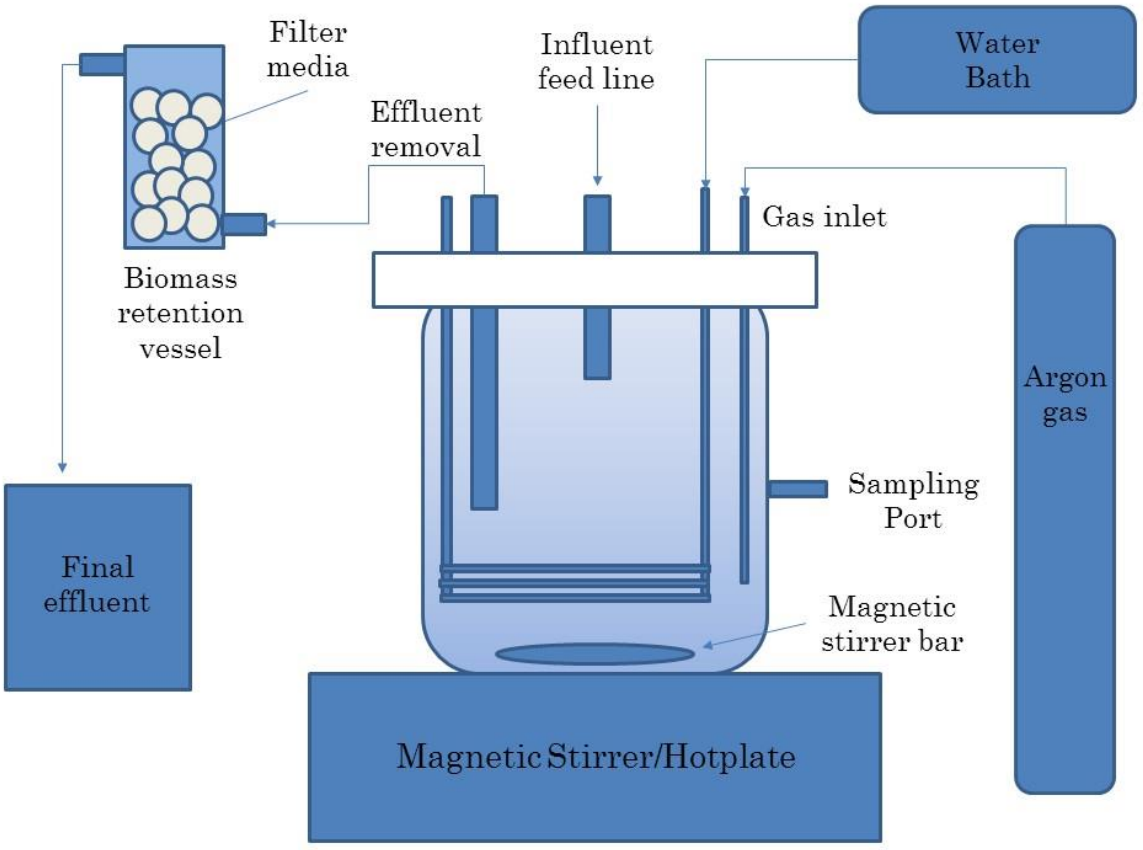

Figure 10: Schematic diagram of the SBR used for anammox enrichment

This reactor was initialized with ~1 g/L of seed inoculum (i.e. 0.1 % w/v), obtained from a parent nitrifier-anammox enrichment reactor established at the University of Colombia (USA), previously described in Section 3.2.1.1 (Park et al., 2010). The SBR in this study was fed with van de Graafs medium (van de Graaf et al., 1997), and purged with Argon

gas (5 L/min for 10 min) at every fill phase. The reactor was operated with an operational cycle time (Table 8) based on total ammonia removal rate during the enrichment period.

$$HRT = \frac{Reactor\ Volume\ (L)}{(Volume\ Decanted\ per\ Cycle\ (L)) \times (Cycles\ per\ day)} \quad (14)$$

Table 8: Reactor operational regime

	Fill Phase	Continuous Agitation Phase	Settling Phase	Decanting Phase	Total Cycle time	HRT
Day 1-250	15 min	64 h 45 min	120 min	300 min	72 h	4.55 days
Day 251-360	15 min	20 h 45 min	120 min	60 min	24 h	1.52 days

For each cycle, 2 L of the reactors total 3 L volume was decanted, providing a hydraulic retention time of 4.55 d for Days1-Day 250, and 1.52 d for Day 251-Day 360 respectively, calculated as per Equation 14 (Shizas & Bagley, 2002).

3.2.4. Analytical Methods

All samples were consistently obtained directly from the reactor during the decanting phase using a 60 mL syringe, filtered through a 0.45 µm cellulose acetate syringe filter (Merck Millipore, USA), and immediately analysed.

3.2.4.1. Nitrogen

The transformation of Nitrogen species are indirect indicators of the metabolism of anammox bacteria, nitrifying bacteria and denitrifying bacteria. Nitrogen in the forms of Ammonia (NH_3–N), Nitrate (NO_3–N), and Nitrite (NO_2^-–N) were measured spectrophotometrically using the Gallery Autoanalyser (ThermoScientific, USA), according to the protocols outlined in Standard Methods (APHA, 2012). The Gallery was calibrated with a linear calibration curve for the NH_3–N and NO_2^-–N, with an R^2 value of 0.998 ± 0.003 and 0.999 ± 0.002 (n = 170) respectively.

The Nitrogen Loading Rate (NLR) can be calculated as the sum of mass fluxes of ammonia (NH_4^+–N) and nitrite (NO_2^-–N) as the mass of total N fed per day (mg N/day) per maximum reactor volume, as shown in Equation 15 below (Bagchi et al., 2010):

$$NLR = \frac{(NH_4^+ - N)_{Influent} + (NO_2^- - N)_{Influent}}{Reactor\ Volume\ .\ Day} \quad (15)$$

The Nitrogen Removal Rate (NRR) can be calculated as the sum of the masses of NH_4^+–N and NO_2^-–N (measured in grams) removed per operating reactor volume per day, as shown in Equation 16 (Bagchi et al., 2010):

$$NRR = \frac{((NH_4^+ - N) + (NO_2^- - N))_{Initial} - ((NH_4^+ - N) + (NO_2^- - N))_{Final}}{Reactor\ Volume\ (L)\ .\ Day} \quad (16)$$

The Specific Nitrogen Removal Rate (SNRR) can be calculated as the sum of NH_4^+–N and NO_2^-–N removed per gram of VSS per day as per Equation 17 (Bagchi et al., 2010):

$$SNRR = \frac{((NH_4^+ - N)(g) + (NO_2^- - N)(g))_{Initial} - ((NH_4^+ - N)(g) + (NO_2^- - N)(g))_{Final}}{Biomass\ (VSS)\ .\ Day} \quad (17)$$

The change in total N is a more accurate measure of Nitrogen removal by the reactor as it also includes the NO_3–N formation within the reactor. This can be calculated with Equation 18 as ΔN (Bagchi et al., 2010):

$$\Delta N = ((NH_4^+ - N) + (NO_2^- - N))_{influent} - ((NH_4^+ - N) + (NO_2^- - N) + (NO_3 - N))_{effluent} \quad (18)$$

3.2.4.2. pH

The pH within the reactor was not actively controlled however both the influent media pH and the reactor effluent media pH were measured electrochemically using the Orion Dual Star Benchtop pH meter (ThermoScientific, USA).

3.2.4.3. Calculating FA and FNA

Free Ammonia (FA) and Free Nitrous Acid (FNA) represent severely inhibitory forms of nitrogen species and are a function of the pH of a solution (Wei et al., 2015; Li et al., 2012). They are calculated as per equations 19 and 20 below:

$$FA\ as\ NH_3\ (mg/L) = \frac{17}{14} \times \frac{Total\ Ammonia\ as\ N \times 10^{pH}}{\exp(\frac{6344}{273+T}) + 10^{pH}} \quad (19)$$

$$FNA\ as\ HNO_2\ (mg/L) = \frac{46}{14} \times \frac{NO_2^- - N}{K_a \times 10^{pH}} \quad (20)$$

In equation 19, T represents temperature in °C, while the K_a in equation 20 is the ionisation constant of HNO_2 (which has a standard value of 4.6×10^{-4}). The FA and FNA values were calculated from the effluent liquid media during each of the reactor decanting phases.

3.2.4.4. DO

In order to measure the DO within the reactor sample with minimal atmospheric contamination, the DO in the reactor was measured electrochemically using the YSI 556 Handheld Multiparameter Instrument (Xylem Inc., USA) and coupled to the hexane overlay technique outlined by Shriwastav et al. (2010). Briefly, the YSI DO probe was inserted into a flask containing 200 mL of hexane and 300 mL of headspace. The headspace on the flask was purged with Argon gas for 10 min, supernatant was siphoned directly from the reactor into the flask under the hexane layer. The experiment was normalized using a saturated solution of sodium sulphite, and a water sample that had been saturated with pure oxygen gas as per Standard Methods respectively (APHA, 2012).

3.2.4.5. Biomass quantification

Reactor biomass was measured according to Standard Methods (APHA, 2012), and was previously described in Section 3.2.1.2.

3.3. Results and Discussion

3.3.1. Characteristics of Seed inoculum

Visual inspection of the seeding sludge displays a reddish colouration that is characteristic of concentrated anammox bacterial sludge (Figure 11A) (Zhang et al., 2015a; Fujii et al., 2002; Shen et al., 2012). This reddish tinge is due to an accumulation of an intracellular heme *c* protein group, and is commonly observed in mature anammox bacterial cells (Tang et al., 2011; Kartal & Keltjens, 2016). A large proportion of the remaining seed sample is pale brown, which is typical of conventional activated sludge. Since this seed inoculum was obtained from an anammox-CANON reactor system, the remaining brown sludge fraction is most likely to contain young anammox bacterial cells and a mix of other heterotrophic and autotrophic microorganisms predominated by AOB and NOBs (further characterised in Chapter 4). Additionally, this seed sludge was characterized using Phase contrast light microscopy for determination of the floc

structure (Figure 11B and Figure 11C) and FISH (Figure 12) for visual confirmation of the presence of anammox bacteria.

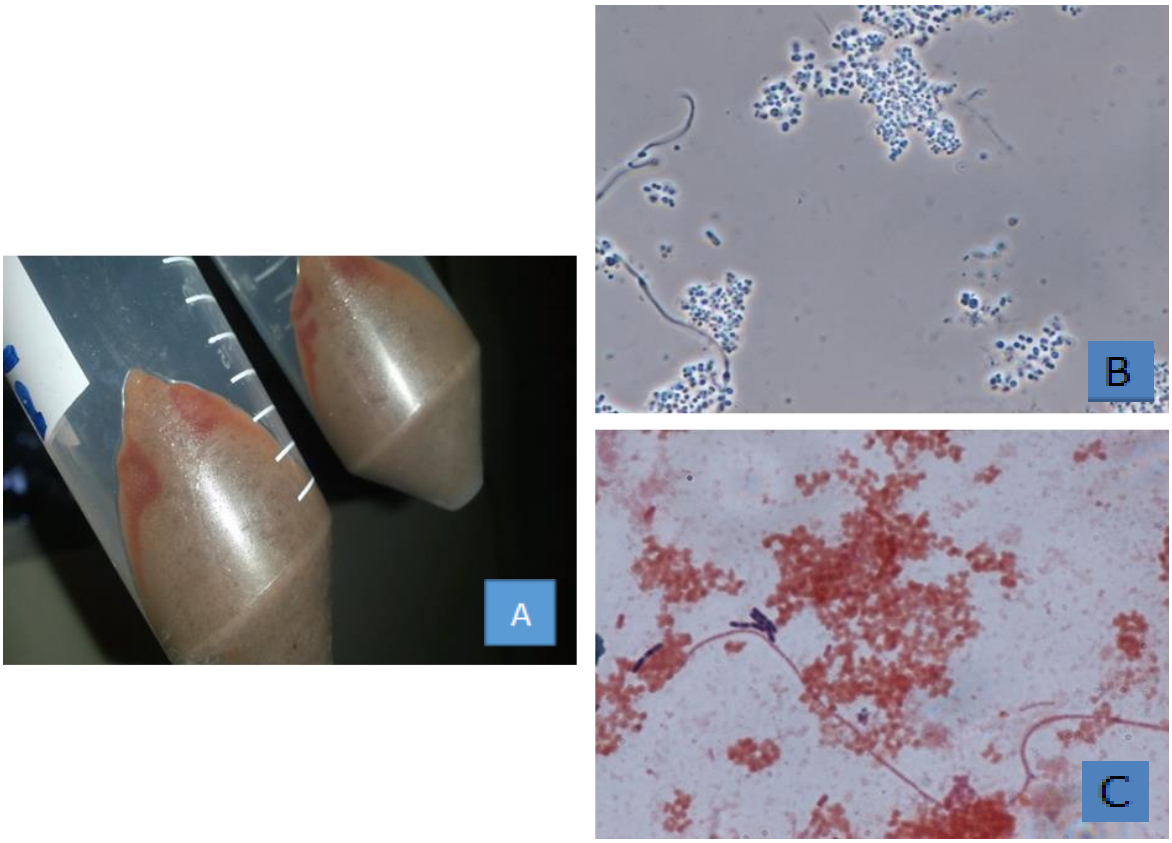

Figure 11: (A) Anammox seed sludge centrifuged at 6000 x *g* for 4 min; (B) Phase contrast of a wet mount at 1000x magnification; and (C) Gram Stain of the seed sample at 1000x magnification

Phase contrast microscopy (Figure 11B) and Gram staining (Figure 11C) displays discrete compact flocs composed of primarily circular clusters of cells that are predominantly Gram negative. Certain species among the nitrifiers are known to be Gram negative cocci, namely the AOB *Nitrosococcus*, and the NOB *Nitrococcus*. Planctomycetes are generally considered an odd species as they lack peptidoglycan cell walls, and thus have no true Gram-stain classification. The anammox bacteria, being members of the Planctomycetes phylum had also been assumed to lack peptidoglycan-containing cell walls due to an insensitivity to peptidoglycan synthesis-targeting antibiotics, and an incomplete peptidoglycan synthesis pathway (van Teeseling et al., 2014; Lindsay et al., 2001; Fuerst, 2013). Conversely, current research using an enriched *K. stuttgartiensis* culture as an anammox bacterial model suggests that anammox bacteria does indeed contain a thin peptidoglycan layer in its cell wall, reclassifying anammox bacteria as Gram negative (van

Teeseling et al., 2015). Despite Figure 11C clearly depicting Gram negative cocci, simple microscopic imaging and differential staining of this seed culture cannot be used as a conclusive indicator of the presence of anammox bacteria since it is composed of a mixed bacterial consortia.

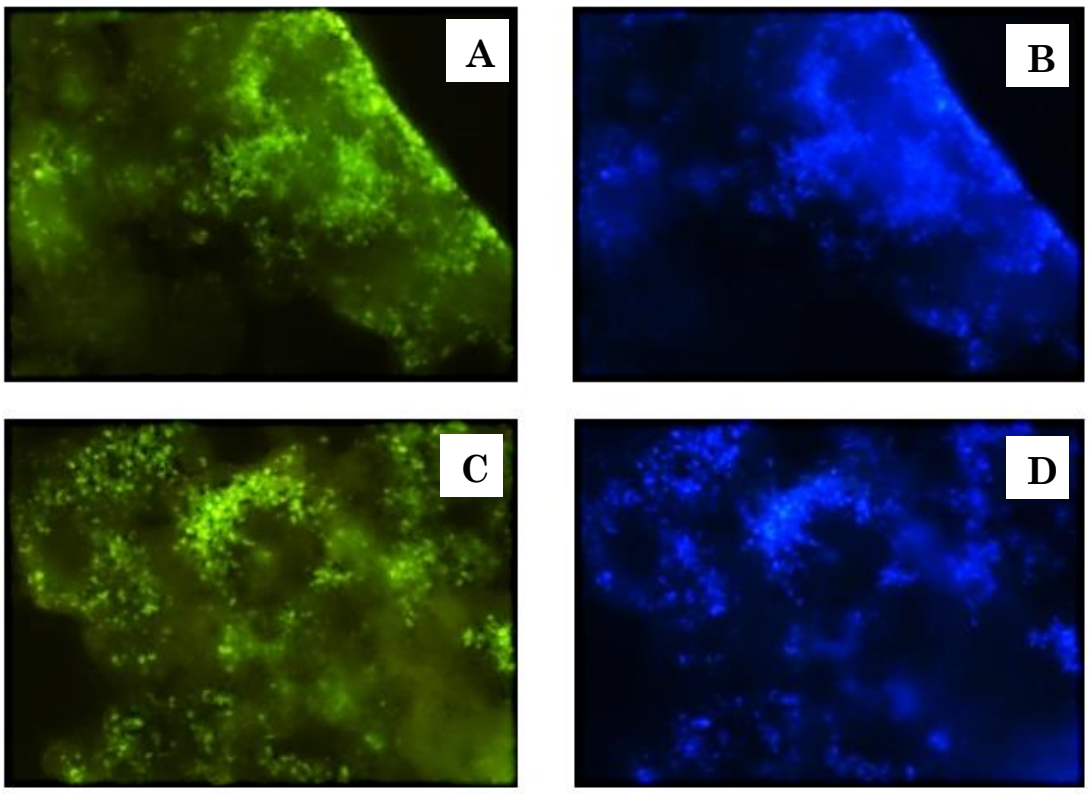

Figure 12: FISH images taken at 1000x magnification from the seed culture. (A & C) The green colour indicates all known anammox species hybridized with the AMX368 FAM-labelled probe. (B & D) The blue stain depicts total microbial population hybridized with DNA-intercalating DAPI fluorescent dye.

The application of FISH to the seed inoculum overcomes the ambiguity of differential staining, and light microscopy, by utilising DNA-based fluorescent probes that specifically target the anammox population therein. Figure 12 displays bacterial cells with a green fluorescence which indicate successful probe binding to these cells. The AMX368 fluorescent probe used is specific for all anammox bacterial species, and thus these positively fluorescing cells represent the total anammox population within the seed sample. The total microbial population is counterstained in blue using the fluorescent DAPI stain. By comparing the positive green fluorescence of the anammox bacteria against the total microbial population with the blue fluorescence, a relative quantification may be obtained. The anammox population represents a large proportion of the total

bacterial population, estimated to be 51 % of total bacteria using the Zeiss Axiovision imaging software. This high proportion of anammox bacterial cells relative to the total microbial population was expected, as the seed culture was obtained from an anammox-CANON reactor, and represents a good starting inoculum for mass cultivation and further enrichment in another reactor system.

3.3.2. Initialisation and operation of the SBR system

An SBR system, as utilized in this study, is a common reactor type for anammox bacterial propagation, and has been successfully applied to numerous anammox enrichment studies (Dapena-Mora et al., 2004a; Jin et al., 2008b; Tao et al., 2012; Wang et al., 2012). The SBR systems display sufficient sludge retention with minimal biomass washout, while HRT can be rapidly modified based on biomass activity- thus allowing for efficient nutrient loading. The performance of the SBR in this study was monitored for 366 days after inoculation with the anammox bacteria-rich seed culture. No aeration or organic carbon was supplied to the reactor during its operation, while temperature was controlled at 35°C ± 1°C with a hot plate and water bath. The pH was not actively controlled within the reactor during the course of the experiment, however the influent pH was maintained at 7.2 - 7.4, and the effluent pH was monitored over the course of the enrichment (Figure 19). The DO was also not controlled within the reactor, however it was measured at reactor start up to be <0.5 mg/L after 1 day of operation, with the DO of the influent media being 1.08 mg/L ± 0.34 mg/L. This decrease could be attributed to DO utilisation by the aerobic proportion of the constituent microbial community within the reactor, as this seed sludge was sourced from a reactor system that also contained aerobic nitrifiers.

The Hydraulic Retention Time (HRT) of the reactor was not fixed at the initialization of the reactor operation but was regulated according to NH_4^+ removal rates (Figure 14). In standard SBR systems, the number of cycles is generally adapted to achieve the desired effluent limits, thus the HRT of this reactor was modified to suit the NRR performance of the system. In this study, the HRT was decreased from 4.5 d for Phases I and II of the reactor operation, to an HRT of 1.5 d for Phase III. The extremely high HRT for this reactor in comparison to the lower HRTs used by similar studies can be attributed to activity and amount of the seeding biomass (Lan et al., 2011; Cydzik-Kwiatkowska & Wojnowska-Baryla, 2015; Daverey et al., 2013; Tomar et al., 2015). This is corroborated by the decrease in HRT as biomass concentration increases over the duration of the reactor operation (Figure 23). This combination of an infinite SRT with an activity-

dependant HRT (and by extension- NLR) is an appropriate anammox enrichment strategy, especially when attempting mass cultivation from a low seed inoculum concentration (Zekker et al., 2014).

Nevertheless, the SBR configuration does have some limitations when used for a long term enrichment study, namely toxic shock loading of substrates, slow start-up times and difficulty in biomass extraction (Dutta & Sarkar, 2015). The latter is due to the strong tendency of anammox bacteria to attach to surfaces and form biofilms (Botchkova et al., 2014). This phenomenon was observed in the present study as well, wherein the seed inoculum formed a strong attachment to the side walls of the reactor vessel, and only shifted to a planktonic growth phase ~120 days into the experiment. During this period, NRR remained high, and this could be attributed to both anaerobic micro-niches that had formed within the biofilm, as well as the high affinity to nitrogenous substrates displayed by both the anammox bacteria and the AOB populations of the seed inoculum (Strous et al., 1999a; Botchkova et al., 2014).

By regarding the present reactor as a closed system, chemical transformations between the influent and effluent streams could be attributed specifically to the action of the microbial population within the reactor system. The primary substrates for the nitrifier and anammox bacterial populations are NH_4^+ and NO_2^-, hence the change in these compounds is an indirect measure of the metabolic activity of the constituent N-removing microbial population, including the target anammox populations. The total amount of N added to the reactor system can be calculated as the sum of the N-compounds added to the influent stream, and can be referred to as the Nitrogen Loading Rate (NLR), while the amount of N utilised by the microbes within the reactor system can be calculated as the difference between the total N-compounds within the influent and effluent streams, and is referred to as the Nitrogen Removal Rate (NRR). The influent N consisted only of NH_4^+–N and NO_2^-–N, while the effluent N consisted of any unmetabolized NH_4^+–N and NO_2^-–N, as well as the NO_3–N produced by the system.

Table 9 outlines the mean NLR and NRR of the reactor system for 3 distinct phases of operation from Days 1-150, 151-250 and 251-365. These phases represent the stable initiation of the reactor system; a period of loss in performance; and a recovery phase respectively. The reactor was initialized with a relatively low NLR compared to similar studies due to the low biomass starting concentration and shift in reactor operational condition. Since the seed biomass was obtained from an anammox-CANON reactor,

operated in the MBBR configuration, the added aeration and biofilm mode of growth in the parent reactor would allow for greater total N-removal. The present study utilises an anaerobic SBR without carrier particles and thus starting with a low NLR, and gradually increasing this NLR over the course of reactor operation allows the constituent bacterial consortia to slowly acclimatise to higher N concentrations, while simultaneously preventing substrate toxicity (Du et al., 2014).

Consequently, the NLR was incrementally increased from an initial concentration of ~39.26 mg N/L/day to a maximum of 166.48 mg N/L/day during Phase I. In Phase II, the NLR was decreased to an average of 112.15 mg N/L/day due to decreased reactor N removal rates. The NLR was then subsequently increased back to an average of 169.69 mg N/L/day during Phase III due to recovery of N removal activity of the constituent population. These NLR modifications, as well as the HRT reduction, were based on the NH_4^+–N removal rates. Since both anammox bacteria and AOB were the dominant organisms within the seed inoculum (having come from a CANON enrichment reactor), ammonia in the form of NH_4^+–N represents the main electron source for both of these autotrophic populations, and its conversion efficiency is a good indicator of the activity of these two populations. Yu and Jin (2012) noticed that the reactor efficiency can be hampered by a substrate shock to a far greater extent than to hydraulic shocks. They also noticed that recovery to inflow rate shocks is much faster than substrate shock loading; thus modifying the HRT rather than just the NLR alone allowed for acclimatization and mitigation of inhibition. Additionally, since high concentrations of N can be inhibitory to the burgeoning anammox community, controlling the NLR at the rate the total N is utilised by the community allows for the system to mitigate inhibitory metabolite concentrations and prevent shock loading, which may destabilize the system.

Table 9: Average NLR at different phases of reactor operation

Day	Average Daily Influent NH_4^+–N (mg/L)	Average Daily Effluent NH_4^+–N (mg/L)	Average Daily Influent NO_2^-–N (mg/L)	Average Daily Effluent NO_2^-–N (mg/L)	Average NLR (mg/L/day)	Average NRR (mg/L.day)
1-150	65.74	17.30	100.74	4.61	166.48	145.32
151-250	50.31	11.05	61.84	9.25	112.15	91.85
251-365	55.56	10.73	114.12	0.07	169.69	159.21

These NLR and NRR differences between the 3 phases can further be seen in Figure 13 below. Phase 1 (from Day 1 - Day 150) shows an increase in NLR and NRR, with a similar trend for ΔN; Phase 2 (from Day 151 – Day 250) shows a drop in NLR and NRR with a matching depression of ΔN; while Phase 3 (Day 251 – Day 365) displays a strong increase in nitrogen removal activity almost returning to its original Phase 1 values.

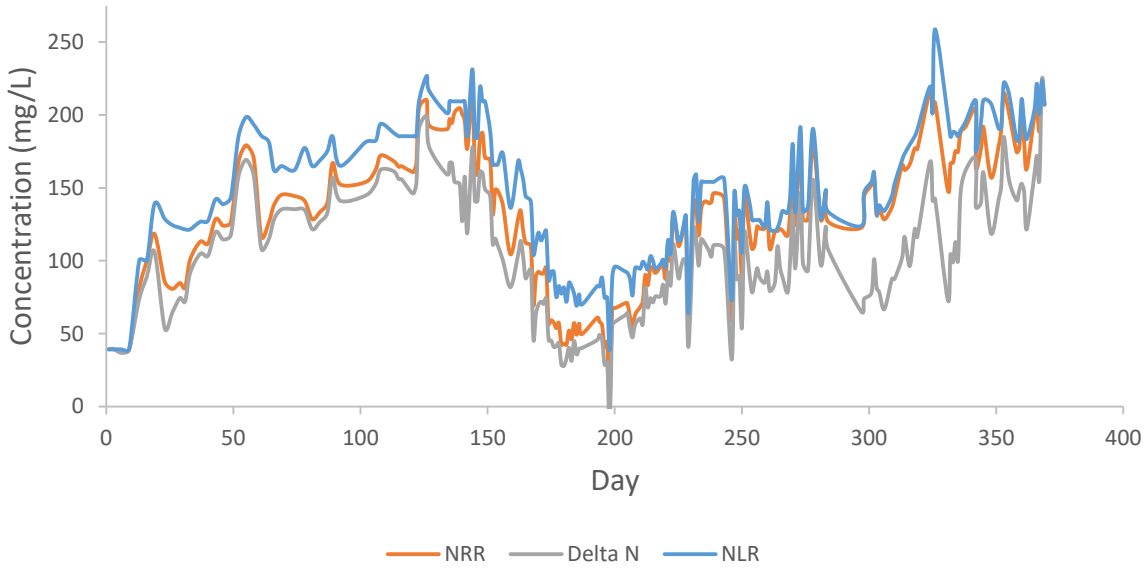

Figure 13: A line graph indicating the trend for Influent N, Effluent N and Delta N

Ammonia represents the primary substrate for both anammox and specific nitrifier bacterial populations, as it is converted to either NO_3 by AOBs under aerobic conditions or N_2 by anammox bacteria under anoxic conditions respectively. Consequently, the change in ammonia removal efficiency by the reactor system over time can be attributed primarily to the metabolic activity of these two constituent populations. Figure 14 depicts the performance of the SBR for NH_4^+–N degradation over the operating period. The reactor shows consistently efficient NH_4^+–N removal capabilities during Phase 1 of the reactor operation, with an average removal of 49.03 ± 19.73 mg/L, and a maximum removal of 85.80 mg/L on Day 137. Phase 2 displays an average NH_4^+–N removal of 39.26 ± 12.75 mg/L and a maximum removal of 68.03 mg/L on Day 222, and Phase 3 displays an average NH_4^+–N removal of 45.16 ± 12.43 mg/L, with a maximal NH_4^+–N removal of 74.55 mg/L on Day 324. The levels of NH_4^+–N removed within this system is similar to those observed in many anammox reactors during the start-up phase, regardless of reactor type.

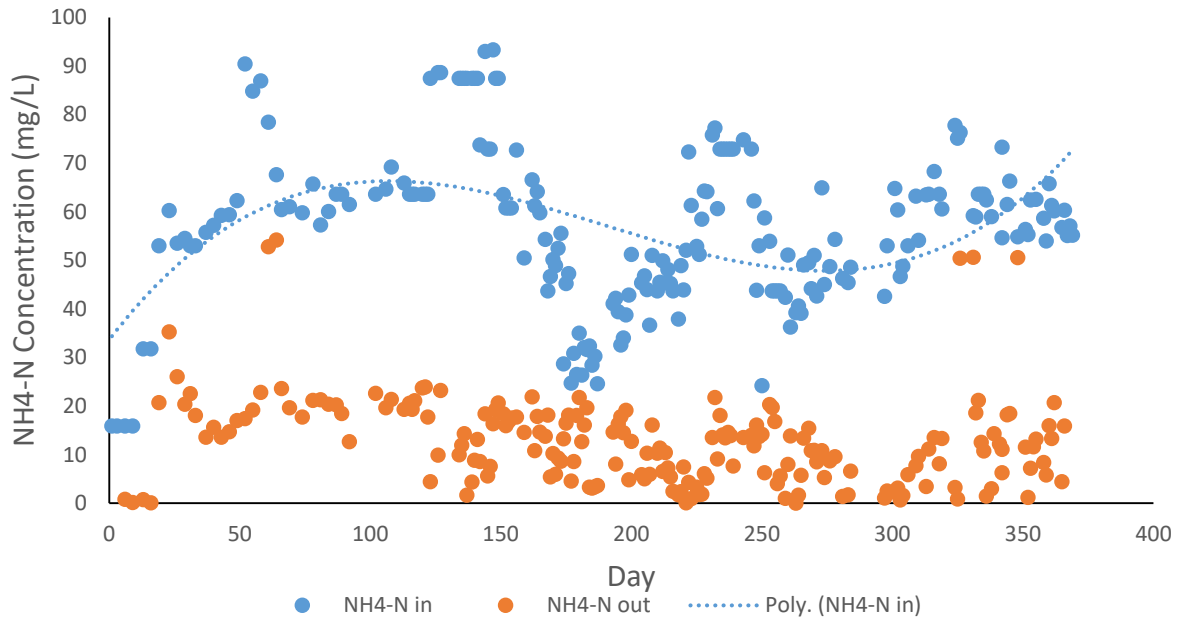

Figure 14: NH_4^+–N degradation by the reactor consortia over the 365 day operational period

Figure 15 depicts the percentage NH_4^+–N removal per day over the reactor operational period. A moving average calculated on a per weekly basis indicates a strong trend towards NH_4^+–N removal over the course of the reactor operational period. Despite some periods of NH_4^+–N removal suppression, the percentage of NH_4^+–N never drops below 40 %. Since an anammox enrichment reactor is designed for maximum retention of biomass, a true steady state in this system could not be achieved. As such, a pseudo-steady-state, defined as a constant nitrogen removal rate per unit of biomass was used as the end point of the reaction (Xing et al., 2013b).

The secondary N-source fed into the reactor was NO_2^-—N, which acts as an electron acceptor for specifically the anammox bacterial population. Nitrite is the primary oxidant in the anammox reaction, and is thus used as measure of anammox activity. The other members of the mixed consortia would use NO_2^-—N to a much lesser degree and would preferentially use O_2 in the case of the nitrifier populations and NO_3 in the case of the denitrifying population. The NO_2^-—N can also be produced during the conventional nitrification pathway by the metabolic action of specific nitrifying communities (i.e.: the AOB). Figure 16 depicts the change in NO_2^-–N concentrations from the influent and effluent over the course of reactor operation while Figure 17 shows the reactor efficiency at NO_2^-–N degradation.

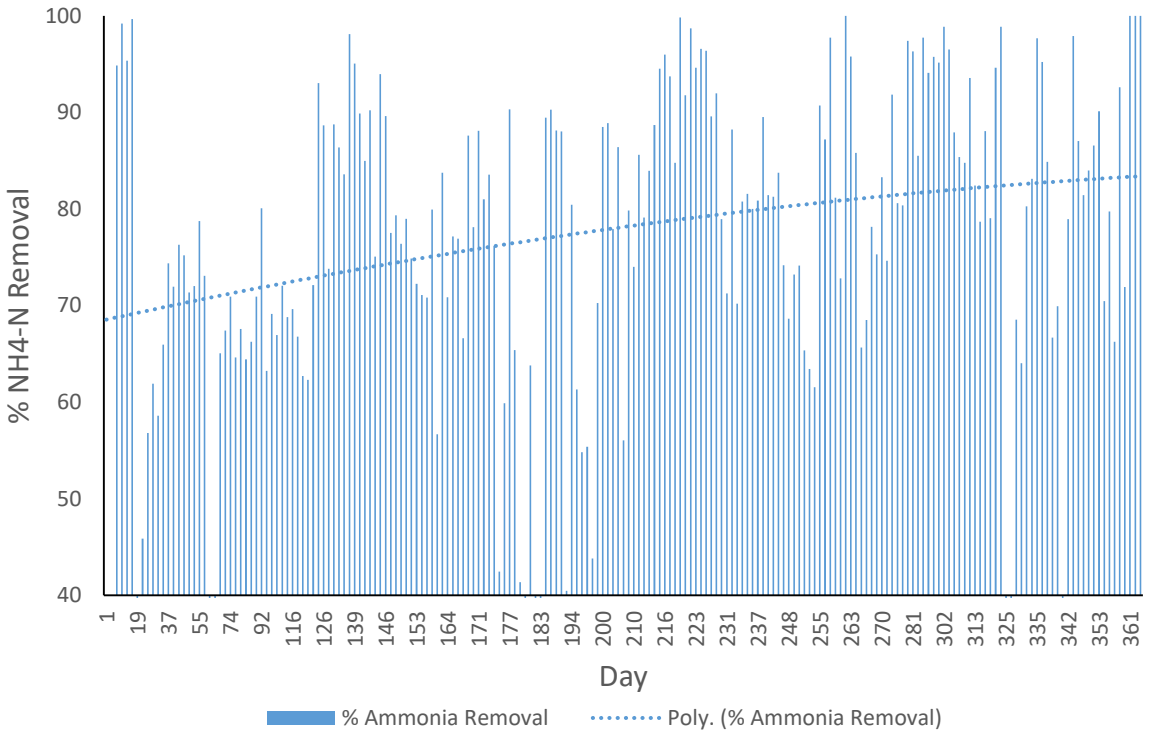

Figure 15: Percentage NH_4^+–N removal during reactor operation

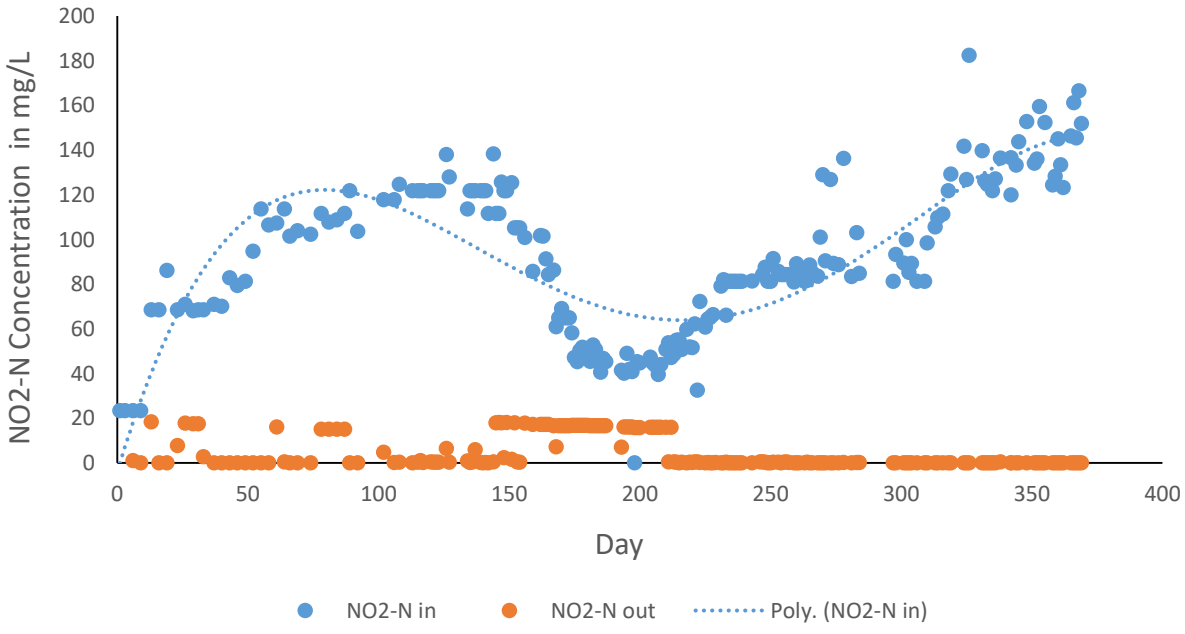

Figure 16: Change in NO_2^-–N concentrations over the enrichment period

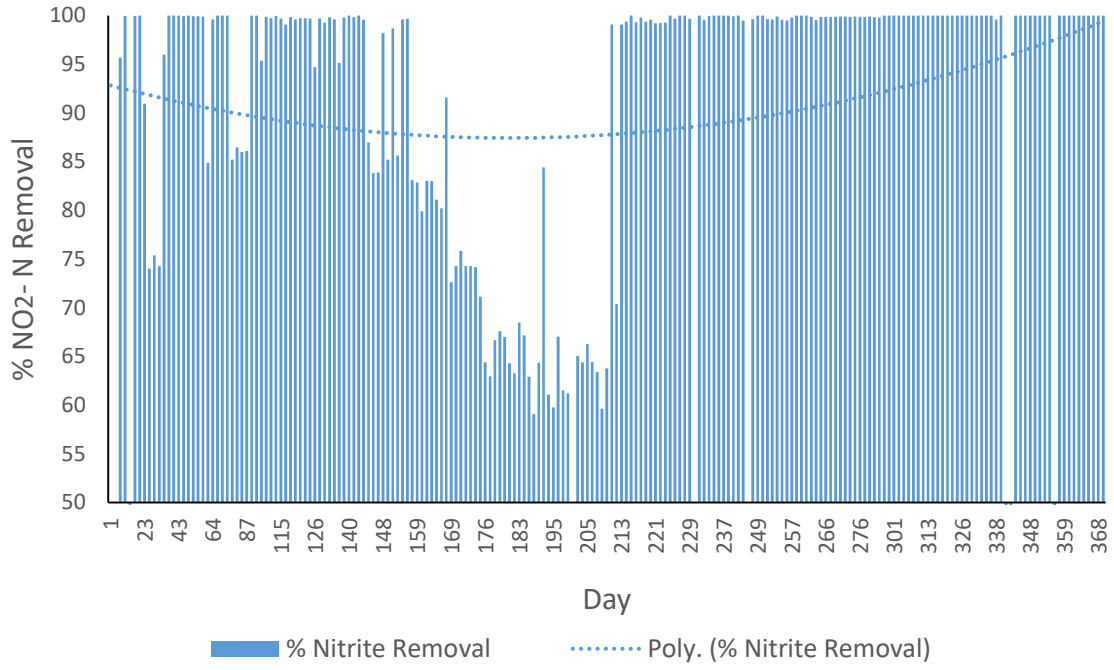

Figure 17: Percentage NO_2^-N degradation during the course of reactor operation

Nitrate is often produced by the complete oxidation of ammonia in the conventional nitrification process, traditionally mediated by the metabolism of NOB. It can also be produced in small quantities as a by-product of the anammox reaction. Figure 18 depicts the NO_3 produced during the period of reactor operation by the constituent microbial consortia.

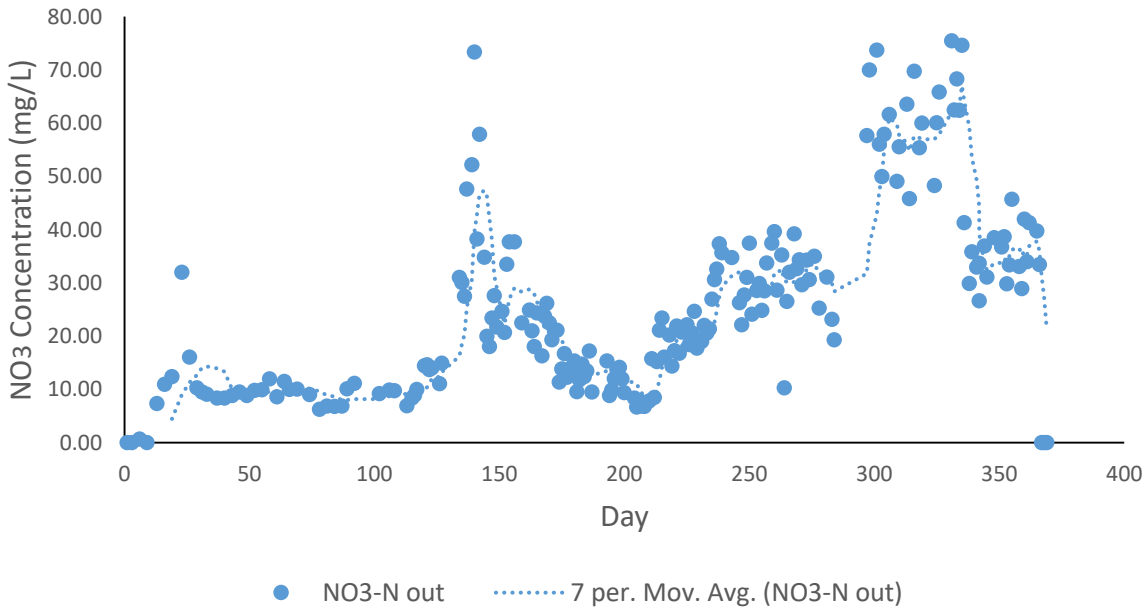

Figure 18: The generation of NO_3–N during the course of the reactor operation

This NO_3 accumulation may also have arisen as a result of the reactor configuration itself. In an SBR, since only a portion of the total liquor is withdrawn as effluent, NO_3 concentrations may continuously increase during long-term operation due to recalcitrance within the system (Dutta & Sarkar, 2015). Ideally, a denitrification process attached to the anammox SBR system can mitigate the NO_3 buildup however denitrification requires addition of an extraneous organic carbon source that my prove inhibitory to the anammox population (Dutta & Sarkar, 2015).

Additionally, the ratio of NH_4^+ to NO_2^- fed into the reactor can influence growth patterns and dominance over the different bacterial groups within the N-removing consortia. Although the standard stoichiometric ratio for anammox bacterial population alone is 1 part NH_4^+: 1.3 parts NO_2^-, the ratios fed to the reactor in this study were substantially higher. Since these ratios were modified to suit the NH_4^+–N and NO_2^-—N removal for the present reactor system, the elevated ratios could be explained by the presence of the mixed consortia within the reactor. As in Table 10, the feed ratio of NH_4^+:NO_2^- varied over the course of the experiment, as did the NO_3:NH_4^+ ratio. Since no NO_3 was fed into the system, the NO_3 produced was generated directly as a result of microbial or chemically mediated transformation of the influent NH_4^+ or the NO_2^- ions. This study displayed a NO_3:NH_4^+ value of 0.26 on Days 1-30, increasing to a maximum of 0.34 from Days 121-150, and subsequently steadily decreasing to 0.19 on Days 331-369. These results are further corroborated by Bagchi *et al.* (2010), who noticed a similar trend with the NO_3:NH_4^+ ratio of 0.85 on Days 1-96 to 0.22 from Day 281 to Day 310 of his experiment, for enrichment of anammox bacteria from an anaerobic digester treating domestic wastewater sludge.

Table 10: Nutrient ratios of the key N-ions during the period of reactor operation

Day	Average Influent NO_2^-/ NH_4^+ ratio	Average Consumed NO_2^-/ NH_4^- ratio	Average Ratio of NO_3 generated to ammonia oxidised
1-30	2.09	2.09	0.26
31-60	1.71	2.06	0.28
61-90	2.31	2.06	0.31
91-120	2.49	2.05	0.32
121-150	2.63	2.03	0.34
151-180	2.83	2.00	0.33
181-210	2.19	1.90	0.31
211-240	1.98	1.84	0.30
241-270	2.52	1.82	0.23
271-300	2.67	1.83	0.20
301-330	2.36	1.86	0.20
331-369	3.11	1.90	0.19

The pH of the system was not actively controlled within the reactor during the operational period. The "pH in" was the pH of the influent media at the time of feeding, and the "pH out" was the pH of the effluent during the decant phase. From Day 290 onwards, the pH of the influent was manually corrected to a constant pH of 7.3 ± 0.2 (Figure 19). An average decrease of 1.28 ± 0.1 pH units was observed within the system between the influent and effluent from Day 1 - Day 150, whereas the converse was observed from Day 150 - Day 227, where the pH had increased by 0.55 ± 0.35 pH units between the influent and effluent. The decrease in pH correlated with a high NRR between days 1-150, while the slight increase in pH between days 150-250 correlated with a suppression in NRR during the same period. Strangely, the pH fluctuated wildly between the influent and effluent from the reactor during day 300-360, despite the influent media pH being strictly regulated at ~7.3. In an N-removal system, an increase in pH is usually observed as a by-product of the anammox reaction, while a decrease in pH implies that the system tends more towards nitrification processes.

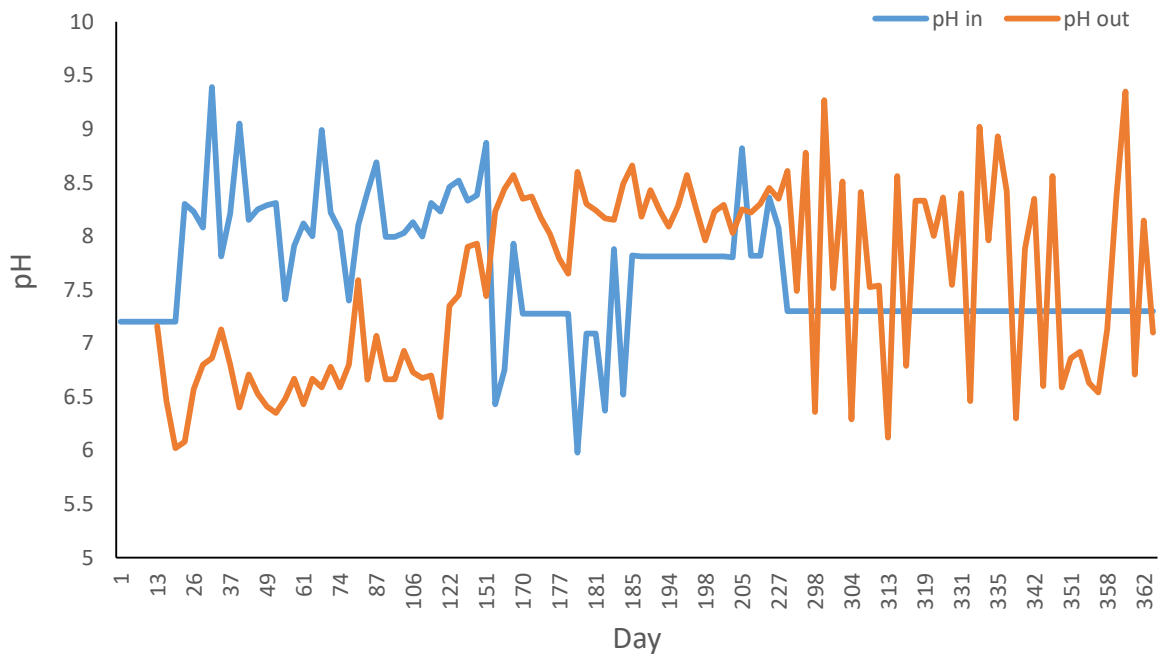

Figure 19: pH flux within the reactor system over the course of operation

Gonzalez-Gil et al. (2014) found that since nitrite and ammonium are equally charged at neutral pH, the anammox reaction does not affect a change to the overall pH value of the system. At higher pH (>7), the concentration of charged ammonium decreases and fully charged nitrite decreases, thus resulting in proton consumption and an overall increase in pH. The anammox and nitrifier bacteria are very sensitive to the pH of the liquor within

the reactor, since a suboptimal pH can induce the formation of FA and FNA. As depicted in Figure 20 and Figure 21, FA only breached the minimum inhibitory threshold of 20 mg/L on a few disparate occasions, for only single 24h periods before being controlled back to sub-inhibitory concentrations, while FNA never reached the inhibitory concentration of 2 mg/L during the period of reactor operation.

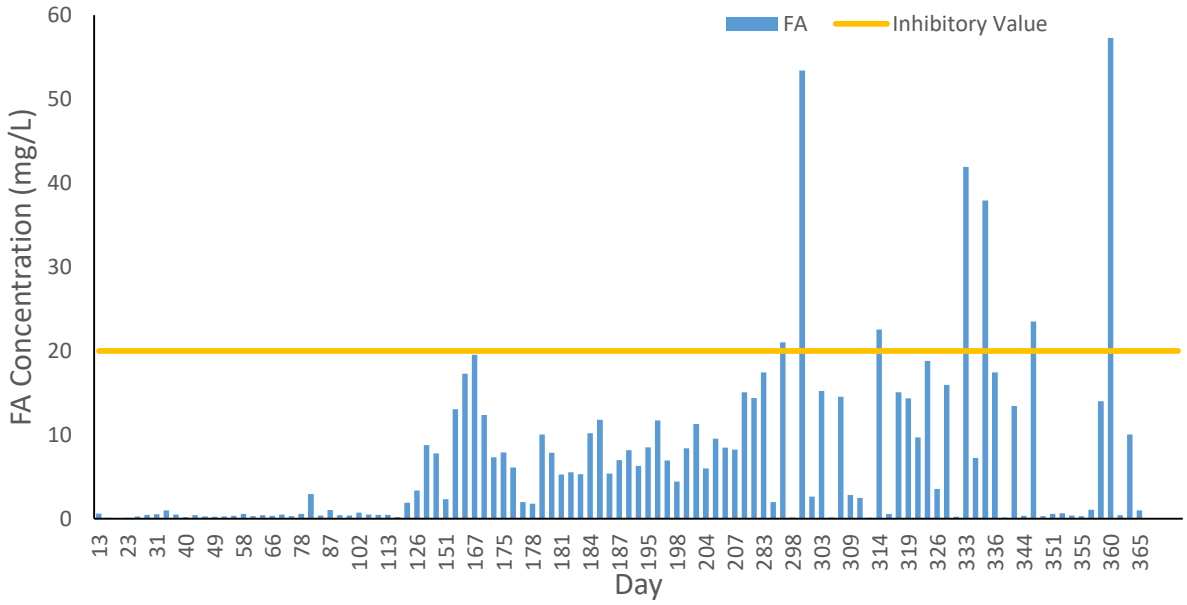

Figure 20: Free Ammonia concentration within the reactor

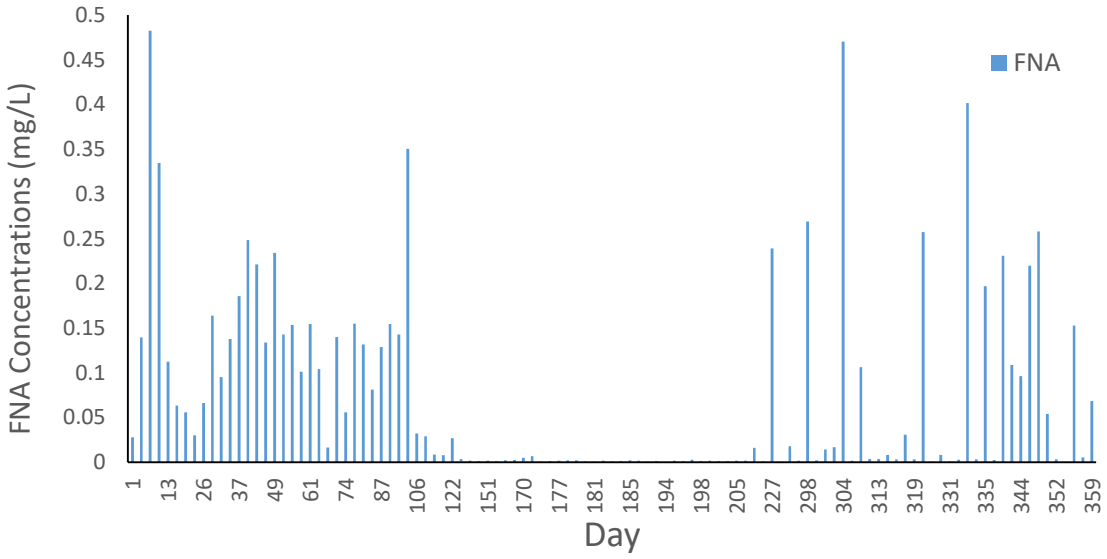

Figure 21: Free Nitrous Acid concentration within the reactor

3.3.3. Physical changes in biomass over the course of enrichment

Successful anammox enrichment has been achieved with a wide range of bioreactor designs. While each reactor design has its own advantages and disadvantages for anammox enrichment, it was shown that reactors which allowed for the maximum biomass retention consistently outperformed those that allowed for washout of biomass (Banihani et al., 2012; Tang et al., 2011; Okamoto et al., 2013; Tsushima et al., 2007b; Park et al., 2010). An SBR system, as utilised in this study, allows for an infinite sludge retention time due to its operational sequence. The initial inoculum of this study was low, at 1.02 g/L ± 0.004 g/L wet biomass, and increased to ~7.30 g/L ± 0.008 g/L over the course of the enrichment (Figure 22 and Figure 23). Aside from ~0.25 g of sludge used for DNA extraction, no other biomass was removed from the system. This indicates significant total biomass growth over the enrichment period, despite the absence of organic carbon and active aeration. This implies that the increase in biomass was attributed primarily to the growth of autotrophic N-removal bacteria within the system.

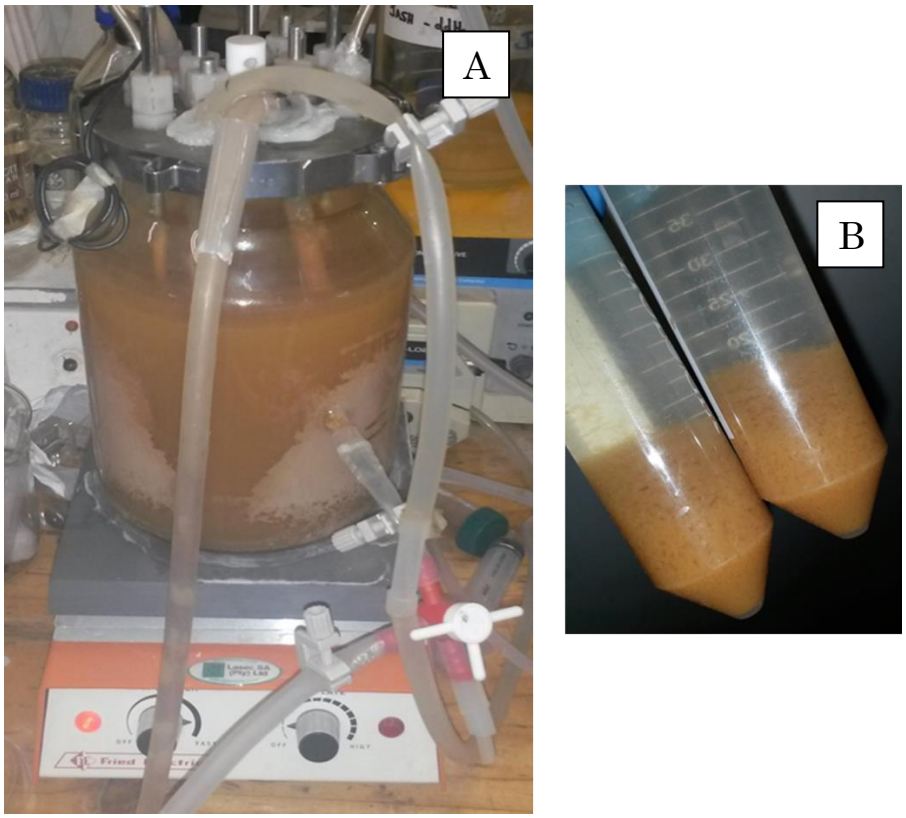

Figure 22: A) Reactor condition and B) biomass change over 300 days of operation

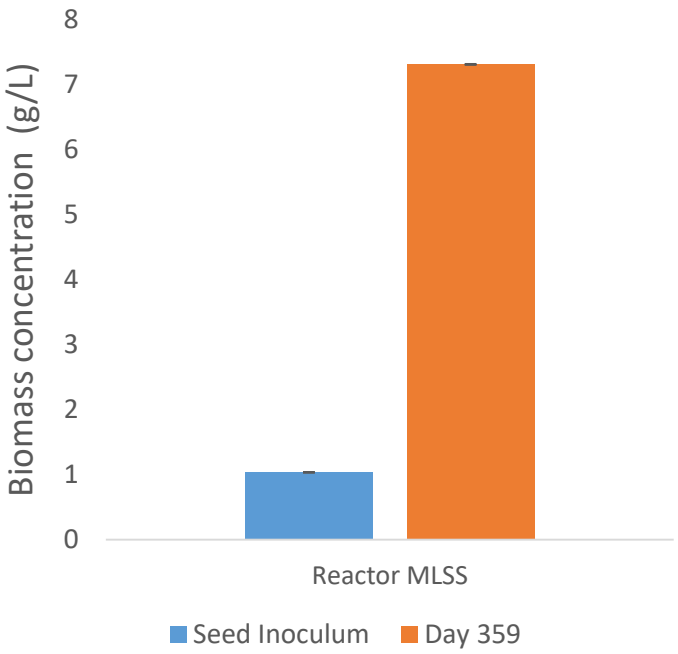

Figure 23: MLSS change within the reactor after 359 days

The anammox bacterial group, as well as the AOB are slow growing organisms relative to the heterotrophic microbes typical within activated sludge systems. These slow growing organisms often possess a higher substrate affinity than the faster growing heterotrophs, defining them as *K*-strategists, thus the low initial biomass inoculum serves to promote the growth of the *K*-strategist anammox and nitrifiers (Regmi et al., 2014). Additional biomass dry weight analysis showing the actual biomass changes over days 1-150, 151-250 and 251-360 would be ideal, however the initial inoculum was so low that removal of biomass severely affected the performance of the system, and resulted in long recovery times.

3.4. Conclusion

By regarding the present reactor as a closed system, nutrient removal between the influent and effluent streams could be attributed specifically to the action of the biological agents within the reactor system. This system was an SBR optimized to remove the N-load from a low C/N influent waste stream. It was initialized with anammox-nitrifier seed sludge from an existing CANON reactor system treating high N loads and this activity was maintained even in the low amount of seeding inoculum used in the current SBR.

An NRR of ~145 mg/L/d was achieved for the first 150 days of operation, which is comparable to the initiation phase of similar existing systems. Despite this being a

relatively low amount of N removal, it is still consistently greater than 90 % N removal efficiency relative to the NLR to this system, which does indicate efficiency. During the next 100 days of operation removal efficiency and NRR both dropped to ~50 % and ~92 mg/L/d respectively. This could be due to inhibition of the constituent microbial population. FA and FNA were investigated as possible causes of this inhibition, however they were consistently below the inhibitory threshold and thus could not be the cause of NRR suppression over the period of 100 days. DO ingress was determined to be the actual cause of this N-removal suppression as the constituent anammox population is reversibly inhibited by the presence of even trace amounts of DO. Days 251-365 displayed a recovery of the system back to an NRR of 159 mg/L/d and an N removal efficiency of >90 % despite constant low DO ingress. This implied that the microbial consortia within the system had adapted to the presence of excess DO.

By enriching for a mixed population of autotrophic N-removing organisms, the consortia had buffered the inhibitory effects of excess DO better than a more enriched anammox population, allowing for system recovery. Nevertheless, the constant NRR during the first phase with minimal DO presence, followed by the NRR suppression and DO ingress in the second phase and recovery in the third phase with constant exposure to DO implies that the same microbial groups may not have been responsible for NRR in the first and third phases. The presence of excess DO may have altered the community structure of the constituent consortia to promote the activity of aerobic N removal organisms, over the anaerobic N removal organisms active during Phase 1. Molecular analysis to determine the community structure follow in Chapter 4 to confirm this hypothesis.

Chapter 4: Community structure of the N-removing microbial consortia within the SBR

4.1. Introduction

Modern BNR wastewater treatment systems rely on the synergistic metabolic action of multiple microbial communities for effective wastewater treatment. These microbial communities arise naturally due to the numerous physical, chemical and biological factors acting on them (Cydzik-Kwiatkowska & Zielińska, 2016). The combination of these factors, collectively regarded as environmental conditions, will have an immense effect on the microbial community makeup. The environmental conditions in an environment impose selection pressures upon the microbial community until only those microbial groups favoured by the prevailing conditions thrive, while others are disadvantaged and eventually outcompeted (Yuan & Blackall, 2002; Adav et al., 2008; Vannecke et al., 2014).

The same phenomenon occurs within bioreactor systems, where a stable microbial population forms due to selection pressures acting on the microbes within the system. Within N-removal reactors, the primary factors of high N loading, pH, and temperature, COD, HRT and SRT all select for the propagation of specialist nitrogen removing microbial populations. In many cases these populations cannot exist in isolation and many anammox enrichment reactors often have anammox species, AOBs, NOBs and denitrifiers all existing concurrently, albeit at vastly different population densities (Kumar & Lin, 2010; Daverey et al., 2014; Wang et al., 2011; Jin et al., 2012a; Li et al., 2009). Even a slight change in the environmental conditions can favour one group over another, resulting in the metabolic or physiological dominance of one group of bacteria within the consortia (Wittebolle et al., 2009).

This chapter outlines the shift in relative population densities of critical N-removing microbial groups during the course of reactor operation, and relate this shift to the overall performance of the reactor using quantitative PCR and high throughput sequencing analysis. The study of these microbial communities and the changes their microenvironments impose on them can provide valuable information to better understand the biology of the nutrient removal processes occurring in wastewater

treatment ecosystems, and allow for the implementation of system conditions that are tailored to promote the growth, and enhance the activity of specific populations.

4.2. Materials and Methods

4.2.1. DNA extraction

Nucleic acids were extracted using the PowerSoil DNA extraction kit (MoBio Laboratories, USA) according to the manufacturer's instructions. Biomass was sampled every 60 days from the reactor. Approximately 0.5g (dry weight) of biomass was used for the genomic DNA extraction.

4.2.2. Nucleic acid quality analysis

Nucleic acid purity was confirmed using the Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, USA), as well as with gel electrophoresis using a 1 % (w/v) agarose gel, run for 1 hour at 75 V, with ethidium bromide (EtBr) staining (final concentration at 10 μg/μL). The final DNA concentrations were determined with the Qubit 3 Fluorometer (ThermoFisher Scientific, USA) as per the manufacturer's instructions.

4.2.3. Primer selection and Polymerase Chain Reaction

The total eubacterial populations, as well as the key nitrifier and anammox populations were tracked using the primer sets outlined in Table 11. All Polymerase Chain Reactions (PCR) were performed using the KAPA Taq 2x Readymix (Kapa Biosystems, South Africa) according to the manufacturer's instructions. The detailed PCR conditions for each of the listed primer sets can be found in Appendix B.

4.2.4. Primer design

Custom primers were designed for the Hydrazine Oxidase, Hydroxylamine Synthase (Subunit A) functional gene targets and for the general anammox bacterial 16S rRNA housekeeping gene. These primers were designed *in silico* using the NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast), IDT Unafold (http://eu.idtdna.com/UNAFold) and Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) web applications (Ye et al., 2012; Untergasser et al., 2012; Owczarzy et al., 2008). Further details on the products can be found in Appendix C.

Table 11: PCR primers used in this study

Target Group	Primer Set	Primer Sequence (5' → 3')	Annealing Temperature (in °C)	Reference
Universal 16S rRNA for total bacteria	27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	53.5 °C	Frank et al. (2008)
Universal 16S rRNA for total bacteria	P338F P518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	55 °C	Marchesi et al. (1998)
All Planctomycetes	PLA46F Univ1390R	GACTTGCATGCCTAATCC ACGGGCGGTGTGTRCAA	58 °C	Neef et al. (1998)
All anammox bacteria	AMX694F AMX960R	GGGGAGAGTGGAACTTCGG GCTCGCACAAGCGGTGGAGC	55 °C	Ni et al. (2010)
Ca. Brocardia and *Ca.* Kuenenia	AMX368F AMX820R	CCTTTCGGGCATTGCGAA AAAACCCCTCTACTTAGTGCCC	56 °C	Schmidt et al. (2003)
ß-*Proteobacteria* ammonia oxidizers	CTO189fAB CTO189fC CTO654r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	57 °C	Kowalchuk et al. (1997)
Nitrobacter spp.	FGPS872 FGPS1269	CTAAAACTCAAAGGAATTGA TTTTTTGAGATTTGCTAG	50 °C	Degrange & Bardin (1995)
Nitrospira spp.	NSR1113F NSR1264R	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG	65 °C	Dionisi et al. (2002)
Ammonia monooxygenase	amoA 1F amoA 2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	55 °C	Rotthauwe et al. (1997)
Hydrazine Synthase subunit A	hzsA526F hzs1857R	TAYTTTGAAGGDGACTGG AAABGGYGAATCATARTGGC	50 °C	Harhangi et al. (2012)
Hydrazine Oxidoreductase	hzoAB1F hzoAB1R	GAAGCNAAGGCNGTAGAAATTATCAC CTCTTCNGCAGGTGCATGATG	52.5 °C	Hirsch et al. (2011)
	hzoAB4F hzoAB4R	TTGARTGTGCATGGTCTAWTGAAAG GCTGACCTGACCARTCAGG	Undetermined	

4.2.5. Generation of clonal amplicons

The successful PCR products were thereafter cloned into *E.coli* (DH5α) strains using T-A cloning through the ThermoScientific InsTAClone PCR Cloning Kit (Thermo Fisher Scientific, USA). The transformation was carried out according to the manufacturer's instructions with the modifications outlined below.

Modifications to the preparation of competent cells: An overnight bacterial culture was incubated in C-medium for 2 hrs, and subsequently pelleted at 10000 x *g*. The pellet was then resuspended in 300 µL of T-solution and incubated on ice for 8 min. Following incubation, these cells were pelleted, resuspended in 120 µL of T-solution and incubated for another 8 min on ice. The C-medium and T-solution are provided as components of the ThermoScientific InsTAClone PCR Cloning Kit (Thermo Fisher Scientific, USA).

Modifications to the ligation mixture: The ligation mixture was made by adding 1.5 µL Vector pTZ57R/T, 6 µL of 5 x Ligation Buffer, 4 µL of purified amplicon, and 0.5 µL of purified T4 DNA Ligase to a final volume of 15 µL. The plasmid vector, 5 x Ligation Buffer and T4 DNA Ligase are provided as components of the ThermoScientific InsTAClone PCR Cloning Kit (Thermo Fisher Scientific, USA).

The remainder of the transformation reaction was carried out as per the instructions of the kit.

4.2.6. Colony PCR and validation of clonal amplicons

Individual colonies of the successfully transformed *E.coli* DH5α cells containing the gene of interest were subcultured onto LB agar plates using isolation streaking and aseptic technique, and incubated at 37 °C overnight. Following incubation, individual colonies were selected from the plate using a sterile needle and transferred into a PCR mixture containing the KAPA Taq 2x Readymix (Kapa Biosystems, South Africa), water and suitable primers for that particular gene of interest (as per Table 11), such that the transformed *E.coli* DH5α colony would act as the template DNA (Figure 24). This reaction mix was again amplified using PCR (at the conditions suitable for the particular gene of interest) to produce sufficient copies of the clonal amplicon.

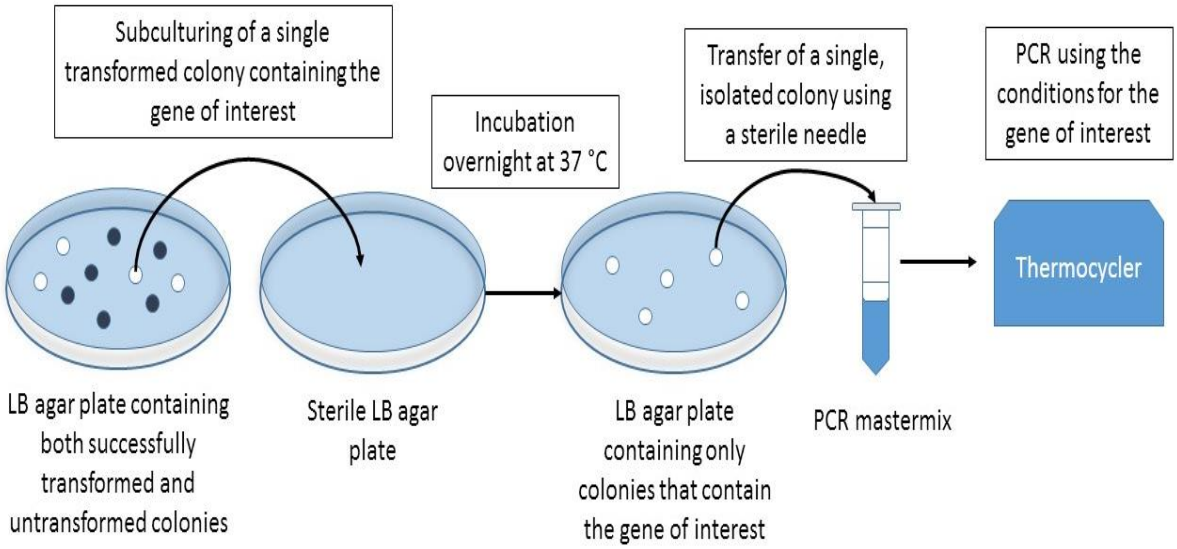

Figure 24: Graphical representation of colony PCR

These clonal amplicons were validated by Sanger Sequencing using the ABI 3500 (Applied Biosystems, USA) as per the manufacturer's instructions using the BigDye Terminator 3.1 chain termination and the BigDye XTerminator kits respectively. Sequences were cleaned and aligned using the ABI GeneMapper Software 5 and parsed against the NCBI database for matching sequences.

4.2.7. Quantitative PCR

The total eubacterial population, the total anammox population, total AOB population, the *Nitrospira* spp., the *Nitrobacter* spp. and total Eubacteria were quantified using quantitative PCR (QPCR) with the CFX96 Touch Real-Time PCR Detection System (BioRAD, USA) according to specific primer sets (summarized in Table 12). The QPCR reaction mixture contained 5 µL of Luminaris Colour HiGreen qPCR Master Mix (Thermo Fisher Scientific, USA), 0.6 µL of each primer (final concentration of 0.3 µM), 1 µL of template DNA (final concentration of 1 ng) and made up to a final volume of 10 µL with molecular grade water. The primers were selected on their specificity to the gene target, in that they produce only single band-amplification products, and their product size had to be less than 500 bp due to limitations of the SYBR green-Fluorescence Resonance Energy Transfer (FRET) based assay used.

Table 12: Primer sets selected for quantification of the AOB, NOB and anammox bacterial populations

Target Population	Target gene type	Primer Name	Product Size
Anammox bacterial	16S rRNA	AMX368F/AMX820R	452bp
Total AOB population	Functional gene	amoA1F/amoa2R	491 bp
Nitrospira spp.	16S rRNA	NSR1113F/NSR1264R	151 bp
Nitrobacter spp.	16S rRNA	FGPS872F/FGPS1269R	397 bp
Total Eubacteria	16S rRNA	P338F/P518R	180 bp

Each QPCR reaction setup included a no-template control containing no genomic DNA, negative controls containing genomic DNA from an unrelated species (either *Bacillus subtilis* genomic DNA or algal *Chlorella vulgaris* total DNA, depending on the primer set tested), and a positive control with a sample known to contain the target gene. For each QPCR assay the target populations were quantified using absolute quantification, and linear standard curves were generated using appropriate purified clonal amplicons, over 8 orders of magnitude, and acceptable standard curves contained:

- a coefficient of determination (R^2) value greater than 0.98;
- an efficiency of 90-110 %; and
- a slope ranging from -3.6 to -3.1 (Awolusi et al., 2016).

The specificity of each QPCR assay was confirmed with regression analysis and melting curve analysis (Table 14 and Appendix E respectively). The standard curves were generated based on ten-fold serial dilutions of the target DNA, prepared from 10^8 to 10^2 copy numbers. Estimation of the copy number of the purified clonal amplicons (Equation 21).

$$\text{Number of copies} = \frac{(\text{Amount in ng x Avogadro's number})}{\text{Length in bp x 1 x } 10^9 \text{ x 650}} \quad (21)$$

The average weight of a base pair (bp) is 650 Daltons and Avogadro's number is 6.022×10^{23}.

4.2.8. Next Generation Sequencing

4.2.8.1. 16S Metagenomic Library Construction

The amplicon library was constructed according to the Illumina MiSeq 16S Metagenomic Sequencing Library protocol (Illumina Inc., 2013) using the primer combination S-D-

Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') targeting the V3-V4 region of the 16S gene directly (Klindworth et al., 2013). The amplicons were sequenced on an Illumina MiSeq platform at Inqaba Biotec (Pretoria, South Africa).

4.2.8.2. Data Cleanup using Mothur

The NGS data from the reactor was analysed using the Mothur software pipeline and the Silva v123 SEED database, according to the MiSEQ workflow (Kozich et al., 2013; Schloss et al., 2009; Quast et al., 2013). The script used was modified to match the 16S rRNA V3-V4 region and can be found in Appendix D.

4.2.8.3. Data analysis using CLC Bio

Illumina MiSeq NGS data was further analysed using the CLC Bio Genomics Workbench ver. 10.0.1, with the included Microbial Genetics Plugin (CLC Bio; Denmark). The forward and reverse reads were merged to yield one high quality representative. Parameters were set at Mismatch cost = 1; Minimum score = 40; Gap = 4; and Maximum unaligned end mismatches = 5. The generated reads were clustered to representative sequences found in the SILVA 16S v123 reference database at 95 % similarity.

Alpha diversity estimates describe the number of species in a single sample, while beta diversity estimates differences in species diversity between samples. To measure alpha diversity, a phylogenetic tree was reconstructed using a Maximum Likelihood approach based on Multiple Sequence Alignment (MSA) of the OTU sequences as generated by the MUSCLE plugin of the CLC Genomics Workbench.

4.3. Results and Discussion

4.3.1. Existing primer validation and new primer design for the detection of anammox and nitrifier bacterial populations

In this study, the individual members of the N-removing microbial consortia that had developed within the SBR system (described in Chapter 3) were identified. The SBR was operated under conditions of high NLR, limited DO and no influent organic carbon. These conditions are known to facilitate the development of an autotrophic N-removing microbial community, and to particularly promote the growth of slow growing anammox

bacteria and nitrifiers (Jetten et al., 2005). Primers targeting the known key members of the N-removing consortia, including the anammox bacteria, were utilised to determine the phylogenetic diversity within the reactor system over the period of its operation. The amplification products of these primer sets can be visualized in Figure 25, which depicts the community structure after ~300 days of enrichment. The amplified single bands in Figure 25 show the concurrent presence of anammox bacterial species; the most common wastewater NOB genera *Nitrobacter* and *Nitrospira*; and AOB all coexisting within the SBR reactor. The presence of these populations explain the high NRR observed within the reactor and could imply the successful enrichment of an anammox-nitrification consortia for autotrophic ammonium removal (Chapter 3: Figure 13).

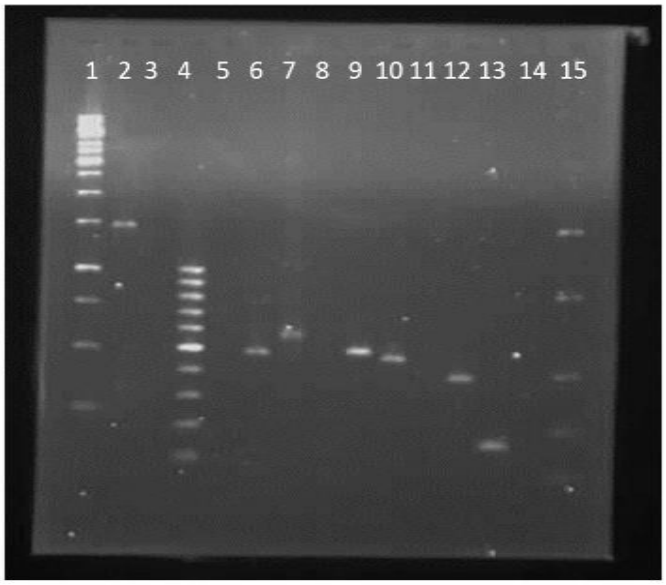

Figure 25: PCR amplification products showing the presence of the main N-removing populations. Lane 1 contains the 1 kbp molecular weight ladder. Lane 2 contains the Planctomycetes specific (PLA46F/1492R) amplicon. Lane 4 contains the 100 bp molecular weight ladder. Lane 6 and 7 contain the anammox-specific amplicons (AMX368F/AMX820R and AMX694F/AMX960R respectively). Lane 9 contains the ammonia monooxygenase amplified product (amoA1F/amoA2R). Lane 10 contains the AOB 16S rRNA targeting amplicon (CTO189fAB, CTO189fC, CTO654r). Lanes 12 and 13 contain the NOB specific amplified products (using the FGPS872F/FGPS1269R and the NSR1113F/NSR1264R primer sets respectively).Lane 15 contains a Low range molecular weight ladder.

The AOB, NOB and anammox bacteria that are present within the SBR are also critical to N-removal within WWTPs. The AOB as a group are responsible for the oxidation of NH_4^+ to NO_2^-, while the *Nitrospira* spp. and *Nitrobacter* spp. are responsible for the conversion of NO_2^- to NO_3. Collectively, these two groups are responsible for conventional nitrification, which requires the presence of DO. Denitrification is the process which

allows for the reduction of NO_3 to N_2 gas and occurs under anaerobic conditions. The presence of both groups of nitrifiers (i.e. the AOB and NOB) implies that DO is present within the SBR system at Day ~300. The presence of DO is inhibitory to both the anammox and denitrification pathway, and thus would not allow the N-removal cycle to go to completion, thus resulting in an accumulation of NO_3 within the system. This is observed with the NRR and NO_3–N analysis (Table 9 and Figure 18 respectively) during this period from Day ~300 to Day ~340. In fact, the SBR reactor performance for NH_4^+–N removal was also depressed during this period (Figure 15). Despite this, the NO_3–N did not equal the NLR over the same period, implying that some N-removal still occurred. As the presence of anammox bacteria was also detected during this period, despite the oxygen ingress, and since anammox bacteria is also capable of $NH4^+$–N oxidation, it is possible that anammox activity still occurred, albeit at a vastly suppressed rate.

Nevertheless, anammox activity cannot be inferred from a PCR of the 16S rRNA gene. The selection of suitable PCR primers targeting functional genes can provide valuable information on the metabolism and activity of the anammox bacterial fraction. The Hydrazine synthase (*hzs*) and Hydrazine oxidoreductase (*hzo*) functional genes encode for enzymes that are critical and unique to the anammox biochemical pathway (Klotz & Stein, 2008). Hydrazine is a metabolic intermediate formed during the anammox reaction, with the hydrazine synthase responsible for its formation and the hydrazine oxidoreductase responsible for its degradation (Strous et al., 2006). The successful amplification of the *hzo* and *hzs* functional genes could provide a far more specific gene target than the 16S rRNA for examining the diversity of anammox bacteria, increasing the detection limit of anammox bacteria and providing activity information (Hirsch et al., 2011; Harhangi et al., 2012; Li & Gu, 2011).

In order to detect anammox bacteria at the functional gene level, the hzsA526F/hzs1857R primer set targeting the hydrazine synthase gene, and the hzoAB1F/hzoAB1R and hzoAB4F/hzoAB4R primer set targeting the hydrazine oxidoreductase gene were also employed (Table 11). Although the hzoAB1F/hzoAB1R primer set described by Hirsch et al. (2011) had successfully amplified a single band from genomic DNA, the nested reaction with the hzoAB4F/hzoAB4R using the PCR product from the hzoAB1F/hzoAB1R showed non-specific binding at multiple target sites (Figure 26). The literature primers hzs526F/hzs1857R for hydrazine synthase also showed non-specific amplification of two distinct target regions, producing an amplification product at both ~350bp and at ~550bp (Figure 27).

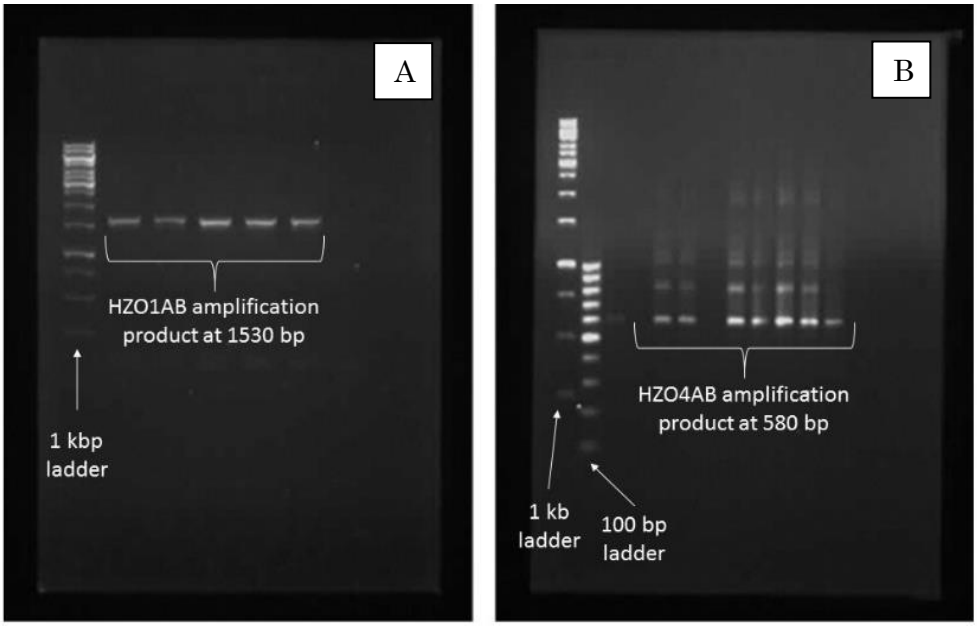

Figure 26: Amplification of the hydrazine oxidoreductase functional gene through nested-PCR. A) Amplification of the hzo1AB gene product at 1550 bp. B) Nested-PCR product of the hzo4AB primer set using the hzo1AB amplification product.

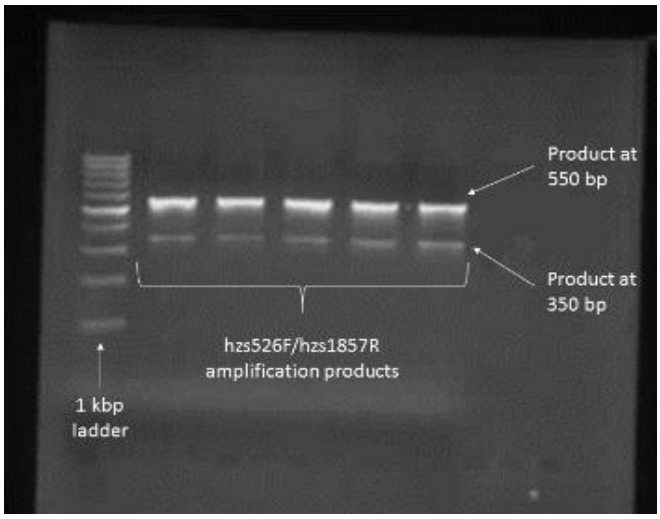

Figure 27: Amplification of the hydrazine synthase functional gene using the hzo526F and hzs1857R primer set shows amplification of two distinct products

In this study, the previously elucidated functional gene primers (hzoAB4F/hzoAB4R and hzs526F/hzs1857R) did not provide the expected single amplification products, but rather multiple amplification products of consistent sizes. A similar result was obtained by Hirsch et al. (2011), who obtained multiple amplification products of different sizes when using another *hzo*-targeting primer set elucidated by Li et al. (2010). Recent analysis of

the *Kuenenia stuttgartiensis* metagenome has revealed that it contains two copies of the gene coding for HZO that share a 77 % nucleotide similarity, along with four structural homologues (Hirsch et al., 2011; Strous et al., 2006; Klotz et al., 2008). These homologues could provide alternate binding sites for the primer sets and could explain the production of multiple amplification products, as observed in this study.

Inappropriate primers may cause incorrect biological conclusions to be drawn, particularly with regard to the amplification of functional genes from a mixed microbial consortia (Lu et al., 2015). Consequently, three additional primer sets respectively targeting the 16S rRNA gene region of anammox bacteria, the Hydrazine oxidoreductase and the Hydrazine synthase subunit A functional genes of anammox bacteria were designed *in silico* (Table 13). These primer sets were designed to anneal at a higher temperature, and to a smaller target region within the respective genes of interest, thus increasing their target specificity, particularly for quantification purposes.

Table 13: Custom designed primer details

Primer Name	Target	Primer Sequence (5'-3')	Tm (in °C)	Expected Product Size
AMX 1F	All anammox 16S rRNA	GCCTTGCAAGTCAGTTGTGA	60	213 bp
AMX 1R		CGGGGTATCTAATCCCGTTT	60	
HZO F	Hydrazine Oxidase gene	AACGTGCAGAGGCTTTCAAC	60.4	149 bp
HZO R		GCCTGCAAATTCTCTCTTGC	60.1	
HZS A1F	Hydrazine Synthase (subunit A)	TGAGCAATGATGGCAGACTC	57.97	110 bp
HZS A2R		ATCCGCGCAGAAAAAGTAGA	57.62	

These custom primers were validated against corresponding primer sets for the equivalent gene targets, and their relative efficiencies and specificities were compared. Both the AMX1F/AMX1R and AMX368F/AMX820R primer sets were amplified against the Pla46F/1492R clonal amplicon in a nested PCR reaction, as this would eliminate background noise from non-specific targets. As per the results of the PCR amplification, the anammox 16S rRNA specific primers designed in this study (i.e. AMX1F/AMX1R) to target the 16S rRNA housekeeping gene displayed no amplification and were thus less efficient at selectively amplifying members of the anammox bacterial clade than the literature sets (AMX368F/AMX820R & AMX694F/AMX960R) designed to target the same gene. Similarly, the custom designed functional gene primer sets for both *hzs* and *hzo* displayed less specificity than the literature sourced primer sets. Both the custom designed hzoF/hzoR and hzs1F/hzs2R showed no amplification of the target gene.

Consequently, the anammox functional genes were replaced with the 16S rRNA gene for downstream quantification of anammox bacteria.

4.3.2. Quantification of anammox, AOB and NOB populations over the course of the experiment using quantitative PCR

The N-removal populations are very sensitive to changes in their microenvironments, and even slight changes in environmental conditions may affect the equilibrium at which these organisms exist (Chen et al., 2009; Harmand et al., 2008). Using quantitative PCR (QPCR), the absolute quantities of the anammox, AOB, NOB and total Eubacterial populations were tracked over the 360 day enrichment period through amplification of their unique group-specific gene targets (Figure 28). To ensure accuracy of the quantification, only data that matched the quality control parameters were used in the quantification for each population (4.2.7 and Table 14).

In the current study, the quantitative PCR analysis for each of the key N-removing populations displays a distinct change in average (n = 2) population densities over the enrichment period (Figure 28). The total eubacterial load shows a sharp decrease from Day 1 to Day 90, and this trend is observed with all the population groups quantified (Figure 28D). A decrease in total biomass and microbial population diversity is common during the initialization phase of anaerobic bioreactors, as the seed inoculum needs to adjust and equilibrate to the new environmental conditions within the reactor. As this reactor was operated under conditions of no organic C, no aeration, and high N-loads, a shift towards an autotrophic N-removal population was expected, and the other bacterial populations that could not proliferate under these conditions were gradually washed out of the system. Total eubacteria seems to stabilize over the course of the remainder of the enrichment period, however the *Nitrospira* spp., the anammox bacteria and the AOB all still displayed large fluctuations over the 360 day enrichment period.

Table 14: Summary of QPCR standard curves and amplification quality for each of the key microbial populations

Parameter	*Nitrospira* spp.	AOB	Anammox bacteria	All Eubacteria
Efficiency	91.12 %	93.29 %	92.3 %	97.50 %
R2	0.995	0.997	0.991	0.999
Slope	-3.555	-3.494	-3.521	-3.383
y-intercept	37.144	39.213	34.954	35.998

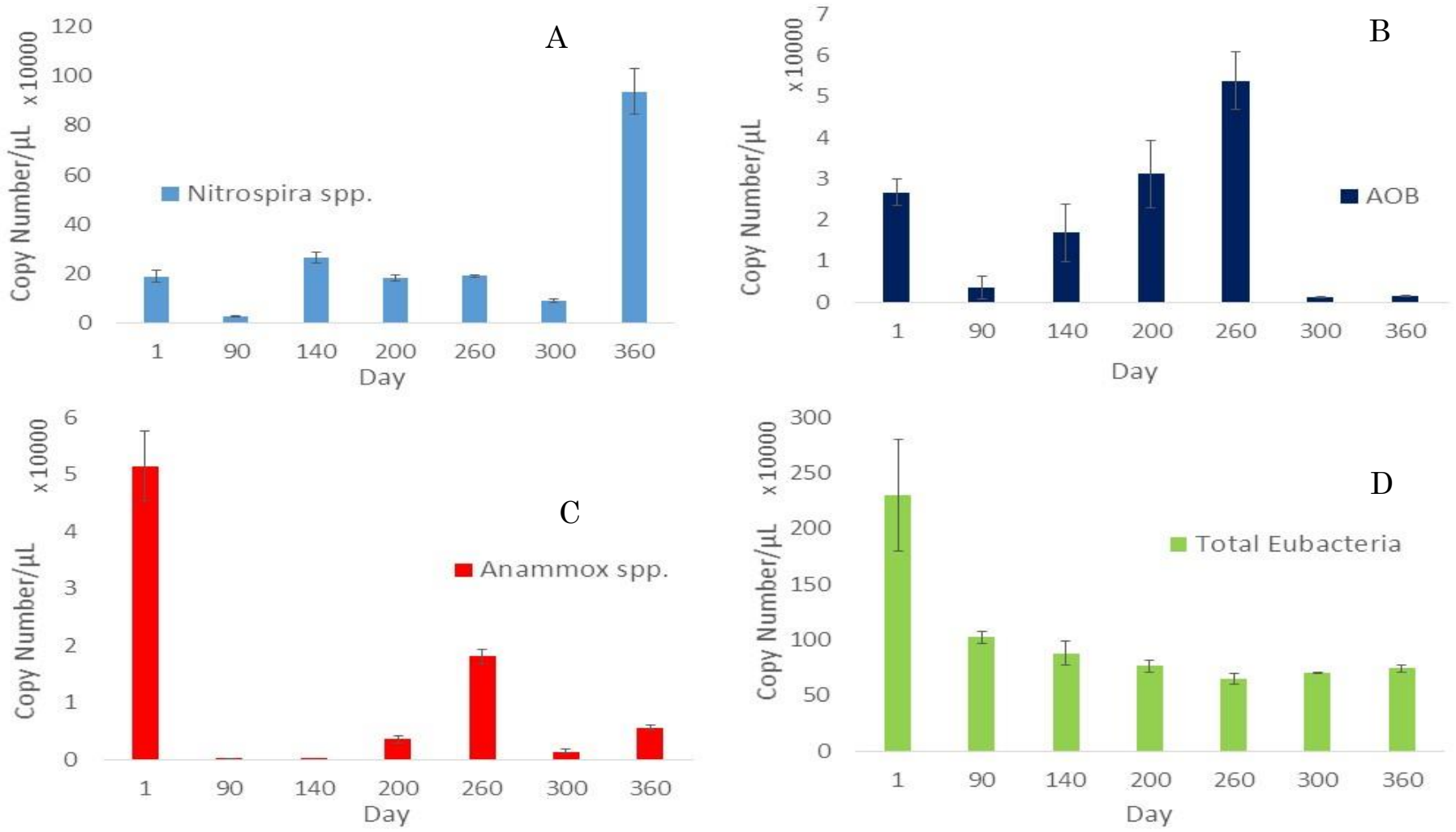

Figure 28: Quantification of the key N-removing microbial populations. A) Population quantification of *Nitrospira* spp. over time. B) Population quantification of AOB over time. C) Population quantification of anammox spp. over time. D) Shift in total eubacterial population over time.

The anammox bacterial copy number decreases drastically from Day 1 to Day 200. At Day 200, a slight increase in population copy number is observed, and this increase continues through to Day 260 (Figure 28C). A similar trend is observed with the AOB population, which displays a large decrease from the starting quantity from Day 1 to Day 90, and thereafter a gradual increase in population density until Day 260 (Figure 28B). The anammox and AOB populations are commonly co-cultured as they metabolically complement each other. Under the limited DO conditions within the reactor during this period, the AOB would have oxidized the provided NH_4^+ to NO_2^-, while the anammox bacteria utilize the produced NO_2^- and any remaining NH_4^+ to produce N_2 gas. Despite this, it is within this same period (i.e. Day 90 to Day 260) that a suppression in NRR is observed within the reactor (Figure 13). It is possible that despite the conditions provided within the reactor being suitable for both anammox and AOB proliferation, they still represented such small fractions of the total population, that their metabolic contributions would be minimal. A direct comparison of copy numbers for the anammox and AOB populations relative to the copy numbers of the total eubacterial load at Day 260 shows that anammox bacteria only comprised 2.78 % of the total population, while AOB comprised 2.13 %.

The *Nitrospira* spp. population also displayed the characteristic decrease in the first 90 days as observed with other populations, however it should be noted that the starting quantity of *Nitrospira* spp. was already higher than the other N-removing populations. After Day 90, the population of the *Nitrospira* spp. remained relatively stable, until the period between Day 300 and Day 360, wherein the population density of the *Nitrospira* spp. rapidly increased (Figure 28A). This increase in the *Nitrospira* spp. population between Day 300 and Day 360 correlated with a leak of oxygen into the reactor system. This oxygen leak caused the DO to increase from less than 0.5 mg/L to ~2.1 mg/L ± 0.2 mg/L (measured at Day 350). Accumulative advantage of the NOB in response to the DO ingress allowed these species to outcompete the AOB within the system, while the excess DO suppressed the growth of anammox bacteria as evidenced in this study.

The *Nitrospira* spp. have previously been found to be the dominant NOB species within biological wastewater treatment systems, and their high substrate affinity makes them typical K-strategists (Siripong & Rittmann, 2007; Dytczak et al., 2008; Awolusi et al., 2015). In this study, their high affinity for DO allowed them to gain a competitive advantage over other R-strategists, including the *Nitrobacter* spp., the AOB and anammox bacteria within the system, even when N-substrates are limiting. Furthermore,

free ammonia and free nitrous acid have been shown to be inhibitory to both anammox and NOB groups (Blackburne et al., 2007). The consistently high *Nitrospira spp.* population fraction, and the rapid growth when suitable DO was available confirmed that the FA and FNA values (Figure 20 and Figure 21) were too low to inhibit the NOB growth, and mitigate the effects of the oxygen ingress (Blackburne et al., 2007).

4.3.3. Total population analysis change from Day 1 to Day 350 using 16S rRNA community analysis

The total genomic DNA extracted from the initial seed sample (taken as Day 1) and the enriched reactor sample after 350 days of operation were sequenced using the Illumina MiSeq platform to assess the total microbial shift within the reactor. From the sequences obtained, the unique reads were extracted, and sequences displaying chimaeras or homopolymers were discarded to generate 15568 unique reads from the sample at Day 1, while only 5265 unique reads were generated from the enrichment reactor at Day 350.

All sequences reads were trimmed to the same length to ensure even coverage for Operational Taxonomic Units (OTU) clustering, and reads that were repeated were filtered out to avoid biased representation of the original sample diversity (Table 15). These usable sequence reads had a mean length of 300 bp and represented the interquartile range between the generated reads. The quality of the reads as a Phred score (Figure 29). The Phred score is a rating assigned to the probability of an error in base calling during sequencing. A Phred score of 10 represents a 90 % base call accuracy, and a score of 40 indicates a 99.99 % base call accuracy (Illumina, 2011). The Phred score of the trimmed sequences display a bell curve between 10 and 40, indicating high quality trimmed reads (Figure 29).

Table 15: Predicted OTU abundances based on merged and trimmed sequences

Sample	Total number of reads	Number of merged reads	Number of reads after trimming	Number of unique reads after filter	Reads used in OTU clustering
Day 1	140812	33,458	15,776	15568	208
Day 350	55956	12.074	5286	5,265	21

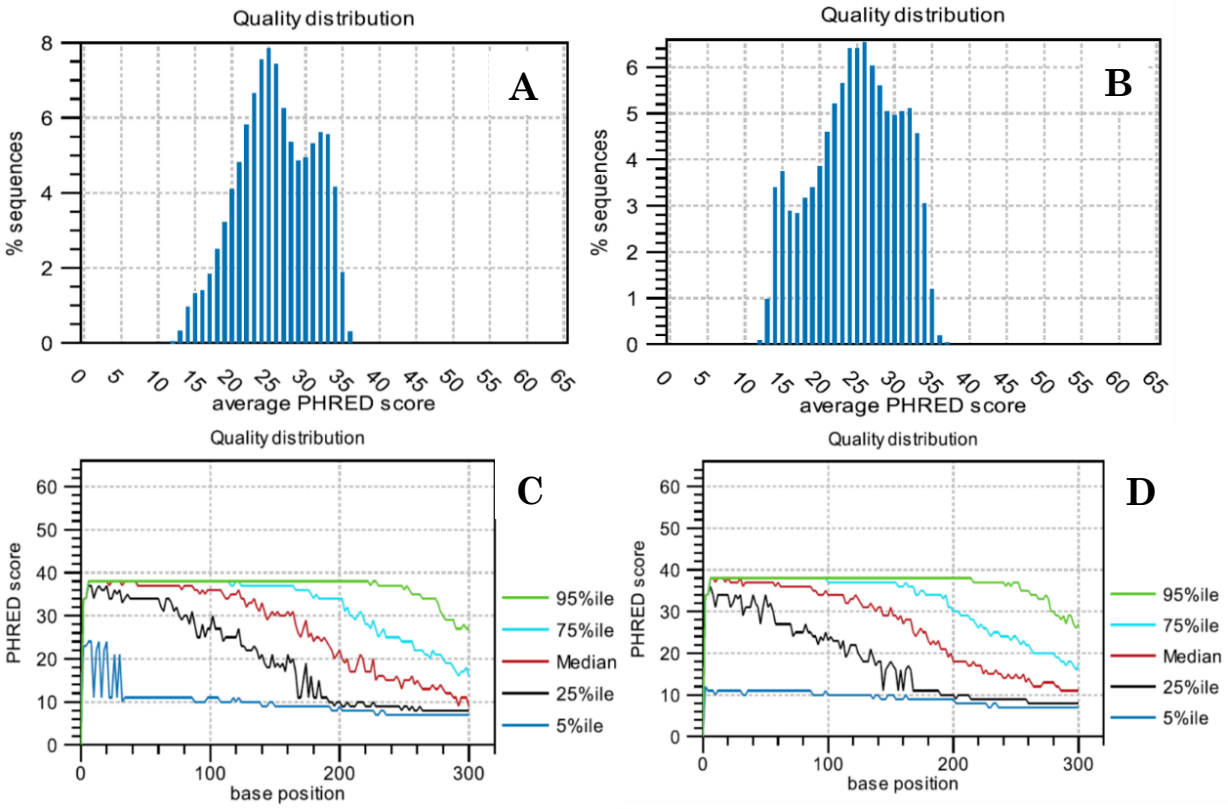

Figure 29: Sequencing quality of reads. The quality distribution curves (A) and (B) represent the PHRED distribution for the initial and final samples respectively, while (C) and (D) display the PHRED interquartile ranges for base-call distribution

Only the reads that passed quality control analysis were taken for OTU clustering using the UPARSE algorithm, producing 208 OTUs for the Day 1 seed sample and 21 OTUs for the enrichment reactor sample at Day 350. These OTU sequences were annotated against the SILVA v123 database modified to remove all non-16S rRNA sequences as well as all sequences pertaining to Archaea and Eukaryota using ARB (Ludwig et al., 2004). The NGS results show a significant shift in the population structure from Day 1 to Day 350 (Figure 30). A comparison of the population at Day 1 and Day 350 displays a shift in the relative abundance of populations already present, rather than show new species. The Planctomycetes phylum, to which anammox bacteria belong, shows dominance at Day 1, which is to be expected due to the source of the inoculum, while other populations are present, albeit at minimal relative quantities. At Day 350, the populations that exhibited a minimal abundance in the initial sample have increased significantly, while the relative abundance of the Planctomycetes phylum decreased (Figure 30).

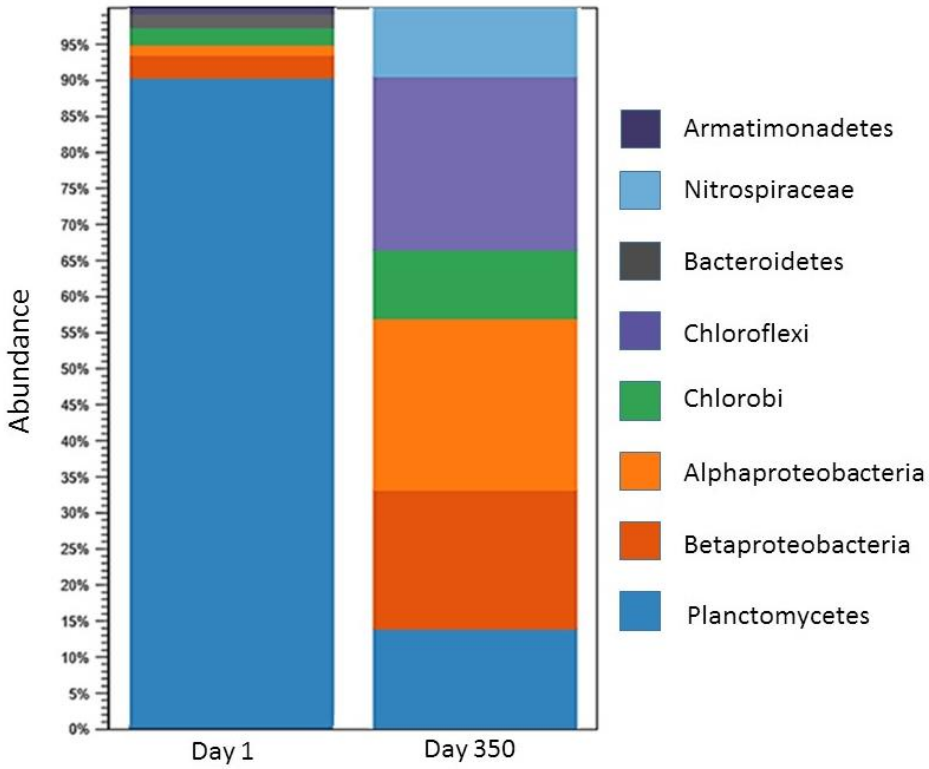

Figure 30: Total population shift at the Phylum-level from Day 1 to Day 350 for the most dominant known organisms present.

Additionally, Alpha diversity (α-diversity) analysis was performed to confirm the results of this alignment (Figure 31). The α-diversity is a measure of the species richness within a single environment, and in this instance represents the species diversity within the reactor system, and how it has shifted over the operational period. Species richness estimates the total number of species present in a community, where the *Chao-1* index is calculated based on the number of rare OTUs found in the sample (Figure 31A). From analysis of the rarefaction curves in Figure 31A, it can be observed that the point at which the number of OTUs does not increase with further sampling is the point at which sufficient samples have been taken to accurately characterize the community. The sample at Day 1 contains fewer unique OTUs due to dominance of a few microbial groups, and this can be further observed using the Shannon Entropy in Figure 31B. The Shannon Entropy is able to combine species richness and abundance into a single measure of evenness (Figure 31B). In the initial sample (Day 1), the community was numerically dominated by a few species (i.e. the anammox and AOB bacterial communities) and displays low evenness. After 350 days of operation in the current study, with concomitant exposure to DO, the abundance was distributed evenly across all species present, thus exhibiting high evenness, and a plateau on the curve for Day 350.

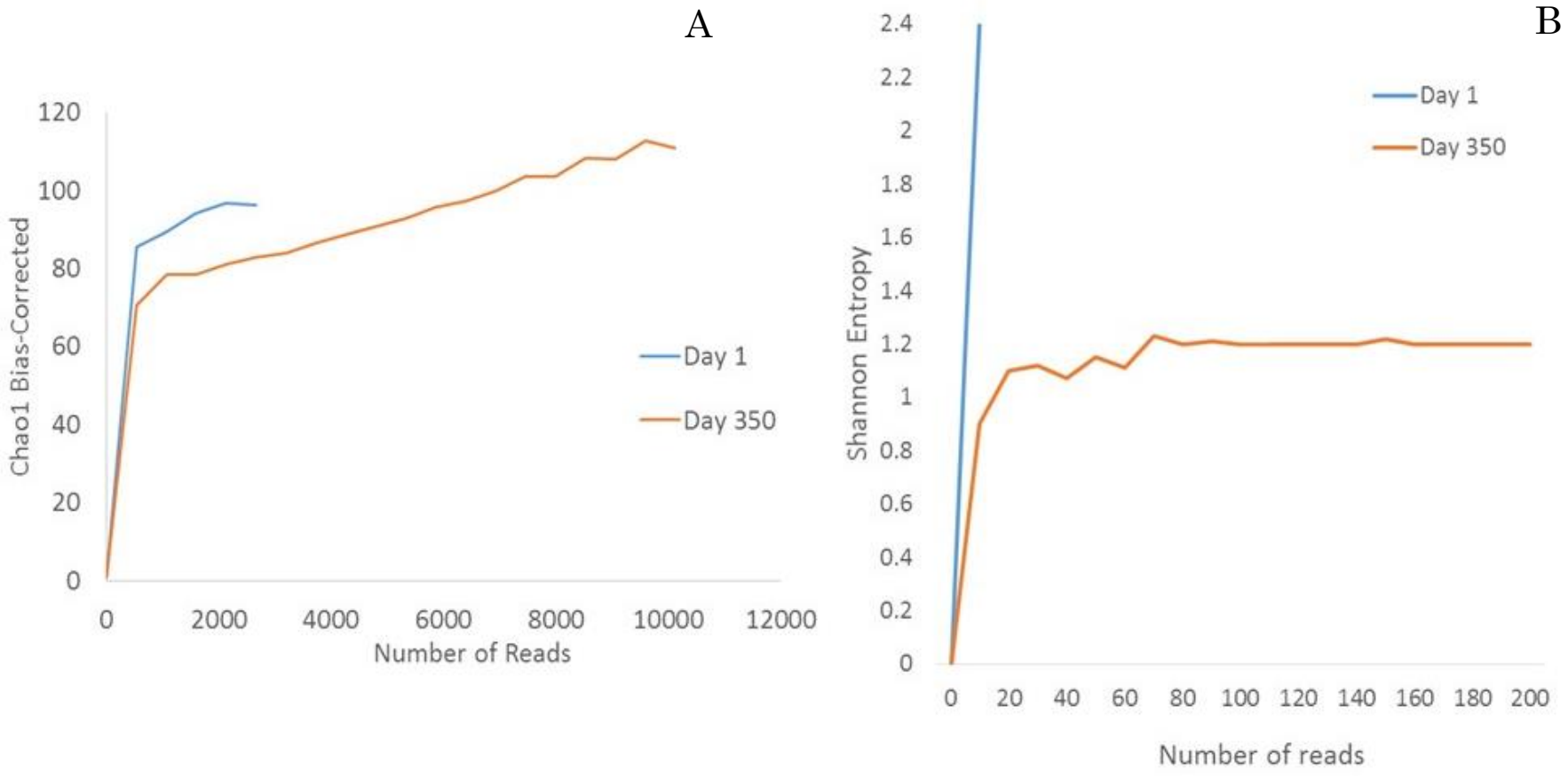

Figure 31: Alpha diversity analysis of the samples with A) Alpha diversity using Chao-1 Bias Corrected; and B) Shannon Entropy rarefaction curves to measure community evenness

The 16S rRNA population analysis corroborated the findings of the QPCR analysis, and indicated a shift in community diversity and abundance. The diversity and abundance of microorganisms within a system are governed by the environmental conditions acting within that environment, and these populations can be greatly influenced by stress effects. Microbial communities may survive transient exposure to stress conditions, however a shift in population diversity is not uncommon in systems operating for long periods under stress, and it is these selfsame stress conditions that shape the dominant organisms (Harmand et al., 2008). In this study, transferring the seed inoculum from an aerated MBBR to an anaerobic SBR system only allowed for growth of those organisms most suited to the current prevailing conditions. The oxygen ingress that had occurred after Day 300 would have also contributed greatly to the population shift. Additionally, this increased DO would have contributed to the increase in biomass, since aerobic orgnisms have a higher growth rate than anaerobic organisms and would replicate at a greater rate, as previously observed in Figure 23.

The Day 1 sample represented the state of the microbial population in the seed inoculum, which showed a dominance of the family Planctomycetes, to which anammox bacteria belong, while the Day 350 sample represents the state of the microbial population after 350 days under the SBR environmental conditions (Figure 30). The sample at Day 350 shows a shift in the entire community composition towards the Nitrospiraceae, of which Nitrospira spp. are a member, when compared to the Day 1 samples. The rapid increase in *Nitrospira* spp. between Day 300 and Day 360 correlates with an increase in NO_3-N within the reactor as well as a decrease in AOB and anammox populations, but only minimal suppression of ammonia removal capabiltiy. Interestingly, despite the suppression of the key ammonia removal populations, the average ammonia removal rate during this period actually increased compared to the previous 100 days. Recent research into *Nitrospira* spp. have indicated that some species within this group are capable of complete ammonia oxidation (comammox), which is the aerobic oxidation of ammonia directly to nitrate (van Kessel et al., 2015; Chao et al., 2016). Additionally, some members of the phylum Chloroflexi have been shown to carry out nitrite oxidation, and may contribute to the overall N-removal within the reactor (Sorokin et al., 2012). These pathways represent new frontiers in N-removal and are not yet well understood.

Conclusion

The composition of the microbial community within a biological wastewater treatment system is critical to the functioning of the system as a whole. An autotrophic N-removal reactor also requires a suitable autotrophic microbial community to function effectively. Microbial composition in any system can be controlled by the environmental conditions acting upon it. In this study, the microbial community was controlled by the limited organic carbon, high NLR and low DO. Analysis of the key N-removal populations over time indicate a population shift from a predominantly anammox and AOB dominated community to a more *Nitrospira* spp. dominated population. The shift in population occurred primarily due to an ingress of excess DO. Since *Nitrospira* spp. are *R*-strategists with a high oxygen affinity, the increase in available DO allowed for a competitive advantage over the other anaerobic and aerobic *K*-strategists. This advantage is clearly observed from day 300 onwards, where the *Nitrospira* spp. population sharply increased, along with a concomitant decrease of the AOB and Anammox populations over the same time period. The 16S rRNA community analysis displayed a similar trend of population shift towards a more aerobic *K*-strategist dominant system. In the initial sample, the Planctomycetes phylum dominated the system, however at Day 350 a significant shift in population abundance was observed. At Day 350, the *Nitrospira* spp. and members of the phylum Chloroflexi seem to dominate the system. New research implicates both of these groups in new N removal pathways, however additional research needs to confirm whether these pathways are indeed active in this reactor. Despite the population shift within the reactor, the reactor remains stable as an autotrophic N-removal reactor.

Chapter 5: Screening for indigenous anammox bacteria within South African biological wastewater treatment systems

5.1. Introduction

Anammox bacteria require strictly anaerobic conditions and the presence of ammonia, nitrite and inorganic carbon to proliferate. Since it occupies such a particular environmental niche, the anammox bacteria was not expected to be ubiquitous, however recent molecular analysis has shown that anammox bacterial phenotypes are more widespread than initially expected. Previous screening attempts have detected anammox-like Planctomycetes in various habitats, such as marine ecosystems, high-salinity lagoons, meromictic salt lakes, alkaline lakes, acid swamps, freshwater systems, brackish water systems, agricultural soil, cattle manure, and activated sludge of wastewater treatment plants (Nozhevnikova et al., 2012; Kuypers et al., 2003; Dapena-Mora et al., 2004b; Dalsgaard & Thamdrup, 2002; Jetten et al., 2003; Wenk et al., 2013; Humbert et al., 2010).

The anammox bacteria that had previously been detected from the activated sludge of wastewater plants are often found at exceedingly low quantities. Applying the anammox process at even pilot scale requires sufficient amounts of anammox-rich biomass, however the current lack of availability of suitably enriched local seed culture is a significant bottleneck to more widespread implementation of the anammox process (Costa et al., 2014; Ni & Zhang, 2013). Since anammox bacteria have been found in diverse environmental niches, identifying local natural reservoirs containing indigenous anammox bacteria is a critical first step towards initialising the anammox process in South Africa for commercial wastewater treatment.

This chapter focuses on an attempt to screen and identify indigenous anammox bacteria from the activated sludge within South African WWTPs using enrichment and molecular techniques. Activated sludge is a complex sample containing many contaminants that may inhibit downstream molecular analysis. Efficient molecular analysis of this complex substrate requires the use of optimised nucleic acid extraction techniques that can produce a high purity and high yield DNA product that minimizes interference from

potential inhibitors. Thus, multiple activated sludge DNA extraction methods were evaluated, and the best method was applied to activated sludge from selected WWTP that could potentially contain anammox bacteria. Nested PCR that first targets anammox bacteria at the family level, and using this product to target anammox bacteria at the species level, greatly amplifies the detection limits for low quantity gene targets such as the anammox bacteria.

5.2. Methodology

5.2.1. WWTP selection

Nine wastewater treatment systems from across the country were selected and screened for the presence of anammox bacteria on the basis of system configuration and operational parameters (Table 16). Activated sludge samples were collected from various points in the aeration and anoxic tanks (specified in Table 16) and pooled together to create a composite sample. These composite samples were transported to the main laboratory in sealed 1 L sampling bottles on ice in a cooler box. Samples that were obtained from the Kraaifontein, Zeekoegat and Scottsdene WWTPs were analysed within 24 hours of collection, while samples from the Kingsburgh, Phoenix, Northern, Amanzimtoti, Mariannridge and Shallcross WWTPs were analysed 3 hours after collection.

Table 16: Overview of WWTP selected for screening (as obtained from the respective WWTP at the time of sampling)

Sample	Source reactor	Design Capacity	Influent type	Operational Parameters (as at time of sampling)							Process Type
				Temperature (°C)	pH	Influent COD (Filtered) (g/L)	HRT (hours)	SRT (days)	TKN (g/L)	DO (mg/L)	
Phoenix WWTP	Aeration tank	25 ML/d	Domestic	24.1	6.50	794.9	15-18	25	-	1.54	Modified Ludzack Ettinger (MLE)
Kingsburgh WWTP	Aeration tank	7.2 ML/d	Domestic	24.3	7.50	671.71	15-18	32	6.74	0.22	University of Cape Town (UCT)
Kingsburgh WWTP	Anoxic tank	7.2 ML/d	Domestic	24.8	7.61	671.71	4-6	32	-	< 0.1	University of Cape Town (UCT)
Shallcross WWTP	Aeration tank	2 ML/d	Domestic	25.1	7.57	622.71	10-15	25	5.35	1.21	Conventional Activated Sludge (CAS)
Northern WWTP	Anoxic tank	70 ML/d	Domestic	27.2	7.43	-	13-18	35	-	0.25	MLE
Mariannridge WWTP	Aeration tank	8 ML/d	Industrial	23.9	7.87	461.48	10-15	25	17.95	5.3	CAS
Amanzimtoti WWTP	Aeration tank	22 ML/d	Industrial	26.3	7.64	365.66	10-15	28	1.42	0.85	CAS
Kraaifontein WWTP	Aeration tank	12.5 ML/d	Domestic	18	7.67	811.4	12	12-15	91.34	-	UCT
Scottsdene WWTP I	Aeration tank	12 ML/d	Domestic	15	8.20	960.8	22	15	127	3.06	MLE
Scottsdene WWTP II	Aeration tank	7.5 ML/d	Domestic	15	8.20	960.8	22	15	-	-	3 stage Phoredox/ UCT/ Johannesburg Process (JHB)
Zeekoegat WWTP I and II	Aeration tank	45 ML/d & 40 ML/d respectively	Domestic	12	7.40	398.9	-	20	34.89	< 2.0	3 stage Phoredox/ UCT/ JHB

5.2.2. Wastewater sludge DNA extraction optimization

Anoxic sludge obtained from the Kingsburgh WWTP was used to test the efficiency of seven different extraction methods, based on the quantity of DNA extracted from a fixed amount of sample (~0.5 g dry weight), and the quality of the extracted DNA based on spectrophotometric readings. The efficiency of the three best extraction methods on anammox bacteria were further confirmed with known anammox-containing biomass from an anammox enrichment reactor, to validate its effectiveness at extracting DNA from any the anammox bacteria that may be present within the complex environmental samples.

5.2.2.1. DNA extraction protocol I

Approximately 0.5 g (dry weight) of sludge samples were added to 2 mL microcentrifuge tubes and centrifuged at 5000 rpm for 3 min at 4 °C. The pellets were washed twice with 1x Phosphate Buffered Saline (PBS) solution, and resuspended in 75 µL of 1 x Tris/ Ethylenediaminetetraacetic acid (TE) buffer and 25 µL of 10 % Sodium Dodecyl Sulphate (SDS). Cell lysis was initiated by incubating these tubes for 2 hours at 65 °C. Thereafter 500 µL of lysis buffer (10 % SDS, 0.1 M NaCl, 0.5 M Tris-HCl) was added and the samples were mixed by vortexing. The sludge samples underwent a secondary lysis using a freeze-thaw method (i.e.: by immersion of the tubes in a dry-ice/ethanol slurry for 2 min followed by immersion in a 65 °C water bath). This lysis step was repeated 5 times. The cell debris was extracted by the addition of Phenol/Chloroform/Isoamyl Alcohol (P/C/I) (25:24:1). The tubes were mixed by inversion, and centrifuged at 5700 rpm for 5 min. The aqueous upper layer was transferred to a fresh tube, and nucleic acids were precipitated by the addition of 0.6 volumes of isopropanol and incubating at -20 °C for 1 hour. The DNA was pelleted at 12000 rpm (5 min), washed in 70 % ethanol, air dried and stored in TE buffer at -20 °C.

5.2.2.2. DNA extraction protocol II

DNA extraction was carried out according to the protocol outlined by Orsini and Romano-Spica (2001), with slight modifications. Approximately 0.5 g (dry weight) of the activated sludge samples were aliquoted into 2 mL micro-centrifuge tubes and centrifuged at 5600 rpm for 4 min at 4 °C. The pellets were washed twice with a 1x PBS solution, and once with a wash solution (50 mM Tris-HCl, pH 7.7; 25 mM EDTA pH 8.0; 0.1 % SDS; 0.1 % PVP). The samples were thoroughly vortexed and pelleted at 5700 rpm for 4 min at 4°C after each wash step. Fifty microliters of the lysis solution (50 mM Tris-HCl (pH 7.2); 25

mM EDTA (pH 8.0); 3 % SDS; 1.2 % PVP) was then added to each respective tube and vortexed thoroughly. The tubes were then heated in a standard domestic microwave oven (800 watt for 35 seconds) in the presence of 50 mL dH_2O to act as a dummy load. A further 400 µL of pre-warmed (~65 °C) extraction solution (10 mM Tris-HCl, pH 8; 1 mM EDTA pH 8.0; 0.3 M Sodium Acetate; 1.2 % PVP) was added to the tubes and the samples was gently mixed by inversion. Thereafter 1 mL of a freshly made phenol/chloroform/isoamyl alcohol mix (25:24:1) was added to the tubes. These tubes were gently mixed by inversion, and centrifuged at 10000 rpm at room temperature. The upper aqueous layer was transferred to a fresh set of tubes, and the chloroform/isoamyl alcohol mix (24:1) was repeated. The upper aqueous layer was again transferred to a fresh set of tubes, and the DNA was precipitated by the addition of 50 µL of 3 M Sodium Acetate and 1.5 volumes of 100 % Ethanol, and stored at -20 °C overnight. The precipitated genomic DNA was pelleted by centrifugation at 12300 rpm at 4 °C for 5 min, washed twice with 70 % Ethanol and air dried. The final DNA pellet was stored in 1 x TE buffer (pH 7.5) at -20 °C.

5.2.2.3. DNA extraction protocol III

The above nucleic acid extraction method was modified from the protocol elucidated by Purkhold et al. (2000). Approximately 0.25 g (wet weight) of sludge sample was aliquoted into 2 mL micro centrifuge tubes, and 625 µL of DNA Extraction Buffer (100 mM Tris-HCL (pH 7.4); 100 mM EDTA (pH 8.0); 100 mM $NaPO_4$ (pH 7.6); 1.5 M NaCl; 1% CTAB) was added to each tube. The tubes were vortexed thoroughly, and a further 50 µL of Enzyme Mix 1 (Lysozyme (14.2 mg/mL) and ß-Glucuronidase (10.6 mg/mL)) was added to each tube. The tubes were incubated for 30 min at 37 °C. Following this incubation, a further 50 µL of Enzyme Mix 2 (Proteinase K (10 mg/mL); Protease (10 mg/mL); Pronase (40 mg/mL)) was added to each tube and incubated for an additional 30 min at 37 °C. Thereafter, 75 µL of 20 % SDS was added, and the sample was incubated for 2 hrs at 65 °C. Six hundred microliters of Phenol/Chloroform/Isoamyl alcohol (25:24:1) was added to each tube and these were incubated at 65 °C for 2 hrs. After incubation, the samples were centrifuged at 10000 x *g* for 10 min (room temperature), and the aqueous layer was transferred to a new tube. Further purification was achieved by repeating the Chloroform/Isoamyl alcohol step, and nucleic acids were precipitated using 0.6 volumes of isopropanol and incubating for 1 hr at room temperature. The DNA was pelleted by centrifugation at 10000 x *g* (4 °C for 10 min) and stored in TE buffer at -20 °C.

5.2.2.4. Commercial DNA extraction kits

The GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, USA), the FastDNA Spin Kit for Soil (MP Biomedicals, USA), the ZR Soil Microbe DNA MiniPrep (Zymo Research, USA), and the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) were also compared. These kits were used in accordance with the manufacturer's specifications, and no extra modifications were made.

5.2.3. PCR amplification

The extracted DNA from each sample was subsequently used to screen for the presence of anammox bacteria using nested PCR, as outlined by Tal *et al.* (2005). The primer set used for the first reaction was PLA46F and the universal primer 1492R. The second (nested) reaction used the anammox specific AMX368F and AMX820R, with the template being the amplicon product from the first reaction. Both reactions were run using the KAPA *Taq* 2x ReadyMix (KAPA Biosystems, USA), according to the reaction conditions outlined in Table 17, using the Bio-Rad T100 Thermal Cycler (Bio-Rad, USA). The reaction cycle parameters of the PLA46F/1492R set included an initial denaturation step of 4 min at 94 °C, followed by 40 cycles of amplification; each cycle consisted of denaturation at 94 °C for 45 s, primer annealing at 59 °C for 50 s, and primer extension at 72 °C for 3 min. The reaction cycle parameters of the AMX368F/AMX820R were largely the same, however the primer extension step was 72 °C for 1 min (Dale et al., 2009).

A negative control (using *E. coli* DNA as the template), a no template control (using water as the template) and a positive control (using enriched anammox bacterial culture DNA) were used.

Table 17: Nested PCR conditions

Primer Set	Primer Concentration	Template Concentration	Product Size
PLA46F/1492R	300pM	10 ng of gDNA	~1350bp
AMX368F/AMX820R	250pM	1 µL of PLA46F/1492R PCR product	~450bp

5.2.4. Nucleic acid and PCR product validation

Extracted nucleic acids and amplified PCR products were validated using agarose gel electrophoresis and spectrophotometry as described in 4.2.2. Genomic DNA was evaluated using a 1 % agarose gel and PCR products were evaluated using a 1.4 % agarose gel respectively.

5.2.5. Anammox enrichment from Activated Sludge

5.2.5.1. Selection of seeding sludge

A subset of the above WWTP samples were further enriched (as described in 5.2.5.2) to determine whether anammox bacteria may arise from the sample when appropriate conditions are provided. Activated sludge samples were collected from the Shallcross, Kingsburgh and Northern WWTPs and inoculated into individual reactor systems. These WWTP all treat domestic wastewater and were selected due to their proximity and accessibility.

Table 18: Rationale behind the WWTP selection

Sample source	Rationale
Kingsburgh WWTP	• Plant was recently recommissioned after a short period of shutdown and the constituent bacterial community is in the process of restabilising. • Aeration is only used intermittently thus possibly creating anoxic pockets within the aeration tank. • Good N removal despite low DO in the aeration zone may indicate SNAD activity.
Northern WWTP	• Anoxic reactor shows strong denitrification capacity, with no addition of organic carbon by operators, which could indicate autotrophic N removal.
Shallcross WWTP	• Intermittent aeration may create possible dead zones/anoxic niches • Complete N removal despite no defined anaerobic or anoxic tank

5.2.5.2. Enrichment reactor initialisation and operation

A series of enrichment reactors were established for the enrichment of anammox bacteria from selected activated sludge samples (Table 19). Although the reactors were each of different sizes and configurations, each of the reactors were initialised with identical

seeding ratios of 30 % (v/v) of the respective activated sludge mixed liquor (Figure 32). Additionally, these reactors were all fed using the same media (i.e.: van de Graafs media as described in 3.2.2).

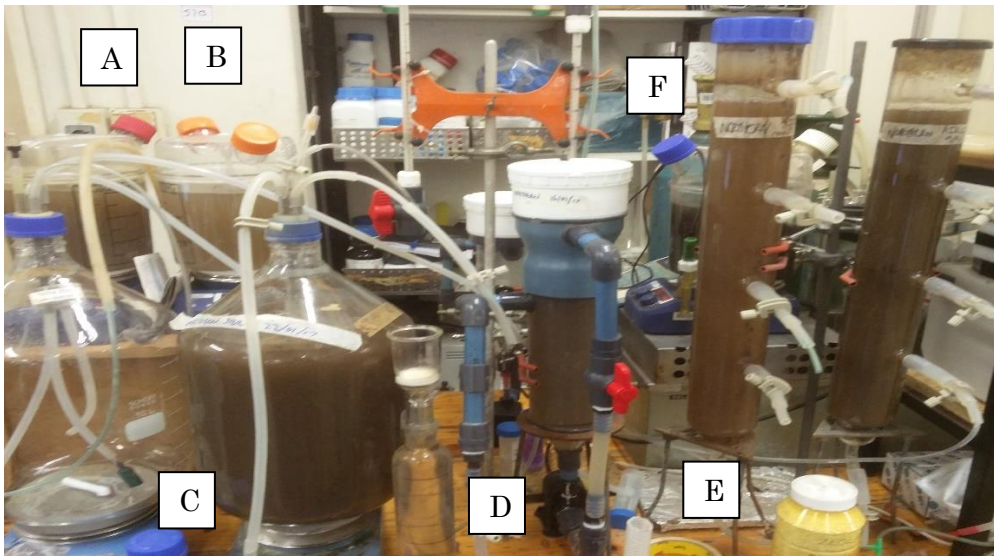

Figure 32: Multiple reactors were established for the enrichment of anammox bacteria from activated sludge. A) Kingsburgh Aeration 3 L SBR. B) Kingsburgh Anoxic 3 L SBR. C) Northern SBR. D) Northern UASB. E) Northern Gaslift. F) Shallcross MBBR

All reactors were operated at ambient temperature, with no active pH or DO control, and samples were taken every 4 days. The pH, DO, and temperature were measured prior to addition of new media.

Table 19: Summary of reactors established for anammox enrichment

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
	Airlift	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

In all instances, the 3 L SBR used was the Corning Disposable Spinner Flask (Corning, USA), and the MBBR utilised this same spinner flask with the addition of Kaldnes K1

filter media (AnoxKaldnes, Norway). The Northern anaerobic Airlift reactor utilized continuous sparging of Argon gas (2 L/min). The Northern 10 L SBR consisted of a glass Schott bottle (Schott, Germany) 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 and upflow was achieved with a Waterfall 700 L/h water pump (Figure 33).

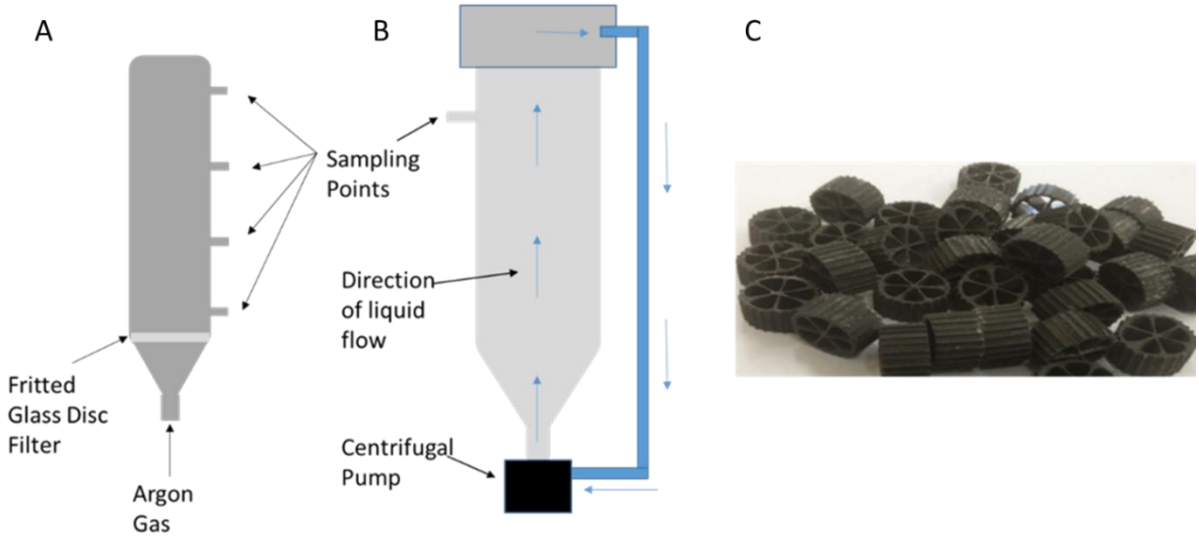

Figure 33: Reactor configuration for A) the Airlift reactor; B) the UASB reactor; and C) the Kaldnes K1 carrier beads used for the MBBR

5.2.5.3. Physicochemical analysis of the enrichment reactors

The pH, temperature and DO were analysed in all reactors using the YSI 556 MPS multiparameter instrument (Xylem, USA).

Samples were also analysed spectrophotometrically for NH_4^+–N, NO_2^-–N, NO_3–N, and PO_4^{2-} using the Gallery Autoanalyser (ThermoScientific, USA) as previously described in 3.2.4.

5.2.5.4. Molecular Analysis of the enrichment reactors

DNA extraction was performed and evaluated as explained in chapter 4, and a nested PCR amplification using the PLA46F/1492R and the AMX368F/AMX820R primer sets as described in 5.2.3.

5.3. Results and Discussion

5.3.1. Optimisation of nucleic acid extraction from wastewater sludge

Activated sludge represents a complex matrix from which to extract DNA for downstream applications (Yeates et al., 1998; Vanysacker et al., 2010). Nucleic acids extracted from activated sludge can often contain humic acids, organic matter and other phenolic impurities that could affect the detection limits of PCR, particularly with regard to the detection of low abundance genetic signatures within a sample, such as anammox bacterial DNA (Harms et al., 2003; Talbot et al., 2008). Furthermore, due to the highly variable chemical composition of wastewater sludge, analysis and extraction methods need to be optimized on a per-sample basis. By selecting a DNA extraction method that will yield a suitable amount of sufficiently high quality genomic DNA, the inherent biasness of the genomic DNA extraction procedure from a complex sample may be minimized.

Seven DNA extraction methods, including both modified manual methods and commercial kits, were applied to activated sludge obtained from the Kingsburgh WWTP (Figure 34). The Kingsburgh WWTP activated sludge sample was selected to approximate a typical wastewater sludge, as this plant treats solely domestic wastewater and has little variability in its influent composition. Despite the consistency of the wastewater sample, each extraction method utilised has shown distinctly different extraction efficiencies (Table 20).

The most efficient DNA extraction procedure would produces a crisp, well-defined single band of DNA visible near the top of the agarose gel, with minimal streaking down the gel as this is most suitable for downstream PCR analysis. Out of the extraction methods tested, only the Microwave extraction method, the FastDNA Soil Microbe extraction kit, and the PowerSoil DNA extraction kit respectively display a single band of DNA with minimal shearing (Figure 34). The other methods tested display a high degree of shearing of the DNA, possibly due to the harsh lysis methods employed in some of these extraction protocols.

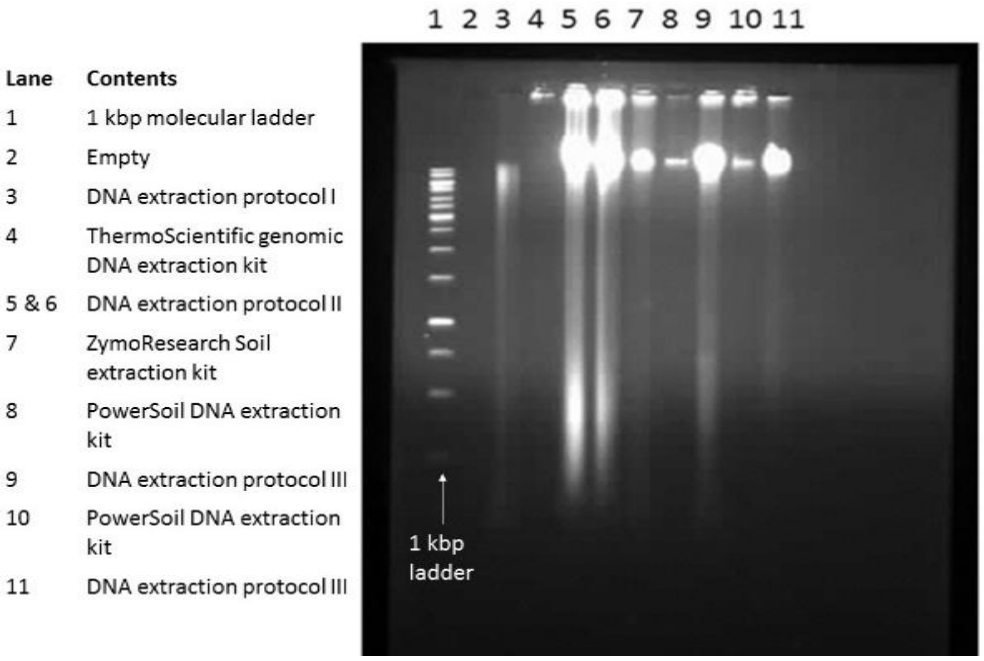

Figure 34: Electrophoretic comparison of the different DNA extraction methods.

In addition to agarose gel analysis, purity of extracted nucleic acids was determined by the sample absorbance (A) ratios at A260 nm/A280 nm and A260 nm/A230 nm. The ideal ratios for pure DNA is an A260/A280 of ~1.8 and an A260/A230 of ~2.0, and deviation from these figures can indicate possible protein, carbohydrate or phenol contamination (Matlock, 2012). DNA Extraction Protocol I displayed low yields and a low purity of 26.10 ng/µL and an A260/A280 of 1.50, while ThermoScientific kit and the ZymoResearch kit each displayed an extraction quantity of less than 10 ng/µL, and can be considered failed extractions (Table 20). DNA Extraction Protocol III displayed the highest nucleic acid yield out of all protocols tested, however it displayed A260/A280 than the PowerSoil DNA extraction kit, the FastDNA soil extraction kit and the microwave extraction methods (Table 20).

Table 20: Empirical comparison of DNA extraction quality (n = 2)

Extraction Protocol	**Quantity (ng/µL)**	**Purity**	
		260/280	**260/230**
DNA Extraction Protocol I	26.10 ± 6.37	1.50 ± 0.01	1.89 ± 0.02
DNA Extraction Protocol II	940.50 ± 16.4	1.86 ± 0.02	1.96 ± 0.02
DNA Extraction Protocol III	1165.10 ± 18.38	1.65 ± 0.01	1.60 ± 0.04
ThermoScientific Genomic DNA Extraction kit	7.65 ± 0.5	2.22 ± 0.06	1.31 ± 0.01
FastDNA Soil Microbe Extraction kit	318.85 ± 103.03	1.90 ± 0.01	1.06 ± 0.01
ZymoResearch Soil Extraction kit	4.7 ± 1.98	1.4 ± 0.02	0.65 ± 0.01
PowerSoil DNA Extraction kit	722.46 ± 9.31	1.92 ± 0.01	2.12 ± 0.01

The three best wastewater sludge extraction protocols, i.e. DNA Extraction Protocol II, the FastDNA soil microbe extraction kit and the PowerSoil DNA extraction kit were further applied to a reactor sample known to contain anammox bacteria, in order to validate the effectiveness of these extraction methods for sensitive anammox bacterial cells (Table 21 and Figure 35). All three methods tested produced good nucleic acid yields with a good purity, for the anammox-rich biomass, and thus all three methods could be suitable for PCR-based anammox bacterial detection. The PowerSoil DNA extraction kit was marginally better than the FastDNA Soil Microbe kit and DNA Extraction Protocol II as it produced a sufficiently high nucleic acid yield with the closest to the ideal purity ratios of ~1.8 and ~2.0 for the A260/A280 and A260/A230 ratios respectively. Consequently, the PowerSoil DNA extraction kit was chosen as the preferred DNA extraction method for anammox screening from environmental samples.

Table 21: Extraction efficiency on an enriched anammox sample (n = 2)

Extraction Protocol	**Quantity (ng/µL)**	**Purity**	
		260/280	**260/230**
DNA Extraction Protocol II	214.45 ± 23.26	1.74 ± 0.02	2.06 ± 0.01
FastDNA Soil Microbe kit	426.25 ± 48.86	1.91 ± 0.01	1.07 ± 0.01
PowerSoil DNA kit	421.8 ± 25.03	1.89 ± 0.01	2.01 ± 0.01

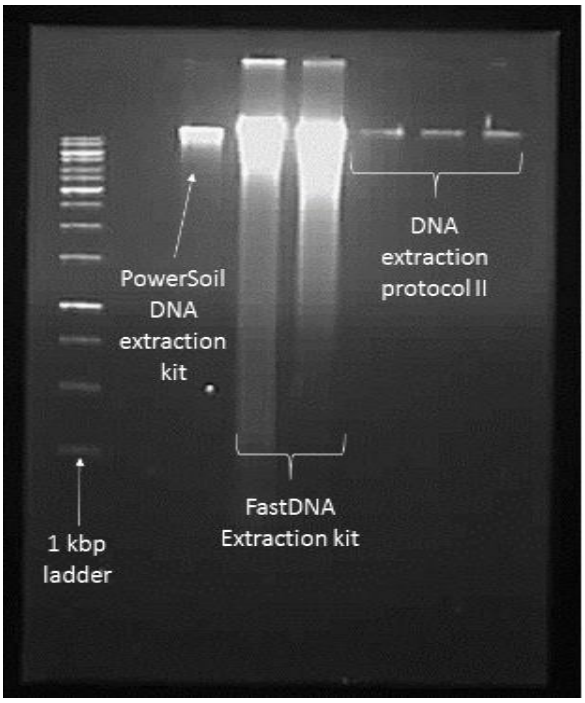

Figure 35: Validation of extraction methods against enriched anammox sample

5.3.2. Molecular screening for an indigenous anammox bacterial strain from local WWTPs

Anammox bacteria have been previously identified in a variety of environments, including the activated sludge of some WWTPs. In the South African context, anammox bacteria were only previously successfully detected within two locations; the trickling filters at the Daspoort WWTP (Tshwane, South Africa) and the anoxic tank at the Zeekoegat WWTP (Tshwane, South Africa) (Wilsenach et al., 2014; Tikilili & Chirwa, 2016). Both of these sites are similar in that they are limited in dissolved oxygen, however these WWTPs have very different process configurations and operational parameters. This implies that anammox bacteria may be also be found in other WWTPs of different process configurations that also possess oxygen minimal zones.

In this study, 11 wastewater treatment systems from across South Africa were selected on the basis of their process configuration, influent type and performance data (Table 16), and molecularly screened for the presence of anammox bacteria. The wastewater treatment systems included:

- the Zeekoegat WWTP from which anammox bacteria had previously been detected (Tikilili & Chirwa, 2016). This WWTP has granular sludge formation facilitated by bubble aeration and composite samples were taken from the aeration basin while the WWTP was operated in the 3-stage Phoredox configuration (which includes an anoxic zone).
- The Scottsdene I and the Phoenix WWTPs both utilise the MLE process, while the Kingsburgh WWTP, Scottsdene II WWTP, and Kraaifontein WWTP all operate with the UCT process. Both the MLE and UCT process types contain a defined anoxic zone containing minimal DO, and allows for internal recycling of oxidised N species, including NO_3 and NO_2^-.
- The Shallcross, Amanzimtoti and Mariannridge WWTPs which all operate with a Conventional Activated Sludge (CAS) process. Although the CAS systems do not include defined anaerobic or anoxic zones, the aeration tanks are operated with intermittent surface aeration, thus creating anoxic "pockets" or dead zones within the aeration tanks (Khanitchaidecha et al., 2015).

- The Northern WWTP was originally designed as a CAS process, however it has been modified to include a pseudo-anoxic zone. This plant has strong denitrification activity in its pseudo-anoxic zone despite no additional supplementation of organic C, and could thus have the potential to contain anammox bacteria.

Chemical analysis of the selected WWTPs indicated that they all achieve complete nitrogen removal, with minimal release of NH_4^+ and NO_3 in the final effluent despite the CAS plants not being designed for total N-removal. Additionally, a significant reduction in ammonia was observed in the anaerobic and anoxic tanks of the WWTPs utilising the UCT process, which was unexpected since conventional N removal requires oxygen for ammonia conversion. Furthermore, although anammox bacteria are strict anaerobes, they have been found to survive transient oxygen exposure in the presence of other aerobic organisms (Schmidt et al., 2002; Third et al., 2005; Joss et al., 2011). This was further corroborated by Bae et al. (2010), who also confirmed that anammox bacteria were present in many different types of wastewater sludge, including the sludge from aerobic processes. Since each of the screened WWTPs has an SRT of >24 hours, with sludge continuously cycling throughout the system, any anammox bacteria present within the sludge would find a niche within the centre of the sludge flocs and would thus remain trapped within the sludge flocs regardless of the sampling location (Bitton, 2005). Efficient cell lysis will break the floc and release the total DNA of all organisms present within, and subsequent PCR from the sludge at any point of the system should reveal the microbial composition of the sludge flocs. Table 22 displays the DNA extraction results using the optimized PowerSoil DNA extraction kit, and this DNA was used in downstream PCR analysis.

Table 22: The quality of DNA extracts from each wastewater treatment system selected for screening (n = 2)

WWTP	Sample Location	DNA Concentration (ng/μL)	DNA Purity	
			OD 260/280	OD 260/230
Scottsdene (Process A)	Aeration Tank	598.01 ± 95.60	1.9 ± 0.01	2.07 ± 0.08
Scottsdene (Process B)	Aeration Tank	1221.5 ± 210.29	1.86 ± 0.01	1.96 ± 0.04
Kraaifontein	Aeration Tank	944.65 ± 7.00	1.91 ± 0.01	2.06 ± 0.01
Zeekoegat (Process A)	Aeration Tank	1095.00 ± 28.57	1.93 ± 0.01	1.92 ± 0.03
Zeekoegat (Process B)	Aeration Tank	1043.35 ± 20.57	1.92 ± 0.01	2.02 ± 0.06
Phoenix	Aeration Tank	851.80 ± 28.71	1.81 ± 0.02	1.91 ± 0.11
Kingsburgh	Anoxic Tank	1104.95 ± 70.49	1.87 ± 0.01	1.86 ± 0.01
Northern	Anoxic Tank	181.41 ± 12.26	1.92 ± 0.03	2.21 ± 0.02
Shallcross	Aeration Tank	614.92 ± 18.89	1.82 ± 0.01	1.94 ± 0.01
Mariannridge	Aeration Tank	174.90 ± 10.47	1.96 ± 0.01	2.14 ± 0.02
Amanzimtoti	Aeration Tank	84.43 ± 22.73	1.65 ± 0.05	1.77 ± 0.06

Detection of anammox bacteria had previously been attempted using the fluorescence *in situ* hybridization (FISH) method, however this method is more suitable to visualise anammox bacteria in already enriched cultures, and is difficult to use in complex environmental samples containing low concentrations of the target rRNA molecules (Third et al., 2005; Tsushima et al., 2007a; Kindaichi et al., 2007). A 16S rRNA or functional gene amplification-based approach enables the detection of anammox organisms down to the genus level faster and with a higher sensitivity than the FISH method (Schmid et al., 2005).

A nested PCR reaction that first amplifies the region denoting all Planctomycetes members, and subsequently using this amplified product to amplify all target anammox bacteria within this region, can increase the sensitivity and specificity of the conventional PCR reaction (Kalland, 2009). Despite the increased sensitivity of the nested PCR reaction, all 11 plants screened for anammox bacteria showed a negative result to the presence of both the Planctomycetes phylum, and any anammox bacteria. Only the positive control showed presence of both the Planctomycetes phylum, as well as positive

presence of anammox bacteria. While this does not conclusively prove that anammox does not exist in any of the 11 systems screened, it may be that if there is anammox bacteria within these systems, they occur at concentrations well below the detection limits of the performed PCR.

This negative result is in contrast to the conclusion made by Bae et al. (2010), who stated that anammox bacteria is common in activated sludge, and is more aligned to the findings by Humbert et al. (2010), who did not detect anammox bacteria ubiquitously. Humbert et al. (2010) screened a total of 112 samples collected at 9 different geographical locations, and 82 displayed a positive result for Planctomycetales, and 60 of those tested positive for anammox bacteria (Humbert et al., 2010). Tal et al. (2005), observed that the AMX820 primer did not detect *Scalindua brodae* and *Scalindua wagneri* anammox species. This could be due to the large genetic diversity at the 16S rRNA level, and although the *Scalindua* spp. are predominantly marine anammox bacterial species, similar observations were made by Hirsch et al. (2011); Harhangi et al. (2012); and Han et al. (2013) with respect to some freshwater anammox bacterial species.

Most notably, the Zeekoegat WWTP, in which anammox bacteria had been previously detected, also displayed a negative result for the presence of anammox bacteria. According to Tikilili and Chirwa (2016), their positive amplification results were not obtained directly from environmental screening of the Zeekoegat WWTP sludge, but only after a period of 120 days of blind enrichment in an SBR. A similar trend was noticed by Tsushima et al. (2007); Bae et al. (2010); and Sri Shalini and Joseph, (2013), who also only successfully detected anammox bacteria after a period of enrichment. Sànchez-Melsió et al. (2009) further noticed that successful results with the 16S rRNA-targeting primer sets were only obtained in DNA extracts from advanced anammox bacterial enrichment stages, and that these primer sets regularly failed to detect anammox bacteria in low-concentrated samples.

These findings imply that anammox bacteria may be present in many environments, albeit at extremely low quantities- often below the detection limits of a nested PCR, and that short term enrichment studies are required to increase the template DNA concentration to detectable levels. In order to determine if anammox bacteria is indeed present below detectable levels in the activated sludge samples tested, a further anammox enrichment attempt was performed on a subset of the WWTPs screened above (Table 18).

5.3.3. Enrichment of anammox bacteria from Activated Sludge

Activated sludge from the Shallcross, Northern and Kingsburgh WWTPs were inoculated into various types of enrichment reactors (Table 19). These reactor configurations, i.e. the SBR, MBBR, UASB, and Gaslift reactors respectively, have previously proven to be effective at anammox enrichment (Jin et al., 2008a; Dapena-Mora et al., 2004a; Thuan et al., 2004; Tao et al., 2012). Each reactor was operated for a period of ~100 days under equivalent HRT and NLR conditions (Table 19), and the N-removal performance of each reactor was monitored for NH_4^+–N, NO_2^-–N and NO_3–N (Figure 36, Figure 37 and Figure 38 respectively).

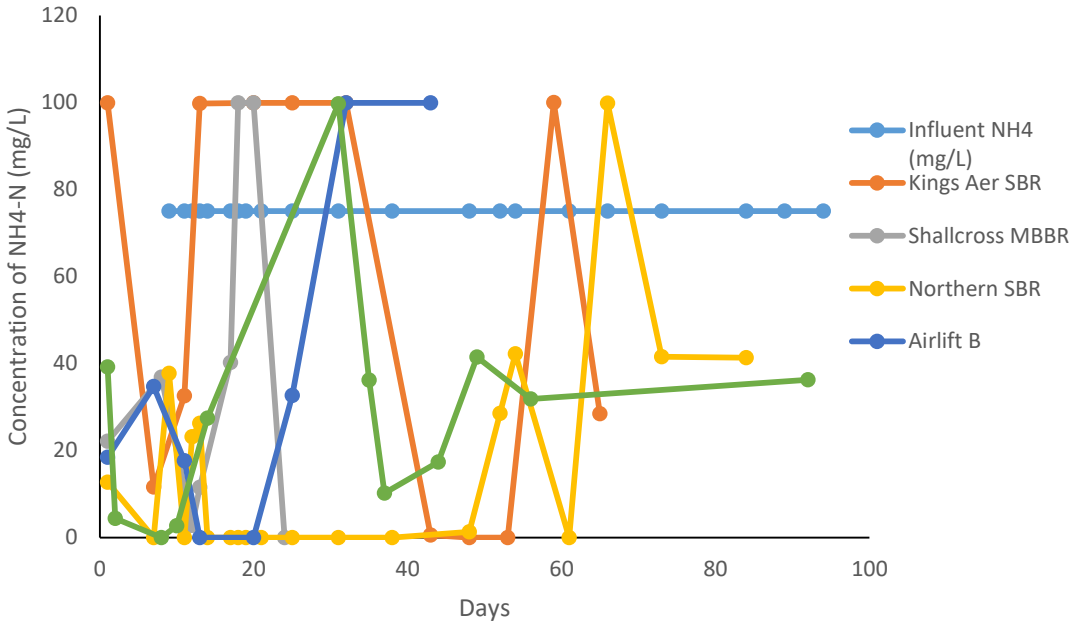

Figure 36: NH4-N change within each of the enrichment reactors

The NH_4^+–N removal values do not show any stability for the reactors sampled. 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 38, which indicates that the NO_3–N produced by all 4 reactor systems displays the same trend of a sharp increase, followed by a rapid decrease 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 fluctuated greatly, and accumulated within the system due an excessive NLR when the overall metabolism of the

system is suppressed due to DO ingress, pH imbalance or temperature changes. Interestingly, this phenomenon was not observed in the mass cultivation study using the already acclimatized anammox-nitrifying consortia which was maintained at a more dynamically controlled NLR (Figure 13; Chapter 3).

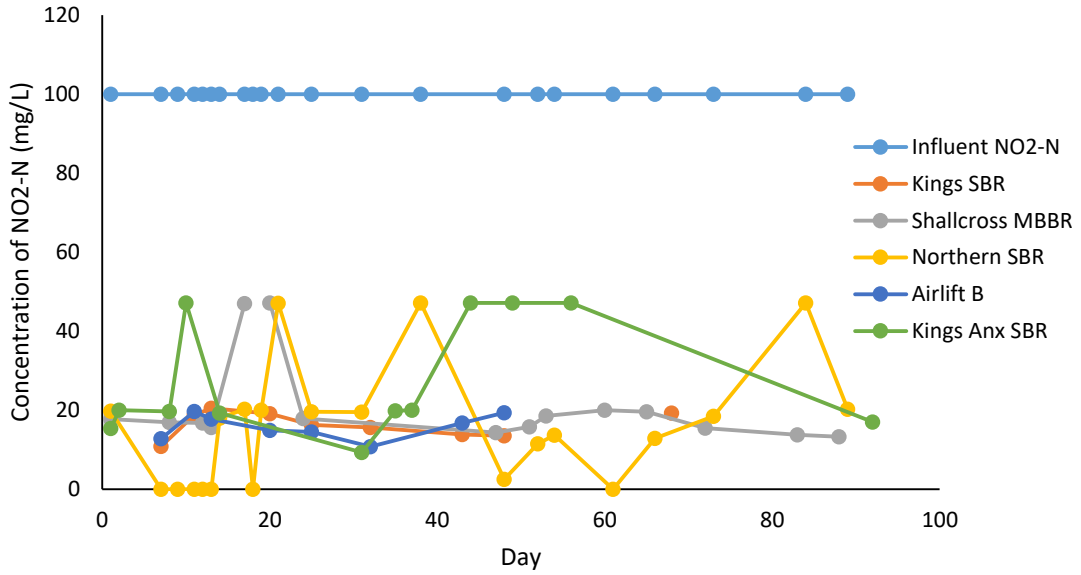

Figure 37: Change in NO_2^-–N for each enrichment reactor

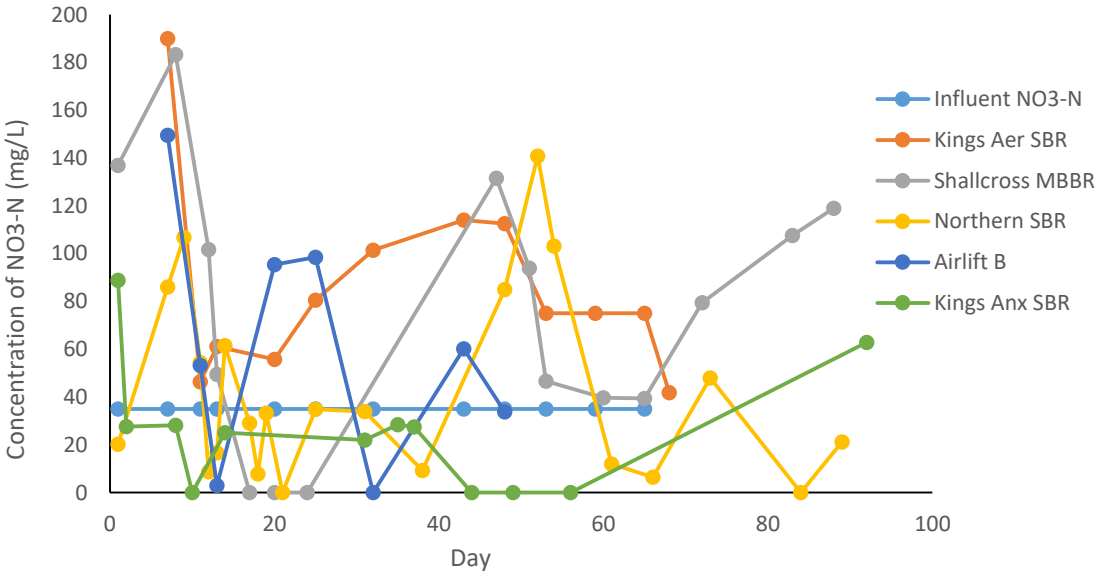

Figure 38: Change in NO_3-N for each enrichment reactor

The pH values of all the reactors displayed a consistent increase above the average influent pH of 7.10 ± 0.10. The largest pH increase occurred within the Airlift and UASB reactors. In the Airlift reactor, the constant argon gas sparging resulted in ammonia and carbonate stripping from the system. These volatiles contained the H^+ ions necessary for

maintain the lower pH, and buffer any change in pH of the system. Conversely, the lowest pH was observed in the Kingsburgh Aerobic SBR at Day 65, where the pH rapidly decreased to a pH of 4.1. This is in the acidic range and is not conducive to N-removal processes or the proliferation of N-removing bacterial populations. The sharp decrease in pH could be due to the metabolic actions of the organisms within the system, since complete ammonia removal is known to decrease pH.

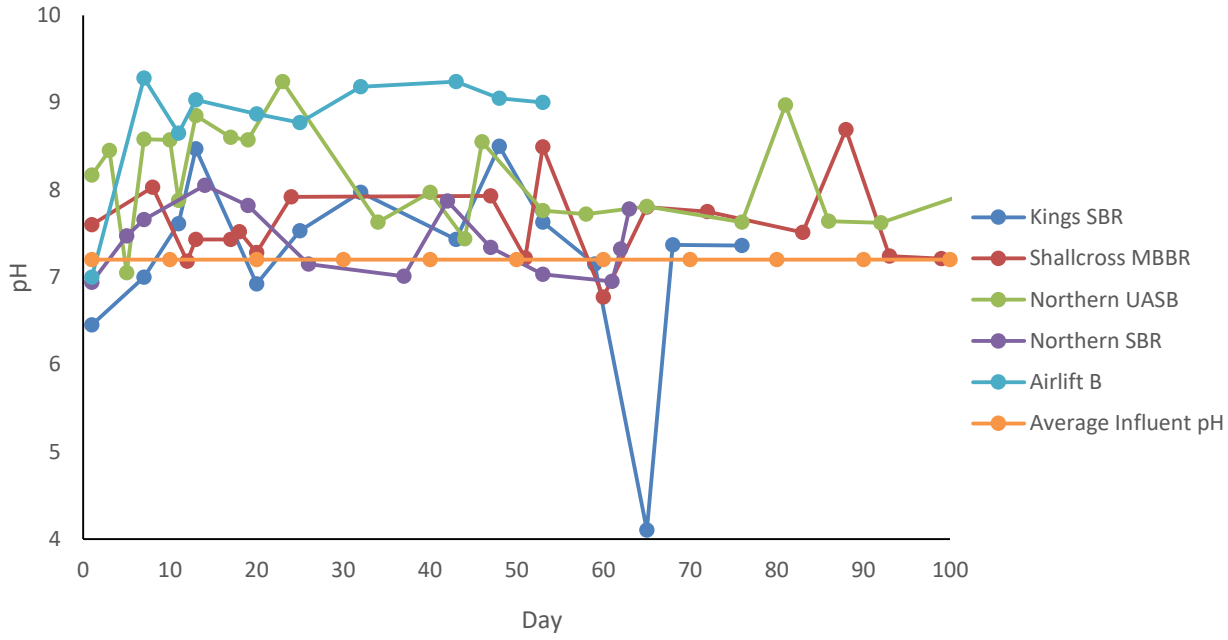

Figure 39: pH of the different enrichment reactors

The chemical results for any of the reactors do not display the characteristic NH_4^+–N, NO_2^-–N utilisation and NO_3–N production that are typical of enriched anammox cultures, or the acclimatized N-removing consortia as observed in the previous SBR (Chapter 3). Previous enrichment of anammox bacteria from activated sludge systems have 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^- at 1:1.3. In a mixed microbial consortia 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, and may overestimate the contribution of anammox in the environment. 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 40 displays a single positive result in screening for anammox bacteria using nested PCR (described in 5.2.3).

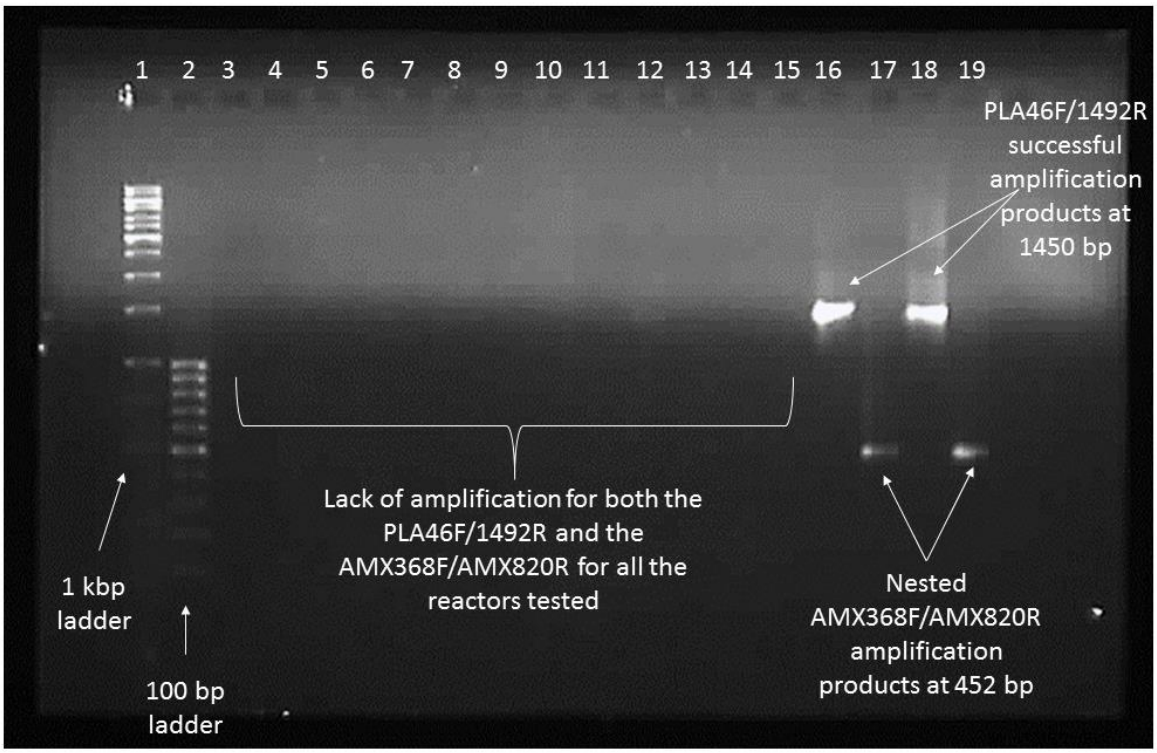

Figure 40: Screening of reactor samples using nested PCR with the Planctomycetes family level primer set (Pla46F/1492R) and nested PCR with anammox specific primer sets (AMX368F/AMX820R). Lane 1 contains the 1 kbp gene ladder, Lane 2 contains the 100 bp gene ladder, Lanes 3-15 show no amplification for either primer set for each of the reactors tested. Lane 16 shows positive amplification for both the Planctomycetes and nested Anammox primer sets for the Kings Aeration SBR. Lane 18 and 19 show the positive controls for the Planctomycetes family level primer set (Pla46F/1492R) and the AMX368F/AMX820R primer set respectively.

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 was positive for anammox bacteria at detectable levels. 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. It is possible that the low DO of the aeration tank at the time of sampling created sufficient nitrite for anammox bacteria to proliferate within the aeration tank without major DO inhibition. 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, 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 is present in detectable quantities, there are other factors at the source inoculum which may affect anammox enrichment at the bench scale.

5.4. Conclusion

Successful enrichment of anammox bacteria from local South African environments represent a major leap towards establishing both local anammox research and the development of anammox-based technology for wastewater treatment. Previous screening attempts across South African wastewater treatment plants may have met with limited success due to the target organism being present below detectable levels. According to the results of this study, anammox bacteria may be found in an environment which exhibit selection factors that promote its growth, however a pre-enrichment must be performed to increase the quantity of this anammox bacteria to detectable levels.

The length of this pre-enrichment will depend on the starting quantities of anammox bacteria within the system, however in contrast to similar research which only detected anammox bacteria after ~120 days of enrichment, anammox bacteria in this enrichment study was detectable after only ~65 days. This is due to the other research waiting for the anammox reaction to make a significant contribution to N removal within the reactors, and approach the NH_4^+: NO_2^- ratio of 1:1.3, before applying sensitive molecular detection techniques. Applying nested PCR to an enrichment sample at ~60 days, despite the reactors not reaching N-removal stability, is sufficient to determine if detectable levels of anammox are present within that environmental sample.

Chapter 6: Final Conclusions and Recommendations

6.1. Final Conclusions

Reactor operational data is commonly used to understand the reaction process at a macro-scale, and the kinetic models generated from these datasets can provide useful insights into optimizing the process performance (Ni et al., 2012). Comparing the reactor kinetics in different kinds of anammox reactors can provide a rational basis for the assessment, control, and enhancement of the anammox reaction (Niu et al., 2016). Furthermore, since DNA based techniques are only able to detect the presence of the target organism, but not discriminate between active, inactive or dead cells, kinetic characterization can provide critical information on the health of the culture (Gilbride et al., 2006).

For anammox enrichment systems the N load in the influent versus the effluent can act as both an important indicator of treatment efficiency of the system itself, and can be used as an indirect measure of the metabolic activity of the constituent microbial populations. Nevertheless, it should be noted that enrichment reactors often contain a mixed microbial population, and as described in Chapter 3 and Chapter 4, these population densities change with changes in reactor operation. The selection pressures imposed by the reactor operational conditions will serve to shape the microbial composition and relative quantities of the populations therein, creating a microbial consortia that will have a similar metabolism and be competing for similar substrates. Relying on the N removal results alone and attributing this to one specific population alone, in a consortia where many members have the capacity to use the same substrates is insufficient. The presence of an undefined side population and the occurrence of inactive or dormant cells at a longer SRT complicate the reaction kinetics of the target population and may result in overestimation of the anammox activity (Lotti et al., 2014). This is particularly problematic during the initial stages of enrichment or mass cultivation when the seeding amount of the target population is low.

The screening of natural environments is routinely applied to isolate organisms of biotechnological value. The anammox bacteria are one such group. Their ability to circumvent the conventional nitrification and denitrification pathways have made its

application to low C/N wastewater remediation extremely attractive. Successfully detecting anammox bacteria within local WWTP could have a three-fold impact:

- The positive detection of anammox bacteria in local WWTP could reveal that the anammox process also acts alongside the currently accepted conventional nitrogen removal mechanisms within these systems. This implies that the nitrogen removal models within these WWTP need to be updated, and could potentially have far-reaching economic implications in the way that that specific WWTP is operated.
- To date, only 6 anammox genera have been elucidated, with 12 recognised species. Since anammox bacteria is such a deeply branched group within the Planctomycetes, it is possible that some anammox species have yet to be elucidated.
- Most importantly, discovery of an indigenous anammox reservoir will provide an easily accessible source of anammox bacterial seed culture, thus allowing for rapid mass culturing of anammox rich biomass for large scale local biotechnological application.

This study was successful at detecting anammox bacteria in a local environment. Comparisons between Chapter 3 and Chapter 5 indicate that the microbial consortia transplanted from an activated sludge system into a low C/N, bench scale reactor system is vastly different from that of seeding sludge obtained from an anammox enrichment reactor. Enrichment from activated sludge and mass cultivation from an acclimatized seed inoculum are indeed different, and require different NLR and operational controls. Utilising the Kingsburgh WWTP activated sludge sample for anammox enrichment would allow for a significantly shorter enrichment time when compared to similar enrichments from other activated sludge. The amount of Anammox bacteria present in the seeding sludge is widely believed to be significant in shortening the required enrichment time (Wang et al., 2011; Ding et al., 2017).

6.2. Observations and Recommendations for future work

- The best type of reactor used for anammox enrichment has been debated in previous studies, however a full parallel comparison has not yet been performed for anammox enrichment. This study had success using SBR systems, however the data obtained from the other reactors was not suitable

to make a valid comparison. Identifying the best reactor type for anammox enrichment from activated sludge still has research value.

- Reactor operational control still represent major critical factors in selectively enriching for specific microbial populations, especially the anammox bacteria from a mixed N-removing consortia. Finding the optimum operation in relation to the both relative microbial population distribution, their kinetics, and nutrient utilisation rates would be more efficient than the current methods of operating a system based on nutrient transformation alone. Future work could incorporate population kinetics and ecosystem models to more efficiently select for specific microbial populations.
- Initial stages of the enrichment displayed a strong tendency of the sludge to form a biofilm on the sidewalls of the reactor. Since anammox and the other N-removing microbial populations exist in such an intimate synergism, and often cannot be grown in the absence of each other, it is possible that some form of intercellular communication occurs to drive this biofilm formation. To date, only genetic evidence points to quorum sensing within these consortia, and practical application of his has yet to be investigated.

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Appendix A: Preparation of Gallery Auto-analyser reagents

Ammonia High Range (10-100 mg/L) Test

Reagent 1:

- 65 g Sodium salicylate + 65 g Trisodium citrate into 400 mL of ddH_2O.
- Lower the pH to < 8.0 with 0.4 % HNO_3
- Add 0.49 g Na-nitroprusside
- Make up to a final volume of 500 mL with ddH_2O

Reagent 2:

- 16 g NaOH in 250 mL ddH_2O
- Add 1 g Na-dichloroisocyanurate
- Make up to 500 mL with ddH_2O

Ammonia High Range Standard (1000 mg/L)

- 3.819 g NH_4Cl in 1000 mL ddH_2O

Ammonia Low Range Reagent (1-10 mg/L) Test

Reagent 1:

- 13 g Sodium salicylate + 13 g tri-sodium citrate in 80 mL ddH_2O and lower the pH to < 8.0 with 0.4 % HNO_3
- Add 98 mg of sodium nitroprusside
- Make up solution to 100 mL.

Reagent 2:

- 3.2 g NaOH in 50 ml ddH_2O
- Add 0.2 g Na-dichloroisocyanurate and make up to 100 mL

Ammonia Low Range Standard:

- 38.16 mg NH_4Cl in 100 mL.

Total Oxidised Nitrogen Test

Reagent 1:

- 0.8 g NaOH in 100 ml ddH_2O

Reagent 2:

- Dissolve 65 mg Hydrazine sulfate in 80 mL ddH_2O.
- Add 0.15 mL Copper sulfate solution (390 mg CuSO4.5H2O in 100 mL H_2O)
- Add 1 mL Zinc sulfate solution (450 mg ZnSO4.H_2O in 100mL)
- Make up to 100 mL

Reagent 3:

- Dissolve 5 mL Phosphoric acid, 0.5 g Sulphanilamide, and 25 mg N-naphthylethylenediamine-HCl
- Dilute to 100 mL with ddH_2O.
- Stored in a dark bottle.

Total Oxidised Nitrogen Standard

- 72.17 mg KNO_3 in 100 mL of ddH_2O

Nitrite-N Test

Reagent:

- Dissolve 5 mL Phosphoric acid, 0.5 g Sulphanilamide, and 25 mg N-naphthylethylenediamine-HCl

- Dilute to 100 mL with ddH_2O.
- Stored in a dark bottle.

Nitrite Standard

- Dissolve 49.25 mg in 100 mL

Appendix B: Detailed PCR cycling times and temperatures

Nitrobacter (FGPS)

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	92C	1 min	
Annealing	50C	1 min	40
Extension	72C	1 min	
Final Extension	72C	5 min	1

Ammonia-oxidase functional gene (amoA

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	2 min	1
Denaturation	95C	30 sec	
Annealing	58C	1 min	35
Extension	72C	1 min	
Final Extension	72C	5 min	1

CTO

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	3 min	1
Denaturation	95C	1 min	
Annealing	57C	1 min	35
Extension	72C	1 min	
Final Extension	72C	10 min	1

Nitrospira (NSR)

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	5 min	1
Denaturation	94C	30 sec	
Annealing	65C	30 sec	40
Extension	72C	30 sec	
Final Extension	72C	15 min	1

All Planctomycetes (Pla46F/1492R)

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	94C	1 min	
Annealing	50C	1 min	30
Extension	72C	1:10 min	
Final Extension	72C	4 min	1

All Anammox bacteria (AMX694F/AMX960R)

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	5 min	1
Denaturation	95C	45 sec	
Annealing	58C	50 sec	35
Extension	72C	1.22 min	
Final Extension	72C	5 min	1

All Eubacteria (Univ338F/Univ518R)

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	5 min	1
Denaturation	94C	1 min	
Annealing	55C	1 min	40
Extension	72C	1 min	
Final Extension	72C	10 min	1

Anammox species- Jettenia and Kuenenia (AMX368F/AMX820R)

Stage	Temperature	Time	Cycles
Initial Denaturation	95C	2 min	1
Denaturation	95C	45 sec	
Annealing	62C	50 sec	35
Extension	72C	1:22 min	
Final Extension	72C	5 min	1

All Eubacteria (Univ27F/Univ1492R)

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	94C	30 sec	
Annealing	57C	45 min	31
Extension	72C	1:30 min	
Final Extension	72C	7 min	1

HZO1

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	94C	1 min	
Annealing	52.5C	1 min	35
Extension	72C	1:30 min	
Final Extension	72C	10 min	1

HZO 4 AB

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	94C	1 min	
Annealing	50-65C	1 min	30
Extension	72C	1:30 min	
Final Extension	72C	10 min	1

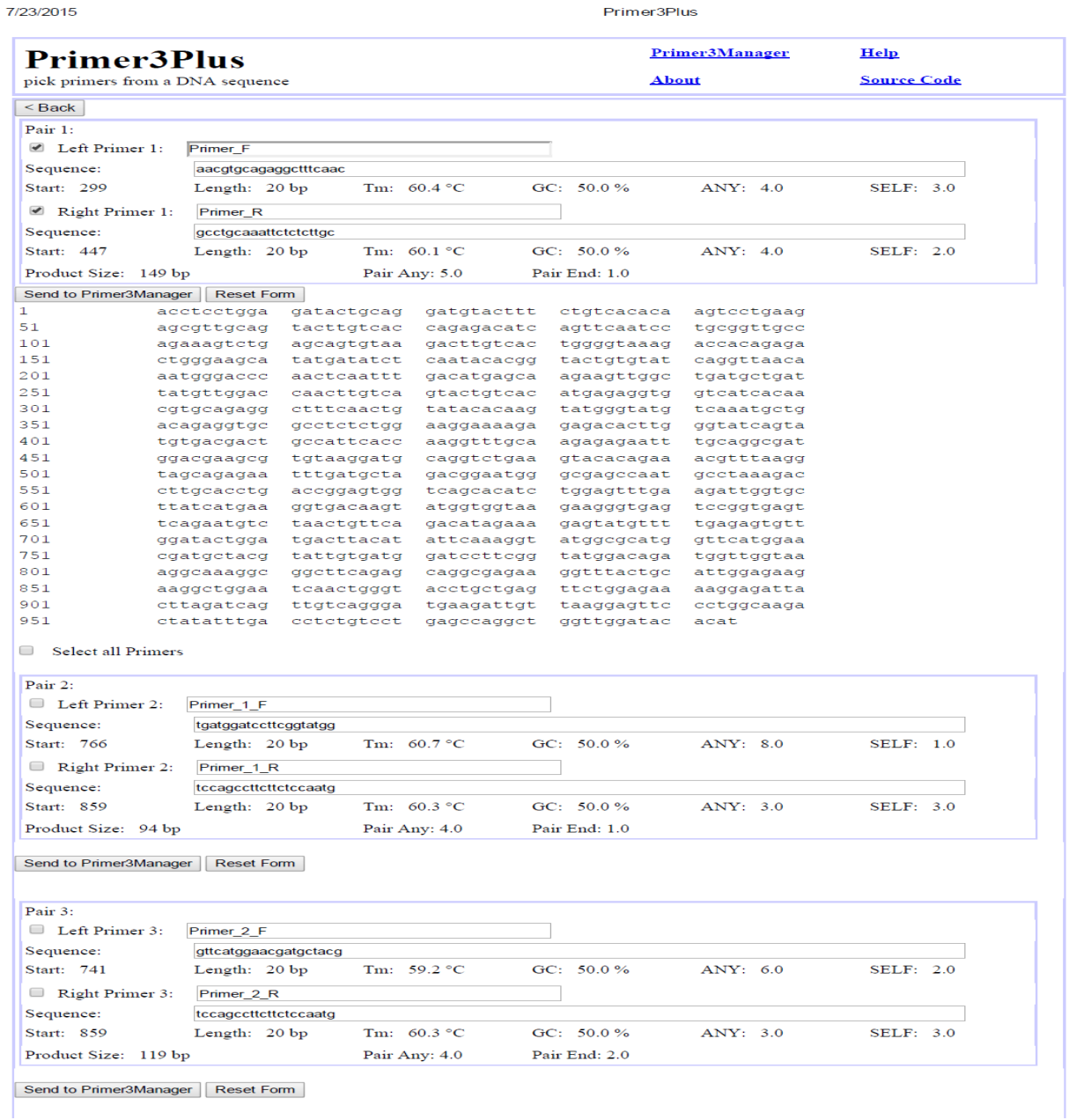
HZS

Stage	Temperature	Time	Cycles
Initial Denaturation	96C	5 min	1
Denaturation	96C	1 min	
Annealing	50C	1 min	40
Extension	72C	1 min	
Final Extension	72C	7 min	1

Stage	Temperature	Time	Cycles
Initial Denaturation	94C	5 min	1
Denaturation	92C	1 min	
Annealing	50C	1 min	40
Extension	72C	1 min	
Final Extension	72C	5 min	1

Appendix C: Primer Design Software Screenshots

HZO primer set:



7/23/2015 Primer3Plus

Primer3Plus
pick primers from a DNA sequence

Primer3Manager | Help
About | Source Code

< Back

Pair 1:
Left Primer 1: Primer_F
Sequence: aacgtgcagaggctttcaac
Start: 299 Length: 20 bp Tm: 60.4 °C GC: 50.0 % ANY: 4.0 SELF: 3.0
Right Primer 1: Primer_R
Sequence: gcctgcaaattctctcttgc
Start: 447 Length: 20 bp Tm: 60.1 °C GC: 50.0 % ANY: 4.0 SELF: 2.0
Product Size: 149 bp Pair Any: 5.0 Pair End: 1.0

Send to Primer3Manager | Reset Form

```
1     acctcctgga  gatactgcag  gatgtacttt  ctgtcacaca  agtcctgaag
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101   agaaagtctg  agcagtgtaa  gacttgtcac  tggggtaaag  accacagaga
151   ctgggaagca  tatgatatct  caatacacgg  tactgtgtat  caggttaaca
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701   ggatactgga  tgacttacat  attcaaaggt  atggcgcatg  gttcatggaa
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951   ctatatttga  cctctgtcct  gagccaggct  ggttggatac  acat
```

Select all Primers

Pair 2:
Left Primer 2: Primer_1_F
Sequence: tgatggatccttcggtatgg
Start: 766 Length: 20 bp Tm: 60.7 °C GC: 50.0 % ANY: 8.0 SELF: 1.0
Right Primer 2: Primer_1_R
Sequence: tccagccttcttctccaatg
Start: 859 Length: 20 bp Tm: 60.3 °C GC: 50.0 % ANY: 3.0 SELF: 3.0
Product Size: 94 bp Pair Any: 4.0 Pair End: 1.0

Send to Primer3Manager | Reset Form

Pair 3:
Left Primer 3: Primer_2_F
Sequence: gttcatggaacgatgctacg
Start: 741 Length: 20 bp Tm: 59.2 °C GC: 50.0 % ANY: 6.0 SELF: 2.0
Right Primer 3: Primer_2_R
Sequence: tccagccttcttctccaatg
Start: 859 Length: 20 bp Tm: 60.3 °C GC: 50.0 % ANY: 3.0 SELF: 3.0
Product Size: 119 bp Pair Any: 4.0 Pair End: 2.0

Send to Primer3Manager | Reset Form

Pair 5:

Left Primer 5: Primer_4_F

Sequence: tggagaagaaggctggaatc

Start: 843 Length: 20 bp Tm: 59.4 °C GC: 50.0 % ANY: 3.0 SELF: 3.0

Right Primer 5: Primer_4_R

Sequence: ttgccagggaactccttaac

Start: 948 Length: 20 bp Tm: 59.2 °C GC: 50.0 % ANY: 5.0 SELF: 2.0

Product Size: 106 bp Pair Any: 4.0 Pair End: 0.0

Send to Primer3Manager | Reset Form

Statistics:	
Left Primer:	considered 2952, GC content failed 57, GC clamp failed 500, low tm 1407, high tm 535, high end compl 2,high 3' stability 53, ok 398
Right Primer:	considered 2945, GC content failed 56, GC clamp failed 501, low tm 1405, high tm 520, high end compl 4,high 3' stability 53, ok 406
Primer Pair:	considered 361, unacceptable product size 345, high end compl 4, ok 12

More about Primer3Plus...

Primer-BLAST Primer-Blast results

NCBI/ Primer-BLAST : results: Job id=9O8G_Pv2UtJt_2nxCN9bjxPyaZ4A7XSb more...

Input PCR template

none

Specificity of primers

Target templates were found in selected database: Nucleotide collection (nt)

Other reports

Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACGTGCAGAGGCTTTCAAC	20	59.34	50.00	4.00	3.00
Reverse primer	GCCTGCAAATTCTCTCTTGC	20	57.73	50.00	4.00	2.00

Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

>KF192664.1 Uncultured anaerobic ammonium-oxidizing bacterium clone M31_Dec09_11C hydrazine oxidase (hzo) gene, partial cds

```
product length = 149
Forward primer  1    AACGTGCAGAGGCTTTCAAC  20
Template        169  ....................  188

Reverse primer  1    GCCTGCAAATTCTCTCTTGC  20
Template        317  ....................  298
```

>KF192663.1 Uncultured anaerobic ammonium-oxidizing bacterium clone M31_Dec09_11B hydrazine oxidase (hzo) gene, partial cds

```
product length = 149
Forward primer  1    AACGTGCAGAGGCTTTCAAC  20
Template        169  ....................  188

Reverse primer  1    GCCTGCAAATTCTCTCTTGC  20
Template        317  ....................  298
```

>KF192662.1 Uncultured anaerobic ammonium-oxidizing bacterium clone M31_Dec09_10A hydrazine oxidase (hzo) gene, partial cds

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - V1K2FNY301R**
Formatting options
Download
Blast report description

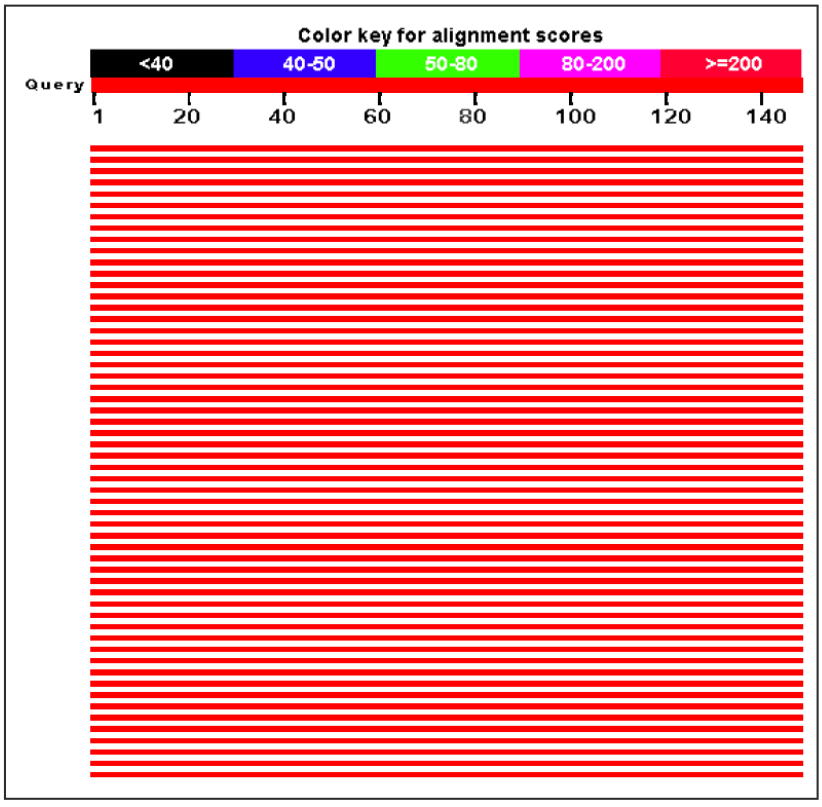
Nucleotide Sequence (149 letters)

RID V1K2FNY301R (Expires on 07-24 19:13 pm)

Query ID	lcl|Query_202669	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.32+
Query Length	149		

Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence



HZS Primer set:

Display Settings: GenBank Send:

Uncultured anaerobic ammonium-oxidizing bacterium clone BS23 hydrazine synthase subunit A (hzsA) gene, partial cds

GenBank: JN703723.1

FASTA Graphics

Go to:

```
LOCUS       JN703723               1284 bp    DNA     linear   ENV 02-FEB-2012
DEFINITION  Uncultured anaerobic ammonium-oxidizing bacterium clone BS23
            hydrazine synthase subunit A (hzsA) gene, partial cds.
ACCESSION   JN703723
VERSION     JN703723.1  GI:364505664
KEYWORDS    ENV.
SOURCE      uncultured anaerobic ammonium-oxidizing bacterium
  ORGANISM  uncultured anaerobic ammonium-oxidizing bacterium
            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1284)
  AUTHORS   Harhangi,H.R., Le Roy,M., van Alen,T., Hu,B.L., Groen,J.,
            Kartal,B., Tringe,S.G., Quan,Z.X., Jetten,M.S. and Op den Camp,H.J.
  TITLE     Hydrazine synthase, a unique phylomarker with which to study the
            presence and biodiversity of anammox bacteria
  JOURNAL   Appl. Environ. Microbiol. 78 (3), 752-758 (2012)
   PUBMED   22138989
REFERENCE   2  (bases 1 to 1284)
  AUTHORS   Harhangi,H.R., Le Roy,M., van Alen,T., Hu,B., Kartal,B.,
            Tringe,S.G., Quan,Z.-X., Jetten,M.S.M. and Op den Camp,H.J.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-SEP-2011) Department of Microbiology, IWWR, Radboud
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            NL-6525 AJ, The Netherlands
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ORIGIN
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       61 tcaatagatg aatttggaag accattcgct tttgacttat acaggcttga tccgcagggt
      121 ggaaagtcaa tggatcgtat ctgcggtcac ttattagtag gtatcgatat gcctaattgt
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      421 tacctgtatt acattgaagc tttggacagt aattctggta taggtaatct tgccagggtt
      481 agctggacta ctcctcatgc taagacgcaa tccaggttga gcaatgatgg cagactctac
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      601 tttggtatct actttttctg cgcggataag ggtacggttt ctgagttagt ttatgatgat
      661 ccagagtgga atgatcatca gcctcagcct gtttatccac gttacaaacc aagatggata
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```

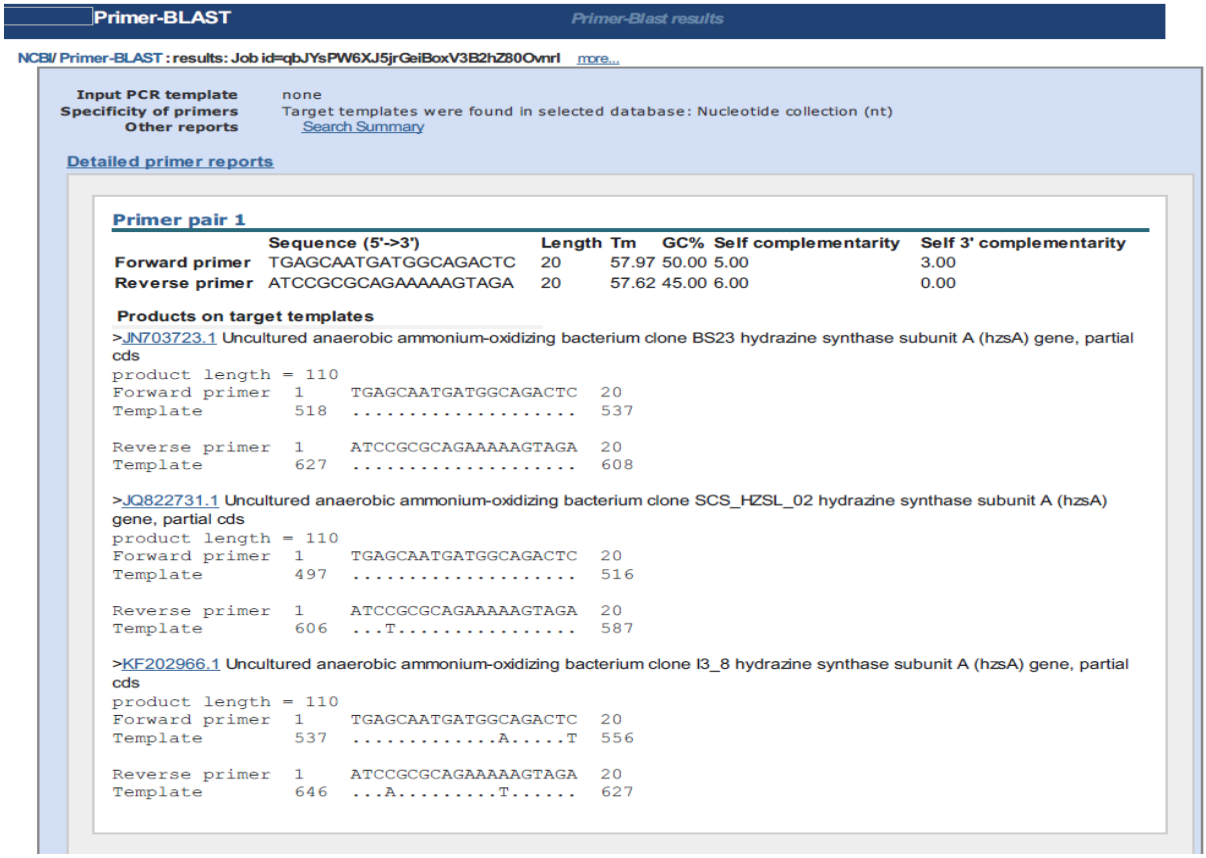
Change region shown

Customize view

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence

Related information
Full text in PMC
Protein
PubMed
Taxonomy

Recent activity
Turn Off Clear
Uncultured anaerobic ammonium-oxidizing bacterium clone I Nucleotide
Candidatus Brocadia sinica JPN1 DNA, contig: brosiA, whole Nucleotide
hydrazine synthase (749) Nucleotide
hzs (18) Nucleotide
Uncultured anaerobic ammonium-oxidizing bacterium clone Nucleotide
See more...



Primer-BLAST — Primer-Blast results

NCBI/ Primer-BLAST : results: Job id=qbJYsPW6XJ5jrGeiBoxV3B2hZ80Ovnrl more...

Input PCR template none
Specificity of primers Target templates were found in selected database: Nucleotide collection (nt)
Other reports Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGAGCAATGATGGCAGACTC	20	57.97	50.00	5.00	3.00
Reverse primer	ATCCGCGCAGAAAAAGTAGA	20	57.62	45.00	6.00	0.00

Products on target templates

>JN703723.1 Uncultured anaerobic ammonium-oxidizing bacterium clone BS23 hydrazine synthase subunit A (hzsA) gene, partial cds

```
product length = 110
Forward primer  1    TGAGCAATGATGGCAGACTC  20
Template        518  ....................  537

Reverse primer  1    ATCCGCGCAGAAAAAGTAGA  20
Template        627  ....................  608
```

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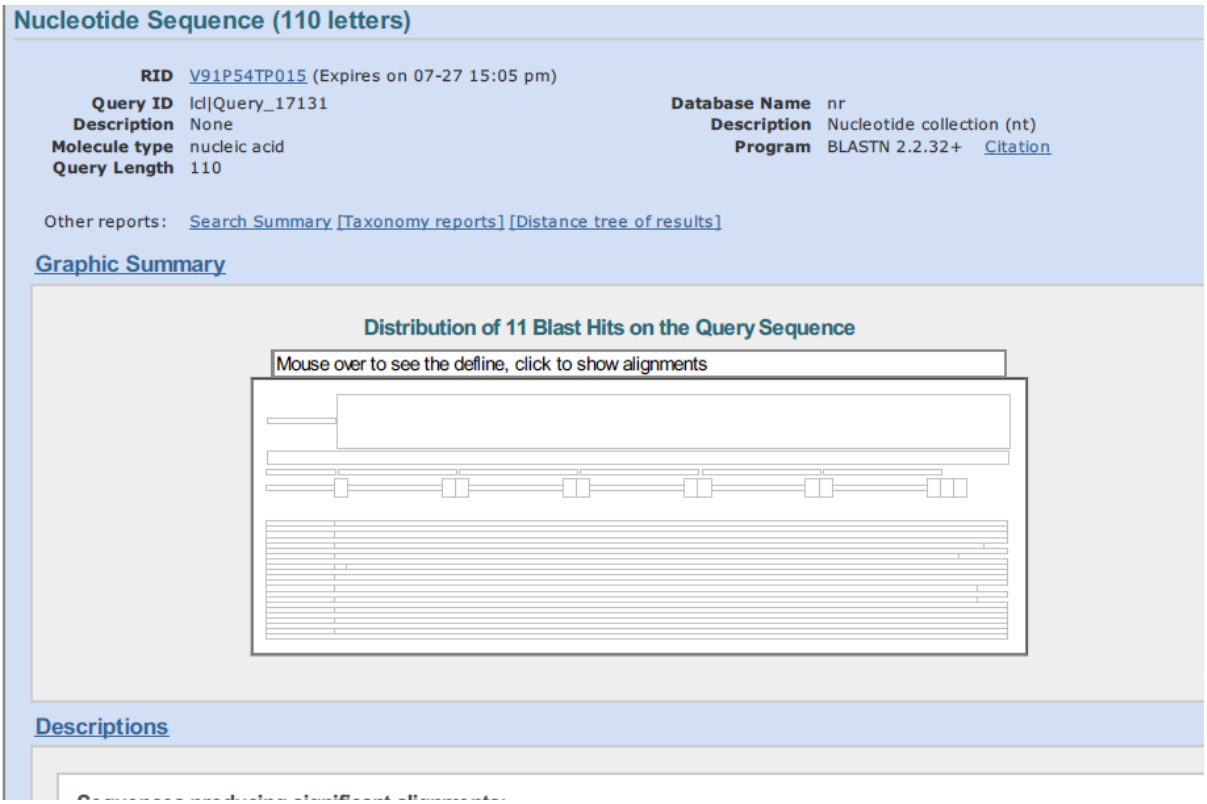
```
product length = 110
Forward primer  1    TGAGCAATGATGGCAGACTC  20
Template        497  ....................  516

Reverse primer  1    ATCCGCGCAGAAAAAGTAGA  20
Template        606  ...T................  587
```

>KF202966.1 Uncultured anaerobic ammonium-oxidizing bacterium clone I3_8 hydrazine synthase subunit A (hzsA) gene, partial cds

```
product length = 110
Forward primer  1    TGAGCAATGATGGCAGACTC  20
Template        537  ............A.....T  556

Reverse primer  1    ATCCGCGCAGAAAAAGTAGA  20
Template        646  ...A.........T......  627
```



Nucleotide Sequence (110 letters)

RID V91P54TP015 (Expires on 07-27 15:05 pm)
Query ID lcl|Query_17131
Description None
Molecule type nucleic acid
Query Length 110

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.32+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

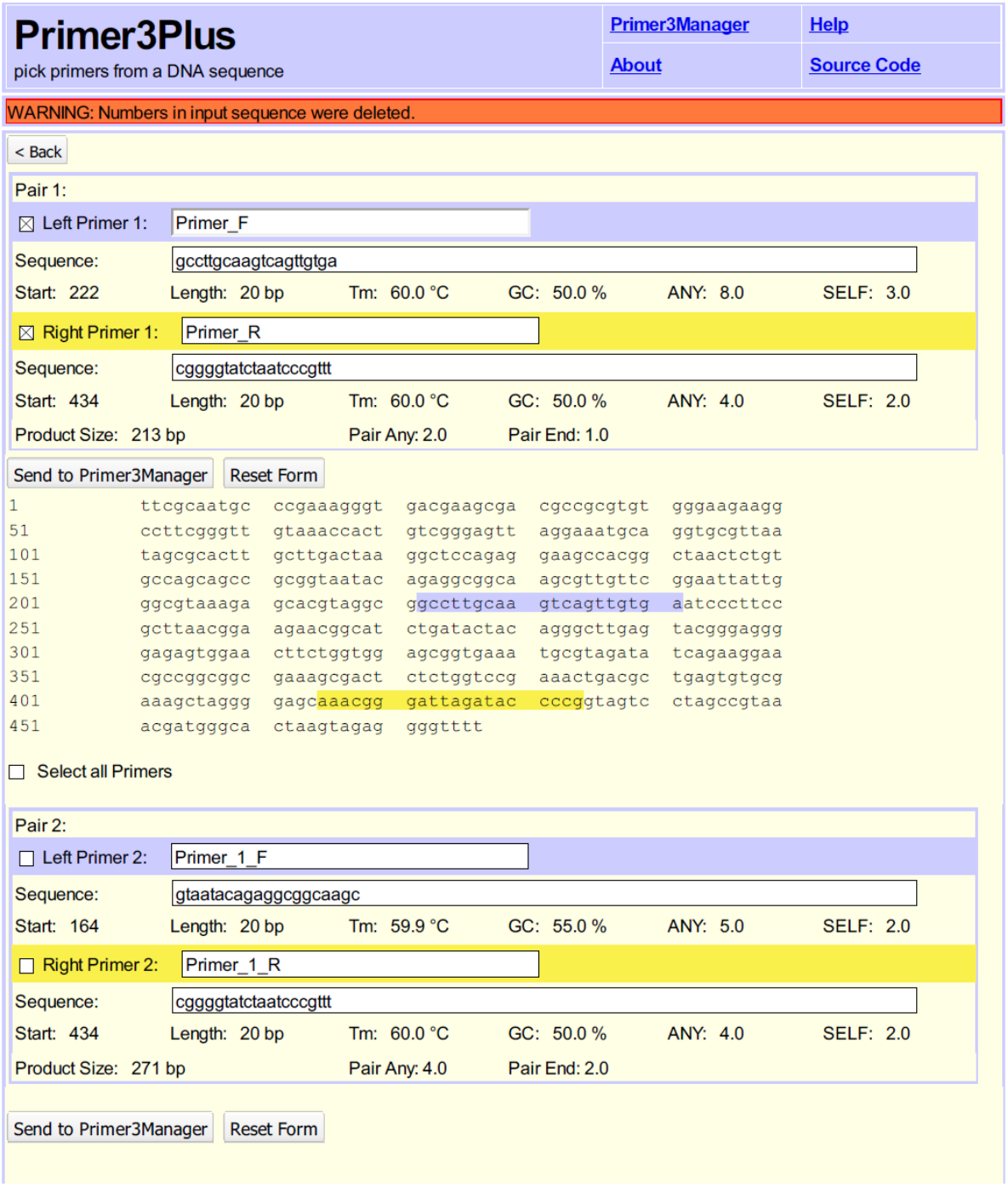
Graphic Summary

Distribution of 11 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments

Descriptions

AMX 16S primer set:



Primer3Plus

pick primers from a DNA sequence

Primer3Manager | Help | About | Source Code

WARNING: Numbers in input sequence were deleted.

< Back

Pair 1:

☒ Left Primer 1: Primer_F

Sequence: gccttgcaagtcagttgtga

Start: 222 Length: 20 bp Tm: 60.0 °C GC: 50.0 % ANY: 8.0 SELF: 3.0

☒ Right Primer 1: Primer_R

Sequence: cggggtatctaatcccgttt

Start: 434 Length: 20 bp Tm: 60.0 °C GC: 50.0 % ANY: 4.0 SELF: 2.0

Product Size: 213 bp Pair Any: 2.0 Pair End: 1.0

Send to Primer3Manager | Reset Form

```
1      ttcgcaatgc  ccgaaagggt  gacgaagcga  cgccgcgtgt  gggaagaagg
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451    acgatgggca  ctaagtagag  gggtttt
```

☐ Select all Primers

Pair 2:

☐ Left Primer 2: Primer_1_F

Sequence: gtaatacagaggcggcaagc

Start: 164 Length: 20 bp Tm: 59.9 °C GC: 55.0 % ANY: 5.0 SELF: 2.0

☐ Right Primer 2: Primer_1_R

Sequence: cggggtatctaatcccgttt

Start: 434 Length: 20 bp Tm: 60.0 °C GC: 50.0 % ANY: 4.0 SELF: 2.0

Product Size: 271 bp Pair Any: 4.0 Pair End: 2.0

Send to Primer3Manager | Reset Form

BLAST®

Basic Local Alignment Search Tool

Home | Recent Results | Saved Strategies | Help

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NCBI/ BLAST/ blastn suite/ Formatting Results - VC5ENUEX015

Your search parameters were adjusted to search for a short input sequence.

Edit and Resubmit | Save Search Strategies | Formatting options | Download | How to read this page | Blast report description

Nucleotide sequence (26 letters)

RID VC5ENUEX015 (Expires on 07-28 19:28 pm)

Query ID lcl|Query_3159
Description None
Molecule type nucleic acid
Query Length 20

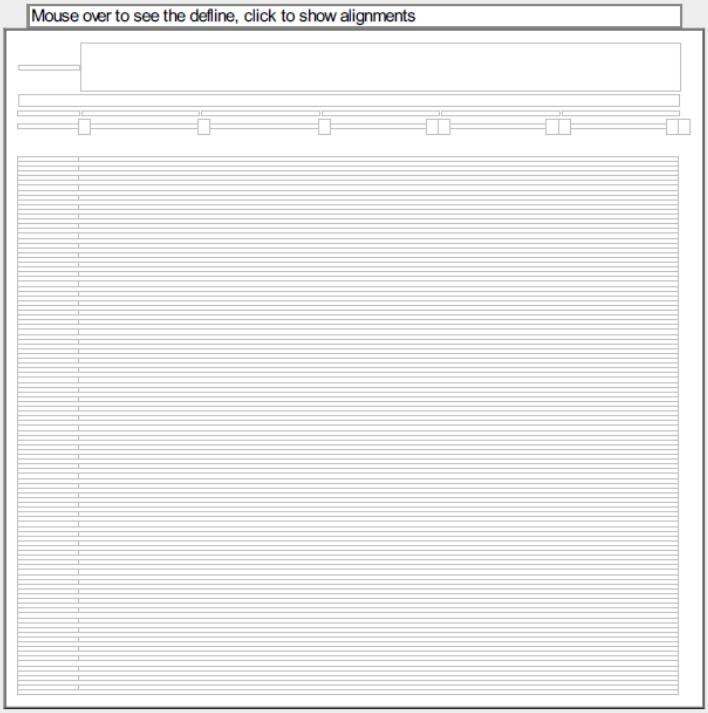
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.32+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured bacterium clone CTRL_1_uncultured_bacterium_6126 16S ribosomal RNA gene, p	40.1	40.1	100%	0.17	100%	KP561276.1
Uncultured bacterium clone OTU40306_AL203_285100 16S ribosomal RNA gene, partial sequ	40.1	40.1	100%	0.17	100%	KP929665.1
Candidatus Kuenenia stuttgartiensis isolate DGGE gel band AAOB-4 16S ribosomal RNA gene,	40.1	40.1	100%	0.17	100%	KP663627.1
Uncultured bacterium clone PREM-368-19 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	0.17	100%	KP329548.1

Uncultured planctomycete clone anammox 16S ribosomal RNA gene, partial sequence

GenBank: JN659913.1

FASTA Graphics

Go to:

```
LOCUS       JN659913                477 bp    DNA     linear   ENV 30-SEP-2013
DEFINITION  Uncultured planctomycete clone anammox 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   JN659913
VERSION     JN659913.1  GI:389552043
KEYWORDS    ENV.
SOURCE      uncultured planctomycete
  ORGANISM  uncultured planctomycete
            Bacteria; Planctomycetes; Planctomycetia; Planctomycetales;
            environmental samples.
REFERENCE   1  (bases 1 to 477)
  AUTHORS   Zeng,T., Li,D. and Zhang,J.
  TITLE     Sequences in anammox biofilter
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 477)
  AUTHORS   Zeng,T., Li,D. and Zhang,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-SEP-2011) School of Municipal and Environmental
            Engineering, Harbin Institute of Technology, Huanghe Road No. 73,
            Harbin, Heilongjiang 150090, P.R. China
FEATURES             Location/Qualifiers
     source          1..477
                     /organism="uncultured planctomycete"
                     /mol_type="genomic DNA"
                     /isolation_source="anammox biofilter"
                     /db_xref="taxon:120965"
                     /clone="anammox"
                     /environmental_sample
     rRNA            <1..>477
                     /product="16S ribosomal RNA"
ORIGIN
        1 ttcgcaatgc ccgaaagggt gacgaagcga cgccgcgtgt gggaagaagg ccttcgggtt
       61 gtaaaccact gtcgggagtt aggaaatgca ggtgcgttaa tagcgcactt gcttgactaa
      121 ggctccagag gaagccacgg ctaactctgt gccagcagcc gcggtaatac agaggcggca
      181 agcgttgttc ggaattattg ggcgtaaaga gcacgtaggc ggccttgcaa gtcagttgtg
      241 aatcccttcc gcttaacgga agaacggcat ctgatactac agggcttgag tacgggaggg
      301 gagagtggaa cttctggtgg agcggtgaaa tgcgtagata tcagaaggaa cgccggcggc
      361 gaaagcgact ctctggtccg aaactgacgc tgagtgtgcg aaagctaggg gagcaaacgg
      421 gattagatac cccggtagtc ctagccgtaa acgatgggca ctaagtagag gggtttt
//
```

Change region shown

Customize view

Analyze this sequence

Run BLAST

Pick Primers

Highlight Sequence Features

Find in this Sequence

LinkOut to external resources

SILVA SSU Database [SILVA]

Related information

Taxonomy

Recent activity

Turn Off Clear

Uncultured planctomycete clone anammox 16S ribosomal F Nucleotide

anammox (17864) Nucleotide

Nucleotide Sequence (110 letters) BLAST

Uncultured anaerobic ammonium-oxidizing bacterium clone I Nucleotide

Candidatus Brocadia sinica JPN1 DNA, contig: brosiA, whole Nucleotide

See more...

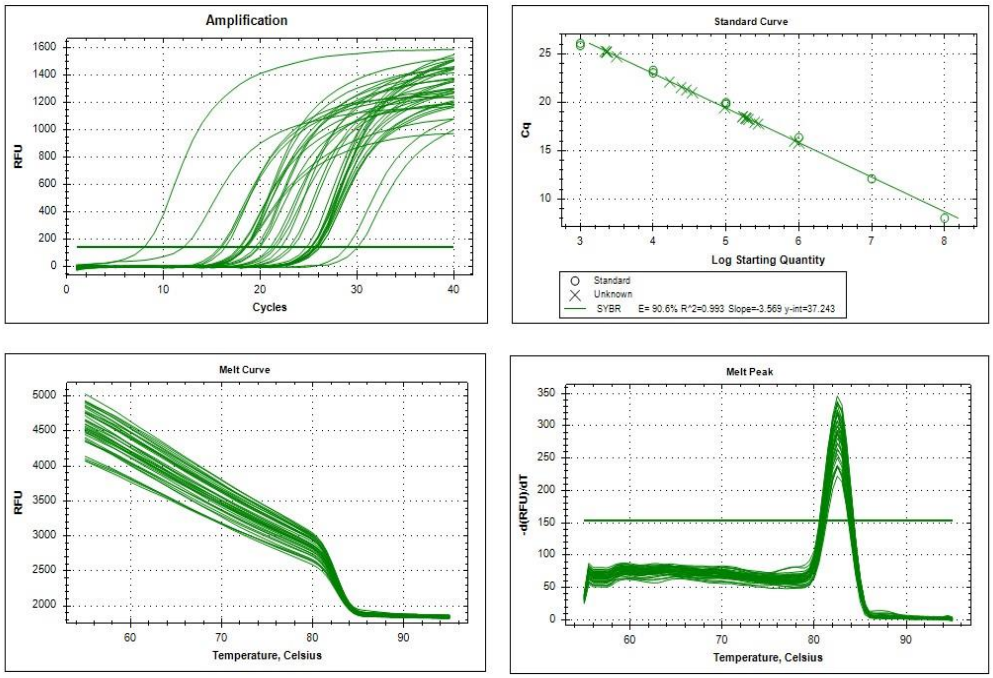
Appendix D: Mothur Script

```
make.contigs(file=JashNGS.files, processors=4)
summary.seqs(fasta=JashNGS.trim.contigs.fasta)
screen.seqs(fasta=current, group=current, maxambig=0, maxlength=520)
unique.seqs()
count.seqs(name=current, group=current)
summary.seqs(count=current)
pcr.seqs(fasta=C:\mothur_1.35.1_64bit\silva.bacteria\silva.seed_v123.align,
    start=6428, end=23450, keepdots=F, processors=4)
system(copy C:\mothur_1.35.1_64bit\silva.bacteria\silva.seed_v123.align
    silvaPCR_DB_V3V4.fasta)
summary.seqs(fasta=silvaPCR_DB_V3V4.fasta)
align.seqs(fasta=C:\mothur_1.35.1_64bit\JashNGS.trim.contigs.good.unique,
    reference=C:\mothur_1.35.1_64bit\silva.bacteria\silvaPCR_DB_V3V4.fasta)
summary.seqs(fasta=C:\mothur_1.35.1_64bit\JashNGS.trim.contigs.good.uniqu
    e.align, count=JashNGS.trim.contigs.good.count_table)
screen.seqs(fasta=current, count=current, start=1046, end=13862, maxhomop=8)
filter.seqs(fasta=current, vertical=T, trump=.)
unique.seqs(fasta=current, count=current)
pre.cluster(fasta=current, count=current, diffs=2)
chimera.uchime(fasta=current, count=current, dereplicate=t)
remove.seqs(fasta=current, accnos=current)
summary.seqs(fasta=current, count=current)
classify.seqs(fasta=current, count=current,
    reference=C:\mothur_1.35.1_64bit\silva.bacteria\silvaseedv123.fasta,
    taxonomy=C:\mothur_1.35.1_64bit\silva.bacteria\silva.seed_v123.tax,
    cutoff=60)
remove.lineage(fasta=current, count=current, taxonomy=current,
    taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)
cluster.split(fasta=current, count=current, taxonomy=current,
    splitmethod=classify, taxlevel=4, cutoff=0.15)
make.shared(list=current, count=current, label=0.03)
classify.otu(list=current, count=current, taxonomy=current, label=0.03)
phylotype(taxonomy=current)

## Genus level ##
make.shared(list=current, count=current, label=1)
classify.otu(list=current, count=current, taxonomy=current, label=1)
system(rename
    JashNGS.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wan
    g.pick.tx.shared JashNGS.Genuslevel.shared)

## Phylum level ##
make.shared(list=C:\mothur_1.35.1_64bit\JashNGS.trim.contigs.good.unique.g
    ood.filter.unique.precluster.pick.seed_v123.wang.pick.tx.list,
    count=JashNGS.trim.contigs.good.unique.good.filter.unique.precluster.uchim
    e.pick.pick.count_table, label=5)
```

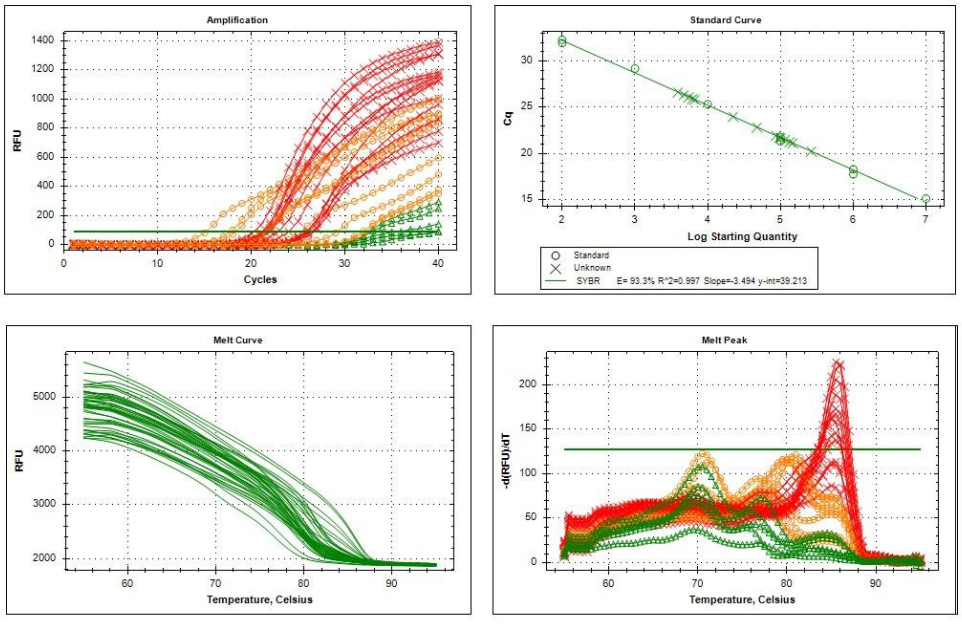
```
classify.otu(list=current, count=current,
    taxonomy=JashNGS.trim.contigs.good.unique.good.filter.unique.precluster.pi
    ck.seed_v123.wang.pick.taxonomy, label=5)
system(rename
    JashNGS.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v1
    23.wang.pick.tx.shared JashNGS.Phylumlevel.shared)
## Rarefaction ##
rarefaction.single(shared=JashNGS.Genuslevel.shared, calc=sobs, freq=100)
rarefaction.single(shared=JashNGS.Phylumlevel.shared, calc=sobs, freq=100)
## Diversity indices ##
summary.single(shared=JashNGS.Genuslevel.shared)
summary.single(shared=JashNGS.Phylumlevel.shared)
```

Appendix E: QPCR Quality control Parameter Data

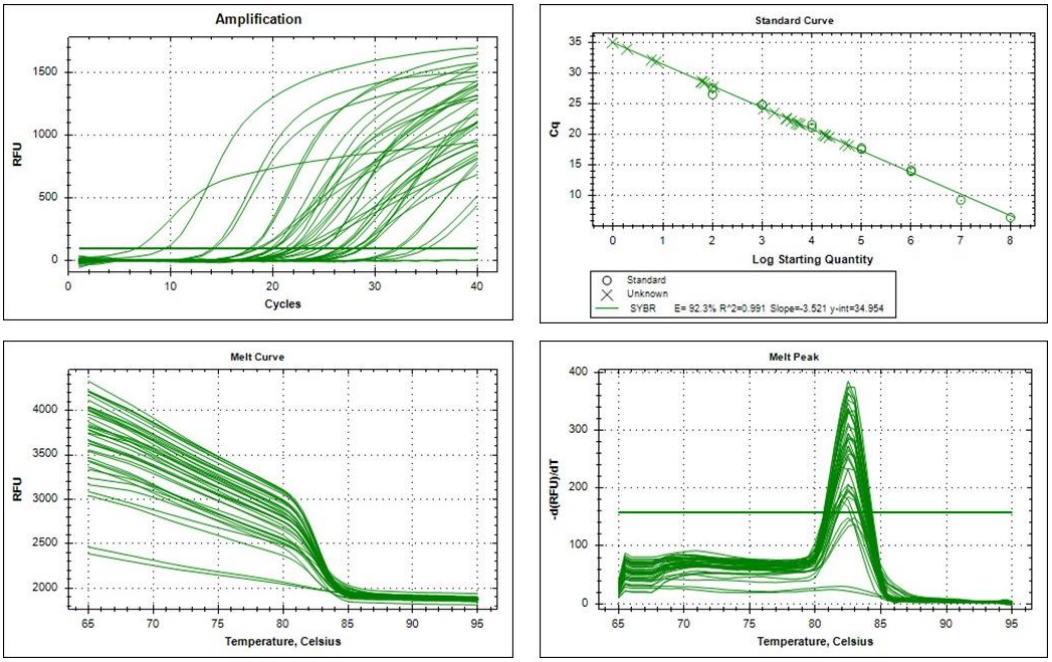
Nitrospira:



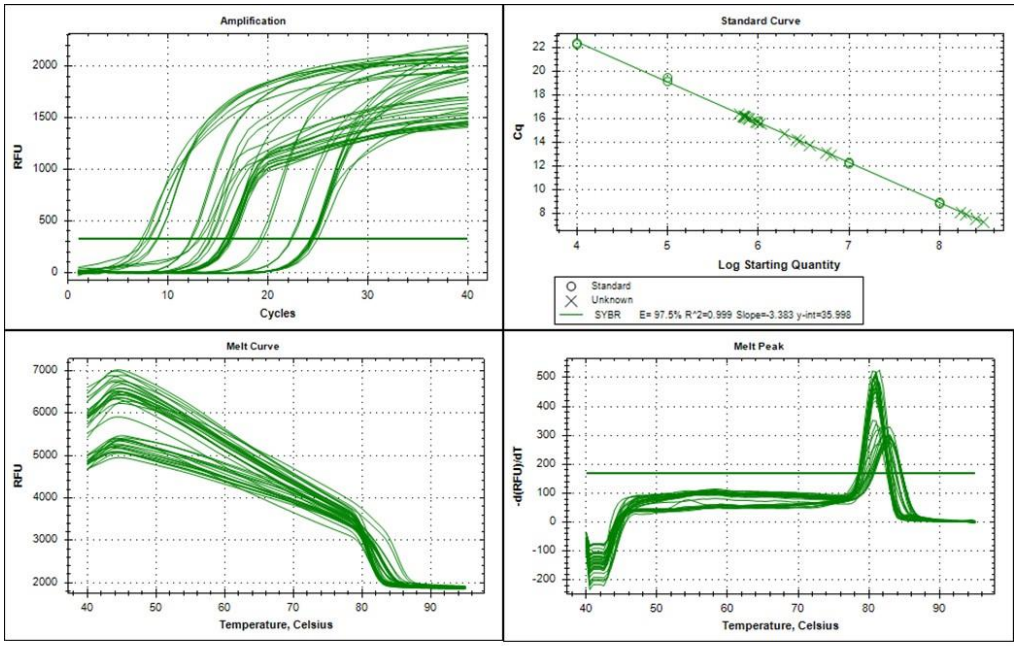
AmoA:



Anammox


Amplification
RFU
Cycles
Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
SYBR E= 92.3% R^2=0.991 Slope=-3.521 y-int=34.954
Melt Curve
RFU
Temperature, Celsius
Melt Peak
-d(RFU)/dT
Temperature, Celsius

EUB


Amplification
RFU
Cycles
Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
SYBR E= 97.5% R^2=0.999 Slope=-3.383 y-int=35.998
Melt Curve
RFU
Temperature, Celsius
Melt Peak
-d(RFU)/dT
Temperature, Celsius