

# Performance and Modelling of Non-granular Anammox Culture for Wastewater Treatment

by

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## ABSTRACT

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**Title: Performance and Modelling of Non-granular Anammox Culture for Wastewater Treatment**

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**Degree:** Philosophiae Doctor (Chemical Technology)

The anammox process is the latest biological process for removing nitrogenous compounds from wastewater. Anammox has been widely studied for the removal of nitrogen from various wastewaters. Despite intensive research for the past two decades to fully understand these processes, there is still some doubt related to the implementation of full-scale systems. Various characteristics of the anammox process, such as the reaction stoichiometry and the kinetic characteristics are still the predominant subjects. Slow bacterial growth and inhibition are the major causes of unsuccessful trials in the lab scale systems, and further prevent up scaling to pilot scale and the implementation of full-scale. Kinetic parameters of interest, such as the substrate affinity constant and maximum specific growth rate, which have been reported in literature, vary widely. Most studies on the anammox processes have been focused on granule-based cell cultures. Parameter values reported in literature are often hindered by mass transfer resistance associated with larger granules of microbial culture. In this study, the enrichment of free cells suspension culture is described. The free-cells suspension of highly active anammox bacteria was further used for detailed kinetic analysis of the anammox process.

Firstly, the existence and diversity of anammox bacteria from various local habitats was investigated. Batch systems were used to enrich anammox biomass from sludge collected from three municipal wastewater treatment works in Pretoria. Anammox activity was tested and detected in two of the three wastewater treatment works after 90 days of primary enrichment. The activity was confirmed by the consumption of both  $\text{NH}_4^+$  and  $\text{NO}_2^-$  in the

system. The presence of anammox bacteria was also confirmed by PCR amplification of the 16S rRNA of the anammox using the anammox specific primers. All clones retrieved were closely related to the *Brocadia* species and were abundant in all habitats tested. The maximum growth rate of anammox for batch experiments was also estimated using a relatively new model.

The denitrifying capability of pre-enriched suspended anammox culture was evaluated using a Sequencing Batch Reactor (SBR) and operated for a period of 120 days. The anammox process achieved high substrate removal with an average total nitrogen removal rate of  $2.6 \text{ gNL}^{-1} \text{ d}^{-1}$ , and reaching a maximum TN removal efficiency of 93%. For ammonium and nitrite, maximum removal efficiencies of 93% and 98%, respectively, were obtained. Stable performance was observed after 10 days of operation. Phylogenetic analysis confirmed the presence of anammox bacteria that are closely related to the *Candidatus Brocadia* species with 16S rRNA sequence similarity of 98%. In addition, a substrate removal model was also employed to simulate and predict the performance of the anammox reactor. According to the model predictions, the maximum substrate removal rate of the reactor should be  $34 \text{ gN} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ . According to the model validation, the modified Stover-Kincannon model was suitable for the nitrogen removal description in the anammox SBR with a high correlation coefficient of  $R^2=0.9739$ .

Further experiments were conducted that focused on improving the anammox process using genetic engineering. In order to improve the anammox nitrogen removal efficiency, *E. coli* was genetically engineered to express the hydrazine oxidoreductase enzyme, a key enzyme in the anammox process. Batch reactors containing simulated wastewater were inoculated with transgenic *E. coli*. After six days of incubation, a drastic removal of nitrite and nitrate was observed with maximum removal efficiencies of 93% and 98%, respectively. The start-up was immediate with the transgenic *E. coli*, as opposed to native anammox, which can take several months. The results obtained from this study have shown genetic engineering technology to be an innovative technology that can speed up the anammox process and improve efficiency and stability. The anammox culture enriched during this study had a biomass specific maximum growth rate of  $0.31 \text{ d}^{-1}$  which was slightly higher than most reported granule base cell cultures in literature. Additionally, the intrinsic half saturation

constant for ammonium and nitrite were detected to be 0.054 and 0.024mg-N L<sup>-1</sup>, respectively.

## Declaration

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

Signature of candidate:..... Date:.....

## Dedication

This thesis is dedicated to

My family.

My late father, who always encouraged me to further my studies and supported me in every way he could.

My mother, for her ongoing support, understanding and patience, and the opportunity she gave me to do this degree.

My kids, Lerato and Mhlengi, to whom I owe so much for their patience and unconditional love.

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## List of Abbreviations

AMO	Ammonium monooxygenase
Anammox	Anaerobic ammonium oxidising
AOB	Ammonium oxidising bacteria
ASM1	Activated sludge model no. 1
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BWA	Burrows wheeler aligner
CaCO <sub>3</sub>	Calcium carbonate
CAI	Codon Adaptation Index
CANON	Completely Autotrophic Nitrogen-removal Over Nitrite
CIAA	Chloroform Isoamyl alcohol (24:1)
CO <sub>2</sub>	Carbon dioxide
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
Eq	Equation
FISH	Fluorescence in situ hybridisation
HAO	Hydroxyl amine oxidoreductase
HCl	Hydrochloric acid
HO	Hydrazine oxidoreductase
HRT	Hydraulic retention time
H <sub>2</sub> O	Water
hzo	Hydrazine Oxidoreductase
MBBR	Moving Bed Biofilm Reactor
ML	Mega litres
MSM	Mineral salt medium
MSR	Miseq reporter
N <sub>2</sub>	Dinitrogen gas
NCBI	National Centre for Biotechnology Information



NGS	Next generation sequencing
NH <sub>3</sub>	Ammonia
NH <sub>4</sub> <sup>+</sup>	Ammonium
NH <sub>2</sub> OH	Hydroxyl amine
NO <sub>2</sub> <sup>-</sup>	Nitrite
NO <sub>3</sub> <sup>-</sup>	Nitrate
NLR	Nitrogen loading rate
O <sub>2</sub>	Oxygen
OLAND	Oxygen-limited autotrophic nitrification/denitrification
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
RBC	Rotating biological contactor
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
SAV	Sequencing analysis viewer
SBR	Sequencing Batch Reactor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHARON	Single reactor system for High activity Ammonium Removal Over Nitrite
SRT	Solid retention time
TF	Trickling filter
TN	Total nitrogen
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket
WWTW	Wastewater treatment works



## Symbol Nomenclature

$A$	total disc surface area on which biomass concentration is immobilised ( $m^2$ )
$b_A$	Specific decay rate for autotrophs ( $day^{-1}$ )
$b_{An}$	Specific decay rate for anammox ( $day^{-1}$ )
$b_H$	Specific decay rate for heterotrophs ( $day^{-1}$ )
$\frac{dS}{dt}$	Substrate removal rate ( $mg \cdot L^{-1} \cdot d^{-1}$ )
$f_p$	Fraction of biomass leading to particulate material
$ix_B$	Nitrogen fraction in biomass ( $g \text{ N} \cdot g^{-1} \text{ COD}$ )
$ix_P$	Nitrogen fraction in endogenous mass ( $g \text{ N} \cdot g^{-1} \text{ COD}$ )
$K_B$	Saturation value constant ( $g \cdot d^{-1} \cdot m^{-2}$ )
$k_h$	Maximum specific hydrolysis rate ( $day^{-1}$ )
$K_{NH_4}$	Ammonium half saturation constant ( $g \text{ NH}_4\text{-N} \cdot m^{-3}$ )
$K_{NO_3}$	Nitrate half saturation constant ( $g \text{ NO}_3\text{-N} \cdot m^{-3}$ )
$K_{O_2,A}$	Oxygen half saturation constant for autotrophs ( $g \text{ O}_2 \cdot m^{-3}$ )
$K_{O_2,H}$	Oxygen half saturation constant for heterotrophs ( $g \text{ O}_2 \cdot m^{-3}$ )
$K_{PO_4}$	Phosphorus half saturation constant ( $g \text{ NO}_3\text{-N} \cdot m^{-3}$ )
$K_S$	Half-saturation coefficient ( $mg/L$ )
$K_{S,h}$	Half saturation coefficient for hydrolysis of XS
$K_{S,H}$	Substrate saturation constant for heterotrophs ( $g \text{ COD} \cdot m^{-3}$ )
$K_\alpha$	Ammonification rate constant ( $m^3 \cdot g^{-1} \text{ COD} \text{ day}^{-1}$ )
$\mu_{max,A}$	Maximum specific growth rate for autotrophs ( $day^{-1}$ )
$\mu_{max,An}$	Maximum specific growth rate for anammox ( $day^{-1}$ )

$\mu_{max,H}$	Maximum specific growth rate for heterotrophs ( $\text{day}^{-1}$ )
$\eta_g$	Anoxic growth correction factor
$\eta_h$	Anoxic hydrolysis correction factor
$[\text{NO}_2^-]_t$	Nitrite concentration at time $t$ ( $\text{mg}\cdot\text{L}^{-1}$ )
$[\text{NO}_2^-]_0$	Initial nitrite concentration ( $\text{mg}\cdot\text{L}^{-1}$ )
$Q$	flow rate ( $\text{L}\cdot\text{d}^{-1}$ )
$S$	Substrate concentration at any time (in this case nitrite) ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_e$	effluent substrate concentration ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_i$	influent substrate concentration ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_{ND}$	Concentration of soluble organic nitrogen ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_{\text{NH}_4}$	Concentration of ammonium nitrogen ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_{\text{NO}_3}$	Concentration of nitrate nitrogen ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_{\text{PO}_4}$	Concentration of dissolved phosphorus ( $\text{mg}\cdot\text{L}^{-1}$ )
$S_S$	Concentration of readily biodegradable COD ( $\text{mg}\cdot\text{L}^{-1}$ )
$t$	Time (d)
$U_{max}$	Maximum substrate removal rate constant ( $\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ )
$V$	reactor liquid volume (L)
$X_{AN}$	Biomass concentration of anammox biomass ( $\text{mg}\cdot\text{L}^{-1}$ )
$X_{AN,0}$	Anammox biomass concentrations at time 0 ( $\text{mg}\cdot\text{L}^{-1}$ )
$X_H$	Concentration of active heterotrophic bacteria ( $\text{mg}\cdot\text{L}^{-1}$ )
$X_{ND}$	Concentration of particulate degradable organic nitrogen ( $\text{mg}\cdot\text{L}^{-1}$ )
$Y_A$	Autotrophic yield ( $\text{g COD}\cdot\text{g}^{-1}\text{ COD}$ )
$Y_{AN}$	Yield coefficient of anammox biomass ( $\text{mg COD}\cdot\text{mg}^{-1}\text{ N}$ )
$Y_H$	Heterotrophic yield ( $\text{g COD}\cdot\text{g}^{-1}\text{ COD}$ )

# CHAPTER 1 :INTRODUCTION

---

## 1.1: Background

Nitrogen compounds are among the most significant pollutants in municipal and industrial wastewater due to their role in eutrophication and their toxicity to aquatic life (Paredes et al., 2007). Destruction and removal of nitrogenous compounds such as ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) is therefore critical to avoid toxic effects and to prevent proliferation of algae in aquatic bodies.

The biological nitrification–denitrification processes are mostly used for the removal of nitrogen from wastewater. In these processes, complete nitrification involves the oxidation of ammonia to nitrite, and then to nitrate, followed by denitrification which involves anoxic reduction of nitrate into nitrite, and further reduction to nitrous oxide, nitric oxide, and finally into  $\text{N}_2$  gas (Zeng et al., 2009). The conventional nitrification–denitrification process for nitrogen removal requires a great deal of energy for aeration during nitrification, and also uses organic carbon to help remove nitrate during denitrification.

Since the late 1990's, the anammox process was discovered in which ammonium was converted to dinitrogen gas under anaerobic conditions with nitrite as the electron acceptor (Mulder et al., 1995). This process is promising as a low-cost alternative to conventional denitrification systems for ammonium removal from concentrated wastewaters (Strous et al., 1997a). The anammox process is autotrophic and therefore there is no need for COD addition to support denitrification (vande Graaf et al., 1996). Additionally, when the anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite while the anammox process combines the remaining ammonium with this nitrite to yield dinitrogen gas. This reduces the oxygen demand in the nitrification reactor and thereby contributing to a reduction in

operational costs. The anammox process has a very low biomass yield and, as a result, minimal sludge is produced. The low sludge production is the third factor that adds up to the significantly lower operation costs when compared to conventional denitrification systems. All the above mentioned characteristics render the anammox process as an appealing option for the removal of ammonium from nitrogen rich wastewaters. Ever since the discovery of the anaerobic ammonia oxidation metabolism, efforts have been made to design anammox into treatment processes with a goal to use it in full-scale systems. The slow growth of anammox bacteria, with the inhibition effects and operational problems, are the main cause of a very long reactor start-up and requires a high sludge retention period (Van der Star et al., 2007). A number of studies have focused on this process over the last few decades so as to understand not only the microbiology and biochemistry of the anammox process, but also how to best design this process for nitrogen removal. It took 3.5 years to achieve the start-up of the first full-scale granular anammox reactor (Van der Star et al., 2007).

Shortening the anammox process start-up period has become an important strategy for successful anammox application. Different types of reactor designs have been used to minimise the wash-out of anammox biomass. These include continuous stirred-tank reactor, anaerobic biological filtrated reactor, sequencing batch reactor (SBR), up-flow reactor, and biofilm reactor (Imajo, Tokutomi & Furukawa, 2004; Isaka, Sumino & Tsuneda, 2007; Strous et al., 1998; van Dongen, Jetten & van Loosdrecht, 2001).

Granular sludge technology is the most commonly used system for the anammox process. A full scale anammox reactor is heavily exploiting the granular based system. However, granular sludge reactor systems are not the most suitable systems for the investigation of the intrinsic properties of the microorganism. Biokinetic parameters, such as substrate affinities, maximum growth rate or maintenance need, cannot be accurately evaluated as they are hindered by the mass transfer limitations within the anammox granule (Chu, van Veldhuizen & van Loosdrecht, 2003; Harremoës, 1977). Therefore, transport limitations

possibly result in the underestimation of the observed maximum specific growth rate ( $\mu_{\max}$ ) and overestimation of the intrinsic affinity constant ( $K_S$ ) (Lotti et al., 2014).

In the present study, the performance of the non-granular anammox free cell culture was investigated. The microbial community of anammox bacteria was analysed using a novel next generation sequencing. The mathematical modelling describing the anammox process in SBR was developed. The developed model was then calibrated and validated using the experimental results. The intrinsic properties of anammox bacteria were also investigated. Efforts to improve the anammox process using genetic engineering technology are also described.

## 1.2: Objectives of the Study

The main objective of this study was to investigate the potential of non-granular anammox free cell culture to achieve high nitrogen removal from nitrogen rich wastewaters. To achieve the primary objective, certain experimental tasks were conducted. These tasks were:

- To isolate and enrich the anammox biomass from sludge of local municipal wastewater treatment plants using batch system.
- To characterise the microbial population obtained in the reactors.
- To evaluate the potential of nitrogen removal in a sequencing batch reactor system (suspended-growth system) using the pre-enriched anammox biomass.
- To develop a mathematical model to describe the developed system in order to provide a solid foundation for the design and operation of this anammox system.
- To investigate the potential of genetic engineering technology for improvement of the anammox process.

### 1.3 Outline of thesis

The thesis is divided into eight chapters. The different chapters of the thesis, and the main contents of each chapter, are summarised below:

**Chapter 1:** This chapter introduces the background and mentions what the research objectives are, and describes the significance of this research and thesis outline.

**Chapter 2:** A literature review about nitrogen removal from wastewater is discussed. Economic analyses of different technologies for nitrogen removal from wastewater are also included. Additionally, the microbiology and application of the latest innovative technology called the anammox process is described. Finally, methods for monitoring the stability of the microbial population in an anammox system are also included.

**Chapter 3:** This chapter describes all the materials and methods used in this study.

**Chapter 4:** This chapter defines the detailed information on the finding of the enrichment of anammox from waste sludge using batch reactors.

**Chapter 5:** This chapter gives full details of the performance of the sequencing batch reactor, and describes microbial population and substrate removal kinetics.

**Chapter 6:** This chapter describes the genetic engineering technology to improve the anammox process.

**Chapter 7:** This chapter covers the modelling of the anammox process and provides key parameters.

**Chapter 8:** This chapter consists of the general conclusions and recommendations.

**Chapter 9:** This chapter describes the engineering significance of the study.

## 1.4 Significance of research

The anaerobic ammonia oxidation process is the latest cost effective alternative for converting reactive nitrogen to dinitrogen gas by anaerobically oxidising ammonia with nitrite as electron acceptor. This autotrophic nature of anammox bacteria makes the anammox process an appealing component of biological nitrogen removal technologies. Unlike the conventional nitrification-denitrification process, the anammox process does not require any organic carbon source. Additionally, the energy requirement is reduced by more than 50% when the anammox process is used, instead of conventional nitrification-denitrification. A number of studies, over the past few decades, have focused on this process to investigate how to best design this process for efficient nitrogen removal. Despite achieving some valuable breakthroughs in understanding the process, there are still some key factors of the anammox process that have not been fully understood. The main challenge of the process is to select the most appropriate type of biomass and the type of reactor to maximise the performance and efficiency of the microbial culture. Reactor designs mostly used for anammox are granule based and attached growth reactor systems. The challenge with these types of reactors is that their performance may be hindered by mass transfer limitation across the biofilm and within the granule, leading to incorrect assessment of the most important parameters. In this study, the main focus was on the use of non-granular suspension anammox culture with no mass transfer limitation for accurate kinetic characterisation of the anammox process. The results from this study provide the intrinsic properties of the culture and contribute greatly to the growing body of knowledge on the anammox process.

## CHAPTER 2 : LITERATURE REVIEW

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### 2.1: Biology of Nitrogen Cycle

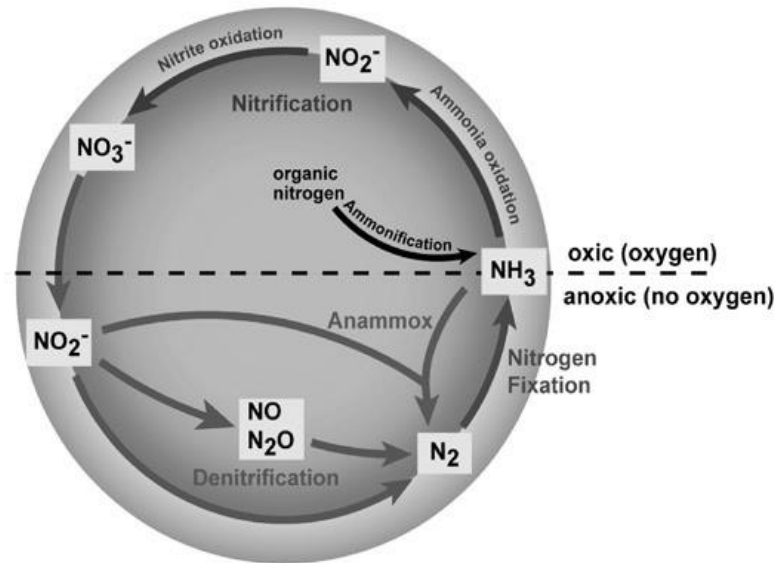
Nitrogen is the most abundant element in the earth's atmosphere and essential element for life found in cells as a building block of proteins and nucleic acids (Canfield, et al., 2005; Yates, 1976). The turnover of nitrogen compounds in the biosphere is known as the nitrogen cycle (Figure 2.1). The nitrogen compound can be found in the environment in numerous forms such as nitrate, nitrite, nitrous oxide and other forms (Jaffe, 1992; Zhang et al., 2011). Although nitrogen is abundant in the atmosphere in its molecular form ( $N_2$ ), it is mainly unavailable in this form to most organisms. (Erisman et al., 2007). Only when nitrogen is converted from dinitrogen gas into ammonia ( $NH_3$ ), does it become available for utilisation by various organisms. The nitrogen cycle is a complex biogeochemical process in which nitrogen is converted from its molecular form ( $N_2$ ) into a form that can be used in biological processes. The nitrogen cycle (Figure 2.1) is known to consist of the following nitrogen transformation reactions (Erisman et al., 2007; Jaffe, 1992; Terada, et al., 2011; Ye & Thomas, 2001):

- Nitrification
- Denitrification
- Dissimilatory nitrate reduction
- Anaerobic ammonium oxidation (anammox)
- Assimilation
- Nitrogen fixation
- Ammonification

It was assumed years ago that the microbial nitrogen cycle was essentially complete and that no other organisms that were involved in the process existed in nature (Strous &



Jetten, 2004). However, recently it has been indicated that its understanding is far from complete. Discoveries, such as anaerobic ammonium oxidation (anammox) (Strous, et al., 1999), provide an example of the fact that there is a huge biodiversity of nitrogen conversions hidden in the microbial world.



**Figure 2.1: The Nitrogen Cycle**

(Source: <http://www.nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632>)

## 2.2: Environmental Problems Related to Nitrogen Discharges

Humans have caused the most remarkable impact on the nitrogen cycle. The latest reports indicate that human activities, such as increased combustion of fossil fuels and increased demand for nitrogen in agriculture and industry, continue to alter the global cycle of nitrogen (Galloway et al., 2008; Vitousek et al., 1997). Because of the increased nitrogen mobility, excess nitrogen from human activities has serious and ongoing environmental consequences for aquatic and terrestrial ecosystems. (Vitousek et al., 1997).

The presence of anthropogenic generated nitrogen compounds in the environment can cause environmental impacts, on the surrounding eco-systems and receiving water bodies or watersheds, such as toxicity or eutrophication of natural water sources. The

widespread nitrogen compounds that may result in a negative effect to the receiving waters bodies are:

- Ammonium ions ( $\text{NH}_4^+$ )
- Nitrite ions ( $\text{NO}_2^-$ )
- Nitrate ions ( $\text{NO}_3^-$ )

The major hazards related to the presence of these compounds in concentrations above the water quality standard may cause the following:

- Dissolved oxygen ( $\text{O}_2$ ) depletion
- Toxicity
- Eutrophication
- Methemoglobinemia
- Deterioration of water aesthetic quality
- Odours from decomposing algae

Concentrations of ammonia as low as  $0.027 \text{ mg NH}_3\text{-N L}^{-1}$  were found to be toxic to aquatic organisms (De L.G. et al., 1989). Methemoglobinemia in infants is the most well recognised health effect resulting from acute nitrite toxicity (Lee, 1970), as it is a fatal blood disorder also known as “blue baby syndrome”. Nitrate is primarily used to make fertiliser, although it is also used to make glass, explosives, and other chemical production and separation processes. Excess nitrate in the soil is most often found in rural and agricultural areas. Nitrate travels easily through the soil, carried by rain or irrigation water into groundwater basins. Nitrate pollution impedes the production of drinking water. During chlorination of drinking water; carcinogenic nitrosamines may be formed by the interaction of nitrite with compounds containing organic nitrogen.

Another problem, due to nitrogen discharge, is eutrophication. Eutrophication is defined as the overloading of natural water sources with nutrients, causing an accelerated growth

of algae and higher forms of plant life, which produce undesirable disturbances to the balance of organisms present in the water and also to the quality of the water system (Tett et al., 2007; Wang & Wang, 2009). Algal blooms affect the water system in two ways. Firstly, they hinder the penetration of sunlight, causing the death of underwater grasses. Secondly, the decomposition of dead algae causes depletion of oxygen, which is normally essential to most organisms living in water. Furthermore, the overload of nutrients and eutrophication make unpleasant conditions, such as odours, that affect the use of lakes and estuaries for leisure. The fundamental process to alleviate eutrophication and algal blooms is nutrient control (Wang & Wang, 2009).

### **2.3: Nitrogen Removal in Wastewater Treatment Plants**

It is extremely important to remove the maximum amounts of nitrogen possible during wastewater treatment because of its contribution to the eutrophication of receiving waters, the toxicity, and the direct threat it poses to aquatic life (Daims, et al., 2006). The nitrogen is mainly present in wastewaters in the form of ammonium ( $\text{NH}_4^+$ ) (van Hulle et al., 2010) and can be removed by physicochemical or biological processes (Zhang et al., 2008). Nowadays, there are several ways to reduce nitrogen content from wastewater. In this section, both the well-established and the innovative treatment technologies for nitrogen removal will be discussed.

Biological treatment to remove nitrogen from wastewater is less expensive and more effective than physicochemical treatments, and thus has been used more often to achieve nitrogen removal from domestic wastewaters (Khin & Annachhatre, 2004). However, in practice, the selection of either a biological or a physiochemical method is determined by the nitrogen concentration of the wastewater. Three ranges of nitrogen concentration in wastewater can be distinguished (Mulder, 2003):

- Diluted wastewater with ammonium concentration up to  $100 \text{ mg N L}^{-1}$  (e.g. domestic wastewater). In this range biological processes, such as activated sludge, are preferred processes based on cost-effectiveness issues.

- Concentrated wastewater with higher ammonium concentrations ranging from 100-5000 mg N L<sup>-1</sup> (i.e. leachate, reject water from dewatering of sludge, slurry from farms, and so forth) (van Hulle et al., 2010). After extensive investigations, the biological treatment nitrification–denitrification process over nitrite is used.
- Concentrated wastewater with ammonium concentrations higher than 5000 mg N L<sup>-1</sup>. In this range physicochemical methods are technically and economically feasible. The main physicochemical processes applied for ammonium removal are:
  - Air stripping
  - Breakpoint chlorination
  - Selective ion exchange

The conventional processes for biological removal of nitrogen are sequential nitrification and heterotrophic denitrification.

## **2.4: Conventional Nitrification/Denitrification**

Biological processes in wastewater treatment are mostly carried out by bacteria. Conventionally, the combined process of nitrification/denitrification is the most commonly used method for municipal wastewater treatment. It is a well-established treatment process based on a sequence of aerobic and anoxic conditions, where the ammonium is first oxidised to nitrate, in the presence of oxygen by nitrification, and then reduced using an organic carbon source as an electron donor during the denitrification process.

### **2.4.1: Nitrification**

Nitrification is a biological process which converts ammonia first to nitrite and then to nitrate. It is mediated by two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) (Terada et al., 2011). The nitrification process is carried out in two sequential

stages, namely the ammonium oxidation to nitrite, and the subsequent oxidation of nitrite to nitrate. The stoichiometry of these reactions is as follows:

1. Ammonia is oxidised to nitrite ( $\text{NO}_2^-$ ) by *Nitrosomonas spp.* bacteria:



These bacteria are also called Ammonium Oxidising Bacteria (AOB).

2. Nitrite is converted to nitrate by *Nitrobacter spp.* bacteria:



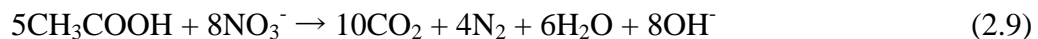
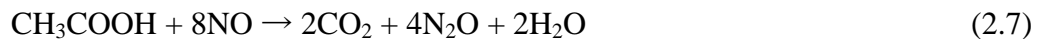
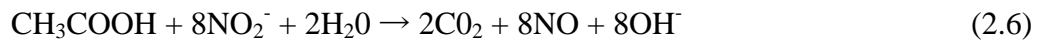
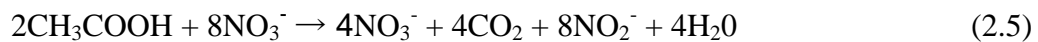
The first step of ammonia oxidation described in Eq. 2.1 is actually a net reaction of two reactions that occur within AOB. The first reaction (Eq. 2.3) involves the enzyme ammonium monooxygenase (AMO), which catalyses the oxidation of  $\text{NH}_4^+$  to hydroxylamine ( $\text{NH}_2\text{OH}$ ).



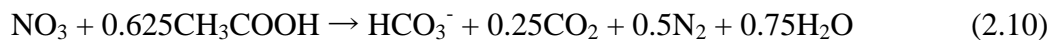
#### 2.4.2: Denitrification

In the denitrification process, the nitrate and/or nitrite present in the wastewaters is reduced to molecular nitrogen in anoxic conditions by the action of general heterotrophic bacteria (such as *Pseudomonas*, *Achromobater*, *Alcaligenes*, *Thiobacillus*, *Bacillus*). The process requires the presence of a source of organic carbon as an electron donor, for example acetic acid or methanol, and nitrate acts as the last electron acceptor in the respiratory chain substituting the  $\text{O}_2$  molecule. Denitrifying bacteria are facultative

organisms that can use either dissolved oxygen or nitrates as a source for metabolism and oxidation of organic matter. In the case of simultaneous presence of dissolved oxygen and nitrates, denitrifying bacteria preferentially use oxygen because the energy generated per unit weight of organic matter metabolised, is higher. Therefore, it is important to keep dissolved oxygen as low as possible (less than 0.3-0.5 mg/L), at least in the microenvironment surrounding the bacteria. The reduction is carried out by subsequent steps through different oxidation states of nitrogen (Eq. 2.5 to 2.8) and the universal stoichiometry with acetic acid as organic carbon source is represented in Eq. 2.9.



Rewriting Eq. 2.9, taking into account the equilibrium of  $\text{CO}_2$ , gives Eq. 2.10.



From the stoichiometry, it can be inferred that the denitrification process creates an increase in the medium alkalinity and that 40% of the organic matter needed is used to reduce nitrate to nitrite.

The main factors which might affect the efficiency of denitrification are:

- DO - as dissolved oxygen increases, denitrification rates decrease, therefore anoxic condition should be maintained.
- Presence of organic matter - the source of available carbon can influence the denitrification rate. The highest rate can be achieved by adding an easily biodegradable and assimilated carbon source, though this may imply costs for its purchase. The highest removal rates occur with the use of effluent from distillery and food industries.
- pH and alkalinity - the optimum pH is between 7.5 and 9.1, but denitrification can occur also at pH between 6 and 7.5. Alkalinity is produced during the process (about 3-3.5 g of alkalinity as  $\text{CaCO}_3$  for each gram of  $\text{NO}_3^-$  reduced).
- Temperature - it affects the growth rate of denitrifying organisms, with greater growth rate at higher temperatures. Denitrification can occur between 5 and 30°C.
- Heavy metals and organic compounds - denitrifying organisms are generally less sensitive to toxic chemicals than nitrifiers, and recover from toxic shock loads quicker than nitrifiers.

In wastewater treatment works, nitrification/denitrification can be performed through the following:

- Suspended-growth biomass processes
  - Conventional activated sludge
  - Sequencing batch reactors-SBR
- Attached-growth systems
  - Trickling filters
  - Rotating biological contactors-RBC

In suspended-growth biomass processes, several schemes and configurations can be adopted. The main distinction is the choice between the following two configurations:

- Separate system configuration, in which nitrification and denitrification are carried out in series (post-denitrification) and in distinct stages with their own clarifier and sludge recycling system. The costs are higher as two clarifiers are needed.
- Combined system configuration, in which biomasses are mixed in a single activated sludge.

If denitrification is carried out after the nitrification (i.e. “post-denitrification”), an external carbon source is usually required, unless other configurations are adopted, as for instance, a post-denitrification with by-pass of part of the incoming wastewater to the anoxic tank where denitrification takes place.

A common scheme in municipal wastewater treatment works is “pre-denitrification”, which provides a denitrification stage followed by a nitrification-oxidation stage with oxidation of organic material and ammonia. The recirculation of nitrates provides the nitrates to the anoxic tank. This configuration requires recirculation ratios up to 4-5 times the inlet flow.

## **2.5: Innovative and Sustainable Technologies for Biological Nitrogen Removal**

The conventional biological nitrogen removal processes are generally used for treating wastewaters with quite low nitrogen concentrations (concentrations of less than 100 mg N.L<sup>-1</sup>) (van Hulle et al., 2010). Some wastewater streams consist of high concentrations of nitrogen, mostly in the form of ammonium. If these streams are returned back to the inlet of the municipal WWTW, they increase the ammonium loading in the mainstream. The conventional biological nitrogen removal process (denitrification-nitrification) is uneconomical and complicated when treating wastewaters containing high nitrogen with low C/N ratio. During the last decade, several new sustainable and cost-effective alternatives have been discovered and studied, and their implementation can be a valid option to treat strong nitrogenous wastewaters characterised by high ammonium



concentrations and low biodegradable organic matter content. Currently, the anammox process is considered to have potential for treating wastewaters with high ammonium concentrations (He et al., 2007; Ma et al., 2011).

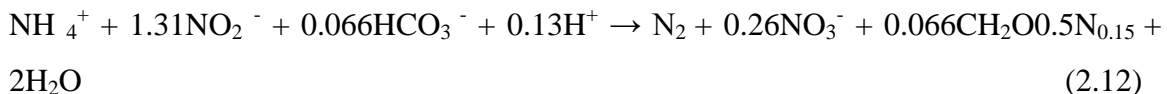
## 2.6: The Anammox Process

### 2.6.1: Discovery and stoichiometry

The anammox process is novel and promising alternative nitrogen removing technique in which ammonium is directly oxidised to dinitrogen gas using nitrite as the electron acceptor under anoxic conditions (Strous et al., 1997b). Until the 1990's, ammonium oxidation was thought to only take place under aerobic conditions (Fux et al., 2002; Jetten et al., 2001). Over two decades ago, Broda predicted the existence of chemolithoautotrophic bacteria capable of oxidising ammonium using nitrite as the electron acceptor on the basis of thermo-dynamic calculations. (Broda, 1977). The predictions were experimentally corroborated by Mulder two decades later in a denitrifying fluidised-bed reactor treating effluent from a yeast plant. That reactor removed an ammonium loading rate of  $0.4 \text{ g NH}_4^+\text{-N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  by this process (Mulder et al., 1995). By nitrogen balances, they hypothesised the following stoichiometry and called the process anammox:



Strous *et al* (1998) optimised the process conditions and found the global equation of the process, based on mass balance over anammox enriched cultures:



Based on phylogenetic analysis, the first discovered anammox organism branched deep into the Planctomycetes phylum and was named *Candidatus Brocadia anammoxidans*

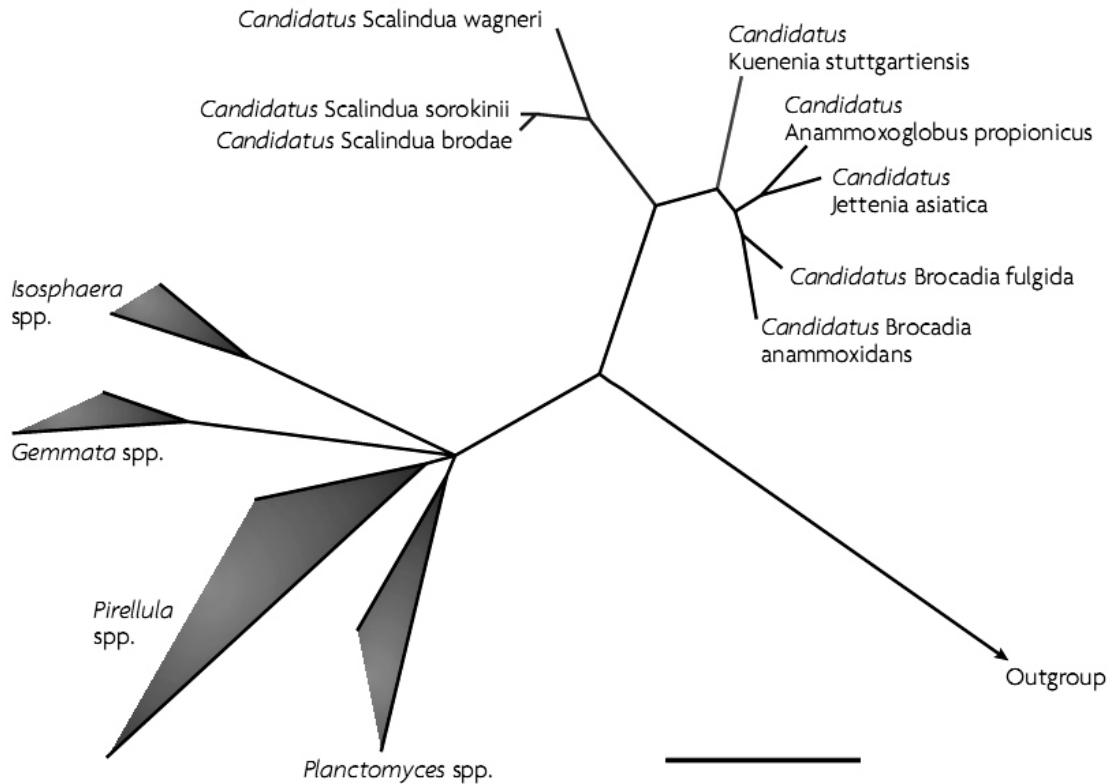
(Kuenen & Jetten, 2001). In follow up studies, the biological nature of the anammox process and the responsible microorganisms were investigated in great detail (Strous et al., 1999). However, the anammox pure cultures have not yet been isolated, but the cultures could be enriched and further purified on selective gradients to sufficient purity (99%) to characterise it (Jetten et al., 2001).

### **2.6.2: Biochemistry of anammox**

Hooper et al., (1997) studied the biochemistry of anammox species *Nitrosomonas europaea* focusing on the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase (HAO). From these studies, a basic knowledge about the metabolic pathway of anaerobic ammonium oxidation in *Candidatus Brocadia anammoxidans*, was obtained. (Van de Graaf et al., 1997) performed a series of experiments with markers to investigate the reaction mechanisms, and possible intermediates and products. The experiments showed that ammonium and nitrite were combined to yield dinitrogen gas. In batch experiments with excess hydroxylamine and ammonium, a transient accumulation of hydrazine was observed, indicating that hydrazine is an important intermediate of the anammox process. The oxidation of hydrazine to dinitrogen gas is known to be mediated by the hydroxylamine oxidoreductase (HAO) enzyme of the aerobic ammonium-oxidising bacterium *Nitrosomonas europaea* (Hooper et al., 1997). In *Candidatus Brocadia anammoxidans* cell extracts, a high HAO activity was observed, indicating that a similar enzyme might be functioning in the anammox mechanism (Schalk et al., 2000). A new type of HAO enzyme with unique amino acid sequences was purified from *Candidatus Brocadia anammoxidans* to homogeneity. This enzyme was also able to catalyse the oxidation of both hydroxylamine and hydrazine and was found to be present only inside a membrane bounded region called ‘anammoxosome’ (Jetten et al., 2002).

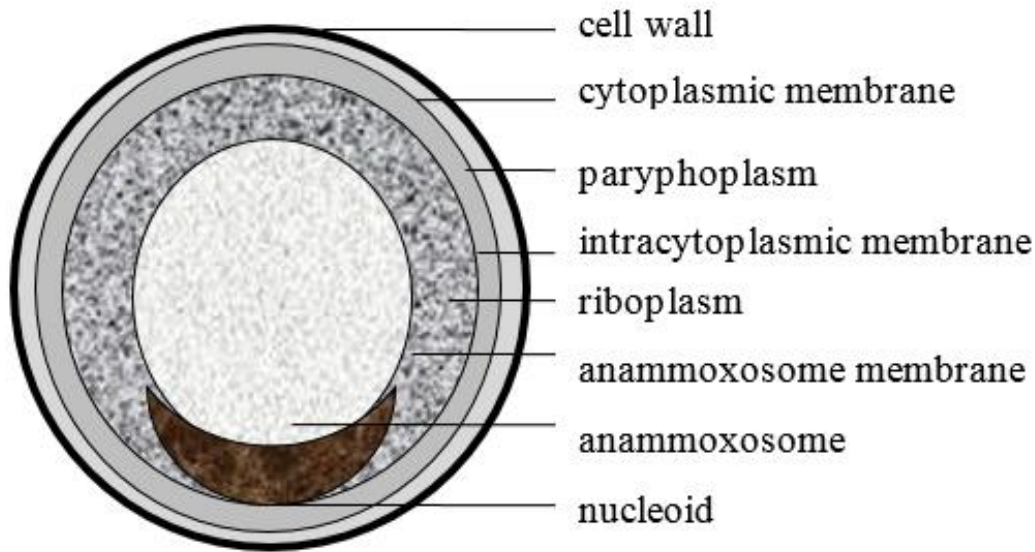
### 2.6.3: Microbiology of anammox

Ever since the anammox process was discovered in Delft and the first anammox organism, *Candidatus Brocadia anammoxidans* was identified, many more of studies have reported the presence of anammox in natural environments such as marine sediments ( Dalsgaard et al., 2003; Kuypers et al., 2003; Thamdrup & Dalsgaard, 2002), marine sponges (Mohamed et al., 2009), estuarine/tidal river sediments (Dale, et al.,g, 2009; Meyer, et al., 2005; Rysgaard et al., 2004; Trimmer, et al., 2003), deep-sea hydrothermal vents (Byrne et al., 2008), hot springs (Jaeschke et al., 2009), and some freshwater ecosystems (Hamersley et al., 2009; Penton, Devol & Tiedje1, 2006; Schubert et al., 2006; Zhang et al., 2007). Thereby, new species other than *Candidatus Brocadia anammoxidans*, were discovered and identified and their 16S rRNA were determined. These were *Candidatus Kuenenia stuttgartiensis*, *Candidatus Scalindua sorokinii*, *Candidatus Scalindua brodae*, *Candidatus Scalindua wagneri*, *Candidatus Brocadia fulgida* and *Candidatus Anammoxoglobuspropionicus* (Kartal et al., 2007; Kuypers et al., 2003; Schmid et al., 2000., 2003). The anammox organisms are similar to each other in their 16S rRNA sequences phylogenetic analyses, which show that they form a monophyletic branch, which consists of five distinct genera with about 90% sequence similarity to each other, within the phylum Planctomycetes. Figure 2.2 shows the relationships between the different families of anammox bacteria and the scale bar represents 10% sequence divergence (Kuenen., 2008). All the species within the order of Planctomycetales lack the peptidoglycan, a virtually universal polymer, found within the Bacteria domain. Instead, they consist of protein as the major constituent of their cell walls. This lack of peptidoglycan is a characteristic shared only with the Chlamydiae and the cell-wall-free mycoplasma within the Bacteria domain, (Lindsay et al., 2001).



**Figure 2.2: 16s ribosomal RNA phylogenetic tree of Anammox bacteria. It shows the relationships of the different families of Anammox bacteria. The scale bar represents 10% sequence divergence (Source: Kuenen, 2008).**

Another structural characteristic of anammox bacteria is the presence of an organelle called anammoxosome (Figure 2.3) which occupies more than 30% of the cell volume (Niftrik et al., 2006). It has been found that the enzyme hydrazine oxidoreductase, which is responsible for the oxidation of the intermediate hydrazine, is present exclusively inside the anammoxosome. Furthermore, this compartment is surrounded by a membrane nearly entirely composed of unique ladderane lipids (Damsté et al., 2002; Niftrik et al., 2006).



**Figure 2.3: Cellular compartmentalization in anammox bacteria (according to Niftrik et al., 2006).**

## 2.7: Applications of Anammox

The discovery and potential contribution of anammox bacteria to  $N_2$  production is important because it may dictate a re-evaluation of nitrogen transformation processes in the global nitrogen budget (Terada, et al., 2011). Some researchers from marine ecosystems suggest that anammox may actually be the major process causing most nitrogen loss in anoxic marine water columns (Dalsgaard et al., 2003, Kuypers et al., 2003). The anammox process has an advantage of low operational costs and has attracted much attention since it was discovered. Anammox has been feasible in both natural and man-made ecosystems.

In particular, ecosystems with low dissolved oxygen concentrations in surface water and the availability of both ammonium and nitrite/nitrate, are considered as prime environments for the anammox process (Terada, et al., 2011). Several studies have been conducted on a variety of ammonium-rich wastewaters (Table 2.1) (Zhang et al., 2008). Degradation of organic matter can generate considerable amounts (mM range) of

ammonium in natural anoxic environments, such as marine sediments (Kuypers et al., 2003, Trimmer, et al., 2003). In the case of water columns and marine ecosystems, nitrate reducing bacteria are the most likely source of nitrite (Dalsgaard et al., 2003, Kuypers et al., 2003). Nitrite can also be produced by aerobic ammonium oxidising bacteria operating at the oxic-anoxic interface of many ecosystems (Schmid et al., 2000). Furthermore, wastewaters from anaerobic treatment of animal waste, known to contain high organic nitrogen content, were also examined for anammox activity (Ahn & Kim, 2004, Hwang et al., 2005, Waki et al., 2007). During anaerobic digestion, ammonium is increased considerably mainly due to protein decomposition.

## **2.8: Factors Affecting the Anammox Process**

### **2.8.1: Effect of temperature**

It has been a common tendency that the anammox process be conducted at relatively high water temperature (i.e., 30-37 °C) (Egli et al., 2001; Jetten et al., 2001; Kartal et al., 2007b; Strous et al., 1997), since the optimal temperature of anammox bacteria obtained from wastewater sludge has been regarded to be around 35 °C. A number of researchers have examined the anammox process at different temperatures (Osaka et al., 2012; Vazquez-Padín et al., 2011; Yang et al., 2011). Isaka, et al., (2007) have reported that they could efficiently operate an anammox reactor at 20-22 °C with a (Strous et al., 1997) relatively high biomass concentration of 20 g VSS L<sup>-1</sup>. From their data, a stable anammox activity about 0.4 g N (g VSSd)<sup>-1</sup> could be estimated. In 2007 Cema and coworkers studied the anammox process at 20 °C in a successfully operating RBC (Rotating Biological Contactor). Toh, et al., (2002) tried to select and enrich an autotrophic anaerobic ammonium oxidation consortium from sludge collected in a municipal treatment plant in Sydney, at two different temperatures, 37 °C and 55 °C. Whereas anammox activities could be successfully detected at mesophilic conditions in batch and continuous cultures, thermophilic anammox organisms could not be cultivated at 55 °C. However, it has recently been observed that the anammox process also occurs at higher temperatures of up to 52 °C in hot springs (Jaeschke et al., 2009) and even at 85 °C in

hydrothermal vents (Byrne et al., 2008). If sufficient anammox activity can be maintained at moderately low temperatures ( $<25\text{ }^{\circ}\text{C}$ ), the anammox process would be applied to a wider range of industrial wastewater than those currently used (Osaka et al., 2012).

### **2.8.2: Effect of pH**

The anammox process was found to occur within a pH range of 6.7 to 8.3, with an optimum at pH 8 (Egli et al., 2001; Jetten, 1999). In contrast, when (Ahn, et al., 2004) was studying the combination of anaerobic digestion and anammox in the same reactor, for the treatment of piggery waste, the pH of the effluent was about 9.3-9.5. They used a lab-scale up-flow anaerobic sludge bed reactor under mesophilic conditions and supplemented the raw piggery waste with nitrite, and the anammox activity in the reactor was not inhibited in spite of the pH values which were out of the ranges reported by (Egli et al., 2001, Jetten et al., 1999).

**Table 2.1: Anammox application to different wastewaters**

Wastewater	Process	NRR (kg N/(m <sup>3</sup> ·d))	Start-up (days)	Scale of reactor	References
Sludge liquor	Partial nitrification-anammox	0.71	110	10 L	van Dongen et al., 2001
Sludge supernatant	Partial nitrification-anammox	2.4	150	2.5 m <sup>3</sup>	Fux et al., 2002
Partially nitrified sludge digestate	Anammox	3.5		3.5 L	Fux et al., 2004
Sludge digestate	Partial nitrification-anammox	9.5	1250	70 m <sup>3</sup>	van der Star et al., 2007
Slaughterhouse wastewater	Nitrification-denitrification	0.031		790 mL+745 mL	Reginatto et al., 2005
Piggery wastewater	Anammox	0.6		1.5 L	Ahn and Kim, 2004
	Partial nitrification-anammox	1.36	~60	1 L	Hwang et al., 2005
Synthetic coke-oven wastewater	Anammox	0.062	~465	1 L	Toh, et al., 2002
Monosodium glutamate wastewater	Anammox	0.46	71	5 L	Chen et al., 2007



### 2.8.3: Effect of oxygen

To fully understand and operate the anammox process, it is important to know the effect of oxygen on the system, because customary anammox is combined with a preceding partial-nitrification step. Strous et al., (1997) confirmed that oxygen reversibly inhibits the anammox process as they did not observe anammox activity in microaerobic conditions (2.0, 1.0 and 0.5% of air saturation). However, Sliekers et al., (2002) and Sliekers et al., (2006) operated an anammox SBR and a gaslift reactor under oxygen-limited conditions and, although the maximum anammox activity decreased, a stable coexistence of anammox and aerobic ammonium oxidisers allowed a completely autotrophic removal of nitrogen in both systems. In this case, the existence of anammox in presence of oxygen is possible because it is consumed by the nitrifying organisms.

### 2.8.4: Effect of substrate and product concentrations

A wide variety of substances have been reported to inhibit the anammox activity. These include substrates (ammonia and nitrite), organic matters (including the toxic and nontoxic organic matter), salinity, heavy metals, phosphate and sulfide (Jin et al., 2012). The inhibitory effects of these anammox inhibitors are related to the anammox species, exposure dosage and operating conditions of the anammox process. Anammox activity is irreversibly inhibited at nitrite concentration higher than 20 mM (Jetten et al., 2001). (Fux et al., 2002) also reported an inhibition of the anammox process in a pilot plant fed with the effluent of a Sharon reactor that treated a sludge digester effluent. In this study, the organisms (*Candidatus Kuenenia stuttgartiensis*) were strongly inhibited by a nitrite concentration of 60 mg  $\text{NO}_2^-$ -N  $\text{L}^{-1}$ . The activity was slowly restored two weeks after the influent nitrogen load was reduced to nearly 50% of the initial value. Batch-scale experimental studies on the effects of nitrite inhibition on anammox bacteria showed a short-term inhibition, with more than 25% maximum nitrite removal rate decrease at concentrations higher than 60 mg  $\text{NO}_2^-$ -NL<sup>-1</sup> and losses of activity were detected with nitrite concentrations higher than 30 mg  $\text{NO}_2^-$ -NL<sup>-1</sup> (Bettazzi et al., 2010). On the contrary, (Egli et al., 2001) showed that *Candidatus Kuenenia stuttgartiensis* has a relatively important tolerance to nitrite concentrations up to 180 mg  $\text{NO}_2^-$ -N  $\text{L}^{-1}$ . A more

recent work studied the short-term inhibitory effects of both substrates and nitrate on the anammox activity (Dapena-Mora et al., 2007).

### **2.8.5: Effect of shear stress**

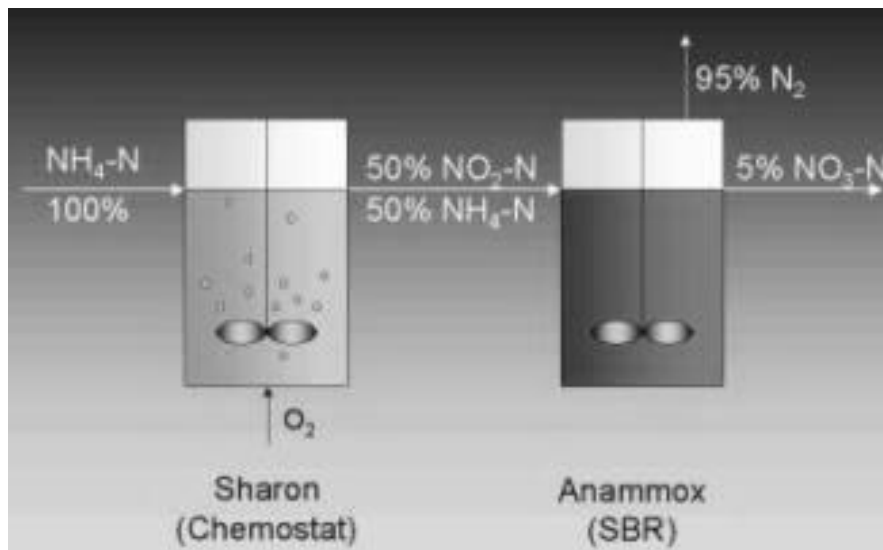
In 2006, Arrojo and coworkers reported that shear stress can cause a decrease in the activity of anammox biomass. According to Arrojo et al (2006), despite the fact that anammox sludge is highly resistant to mechanical stress, it would be not advisable to operate a SBR with specific mechanical stirring power higher than  $0.09 \text{ kW m}^{-3}$ . Moreover, Arrojo et al., (2008) found that anammox biomass is less resistant to shear stress caused by gas-flow mixing. In this case, these authors advise not to operate with specific input power higher than  $0.017 \text{ kW m}^{-3}$ .

## **2.9: Anammox Technologies**

### **2.9.1: Two stages configuration**

Since the anammox process was discovered, it has been used for nitrogen removal in a few incidents in wastewater treatment works (WWTWs) though this application is still considered experimental. The application of the anammox process, combined with nitrification specifically, contributes to the ammonium removal in wastewater systems. Partial nitrification and the anammox processes can be carried out in two different units operated in series. When the partial nitrification and the anammox processes are combined, there are certain factors that need to be taken into account. For instance, looking at the stoichiometry (Equation. 2.12) it can be presumed that anammox bacteria need ammonium and nitrite in a ratio of approximately 1:1.3. To achieve this, half of the ammonium in the wastewater needs to be converted into nitrite by AOB. Therefore, in a two stage configuration, the first reactor is operated under aerobic conditions in order to convert approximately half of the ammonium in the influent into nitrite. The most common system to reach partial nitrification is the Sharon reactor (Hellings, et al., 1999). The second reactor is the anammox anoxic reactor where autotrophic denitrification occurs. This combined process that involves partial nitrification, where half of the ammonium is oxidised to nitrite, followed by anammox, where

the remaining ammonium together with the produced nitrite is converted to  $N_2$ , is mainly called SHARON-Anammox (van Dongen, et al., 2001) (Figure 2.4).



**Figure 2.4: The Sharon-Anammox combined system**

In the Sharon-Anammox process nitrification is carried out in a sequencing batch reactor (SBR) operated at relatively high temperature and controlled SRT. Gali et al., (2007) performed a comparative study to produce the correct influent for the anammox process from anaerobic sludge reject water. They demonstrated that both systems (SBR and Sharon chemostat) were able to achieve the same specific conversion rate ( $40 \text{ mg } NH_4^+-N \text{ (g VSS h)}^{-1}$ ), but the SBR achieved a higher value of absolute nitrogen removal ( $1.1 \text{ g N (L d)}^{-1}$  versus  $0.35 \text{ g N (L d)}^{-1}$ ), due to the different HRT used. The Sharon process showed, however, a better stability. One more recent option is the use of aerobic granules in order to perform the conversion of half of the ammonium into nitrite (Vázquez-Padín et al., 2009). This work reported stable partial nitrification operating a granular SBR at room temperature. The average size of the developed granules was about 3 mm and the reactor was operated along one year. In this case, the two key aspects to obtain partial nitrification were the oxygen diffusion limitation into the granule and the controlled dissolved oxygen concentration in the bulk liquid ( $2.0\text{-}3.5 \text{ mg } O_2 \text{ L}^{-1}$ ). The treatment after partial nitrification, i.e. the anammox step, can be carried out in different reactors like UASB (Upflow Anaerobic Sludge Blanket), similar to the ones used in the anaerobic digestion processes, such as gas-lift, continuous stirred tanks, and so forth. At laboratory scale, the SBR is widely used due to its flexibility of

operation and easy control. (Dapena-Mora et al., 2004) showed that the SBR is a suitable system to grow and enrich anammox biomass in the form of granular sludge.

### **2.9.2: One stage configuration**

Partial nitrification and the anammox processes can also be carried out together in a single unit. This technology has been named with different names such as CANON (Completely Autotrophic Nitrogen removal Over Nitrite (Sliemers et al., 2002; Third et al., 2001), OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) (Kuai & Verstraete, 1998; Philips et al., 2002), and deammonification (Helmer-Madhok et al., 2002; Hippen et al., 1997). In these processes, the aerobic ammonium oxidising bacteria (nitrifiers) with anammox bacteria simultaneously perform a two-step reaction under oxygen-limited conditions. The main difference among them is that the CANON process employs suspended biomass growing in a mixed reaction medium, while the OLAND and deammonification are biofilm processes, thus the biomass is growing on biodiscs or on moving plastic carriers, like Kaldnes rings. The deammonification process is based on the pleasant co-existence and cooperation of aerobic (AOB) and anaerobic ammonium-oxidising (anammox) bacteria in one single reactor. This can be established under oxygen-limited conditions to avoid the inhibition of anammox bacteria by oxygen and to achieve appropriated conditions to obtain partial nitrification. In practice, the main systems that can provide the favourable microaerobic conditions for the co-existence of these two bacteria species are the biofilm system (moving bed biofilm reactors, MBBR), reactors with an intermitted aeration (SBR or RBC), or granular sludge. The Deammonification Moving Bed Biofilm Reactor was developed at full scale in biofilms grown on Kaldnes rings in Hattingen (Rosenwinkel & Cornelius, 2005). Dissolved oxygen concentration is the main operational variable to obtain a stable performance of the system. Diffusional limitations allow oxygen to be completely consumed in the outer layer of the granule or biofilm, the inner part being anoxic. Therefore, partial nitrification is carried out in the external part while anammox bacteria will be growing in the inner layers.

## 2.10: Anammox detection

The discovery of anammox reactions in natural environments and expansion in anammox-related technologies have led to the development of various methods concentrating on detecting anammox bacterial cells/genome (Terada, et al., 2011). For the starting up of the anammox process, it is necessary to employ the analytical methods that detect the presence of anammox microorganisms even in low concentrations. For this purpose the following methods can be used:

- **Fluorescence In Situ Hybridisation (FISH).**

FISH relies on DNA/RNA hybridisations occurring within whole microbial cells in situ. In situ hybridisations with DNA oligonucleotides designed for the detection of specific bacteria are performed with fluorescent-labelled compounds. Currently, FISH is well known as a powerful diagnostic tool with widespread environmental and medical applications. It is a rather fast method, but might not be sensitive enough to detect anammox organisms if they appear in relatively low numbers (below 1000-10,000 cells·mL<sup>-1</sup>) or if the sample is highly autofluorescent. As more anammox microorganisms are discovered, the designed probes to detect these microorganisms increase, permitting the detection of all anammox bacteria or to distinguish between the existing different types (Schmid et al., 2005).

- **The PCR (Polymerase Chain Reaction).**

To detect anammox bacteria in environmental samples, PCR amplification with general 16S rRNA gene-targeted primers and subsequent quantitative or phylogenetic analyses are a central method in common use. The 16S rRNA, or functional gene-based approach without cultivation, is a very powerful technique when anammox bacteria have low activity or samples contain a lot of inert particulates, both of which hamper the application of the fluorescence in situ hybridisation (FISH) method. It enables the detection of anammox organisms down to the genus level even faster and with higher sensitivity than the usual FISH approach (Schmid et al., 2005).

- **The conversion of hydroxylamine to hydrazine**

This is a unique reaction of the anammox process (van de Graaf et al., 1997) that can be used specifically to detect anammox activity in environmental samples (Schmid et al., 2003).

Much more sensitive are tracer experiments with  $^{15}\text{N}$  labelled ammonium. Under anoxic conditions, labelled  $^{15}\text{N}$ -ammonium reacts uniquely, in a 1:1 ratio with unlabelled  $^{14}\text{N}$ -nitrite, to  $^{29}\text{N}_2$  ( $^{14}\text{N}^{15}\text{N}$ ) via the anammox reaction. This method was successfully used to assess the contribution of anammox to the nitrogen conversion in marine and estuarine environments where the cell count of anammox was low (Dalsgaard et al., 2003; Kuypers et al., 2003, Thamdrup & Dalsgaard, 2002; Trimmer et al.; 2003).

Anammox bacteria have lipids with unique properties (Damsté et al., 2002; van Niftrik et al., 2004) that can be used as biomarkers for the presence of these cells in an environmental sample (Kuypers et al., 2003, Schmid et al., 2003).

## **2.11: Characterisation of Total Microbial Population**

Bacteria are key players in nutrient removal and comprise a considerable measure of the genetic diversity on Earth (Whitman, et al., 1998). Different microbial diversity metabolisms such as photoautotrophic and chemoheterotrophic have permitted bacteria to flourish as both free-living and interdependent organisms. Measuring biodiversity includes characterising the number, composition and variation in taxonomic or functional units over a wide range of biological organisations. There are some difficulties in choosing the most suitable unit to measure microbial diversity. Microbial diversity was originally studied through microscopy, and culture on specific liquid and solid media. Performing this type of classification required tedious inspections that relied entirely on morphological characteristics, and bacterial diversity was evaluated mainly by morphotype description of the colony they would form on specific media. Although bacteria are naturally abundant, microbiologists estimated that only 1% of the bacteria counted under the microscope could be cultured on solid or in liquid media, and called this discrepancy the ‘Great plate count anomaly’ (Staley & Konopka 1985). Therefore, the traditional techniques of isolation and cultivation limit the study of bacteria diversity to those that can be cultivated in laboratory media. Due to the limitations of

conventional microbiological methods that are laborious and time consuming (Jasson et al., 2010), different culture-independent methods have been developed to match the conventional microbiology and to acquire a more detailed and precise structure of the total microbial population in the product under survey (Osés et al., 2013).

Various culture-independent molecular methods have been developed to detect many bacterial populations and they provide a more accurate system of taxonomy. The use of molecular techniques in microbial ecology has made it possible for the discovery of several new microorganisms that were previously unknown. Most investigations were mainly based on PCR using specific primers for certain genes in a particular bacterial species. The 16S rRNA is the gene that is normally selected as the target gene because it comprises both variable and conserved regions, permitting the use of primers to conserved regions and more specific primers to amplify 16S rRNA genes from any source to distinguish between various taxa (Kawamura & Kamiya, 2012). However, since universal primers bind to the same conserved area of the 16S rRNA target population, PCR competitive inhibition can occur, in which the DNA of the prevalent bacterial species is much more likely to be amplified than DNA from bacteria that form a negligible amount of the overall mixed population. This method could be biased towards limited common pathogens (Kuang et al., 2009) and also lead to the complete omission of the DNA of minority bacterial species from analysis (Kawamura & Kamiya, 2012). Moreover, the entire bacterial population at a given time cannot be determined by this type of analysis.

Improvements in molecular biology used methods such as fingerprinting methods, which separate rDNA fragments according to their length and/or their nucleotide composition, i.e. PCR-RFLP (restriction fragment length polymorphism) and T-RFLP (terminal restriction fragment length polymorphism analysis). Later on, DGGE (denaturing gradient gel electrophoresis) analysis, which separates amplified partial 16S rDNA fragments of each bacteria based on differences in the GC content and distribution in each fragment, was developed and has been widely used to evaluate the overview of the microbial population in several environmental samples. The FISH (fluorescence in situ hybridisation) approach has also been used. The Sanger sequencing method by cloning 16S rRNA gene fragments, and subsequently sequencing the clones, is also another method for identifying population diversity. However, cloning/sequencing and FISH are not directly compatible with high-

throughput approaches. The most recent and powerful method, metagenomic analysis, has also been applied for the analysis of microbiota. The pursuit to describe microbial communities has now reached a new stage with the development of next-generation sequencing techniques (NGS), leading towards a high-throughput description of the microbial world at a higher level of detail than cloning or sequencing. This method potentially allows for the complete analysis of the entire diversity of the microbial population.



## CHAPTER 3: MATERIALS AND METHODS

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### 3.1: Growth Media

#### 3.1.1: Mineral salt media

Mineral salt medium (MSM) was prepared by dissolving 1.25 g of  $\text{KHCO}_3$ , 0.05 g of  $\text{NaH}_2\text{PO}_4$ , 0.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.006 g of  $\text{FeSO}_4$ , 0.006 g of EDTA in 1L of deionised water ( $\text{dH}_2\text{O}$ ) (Jetten et al., 2005). 1.25 mL of trace elements solution was added per litre of mineral salt solution. The trace elements solution contained (per litre deionised water) 0.4 g of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1 g of KI, 0.2 g of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.4 g of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.2 g of  $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ , 0.4 of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g of NaCl, 0.1 g of  $\text{CoSO}_4$ , 0.1g of  $\text{CaCl}_2$ , 0.01g of  $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  and 0.05 g of  $\text{H}_3\text{BO}_3$ . MSM was supplemented with ammonium and nitrite (at required concentrations) in the form of  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaNO}_2$ , respectively. The prepared medium was sterilised before use by autoclaving at  $121^\circ\text{C}$  at  $115 \text{ kg}\cdot\text{cm}^{-2}$  for 15 minutes.

### 3.2: Reagents

#### 3.2.1: Chemicals

Chemicals used in the preparation of mineral salt medium i.e. potassium bicarbonate, sodium dihydrogen phosphate, magnesium sulphate heptahydrate, calcium chloride dihydrate, ferrous sulphate, ethylenediaminetetraacetic acid, zinc sulphate heptahydrate, copper (II) sulphate pentahydrate, copper chloride, sodium bromide, sodium molybdate, potassium iodide, iron (III) chloride hexahydrate, manganese (II) sulphate monohydrate, sodium molybdate dehydrate, zinc sulphate heptahydrate, sodium chloride, cobalt (II) sulphate, calcium chloride, aluminum potassium sulphate dodecahydrate, and boric acid were all purchased from Merck (Johannesburg, SA). Sulfamic acid, salicylic acid, ortho-phthalaldehyde,  $\beta$ -mercaptoethanol, sulfanilic acid, n-naphthylethylenediamine, sulphuric acid, and phosphoric acid for analysis were purchased from Sigma Aldrich (Johannesburg, SA).

### **3.3: Bacterial cultures**

#### **3.3.1: Collection of sludge and wastewater samples**

Wastewater samples were collected from three wastewater treatment works in Pretoria namely:

- Daspoort wastewater treatment works
- Baviaanspoort wastewater treatment works
- Zeekoegat wastewater treatment works

From Zeekoegat and Baviaanspoort, the sludge samples were collected from both anoxic and anaerobic zones of the activated sludge system. This was done by lowering a sampling jug with an extended handle, of about 1-1.5 m in length, into the reactor. The sludge samples were then transferred to 1 L plastic containers. From Daspoort, the sludge samples were taken from an anaerobic digester from the middle (anoxic) and the bottom (anaerobic) part of the digester. This was done by opening the valve in the middle and bottom part of the digester and collecting the samples into plastic containers. At Daspoort, the samples were also taken from the trickling filter by taking stones covered with biofilm and placing them into plastic sampling containers. The length from which the stones were taken was not measured but they were only sampled based on the attached growth. All the samples were stored in a room temperature and cultured within 24 hours. The remaining samples were stored at 4 °C for future reference.

#### **3.3.2: Wastewater treatment works description**

##### **3.3.2.1: Daspoort wastewater treatment works**

Daspoort wastewater treatment works (DWWTW) consists of two treatment plants i.e. the older “Eastern” Works, and the newer “Western” Works respectively. The Eastern Works is a trickling filter (TF) plant that consists of two sets of TF systems, Modules 1-4 and 5-6 respectively. On the other hand, the Western Works is a conventional activated sludge system which consists of biological nutrient removal (BNR) systems Modules 9-1. DWWTW

receives raw wastewater from the outfall sewer which collects wastewater from the Central Pretoria area. The influent wastewater received by both plants is subject to mechanical screening, grit removal and primary settling in Dortmund type vertical flow settling tanks.

### **3.3.2.2: Zeekoegat wastewater treatment works**

Zeekoegat wastewater treatment works (ZWWTW) is designed for the capacity of 35 MLD. The raw influent undergoes primary treatment i.e. coarse and fine screening, grit removal and primary settling in four primary settling tanks. This is followed by return activated sludge process with two identical biological reactors which are operated as modified University of Cape Town (UCT) process for nutrient removal. The mixed liquor is then diverted to four clarifiers. The overflow of the clarifiers is disinfected by chlorine gas before entering the filtration unit. The influent is released into the pond to allow for sufficient chlorine contact time. The final effluent is discharged into Lake Roodeplaat via an artificial wetland.

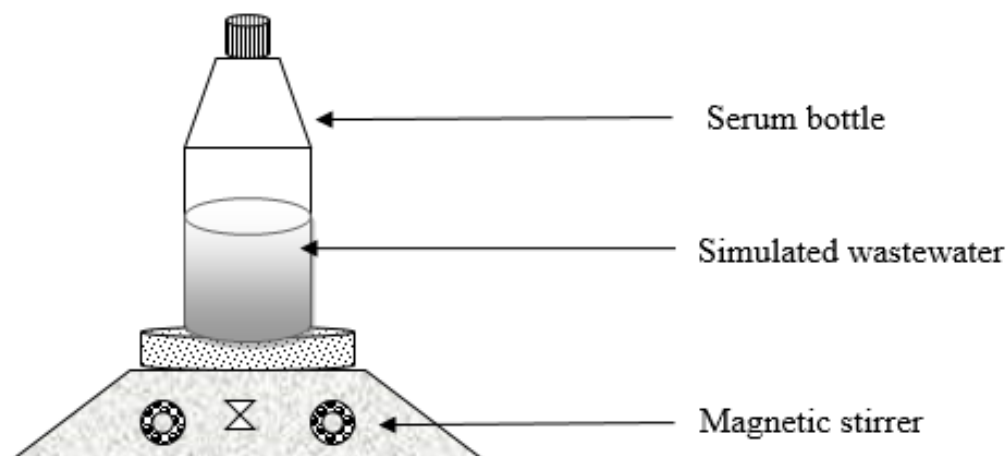
### **3.3.2.3: Baviaanspoort wastewater treatment works**

Baviaanspoort wastewater treatment works (BWWTW) is among the first BNR reactors in South Africa and was constructed in 1977. It has the capacity of 35 MLD. It consists of the primary stage, similar to that of Zeekoegat, but lacks the division tank. It also consists of four biological nutrient removal activated sludge (BNRAS) modules with a total capacity of  $62 \text{ m}^3 \text{d}^{-1}$ . Based on the TKN/COD ratio of the influent, the best suited process configuration was found to be the UCT configuration. However, as the raw influent sewage composition differs, the configuration is also changed to a different configuration that best suits the wastewater composition. The reactors consist of the mechanical aeration system. The final effluent is also discharged into Lake Roodeplaat.

### **3.3.3: Enrichment of anammox from municipal waste sludge**

#### **3.3.3.1: Batch reactor experiments**

A series of batch reactors were set up and used for cultivation and enrichment of anammox bacteria. Each reactor was a 500mL serum bottle containing 300mL of mineral salt medium (MSM). Each reactor was inoculated with 20% of different activated sludge samples from different wastewater treatment works. The sludge samples were collected from Daspoort (anaerobic digester), Zeekoegat and Baviaanspoort wastewater treatment works, from anoxic and anaerobic zones of the activated sludge system. Apart from the samples collected from the two mentioned WWTW, trickling filter samples from Daspoort wastewater treatment works were also included in the primary enrichment experiments. To get rid of oxygen from the reactors and create anaerobic conditions, the reactors were purged with argon gas for 15-20 minutes before being sealed with rubber septa and aluminium crimp seals. The reactors were then shaken on a rotary shaker or stirred on a magnetic stirrer at a speed of 80-100 rpm at a temperature of 31 °C in the dark controlled room. After 60 days of incubation, the synthetic medium was replaced with the freshly prepared one to replace the depleted nutrients. After a total of 90 days of incubation, experiments to test anammox activity were conducted in 500mL reactors using the all the biomass from the primary enrichment reactors as the inoculum. 2mL aliquot samples were withdrawn aseptically and analysed in duplicates according to the analytical methods. Figure 3.1 illustrates the experimental set up for batch experiments.



**Figure 3.1: Schematic representation of experimental set up**

### 3.4: Analytical methods

Ammonium, nitrite and nitrate in the form of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  respectively were analysed calorimetrically according to the following methods:

- Nitrate analysis – 10  $\mu\text{L}$  of saturated sulfamic acid was added to 40  $\mu\text{L}$  of reactor effluent and mixed together. To the mixture, a total of 0.2 mL reagent, containing 5% salicylic acid in 98% sulphuric acid and 2ml 4 M NaOH (4 °C), was added. This solution was analysed in a spectrophotometer at 420 nm after 30 minutes of reaction.
- Ammonium analysis – 760  $\mu\text{L}$  of a solution containing 0.54% ortho-phthalaldehyde, 0.05%  $\beta$ -mercaptoethanol and 10% ethanol in 400mM potassium phosphate buffer (pH 7.3) was added to a 40  $\mu\text{L}$  reactor effluent sample. This solution was analysed in a spectrophotometer at 420 nm after 30 minutes of reaction.
- Nitrite analysis – 950  $\mu\text{L}$  of a reagent containing 1% sulfanilic acid and 0.05% N-naphthylethylenediamine in 1M  $\text{H}_3\text{PO}_4$  was added to 50  $\mu\text{L}$  of reactor effluent. Subsequently, a spectrophotometric analysis at 540 nm was done after five minutes of reaction.

### 3.5: Genetic characterisation of anammox bacteria

#### 3.5.1: Isolation of DNA

Genomic DNAs were extracted from the Sequencing Batch Reactor (SBR). 2 mL samples were withdrawn from the SBR and centrifuged at 6000 rpm for 10 minutes. Pellets were resuspended in phosphate buffered saline (PBS pH 7.4) and centrifuged again. DNA was directly extracted from these samples using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufacturer's instructions.

#### 3.5.2: Polymerase Chain Reaction (PCR)

To detect the presence of anammox in the reactors, PCR amplification targeting the 16S rRNA of the anammox was performed. From the reactors, samples were withdrawn and centrifuged at 6000 rpm for 10 minutes. Pellets were resuspended in phosphate buffered saline (PBS pH 7.4) and centrifuged again. DNA was directly extracted from these samples using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufacturer's instructions. The PCR primers used in this study are primer sets AMX 368F – AMX 820R and AMX 368F – BS 820R (Table 3.1).

**Table 3.1: PCR primers for anammox bacteria related 16S rRNA**

Primer	Target	Sequence (5' - 3')
AMX 368F	All anammox bacteria except for anammoxoglobus propionicus	TTCGCAATGCCCGAAAGG*
AMX 820R	Brocadia anammoxidans, Brocadia fulgida and Kuenenia stuttgartiensis	AAAACCCCTCTACTTAGTGCCC*
BS 820R	Sculindua wagneri and Scalindua sorokinii	TAATTCCTCTACTTAGTGCCC*

\* (Amano et al., 2007)

The PCR reaction mixture (50 µL) contained 25 µL of DreamTaq™ Green PCR Master Mix (2X) (Fermentas Life Sciences), 0.1 µM of each reverse and forward primer, 1 µg of DNA

template, and nuclease free water. DreamTaq™ Green PCR Master Mix is a ready-to use solution containing DreamTaq™ DNA polymerase, optimised DreamTaq™ Green buffer, MgCl<sub>2</sub> and dNTPs.

The thermal cycle program used was an initial denaturation at 95 °C for five minutes, followed by 45 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for two minutes, final extension at 72°C for 10 minutes and hold at 4 °C.

### 3.5.3: Cloning and sequencing

The PCR products were recovered and purified using the ExoSAP amplicon purification kit (Inqaba Biotech) according to the manufacturer's instructions. The purified anammox-specific 16S rRNA genes were cloned into pJET1.2/blunt cloning vector (Thermo Scientific) following the manufacturer's instructions. 16S rRNA gene clone libraries were constructed by transforming Premade Z-Competent TM *E. coli* Cells (Zymo Research) with a vector. The clones were grown on LB agar plates supplemented with ampicillin (100 mg mL<sup>-1</sup>). Clones were randomly selected for PCR amplification and sequencing. Sequencing was carried out with the ABI V3.1 Big Dye kit. Cleaned sequencing products were analysed on the ABI 3500XL genetic analyser (Applied Biosciences, USA) using a 50 cm array and POP7.

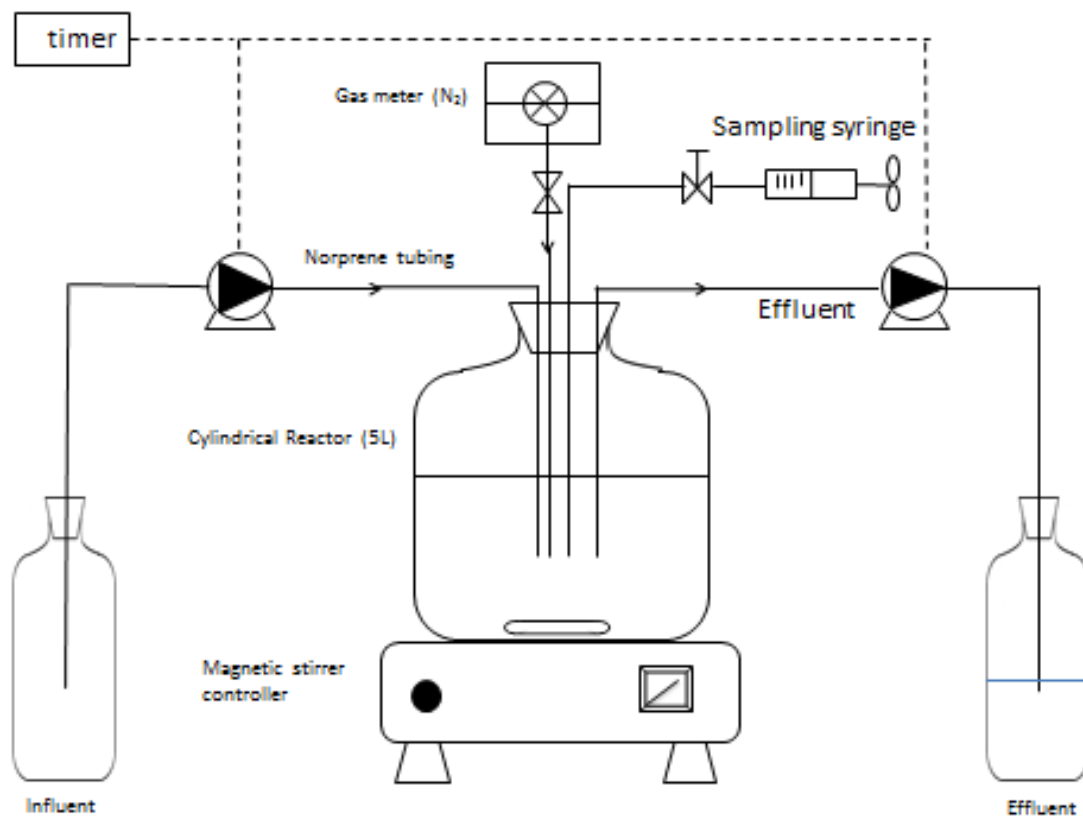
### 3.5.4: Phylogenetic analysis

For phylogenetic analysis, 16S rRNA sequences were compared with available database sequences via NCBI-BLAST search, and the related taxa were obtained from GenBank. The multiple alignments were performed with the CLUSTAL X program (Thompson et al., 1997). Sequences of 16S rRNA gene were edited with the BioEdit program (Hall, 1999). The phylogenetic trees were constructed via the neighbour-joining and maximum-parsimony algorithms with the MEGA 3 program (Kumar, et al., 2004). The similarity of the rRNA gene sequences of the clones were compared with those of other known anammox species.

### 3.6: Sequencing batch reactor experiments

#### 3.6.1: Reactor description

A sequencing batch reactor (SBR) with a working volume of 5L was used (Figure 3.2). The pH was maintained between 7.5 and 8 without specific control. The medium was homogenised by a magnetic stirrer. A set of two peristaltic pumps was used to introduce the feeding solution and to discharge the effluent. Timers controlled the actuations of the pumps and valves and regulated the different periods of the operational cycle. The reactor was flushed continuously with Argon to maintain anaerobic conditions. All the tubing used was Norprene tubing, so as to prevent the diffusion of oxygen inside the system.



**Figure 3.2: Experimental set up for SBR**



### **3.6.2: Inoculum**

The reactor was inoculated with a mixture of settled biomass of anammox bacteria from primary enrichment batch reactors.

### **3.6.3: Operational conditions**

The SBR was operated in 12 hour cycles distributed as follows during operation: 600 minutes of feeding and mixing, 65 minutes of settling, and 20 minutes of effluent withdrawal (Table 3.3). The feeding supplied to the reactor was prepared using the mineral salt medium. The reactor was operated in two different stages, depending on the wastewater composition (Table 3.2). The Hydraulic Retention Time (HRT) was maintained at 0.4 day.

**Table 3.2: Composition of mineral salt medium used as the influent in the SBR**

Compound	Concentration (g/L)
KNO <sub>3</sub>	0.06
KHCO <sub>3</sub>	1.25
NaH <sub>2</sub> PO <sub>4</sub>	0.05
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.3
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2
FeSO <sub>4</sub>	0.006
EDTA	0.006
NaNO <sub>2</sub>	As required
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	As required
Trace elements solution	1.25 mL/L

**Table 3.3: SBR operation conditions**

Feeding + mixing			
Settling			
Draw			
Time (minute)	600	65	20

### 3.7: Biomass characterisation

Total Suspended Solids (TSS) was determined according to the methods 2540D as described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, American Water Works Association & Water Environment Federation, 1995).

#### 3.7.1: Procedure

For the determination of TSS, a well-mixed sample volume was filtered through a weighed glass-fibre filter (Whatman, GF/A, 90 mm of diameter, 1.2 µm of pore size) and the residue

retained on the filter was dried to a constant weight (2h) at 103-105°C. The increase in weight of the filter represented the total suspended solids (TSS).

### **3.8: Characterisation of Microbial Population**

#### **3.8.1: DNA sample isolation**

Genomic DNAs were extracted from the Sequencing Batch Reactor (SBR). 2 mL samples were withdrawn from the SBR and centrifuged at 6000 rpm for 10 minutes. Pellets were resuspended in phosphate buffered saline (PBS pH 7.4) and centrifuged again. DNA was directly extracted from these samples using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufacturer's instructions.

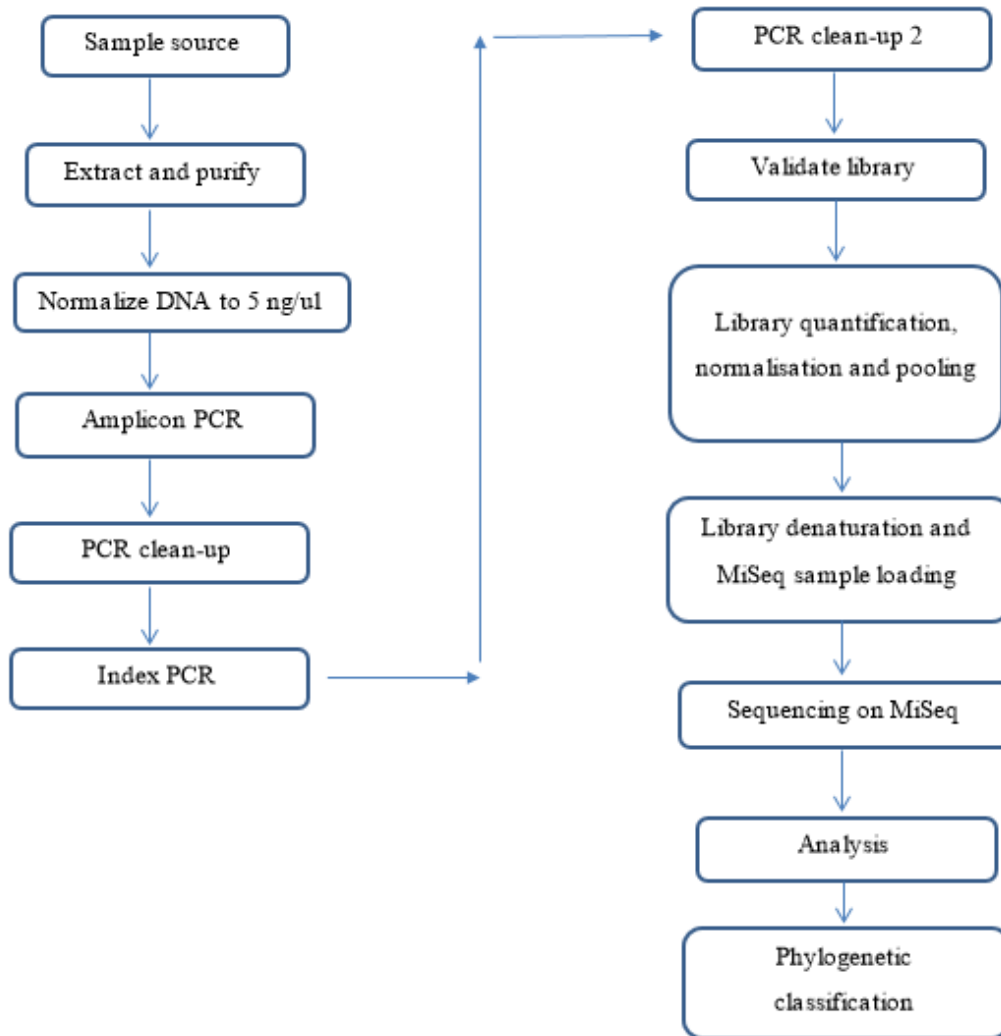
#### **3.8.2: Illumina paired end library generation**

A flow diagram of sample preparation procedures for the Illumina MiSeq Platform is illustrated in Figure 3.3. The V3 and V4 regions of the 16S rRNA, were amplified using the amplicon primers (Table 3.4). These primers were selected from the publication by (Klindworth et al., 2013). Attached to both primers were overhang adapter sequences (Table 3.4) for compatibility with the Illumina index and sequencing adapters. PCR amplifications were carried out with 25µL reaction mixture. Each reaction mixture contained 2.5 µL of genomic DNA (5ng/ µL), 5 µL of each forward and reverse primer (1 µM), and 12.5 µL of 2x KAPA Hifi Hot start Ready mix. The thermal cycle program used was an initial denaturation at 95 °C for 30 seconds, followed by 25 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, final extension at 72 °C for five minutes and hold at 4 °C. The PCR was followed by PCR clean up to purify the 16S V3 and V4 amplicons by removing primer dimers and free primers using AMPure XP beads. The clean-up was followed by Index PCR which attached dual indices and Illumina sequencing adapters using the Nextera XT Index kit. The PCR reaction (50 µL) was composed of 5 µL DNA 5 µL of each Nextera XT index primer 1(N7xx) and 2(S5xx), 25 µL of 2x KAPA Hifi Hot start Ready mix, and 10 µL PCR grade water. The PCR conditions used were an initial denaturation at 95 °C for three minutes, followed by eight cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, final extension at 72 °C for five minutes and hold at 4 °C. The second PCR

clean-up was performed using AMPure XP beads. The quality of DNA libraries was assessed with the use of the Agilent High-Sensitivity DNA Kit (Agilent Technologies, USA).

### **3.8.3: Library denaturing and MiSeq sample loading**

Sodium hydroxide (NaOH) was added to equal volumes of the DNA libraries for DNA denaturation, followed by 50-fold dilutions with pre-chilled HT1 buffer to obtain the DNA libraries at 20 pM. The DNA libraries were further diluted to 8 pM with a total volume of 1 mL with pre-chilled HT1 buffer. Five percent (5%) PhiX, which was used as internal control, was added into the 8 pM denatured DNA solution to observe the efficiency of DNA incorporation during DNA sequencing. Libraries were loaded onto the MiSeq V3 reagent cartridge. For cluster generation, the pooled DNA libraries were bound to the complementary adapter oligos on the surface of the flow cell and then bridge-amplified to form clusters.



**Figure 3.3: 16S paired-end library preparation workflow**

**Table 3.4: Primers for Illumina MiSeq sequencing**

PCR Primer	Sequence
Forward primer	5'-TCGTCGGCAGCGTCAGATGTGTATAAAGAGACAGCCTACGGGNGGCWGCAG-3'
Reverse Primer	5'-GTCTCGTGGGCTCGGAGATGTGTATAAAGAGACAGGACTACHVGGGTATCTAATCC-3'
Forward overhang	5'-TCGTCGGCAGCGTCAGATGTGTATAAAGAGACAG-[locus specific sequence]
Reverse overhang	5'-GTCTCGTGGGCTCGGAGATGTGTATAAAGAGACAG-[locus specific sequence]

### **3.8.4: Sequencing**

Following cluster generation, paired-end dual index 2 x 300 bp sequencing was performed on a Miseq platform (Illumina) with the use of the Miseq V3 reagent kit (Illumina). Clusters were imaged using LED and filter combinations specific to each of the four fluorescently labelled dideoxynucleotides. After the imaging of one tile of the flow cell was complete, the flow cell was moved into place to expose the next tile. The process was repeated for each cycle of sequencing. Sequencing was performed as multiplex two read libraries for 308 cycles (including an additional six cycles of index reads) according to the manufacturer's protocol. After sequencing was complete, the software performed the primary analysis, which included base calling, filtering, and quality scoring.

### **3.8.5: Bioinformatics Analysis**

When the run was complete, the MiSeq Reporter analysis software launched automatically to perform the secondary analysis, which included alignment and variant calling. The quality of the sequence run was monitored by Sequencing Analysis Viewer (SAV) (Illumina, Hayward, USA) and by conducting “resequencing” or aligning of 70 bp–1 Mbp sequence reads to reference sequences using MiSeq Reporter (MSR) (Illumina, Hayward, USA), which used the Burrows–Wheeler Aligner (BWA) (Li & Durbin, 2009, 2010). The reads that did not align or aligned to different references, were identified as “unresolved pairs”. BWA automatically adjusts parameters based on read lengths and error rates, and then estimates insert size distribution. The sequence error rate of 3% for a 200 bp and 5% for a 500 bp can be tolerated by BWA. By MSR default setting, GATK was utilised for variant calling (DePristo et al., 2011; McKenna et al., 2010) when BWA was used for alignment.

## **3.9: Transformation experiments**

### **3.9.1: Construction of codon optimised gene**

The synthetic gene sequence encoding Hydrazine Oxidoreductase (hzo) (GenBank accession no. HQ666192.1) was optimised for high expression in *E. coli*. The genetic codons of hzo gene were optimised by eliminating rare codons and improving the GC content using the

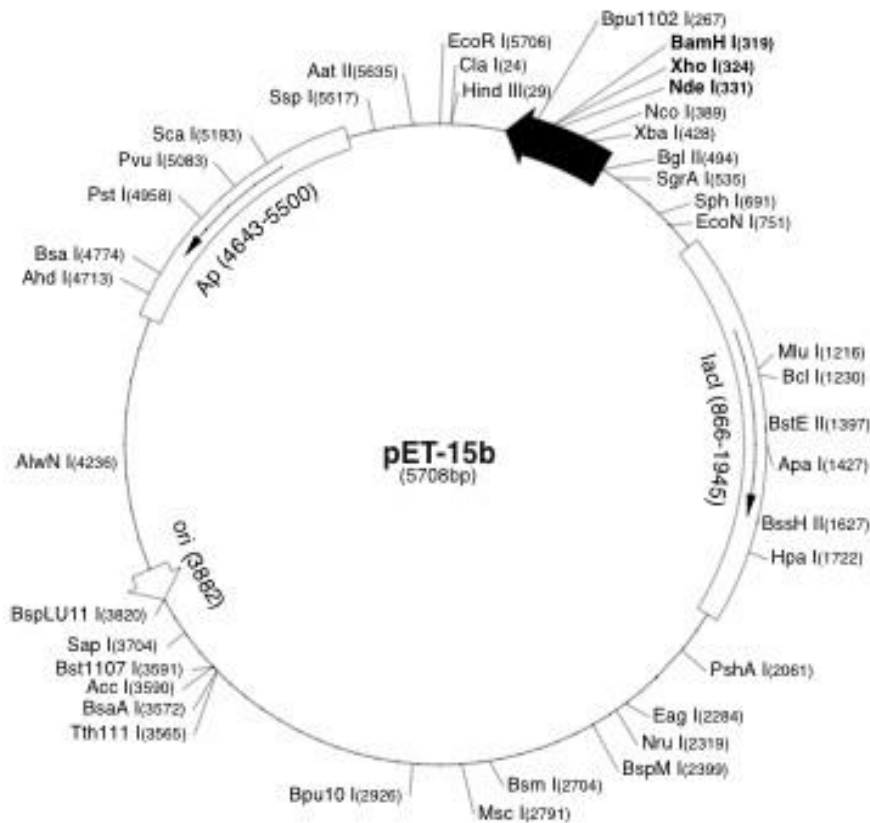
GeneOptimizer® software. The codon-optimised hzo gene was designed to contain restriction enzyme cut sites (Nde I and BamH I) at 5' and 3' respectively. Two sequences were synthesised using Geneart (Life Technologies, CA, USA). One of the genes was tagged with 6xHis encoding sequences on the C-terminal preceded by the *BamH I* restriction site.

### **3.9.2: Cloning of optimised hzo gene into a pET15b vector**

#### **3.9.2.1: Preparation of plasmid vector**

The pET system (pET15b) (Figure 3.4) was used for cloning the synthesised genes. It is a T7 promoter-driven system and is the most remarkable system thus far established for the cloning and expression of recombinant proteins in *E. coli*. Firstly, the pET15b vector was treated with Nde I and BamH I to linearise the supercoiled plasmid. To do restriction enzyme digestion of plasmid DNA, 3 µg of pET15b, 3 µL of 10x restriction enzyme buffer, 10-20 U of each restriction enzyme, and nuclease-free water to a final volume 30 µL were pipetted into a sterile Eppendorf tube. The reaction mixture was incubated at 37 °C for two to four hours. To verify the complete digestion of the DNA, the restriction mixture was analysed, together with a sample of uncut DNA, by agarose gel electrophoresis in the presence of an appropriate molecular weight marker. When digestion was complete, diluted 0.05 U of calf intestinal phosphatase was added to the remaining digestion solution to stop the reaction and incubated for 30 minutes. The reaction mixture was then extracted with 1 volume of TE-buffered phenol, 1 volume of TE-buffered phenol: CIAA (1:1) and 1 volume of CIAA [CIAA is chloroform: isoamyl alcohol (24:1)]. The reaction mixture was then precipitated with 0.1 volume of Na acetate and 2 volumes of ethanol. The reaction mixture was then centrifuged at 12000 x g for 10 minutes. The pellet was then rinsed with 70% ethanol, air dried and resuspended in 30 µL of TE buffer. The prepared vector was then stored at -20 °C until use.





**Figure 3.4: pET 15b plasmid**

(Source: <http://ecoliwiki.net/colipedia/images/0/01/PET15b.jpg>)

### 3.9.2.2: Ligation of target gene insert into plasmid

The ligation of the target gene inserts into the prepared pTE15b was achieved by using T4 DNA ligase. Two ligation reaction mixtures (20  $\mu$ L) contained 2  $\mu$ L of 10 $\times$  ligase buffer, 2  $\mu$ L of 100mM DTT, 1  $\mu$ L of 10mM ATP, 2  $\mu$ L of 50 ng/ $\mu$ L prepared pET 15b, 1 $\mu$ L diluted T4 DNA ligase, 100 ng of each target gene insert, 1-5  $\mu$ L of 50 ng vector DNA, and nuclease-free water. The ligase was added last. The ligation reaction mixtures were incubated for 16 hours at 16 $^{\circ}$ C. The synthetic gene with a C-terminal His-tag was inserted between the Nde I and BamH I sites. The other synthetic gene without the His-tag was inserted into the enzyme digestion sites and positioned downstream, in the same frame with the sequence that encodes N-terminal His-tag fusion peptide. Two pET15b-hzo plasmids with N- and C- 6x His tags were obtained.

### 3.9.3: Transformation

After ligation, constructs were transformed into *E. coli* DH5 $\alpha$  competent cells. To transform the *E. coli* DH5 $\alpha$  competent cells with DNA, 50  $\mu$ L of the competent cells were added to a pre-chilled Eppendorf tube and mixed with 5  $\mu$ L of the ligation reaction mixture. For the control 2.5  $\mu$ L of pUC19 was mixed with cells. After incubation on ice for 30 minutes, the tubes were subjected to heat shock by incubation at 42  $^{\circ}$ C for exactly 30 seconds. After chilling the tubes on ice for two minutes, 250  $\mu$ L of pre-warmed SOC (37  $^{\circ}$ C) was added to the tubes, followed by incubation at 37  $^{\circ}$ C for one hour. Aliquots (200  $\mu$ L) of the transformation mixtures and 20  $\mu$ L of pUC19 control were plated onto LB agar plates supplemented with 100 $\mu$ g/mL ampicillin. The agar plates were incubated overnight at 37  $^{\circ}$ C until the colonies developed to a visible size. The number of colonies that formed were counted and the transformation efficiency calculated as follows:

$$\rightarrow \text{transformation efficiency} = \frac{\# \text{colonies}}{\mu\text{g} \text{ DNA}} \times \frac{\text{final volume}}{\text{plated volume}}$$

### 3.9.4: Screening of transformants/plasmid extraction

Putative recombinant transformants were randomly selected from LB agar plates and were inoculated into 20 mL of LB broth containing 100 $\mu$ g/mL ampicillin and incubated overnight at 37  $^{\circ}$ C with shaking at 250 rpm. The plasmid DNA was then extracted from the respective cultures by alkaline lysis method as follows: After incubation, cells at 2 mL aliquots of each culture were collected in Eppendorf tubes by centrifugation at 13000 rpm for one minute. The supernatant was discarded and the bacterial pellet was suspended in 100  $\mu$ L of solution 1 (50 mM glucose; 25 mM Tris-HCL, pH 8.0; 10 mM EDTA, pH 8.0) before incubation on ice for five minutes. Two hundred microliters (200  $\mu$ L) of solution 2 (1% (w/v) SDS; 0.2 N NaOH) was then added and the tubes were placed on ice for five minutes. The tubes were gently inverted to avoid the shearing of DNA. Following the addition of 150  $\mu$ L of solution 3 (3 M sodium acetate, pH 4.8), the tubes were incubated on ice for 15 minutes and then centrifuged at 13000 rpm for 15 minutes. The plasmid DNA was precipitated from the

supernatant by adding 2 volumes of 96% ethanol at -20 °C for one hour. Precipitated plasmid DNA was collected by centrifugation at 13000 rpm for 15 minutes and washed twice with 70% ethanol to remove residual salts from the DNA. After drying the pellet under vacuum for five minutes to remove the excess ethanol, the pellets were resuspended in 1x TE buffer. The plasmid DNA were analysed by agarose gel electrophoresis on a 0.8 (w/v) agarose gel.

### **3.9.5: Restriction enzyme digestion of DNA**

Isolated recombinant constructs were digested by double digestion method using Nde I and BamH I in order to verify the presence and the size of the target gene. The reaction mixture containing, 1 µg of DNA, 1 µL restriction enzyme digestion buffer, 5-10 U of the restriction enzymes, and nuclease free water to a final volume 15 µL was mixed into a sterile Eppendorf tube. The reaction mixture was incubated at 37 °C for one hour. Resultant digested products were analysed by agarose gel electrophoresis in the presence of an appropriate molecular weight marker.

### **3.9.6: DNA sequencing**

To verify the precision of the cloned insert DNA, an automated DNA sequencing was performed using primers directed against the T7 promoter and T7 terminator sites on the pET15b vector (Promega, USA). For this purpose, an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.0 (Applied Biosystems, Perkin-Elmer) was used according to the manufacturer's instructions. Single primer reactions were set up containing 200 ng of template DNA, 2 µL BigDye Terminator Ready Reaction Mix and 3.2 pmol of T7 promoter or T7 terminator primer, 1 µL BigDye Sequencing Buffer, and Nuclease free water made up to a final volume of 10 µL mixed together. Cycle sequencing was performed using the following cycling conditions: 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 15 seconds and extension at 60 °C for four minutes. To remove the unincorporated dye terminators, the sequencing reaction mixtures were made up to 100 µL with 70% ethanol and centrifuged at 16000 x g for 30 minutes. The supernatant was discarded and the pellet washed in 250 µL 70% ethanol and centrifuged again. Finally the sequences were analysed using an ABI Prism 310 Genetic analyser from Perkin Elmer.

Sequence analysis and comparison to the reference gene sequence was performed on Vector NTI 9.1.0 (Invitrogen) and BioEdit Sequence Alignment Editor.

### **3.9.7: Expression of recombinant hydrazine xidoeductase**

The recombinant pET15b plasmid was isolated from *E. coli* DH5 $\alpha$  and transformed into chemically competent *E. coli* BL21 (DE3) Lys. A single colony of a transformed *E. coli* BL21 was inoculated into 5 ml LB broth containing of 100  $\mu$ g/mL ampicillin and then incubated for 16 hours at 37 °C with shaking at 225 rpm. An aliquot of the cell culture was diluted 1:100 into another fresh LB broth and incubated at 37 °C with shaking (225 rpm) until an OD<sub>600</sub> of 0.5 was reached. Recombinant protein expression was induced using a freshly prepared filter sterilised 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG). The induced culture was then incubated for 5 hours with shaking (225 rpm) at 37 °C.

#### **3.9.7.1: Preparation of lysate**

For expression of recombinant protein, the bacterial cells were harvested aseptically by centrifugation at 6000 rpm for 10 minutes. For the preparation of cleared *E. coli* lysate, the bacterial cell pellets were resuspended in standard ice cold bacterial cell lysis buffer (50mM K<sub>2</sub>PO<sub>4</sub> (pH 7.8), 400 mM NaCl, and 100 mM KCl, 10% glycerol, 0.5% Triton X-100. 10 mM imidazole) containing 1 mg/mL lysozyme and incubated on ice for 45 minutes. After the lysis step, the cell lysate was sonicated. The sonicated lysate was then centrifuged at 16,000 rpm for 20 minutes at 4 °C and the supernatant was collected in fresh micro-centrifuge tubes and stored at -20 °C until analysis by SDS-PAGE and Western blotting was done.

#### **3.9.7.2: SDS-PAGE analysis**

Proteins were resolved under denaturing conditions on 12% Tris-glycine gel which was thereafter stained with Coomassie blue R-250 in order to visualise the proteins using the following protocol.

Protein samples were mixed (v/v) with Reducing Treatment Buffer (125 mM Tris-HCL (pH 6.8), 4% (m/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, 0.05% (v/v) Bromophenol Blue in 10 ml Ultra-pure water) and denatured at 98°C for 10 minutes. Appropriate amounts were loaded onto a 12% separating gel (12% polyacrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) with stacking gel (5% polyacrylamide; 0.0125 M Tris-HCl, pH 6.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) alongside Protein Ladder (Life technologies) to estimate the protein molecular weight. SDS-PAGE was conducted at 60 V and 10 mA for the stacking gel and at 110 V and 20 mA for separation of protein bands in the Minigel G-41 system (Biometra, Germany) consisting of SDS running buffer (0.1% (v/v) SDS; 0.25M Tris; 192mM Glycine buffer, pH 8.3). To visualise the protein bands on a SDS-PAGE, gels were stained with staining solution (0.125% Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid in dH<sub>2</sub>O) for 30 minutes. Protein bands were thereafter fixed with 25% methanol and 10% acetic acid and then de-stained with 25% methanol to reduce background staining

### **3.9.7.3: Western blotting**

Following the protein separation by SDS-PAGE, the proteins were transferred by electrophoresis at 100 V and 400 mA for 90 minutes to a Hybond-C+ nitrocellulose membrane in transfer buffer (0.025 M Tris, pH8.3; 0.15 M glycine; 20% (v/v) methanol) using a protein immunoblotting system (Cleaver Scientific Ltd, UK). The unoccupied binding sites on the membranes were then blocked with 5% (w/v) skimmed milk in PBS for one hour with shaking. The membranes were then incubated in a 1:8000 dilution of mouse anti-His-tag IgG (Primary antibody) in PBS/T (PBS containing 0.05% Tween 20) for 16 hours with shaking at 225 rpm. The membranes were then washed three times for five minutes each wash in 20 ml Wash Buffer (0.05 Tween 20, 1x PBS) before incubation for one hour at room temperature with HRP- conjugated secondary antibodies in PBS/T at 1:10,000. Antibody binding was detected using an enhanced chemiluminescence HRP substrate detection kit (Millipore; Watford, UK). Thereafter, the membranes were washed in distilled water (dH<sub>2</sub>O) and dried. Imaging was carried out by scanning the membranes using the VersaDoc Imaging System (Biorad).

## CHAPTER 4: ENRICHMENT OF ANAMMOX BIOMASS

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### 4.1: Introduction

One of the legacies of the current dominance of the human kind on earth is the evident impact on nitrogen, phosphorous and sulphur cycles. Latest reports indicate that human activities, such as the increased combustion of fossil fuels and increased demand for nitrogen in agriculture and industry, continue to alter the global cycle of nitrogen (Galloway et al., 2008, Vitousek et al., 1997). Because of the increased nitrogen mobility, excess nitrogen from human activities has serious and ongoing environmental consequences for aquatic and terrestrial ecosystems (Vitousek et al., 1997).

It is extremely important to remove nitrogen to meet the minimum disposal requirements during wastewater treatment because of its contribution to the eutrophication of receiving waters, and the toxicity and direct threat it poses to aquatic life (Daims, et al., 2006). The nitrogen in wastewater is present mainly as ammonium ( $\text{NH}_4^+$ ) (van Hulle et al., 2010), which can be removed by physicochemical or biological processes (Zhang et al., 2008). Currently, there are several methods that are used for the removal of nitrogen from wastewater. For example, physical methods, such as membrane processes or ion exchange can be used to remove ammonium using a cation exchange resin (Bódalo et al., 2005; Jorgensen & Weatherley, 2003, Lahav & Green, 1998; Sarioglu, 2005). The disadvantage of the physical processes is that, in both cases, a highly concentrated brine or a highly acidified effluent stream is produced which requires further neutralisation before disposal. On the other hand, biological processes, including nitrification-nitration-denitrification, can be employed as a cleaner process (Bertanza, 1997; Mahne, et al., 1996; Park & Yoo, 2009; Peng & Zhu, 2006). Biological nitrogen removal is considered less expensive and more effective than physicochemical treatments and has thus been used more often to achieve nitrogen removal from domestic wastewaters (Khin & Annachhatre, 2004).

The conventional biological nitrogen removal processes are generally used for treating wastewaters with nitrogen concentrations that are quite low (concentration less than 100 mg

$\text{N.L}^{-1}$ ) (van Hulle et al., 2010). Some wastewater streams consist of high concentrations of nitrogen, mostly in the form of ammonium. If these streams are returned back to the inlet of the municipal WWTW, they increase the ammonium loading in the mainstream. Conventional biological nitrogen removal processes (denitrification-nitrification) are uneconomical and complicated when treating high nitrogen containing wastewaters with low C/N ratio. During the last decade, several new sustainable and cost-effective alternatives have been discovered and studied, and their implementation can be a valid option in terms of treating strong nitrogenous wastewaters characterised by high ammonium concentrations and low biodegradable organic matter content. Currently, the anammox process is considered to have potential for treating wastewaters with high ammonium concentrations (He et al., 2007; Ma et al., 2011).

Over the past few years, the main focus has been on shortening the start-up period and enhancing the activity of anammox bacteria. In an effort to do this, (Li et al., 2016; Li, Lu & He, 2015) used a novel osmotic anammox system where anammox was linked to forward osmosis so as to remove nitrogen. On the other hand, (Yin et al., 2016) added reduced graphene oxides into an anammox reactor in order to accelerate anammox growth, enhance the activity, and simultaneously improve the start-up period.

At the inception of this study, very little information was available regarding the distribution, diversity, and abundance of anammox bacteria in the South African wastewater treatment works. Therefore, the principal aim of this study was to investigate the extent of existence, the culture community analysis, and the location of anammox activity in selected wastewater treatment works.

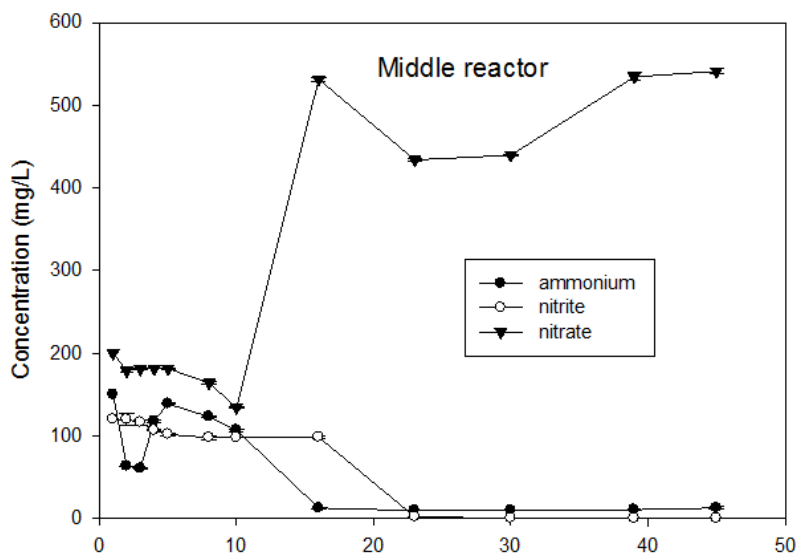
## **4.2: Results and Discussions**

### **4.2.1: Batch experiments**

Various attempts for anammox enrichment were performed from different sludge samples using batch reactors. The sludge samples that were used for starting anammox enrichment originated from three municipal wastewater treatment works. A period of 90 days was used for enriching anammox biomass due to the slow growth of anammox bacteria. This was done

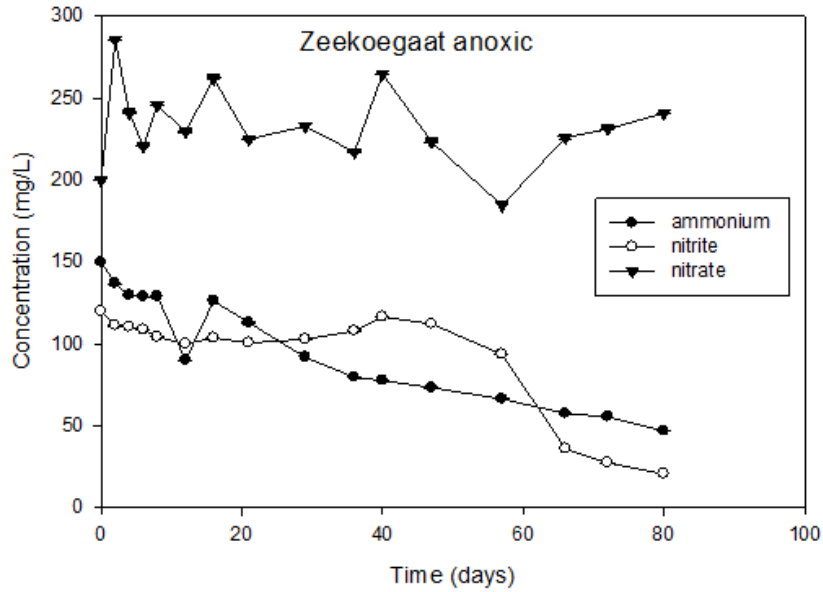
to allow for enough time for anammox growth to large numbers if it was initially present in the sludge samples. The results for the anammox activity obtained by calorimetric analysis of ammonia, nitrite and nitrate are presented below (Figure 4.1 to Figure 4.3).

In Figure 4.1, the middle reactor represents the results for the reactor inoculated with the Daspoort sample collected from the middle part of an anaerobic digester. In Figure 4.2, the Zeekoegat anoxic represents the results from the reactor inoculated with sludge samples obtained from the anoxic zone of the secondary stage of the Zeekoegat treatment plant. In Figure 4.3, the control reactor represents the results for the reactor that was not inoculated with sludge.

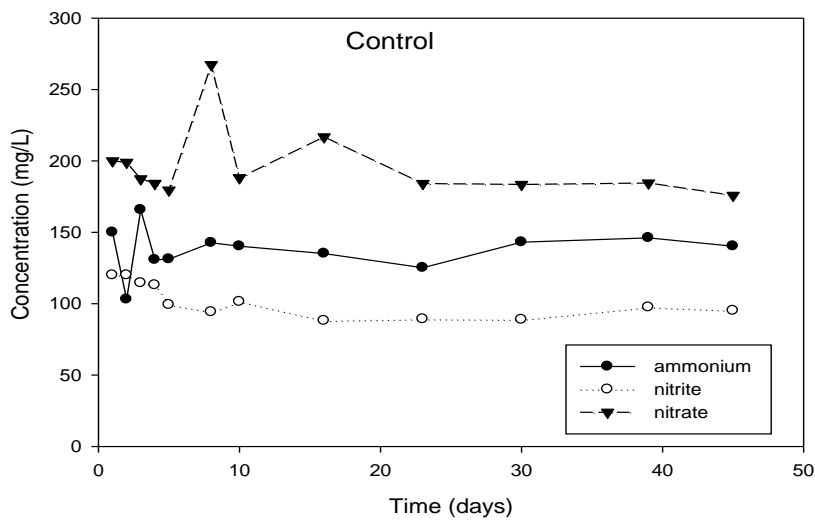


**Figure 4.1: Middle reactor (Daspoort sample)**





**Figure 4.2: Zeekoegaat anoxic**

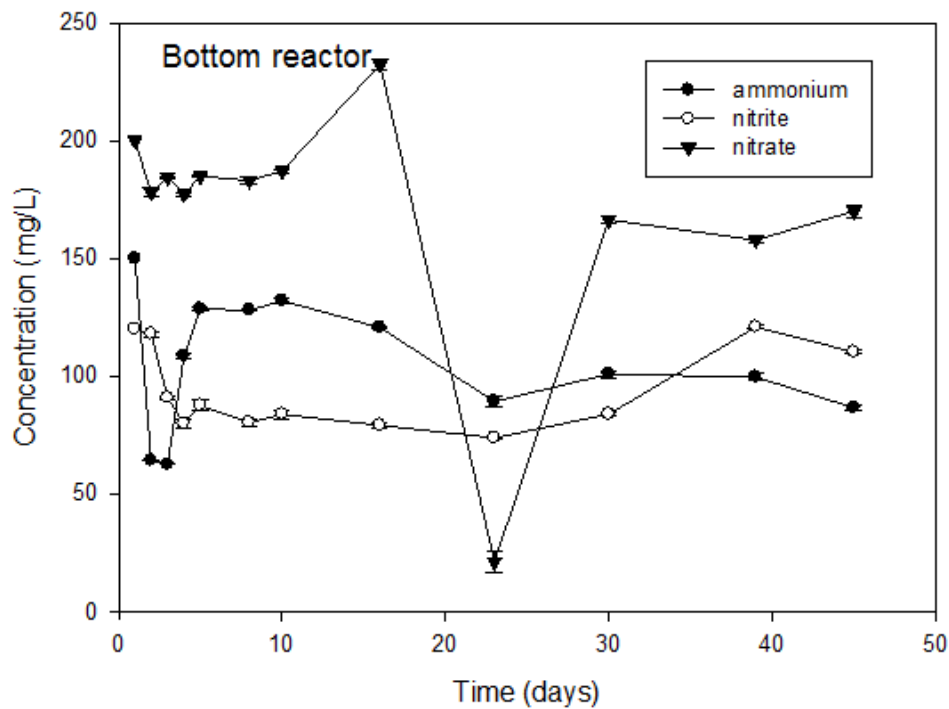


**Figure 4.3: Control reactor**

The successful enrichment results are illustrated in Figure 4.1 and Figure 4.2. Firstly, simultaneous ammonium and nitrite removal were observed in these reactors. Additionally, an increased production of nitrate was also observed at the same time. There was no consumption of ammonia and nitrite observed in the rest of the tested samples for the duration of the experiments (Figure 4.4, Figure 4.5 and Figure 4.6). This observation suggested the absence of anammox activity in these reactors. There was also no significant

change in the control reactor (Figure 4.3) where there were no cells added. Out of the seven enrichment attempts that were performed, only three were successful.

In Figure 4.4, the bottom reactor represents the results for the reactor inoculated with the Daspoort sample collected from the bottom part of an anaerobic digester. In Figure 4.5, the Zeekoegat anaerobic represents the results from the reactor inoculated with sludge samples obtained from the anaerobic zone of the secondary stage of the Zeekoegat treatment plant. In Figure 4.6, the Baviaanspoort anaerobic represents the results from the reactor inoculated with sludge samples obtained from the anaerobic zone of the secondary stage of the Baviaanspoort treatment plant.



**Figure 4.4: Results for Daspoort bottom sample**

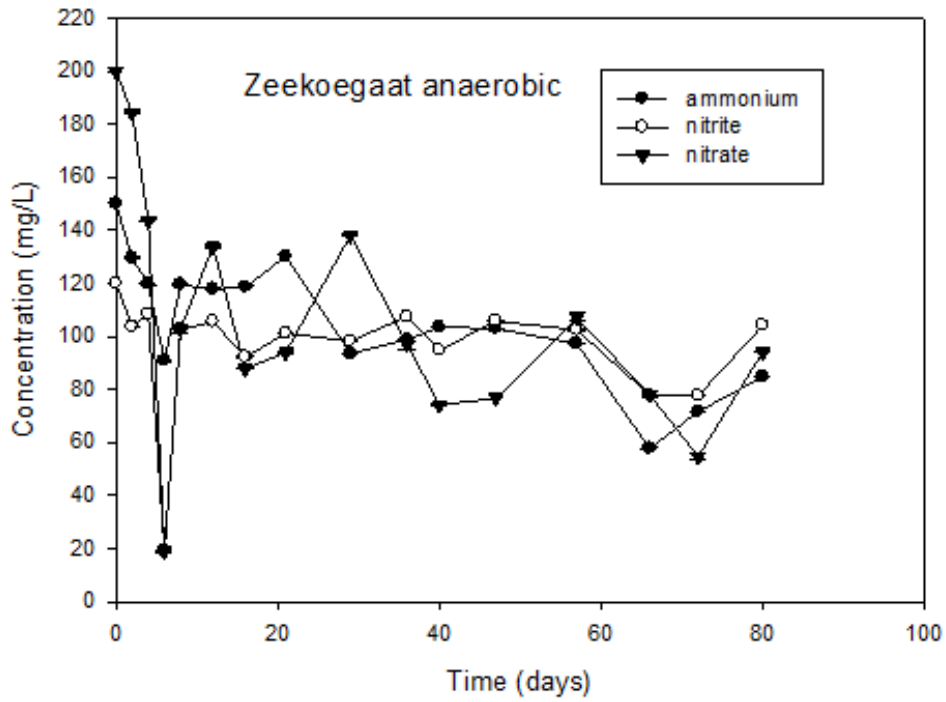


Figure 4.5: Results for sample from Zeekoegat anaerobic zone

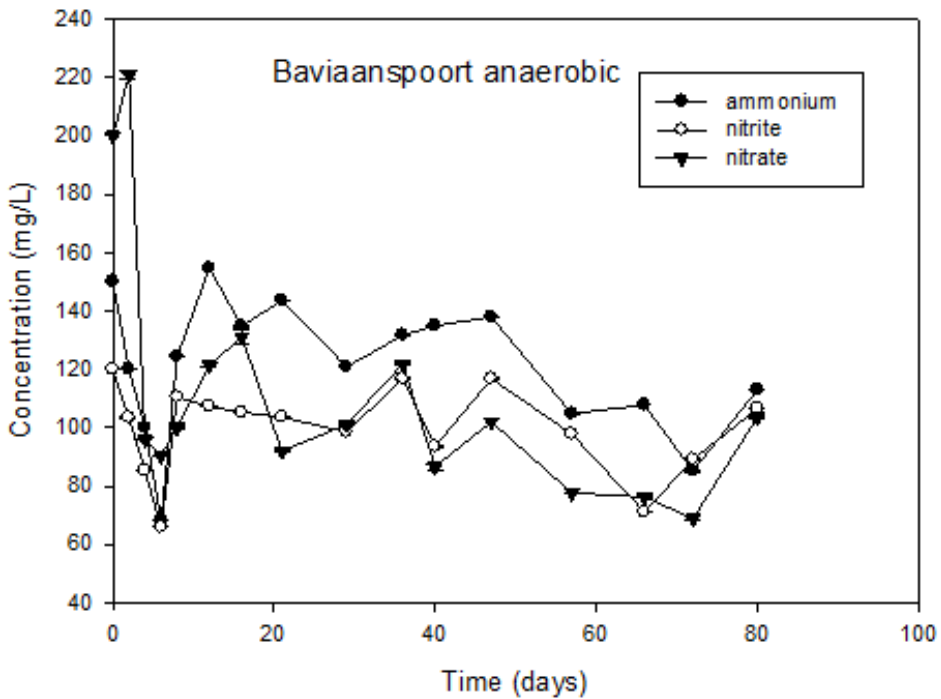


Figure 4.6: Baviaanspoort anaerobic

Notably, because the cultures were pre-enriched before testing the anammox activity, it took only 22 days for the complete consumption of the substrates in the Daspoort culture. In

contrast, when (Dapena-Mora et al., 2004) was enriching anammox using municipal sludge, it took two months of reactor operation just to observe noticeable consumption of  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . Recently, it took 40 days for (Chen et al., 2016) to start up a reactor using pre-enriched mixed denitrifying-anammox sludge. However, it took just over 80 days for the Zeekoegat culture for complete consumption. This was attributed to low anammox biomass concentration and confirmed by PCR results which showed a very faint positive band on a gel (Figure 4.9). Furthermore, (Qin & Zhou, 2009) reported that high concentrations of anammox bacteria translate into a higher rate of nitrogen conversion.

Successful enrichment could also be identified by visual observation through the colour change of the biomass. Anammox bacteria are characterised by a reddish brown colour, which was seen in reactors with successful enrichment (Figure 4.7). The red colour is due to the heme c group of the protein cytochrome c that plays an important role in the anammox metabolism. There was no colour change observed in the reactors with unsuccessful enrichment. The reactor contents remained black like the colour of the sludge (Figure 4.8).



**Figure 4.7: Visual observation of cultures with successful enrichment**

Anammox was also detected in a trickling filter sample and was only confirmed by PCR. These results are in accordance with those reported by Schmid and co-workers (2000) who also detected anammox bacteria with high anaerobic ammonium oxidation activity in a trickling filter. It was noteworthy that enrichment was observed mostly from samples that were collected from anoxic conditions. This observation could be a characteristic associated with the favourable conditions of anammox bacteria. A summary of all the enrichment experiments is shown in Table 4.1.



**Figure 4.8: Visual observation of cultures with unsuccessful enrichment**

**Table 4.1: Summary of enrichment experiments**

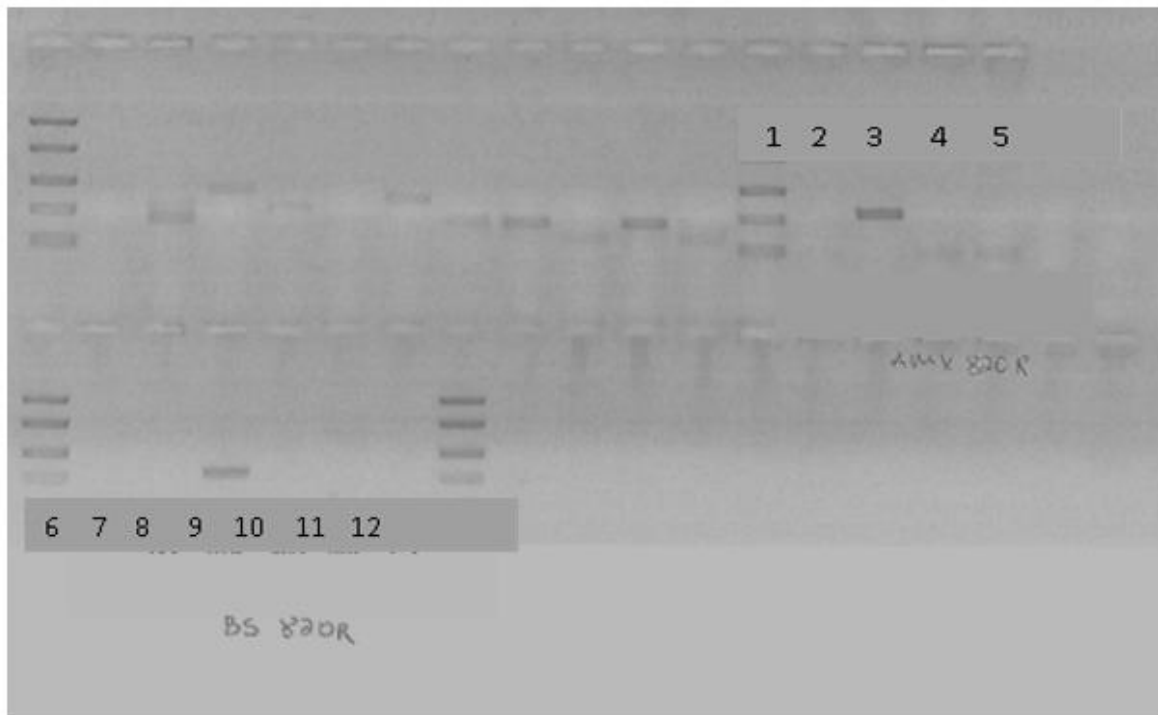
WWTW	Zone of Sampling	Enrichment
Daspoort	Trickling Filter	Yes
Daspoort	Anaerobic digester (middle)	Yes
Daspoort	Anaerobic digester (bottom)	No
Baviaanspoort	Anoxic	No
Baviaanspoort	Anaerobic digester	No
Zeekoegat	Anoxic	Yes
Zeekoegat	Anaerobic digester	No

#### 4.2.2: PCR Identification and Phylogenetic Analysis

After a successful enrichment was obtained, PCR was performed using anammox-specific primer sets Amx368F-Amx820R and Amx368F- BS820R. The PCR for the middle, trickling filter and Zeekoegat anoxic reactors DNA extracts resulted in positive amplicons for both primer sets (Figure 4.9 and Figure 4.10). These results were the second confirmation of the

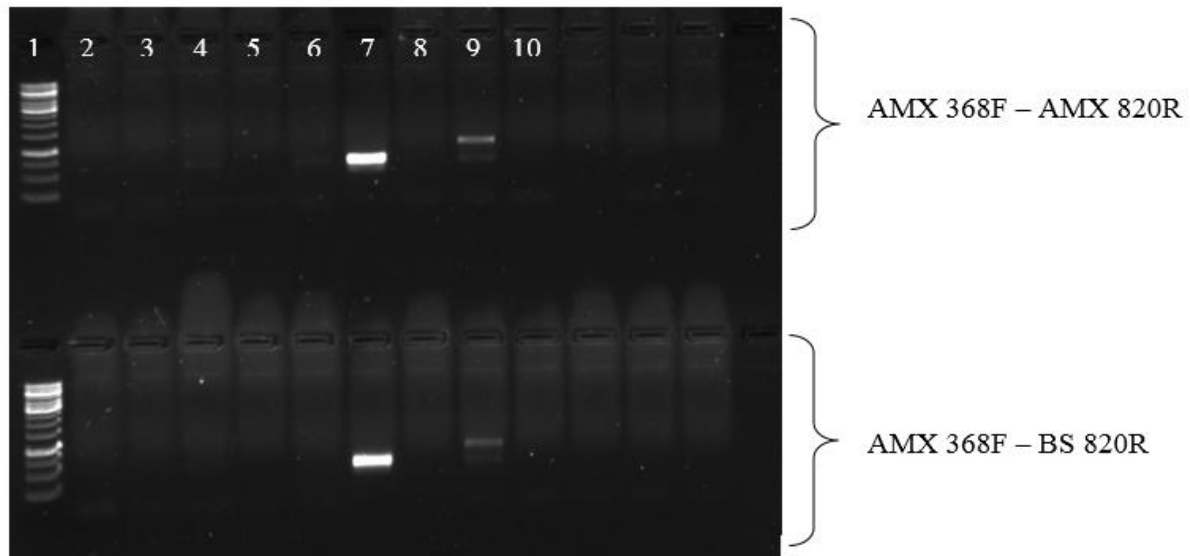
presence of anammox from the two reactors including the trickling filter. On the other hand no positive results were observed for all other reactors.

Figure 4.9, shows the PCR products amplified by primer sets Amx368F-Amx820R and Amx368F-BS820R. Lanes 1 to 5 are Amx368F-Amx820R and lanes 6 to 12 are Amx368F-BS820R. Lanes 7 and 12 are negative controls, lanes 2 and 8 are bottom reactors, and lanes 3 and 9 are middle reactors.



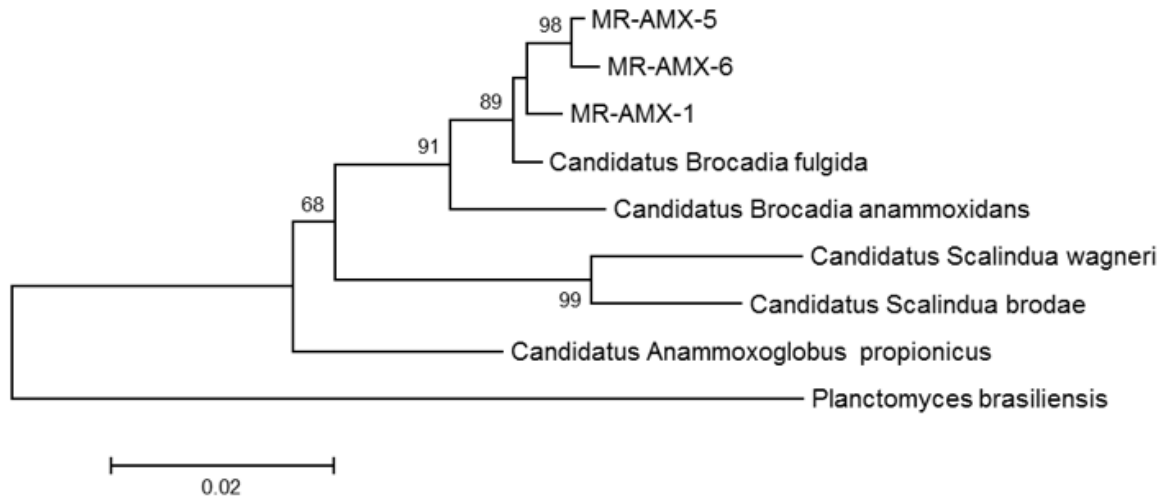
**Figure 4.9: PCR products amplified by primer sets Amx368F-Amx820R and Amx368F-BS820R**

Figure 4.10 shows the PCR products amplified by anammox specific primer sets Amx368F-Amx820R and Amx368F-BS820R. The top lanes are Amx368F-Amx820R and the bottom lanes are Amx368F-BS820R. Lanes (1) Marker, (2) negative controls (4) Baviaanspoort anoxic zone, (5) Zeekoegaat anaerobic zone, (6) Baviaanspoort anaerobic zone, (7) Trickling filter (9) Zeekoegaat anoxic zone, (10) negative control.

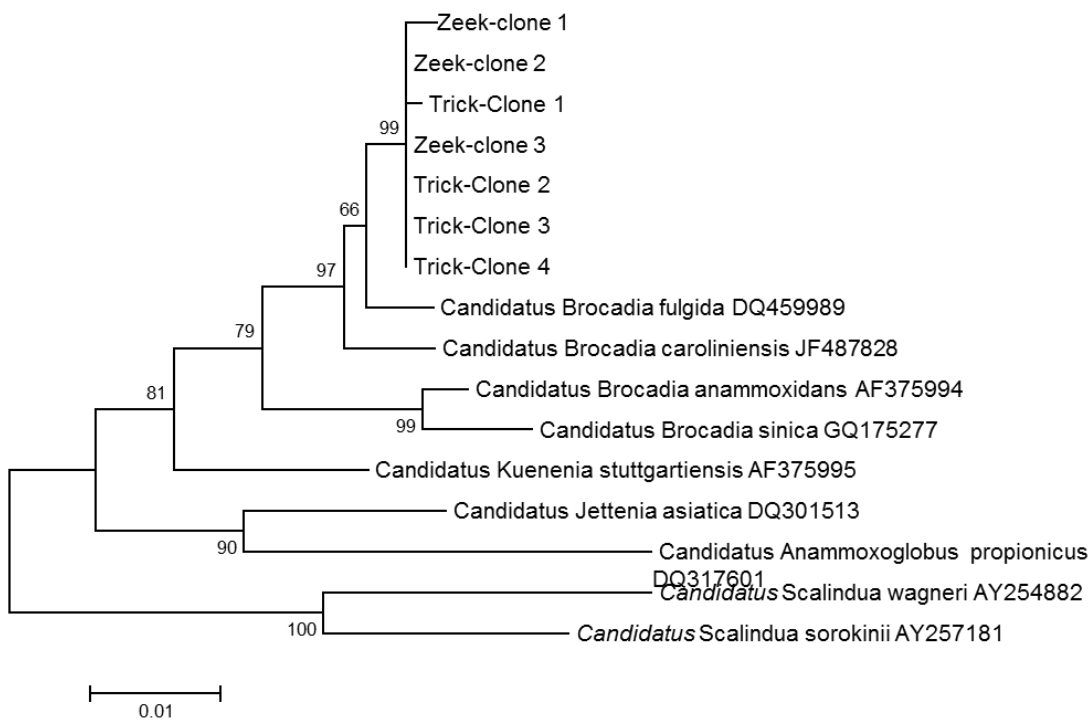


**Figure 4.10: PCR products amplified by anammox specific primer sets Amx368F-Amx820R and Amx368F-BS820R**

Sequencing of the cloned 16S rRNA fragments resulted in the detection of sequences which were highly similar to known anammox bacteria. Figure 4.11 and Figure 4.12 show the phylogenetic trees obtained from the sequencing of the retrieved clones. These results showed that anammox bacteria from an anaerobic digester (middle reactor), trickling filter and Zeekoegat WWTW were all closely related to the *Brocadia* species (*fulgida*, *anmmoxidans* and *caroliniensis*) (Figure 4.11 and Figure 4.12). Previously (Hu et al., 2011) enriched *Brocadia fulgida* from a peat soil. Correspondingly, (Sánchez Guillén et al., 2016) found that *Brocadia fulgida* was the dominant anammox strain throughout the long-term operation of a sequencing batch reactor seeded with granular sludge for sewage treatment. (Dapena-Mora et al., 2004a) likewise performed anammox enrichment from municipal activated sludge and found *Kuenenia stuttgartiensis* as major anammox species in the enrichment culture. In contrast, (Schmid et al., 2000) isolated *Kuenenia stuttgartiensis* from the trickling filter biofilm. This indicates that each anammox species can occupy diverse ecological niches. There is still no well-defined niche definition for the anammox species, however, there are reports that *Brocadia* and *Kuenenia* species are commonly found in wastewater treatment works and bioreactors (Schmid et al., 2005). Based on the phylogenetic tree analysis, it was clear that the South African wastewater treatment works (in the Pretoria region) were dominated by *Brocadia* species.



**Figure 4.11: Phylogenetic tree showing relationship of anaerobic digester enrichment culture 16S rRNA gene clone to other anammox bacteria. The scale bar represents 2% sequence divergence**



**Figure 4.12: Phylogenetic tree showing relationships of trickling filter and Zeekoegat enrichment cultures 16S rRNA gene clone to other anammox bacteria. The scale bar represents 1% sequence divergence**



### 4.3: Kinetic Model of Batch Experiments

#### 4.3.1: Model Development

The model was initially developed for determining the maximum specific growth rate for autotrophic bacteria in nitrification (Sözen, et al., 1996). The model was confirmed by (Melcer, 2003) and it was termed a high F/M model. In the present study, the model was adopted and modified to best describe the anammox process. The model has the assumption that nitrite consumption is the best signal for anammox growth. A mathematical expression was then derived to correlate nitrite consumption to biomass (anammox) growth rate.

#### 4.3.2: Biological reactions

In the calculations of the maximum growth rate of anammox ( $\mu_{An}$ ), it was assumed that the consumption of nitrite was related to anammox growth. The rate expression to correlate nitrite consumption to biomass production was as follows:

$$\frac{dS_{NO_2}}{dt} = -\frac{\mu_{An} X_{An} S_{NO_2}}{Y_{An} (K_{NO_2} + S_{NO_2})} \quad (4.1)$$

In the case where  $S_{NO_2}$  is high enough not to be a rate limiting (i.e. in high F/M ratio), Eq. 4.1 may be simplified as:

$$\frac{dS_{NO_2}}{dt} = -\frac{\mu_{An} X_{An}}{Y_{An}} \quad (4.2)$$

For this condition, the growth of anammox biomass can be expressed as:

$$\frac{dX_{An}}{dt} = (\mu_{An} - b_{An}) X_{An} \quad (4.3)$$

In batch reactors, the concentration of biomass can be calculated by integration of Equation. 4.3 to obtain the following expression:

$$X_{An} = X_{A0} \exp(\mu_{An} - b_{An})t \quad (4.4)$$

Substituting Eq. 4.4 into Eq. 4.2 gives:

$$\frac{dS_{NO_2}}{dt} = -\frac{\mu_{An} X_{An,0}}{Y_{An}} \exp(\mu_{An} - b_{An})t \quad (4.5)$$

Integration of Eq. 4.5 gives the following expression:

$$S_{NO_2,t} - S_{NO_2,0} = -\left[ \frac{\mu_{An} X_{An,0}}{Y_{An} (\mu_{An} - b_{An})} \exp(\mu_{An} - b_{An})t - \left( \frac{1}{\mu_{An} - b_{An}} \right) \frac{\mu_{An} X_{An,0}}{Y_{An}} \exp(\mu_{An} - b_{An})0 \right]$$

$$S_{NO_2,t} - S_{NO_2,0} = -\left[ \frac{\mu_{An} X_{An,0}}{Y_{An} (\mu_{An} - b_{An})} \exp(\mu_{An} - b_{An})t - 1 \right] \quad (4.6)$$

$Y_{An}$  = the yield coefficient of anammox biomass (mg COD/mg N)

$X_{An}$  = the biomass concentration of anammox biomass (mg/L)

$\mu_{max,An}$  = growth rate of anammox biomass ( $h^{-1}$ )

$S_{NO_2}$  = substrate concentration at any time (in this case nitrite) (mg/L)

$K_s$  = half-saturation coefficient (mg/L)

### 4.3.3: Model simulation

The simulation was performed using a computer program for the identification and simulation of aquatic systems (AQUASIM 2.0) (Reichert, et al., 1998). Batch experiments of cultures obtained from two wastewater treatment works (Daspoort and Zeekoegat) were conducted to evaluate the rate equations of kinetic constants for processes taking place in the batch reactors. The performance of the mathematical model and the utility of different parameters were then evaluated by simulating the biotransformation of nitrogen compounds by anammox bacteria.

#### 4.3.4: Model calibration

During model calibration, certain parameter values of the model were adjusted in order to generate the results that were in agreement with the experimental data. In the present study, the calibration approach was to fit the model simulation data on the nitrite experimental data obtained during the batch experiments. The maximum growth rate of anammox was determined by performing a nonlinear regression analysis on Equation. 4.6 using AQUASIM program. By using Equation. 4.6 and the experimental data, three biokinetic coefficients ( $b_{An}$ ,  $\mu_{maxAn}$ , and  $Y_{An}$ ) were predicted.

#### 4.3.5: Estimation of maximum growth rate

The maximum growth rate values predicted by the model are presented in Table 4.2. The growth rate for Daspoort and Zeekoegat cultures were estimated as  $0.101 \text{ h}^{-1}$  and  $0.023 \text{ h}^{-1}$  respectively. These values are considerably higher than those reported by Strous et al. (1998) and Van de Graaf et al. (1996). Although the maximum growth rates for the present study were higher than those in literature, the culture from Zeekoegat had lower growth rate than the culture from Daspoort. Strous et al., (1998) attributed the low anammox growth rate to low substrate consumption rate. This was confirmed in the present study, as it took more than 80 days for nitrite to be completely consumed with the Zeekoegat culture, while it took only 45 days for nitrite consumption with the Daspoort culture. This means that the low growth rate for Zeekoegat was due to low nitrite consumption rate.

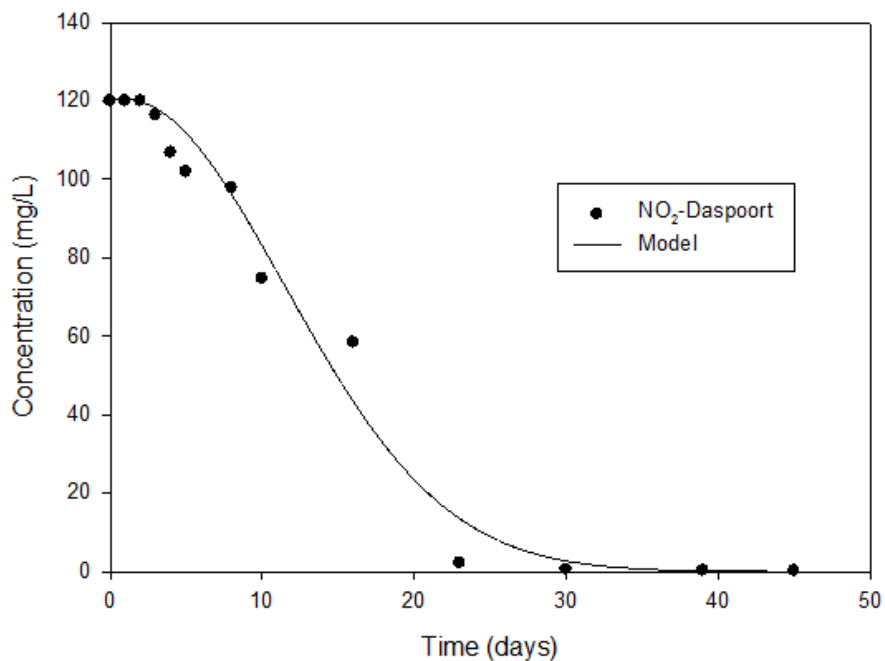
**Table 4.2: Predicted parameters using high F/M growth model**

Culture	$X_{An}$ ( $\text{mg.L}^{-1}$ )	$\mu_{maxAn}$ ( $\text{h}^{-1}$ )	Decay ( $\text{h}^{-1}$ )	Yield ( $\text{mg cell mg}^{-1}$ N)
Daspoort	8.29336	0.1011	0.004899	9.89097
Zeekoegat	8.23959	0.02387	0.00032	9.53319

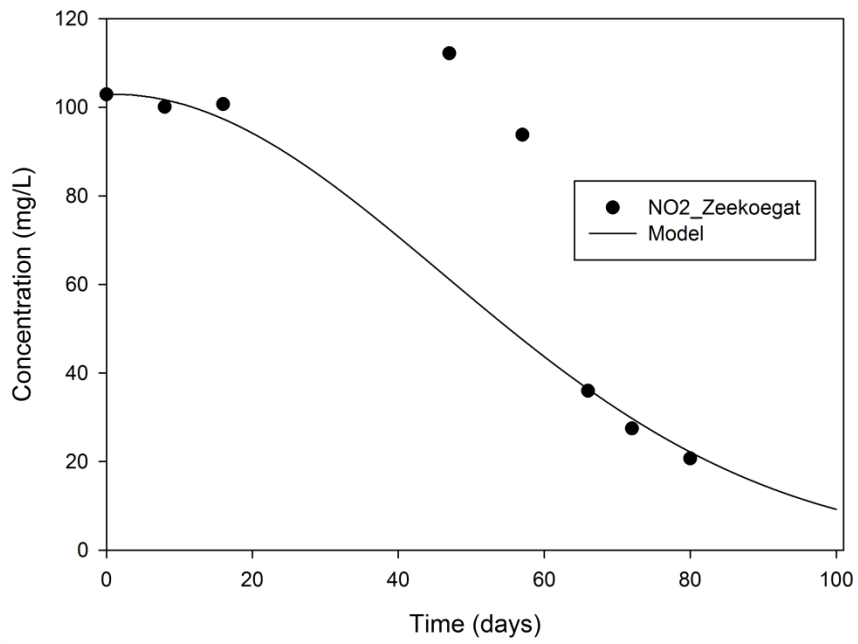
The yield coefficients for anammox biomass were calculated to be  $9.9 \text{ mg biomass mg}^{-1} \text{ N}$  and  $9.5 \text{ mg biomass mg}^{-1} \text{ N}$ , whereas the decay rate coefficients were  $0.0049 \text{ d}^{-1}$  and  $0.0003$

$d^{-1}$  for Daspoort and Zeekoegat, respectively. Based on these values, the anammox concentration was calculated to be  $8 \text{ mg.L}^{-1}$  on both experiments.

In assessing the validity of the model, the model predictions were compared to measured experimental data. According to Figure 4.13 and Figure 4.14, the model predicted values fit to the measured experimental data quite well for the Daspoort and Zeekoegat experiments, respectively. However, there was a bit of deflection in the Zeekoegat experiment (Figure 4.14) due to the instability of the reactor at the beginning of the experiment. The good agreement between the model and measured data is evidently demonstrating the validity of the model developed in this study. Therefore, this model is suitable for estimating the maximum growth rate for anammox in batch experiments.



**Figure 4.13: Comparison between measures and predicted values of  $\text{NO}_2^-$  – Daspoort sample**



**Figure 4.14: Comparison between measures and predicted values of NO<sub>2</sub><sup>-</sup> – Zeekoegat sample**

#### 4.4: Summary

In this chapter, the results from the enrichment and evaluation of anammox cultures for municipal waste sludge are presented. The suitable conditions to isolate and develop anammox biomass from WWTW sludge were analysed, for further application of the process. For this purpose, sludge samples from different plants were collected and used to inoculate batch reactors to determine the presence and activity of anammox. After several attempts, anammox biomass was enriched from sludge samples collected from the Daspoort and Zeekoegat municipal WWTW and the trickling filter. Anammox activity was detected after 90 days of operation which was identified by simultaneous consumption of  $\text{NO}_2^-$  and  $\text{NH}_4^+$  in the system. This presence of anammox bacteria was also confirmed by the PCR amplification of the 16S rRNA of anammox bacteria. Phylogenetic analysis indicated that the anammox were closely related to the Brocadia species. The maximum specific growth rate for batch experiments was estimated using a new model.

# CHAPTER 5: PERFORMANCE OF SEQUENCING BATCH REACTOR

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## 5.1: Introduction

Nitrogen compounds in wastewater have serious unfavourable effects on the environment. In order to minimise the negative effects on the environment, wastewater must be treated to remove nitrogen compounds before being discharged as they can be toxic to aquatic life, causing hypoxic conditions and eutrophication in receiving water bodies (Daims, et al., 2006; Gong et al., 2008; Howarth & Marino, 2006; Smith & Schindler, 2009). There are numerous techniques that are employed to remove nitrogenous compounds. However, biological processes are considered to be most effective and inexpensive, and are utilised widely in comparison to the physical-chemical processes (Peng & Zhu, 2006; Wiesmann, 1994; Zhu et al., 2008).

Various innovative biological nitrogen removal processes, such as the so called Single reactor High activity Ammonia Removal Over Nitrite (SHARON) process, the Completely Autotrophic Nitrogen removal Over Nitrite (CANON) process, de-ammonification, and the Oxygen-Limited Autotrophic Nitrification-Denitrification (OLAND) process, have been developed (Verstraete & Philips, 1998). However, the latest biological process that has drawn a lot of attention is the anaerobic ammonium oxidation (anammox) process. The anammox discovery initiated an additional and alternative treatment process for nitrogen, and it formed a new component of the biological nitrogen cycle. In this process, ammonium is oxidised autotrophically using nitrite as an electron acceptor under anaerobic conditions. The anammox process is considered to be an economical and low energy alternative to the conventional biological nitrogen removal, which is generally accomplished through successive aerobic autotrophic nitrification and anoxic heterotrophic denitrification (Cho et al., 2010). The anammox process utilises less oxygen for nitrification and does not require an external carbon source for denitrification (van Dongen, et al., 2001). This leads to cost saving for aeration and the prevention of an incomplete conversion of organic matter if an additional

carbon source was initially added. As a result, the anammox process can save up to 90% of operation costs when compared to conventional nitrogen treatment processes (Jetten et al., 2001).

Nevertheless, the anammox process has a shortcoming of a long experimental start-up that is due to the slow growth rate of anammox bacteria (Hao et al., 2014). Therefore, it is very essential to maintain adequate amounts of anammox bacteria in reactors for the successful establishment of the anammox processes. Having the correct reactor type is also very important during the start-up of the anammox process. A wide range of reactors have been studied by different researchers to achieve quick start-up and optimise the anammox process. These include the sequencing batch reactor (SBR) (Dapena-Mora et al., 2004; Strous et al., 1998), Fluidised bed reactors (Mulder et al., 1995; Strous et al., 1997; van de Graaf et al., 1996), rotating biological contactors (Egli et al., 2001), fixed bed biofilm reactors (Fux et al., 2004; Kindaichi et al., 2007; Strous et al., 1997a; Tsushima et al., 2007), up-flow anaerobic sludge blanket reactors (Ahn, Hwang & Min, 2004), membrane sequencing batch reactors (Trigo et al., 2006), and gas lift reactors (Dapena-Mora et al., 2004b; Sliemers et al., 2006). However, SBRs have proven to be more suited for the long-term enrichment, cultivation and quantitative analysis of a very slow growing microbial community (Strous et al., 1998).

Granular sludge technology is the most commonly used system for wastewater treatment. Various studies have also used granular biomass for the anammox process (Ni et al., 2009; Ni et al., 2010b; Ni & Yang, 2014; Xiong et al., 2013). The most attractive characteristics about the granular biomass are its regular, dense, and strong microbial structure with good settling properties. These qualities result in high biomass retention, which is extremely important in the anammox process. Anaerobic granular sludge has been reported as the most suitable inoculum for anammox start-up (Xiong et al., 2013). However, anaerobic granulation has some drawbacks, which include a long start-up period. Disintegration of granules, resulting in instability of granular sludge, during a long-term operation period has been reported (Morgenroth et al., 1997; Mosquera-Corral et al., 2011). Additionally, anaerobic granulation technology is not suitable for nutrient removal from wastewater. The study was therefore, aimed at investigating the feasibility of the start-up of the anammox process for nitrogen removal in a sequencing batch reactor using a pre-enriched, non-granular anammox biomass.



As a follow up to this process, the performance of the reactor in terms of substrate removal was also studied.

## 5.2: Results and discussion

### 5.2.1: Nitrogen removal performance of the anammox reactor

The Sequencing Batch Reactor (SBR) for an anammox process was started-up and operated for 120 days. The experimental period of the reactor was divided into two stages. In the first stage, the initial nitrogen loading rate (NLR) was  $5 \text{ g N L}^{-1}\text{d}^{-1}$  and the second stage was distinguished by an increase of NLR to  $6.3 \text{ g N L}^{-1}\text{d}^{-1}$ . The NLR was elevated by increasing the concentrations of nitrogen compounds. Only  $\text{NH}_4^+$  concentration was increased during NLR increase and nitrite concentration remained constant throughout the experiment so as to avoid its inhibitory effect. The adverse effects of nitrite were first reported by (Strous, et al., 1999), who found a great, yet reversible, inhibition of the process at  $100 \text{ mg N L}^{-1}$ . (Wett, 2007) also reported nitrite concentrations as low as  $30 \text{ mg N L}^{-1}$  to be inhibitory for the process. The anammox activity was measured by simultaneous consumption of ammonium and nitrite, and the production of nitrate in the reactor. The performance of the reactor is presented in Figure 5.1.

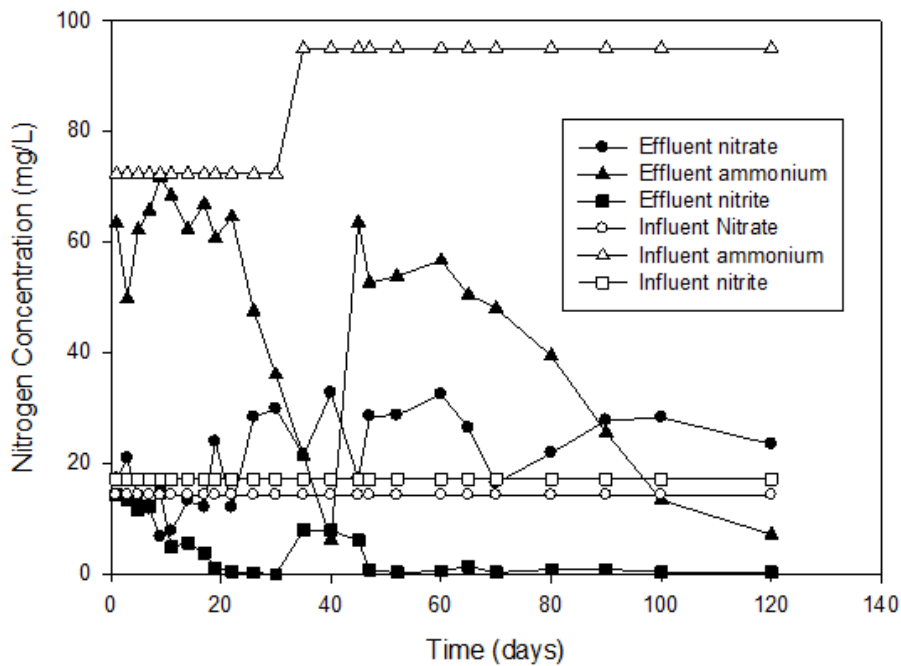
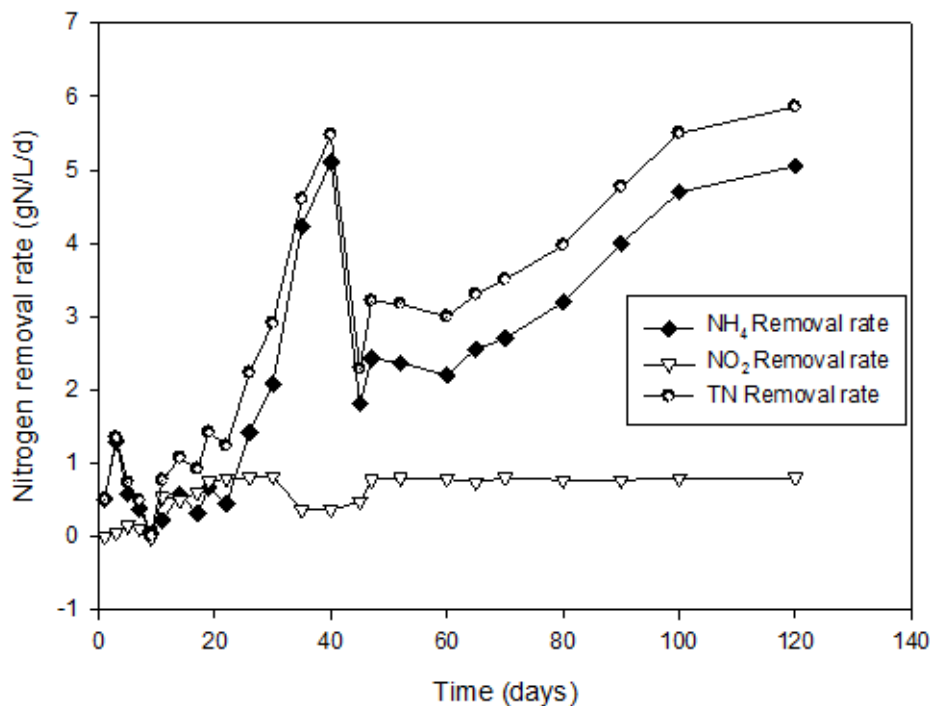
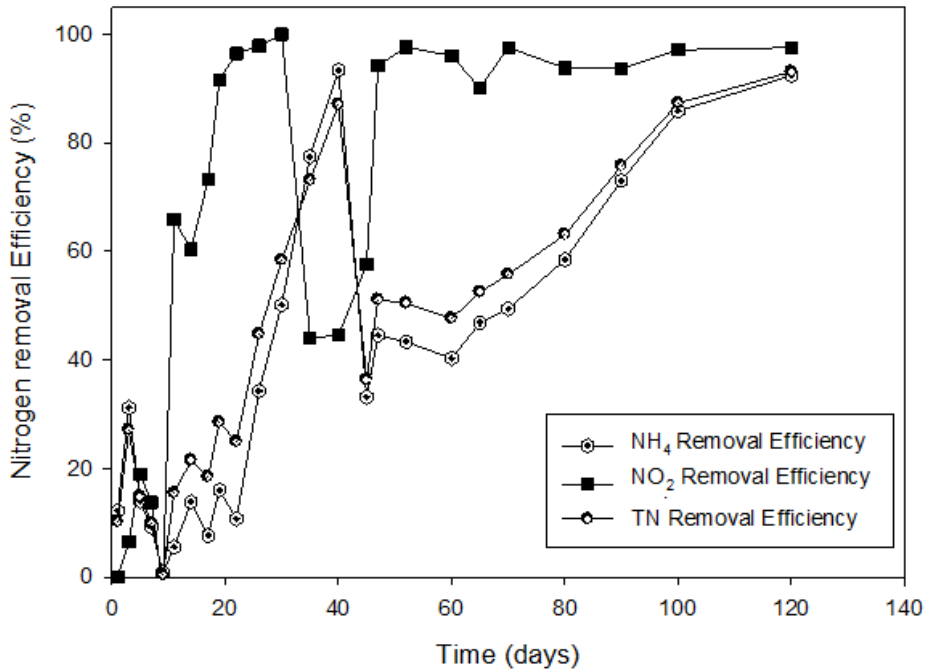


Figure 5.1: Performance of anammox sequencing batch reactor

For the first nine to 10 days, the reactor was unstable with nitrate concentrations declining and ammonium concentrations rising a little above the influent concentrations. The ammonium increase was also seen in other studies (Chamchoi & Nitorisavut, 2007; Gao et al., 2014; Zhao et al., 2007). After 10 days of operation, a gradual decrease of both nitrite and ammonium concentrations in the effluent, with a significant increase in nitrate concentrations, was observed. On day 40 of operation, the total nitrogen removal rate was  $3 \text{ g}\cdot\text{N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  (Figure 5.2). At the same time, the ammonium and nitrite removal efficiency were 50% and 100%, respectively (Figure 5.3). (Xiong et al., 2013) observed a total nitrogen removal rate of  $3.7 \text{ g}\cdot\text{N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  only after 148 days in the granular sludge anammox reactors. In the second stage, the effluent concentration of ammonium increased when the inlet NLR was elevated from  $5 \text{ g}\cdot\text{N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  to  $6.3 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  (after day 40), and they later started to decrease gradually (Figure 5.2). After 45 days of operation, nitrite consumption improved remarkably, with nitrite almost completely consumed most of the time (Figure 5.1). The average removal rate for nitrite was  $0.6 \text{ g}\cdot\text{N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  (Figure 5.2) and reaching up to 98% nitrogen removal efficiency (Figure 5.3).



**Figure 5.2: Nitrogen removal rate for an anammox reactor**



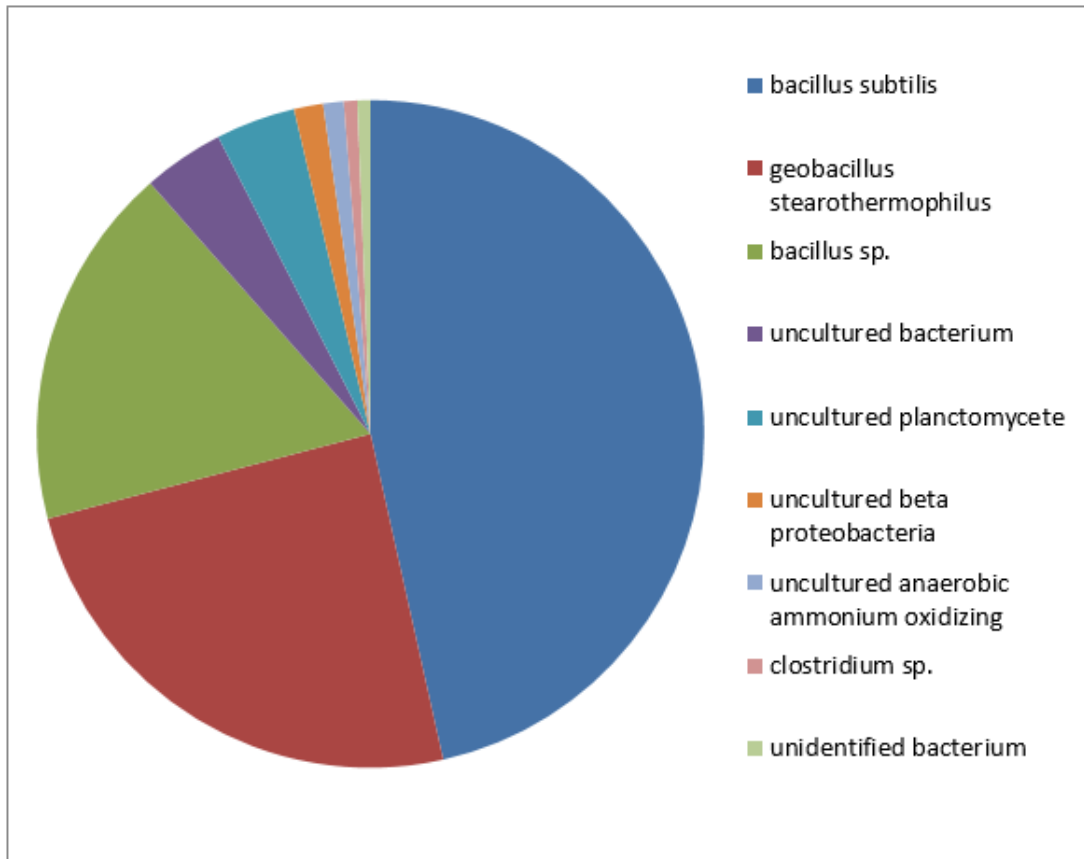
**Figure 5.3: Nitrogen removal efficiency for an anammox reactor**

The total nitrogen (TN) removal rate was slightly low during the first stage of operation, with an average removal rate of about  $1.142 \text{ g N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ . However, in the second stage, an average removal rate for TN was  $4.057 \text{ g N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ . Towards the end of the experiment, the TN removal efficiency increased dramatically, reaching about 94% on day 120 of operation (Figure 5.3) thereby indicating a great amount of anammox activity. The nitrogen removal efficiency was similar to the previously reported for the granular sludge anammox (Ni et al, 2010). However, this TN removal efficiency was reached after 250 days of operation. Moreover, (Ni & Yang, 2014) achieved a TN removal efficiency of only 72% after 22 days in the granular sludge reactor.

### 5.2.2: Characterisation of Microbial Population

In order to identify the main microorganisms forming part of the microbial population in the reactor, metagenomics, using next generation sequencing, was performed. A clone library was done with the sample taken towards the end of reactor operation. Microbial population analysis was done to determine the level of enrichment over the period of reactor operation. Figure 5.4 shows the population distribution obtained after data analysis using MiSeq reporter. The majority of the observed sequences were from known bacteria origin. The

*Bacillus subtilis* were the most dominant genus (44%) in the total microbial community. *Geobacillus stearothermophilus*, which presented 24% of total population, was the second largest genus, followed by *Bacillus* species, which presented 17% of the total population. *Bacillus* species are aerobic or facultative anaerobic bacteria that recalcitrant in nature and spore-forming. The overabundance of *Bacillus* species in the reactor is attributed to their ability to survive in most environmental conditions, and they are rapid growers. As a result, the population of *Bacillus* species is drastically increased through the cycle of sporulation, germination and proliferation. Additionally, their predominant growth is thought to be due to their decomposition activities (Mori et al., 2004). However, the overabundance of *Bacillus* species was not attributed to nitrogen removal in the system. Grouping the three types of *Bacillus* makes the *Bacillus* species the most predominant in the reactor. Planctomycetes, to which anammox bacteria belong, presented a considerable amount of 4% of the total microbial population. In addition, 1% of uncultured ammonium oxidising bacteria was also observed. This made it possible for anammox bacteria to be the 4<sup>th</sup> largest group of bacteria, presenting a total of 5% in the reactor. All other species were present in irrelevant numbers (Table 5.1) and were also not attributed to nitrogen removal in the system.



**Figure 5.4: Dominant bacterial species in SBR**



**Table 5.1: Diversity of microbial population in SBR**

<b>Bacterial species</b>	<b>Cluster count</b>	<b>Percentage</b>
<i>Bacillus subtilis</i>	52192	44.18
<i>Geobacillus stearothermophilus</i>	27396	23.19
<i>Bacillus</i> sp.	19769	16.73
Uncultured Bacterium	4412	3.73
Uncultured Planctomycete	4331	3.66
Uncultured Beta Proteobacteria	1579	1.33
Uncultured anaerobic ammonium oxidising	1116	0.94
<i>Clostridium</i> sp.	730	0.61
Unidentified Bacterium	709	0.60
Uncultured <i>Nitrospira</i>	680	0.57
<i>Burkholderia pseudomallei</i>	511	0.43
Uncultured soil	487	0.41
<i>Nocardia</i> sp.	431	0.36
<i>Bifidobacterium</i> sp.	389	0.32
<i>Pseudomonas aeruginosa</i>	366	0.31
<i>Achromobacter xylosoxidans</i>	259	0.22
<i>Agrobacterium tumefaciens</i>	251	0.21
<i>Pseudoxanthomonas</i> sp.	218	0.18
<i>Nitrosomonas</i> sp.	210	0.17
<i>Betaproteobacterium</i>	198	0.17
<i>Nitrospira</i> sp.	186	0.16
Endosymbiont of <i>Syntrophobacter</i> sp.	178	0.15
<i>Syntrophobacter</i> sp.	177	0.15
<i>Bos taurus</i>	176	0.15

Bacterial species	Cluster count	Percentage
<i>Alphaproteobacterium</i>	175	0.15
<i>Conexibacter woesei</i>	168	0.145
<i>Bacillus licheniformis</i>	148	0.13
<i>Pirellula staleyii</i>	147	0.123
Uncultured Bacteroidetes	137	0.12
Uncultured Rubrobacteridae	136	0.12
<i>Rhodococcus ruber</i>	120	0.10
<i>Opitutus</i> sp.	115	0.09
Uncultured Actinomycete	19	0.02

### 5.3: Substrate removal Kinetics

#### 5.3.1: Modified Stover-Kincannon model

The Stover-Kincannon model is the most used mathematical model for determining the substrate removal rate as a function of substrate loading rate. The model was initially used for the attached growth biomass performance in a rotating biological contactor (Stover & Kincannon, 1982) using the following equation:

$$\frac{dS}{dt} = \frac{Q(S_i - S_e)}{A} = \frac{U_{\max} \frac{QS_i}{A}}{K_B + \left(\frac{QS_i}{A}\right)} \quad (5.1)$$

Where  $\frac{dS}{dt}$  is the substrate removal rate ( $\text{mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ ),  $Q$  is the flow rate (L/d),  $V$  is the reactor liquid volume (L),  $S_i$  is the influent substrate concentration,  $S_e$  is the effluent substrate concentration (mg/L), and  $A$  is the total disc surface area on which biomass concentration is

immobilised ( $m^2$ ).  $K_B$  represents the saturation value constant ( $g/d.m^2$ ), whereas  $U_{max}$  is the maximum substrate removal rate constant ( $g.L^{-1}.d^{-1}$ ).

The original model was later modified and used to predict the bioreactor performance (Yu, et al., 1998). In this approach, the suspended biomass concentration was compared to the attached biomass. When the surface area ( $A$ ) is replaced by reactor volume ( $V$ ), the Stover-Kincannon model is modified as follows:

$$\frac{dS}{dt} = \frac{U_{max} \frac{QS_i}{V}}{K_B + \left(\frac{QS_i}{V}\right)} \quad (5.2)$$

For this equation, units of  $K_B$  change to  $g/L.d$

Eq. 5.2 can be linearised as follows:

$$\frac{1}{dS/dt} = \frac{V}{Q(S_i - S_e)} = \frac{K_B}{U_{max}} \cdot \frac{V}{QS_i} + \frac{1}{U_{max}} \quad (5.3)$$

If  $\frac{1}{dS/dt}$  is deduced as  $V/Q(S_i - S_e)$ , the inverse of the removal rate and is plotted against  $V/QS_i$ , the inverse of the loading rate, a straight line plot is obtained. From this plot, the slope gives  $\frac{K_B}{U_{max}}$  and the intercept of the straight line gives  $\frac{1}{U_{max}}$

The substrate balance for the reactor can be expressed as follows:

$$Q.S_i = \frac{dS}{dt}.V + QS_e \quad (5.4)$$

Replacing Eq. 5.2 in the above equation gives:



$$Q \cdot S_i = \frac{U_{\max} \frac{QS_i}{V}}{K_B + \left(\frac{QS_i}{V}\right)} \cdot V + QS_e \quad (5.5)$$

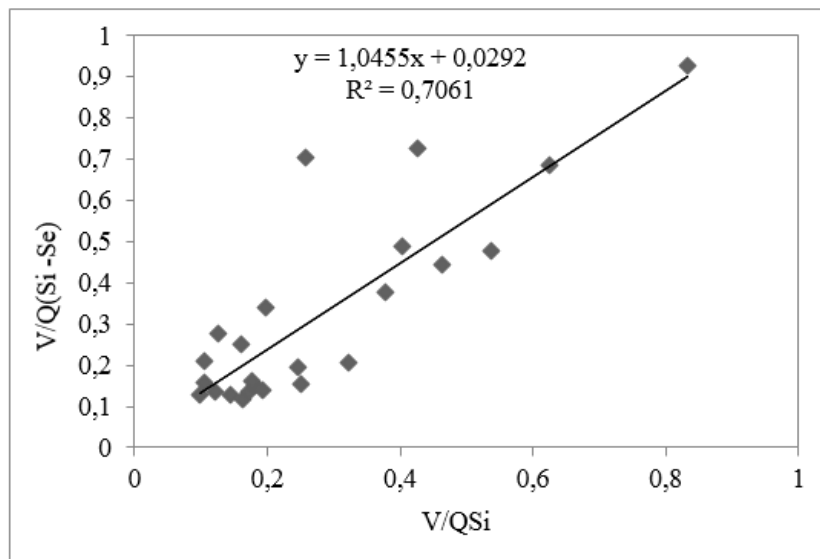
This equation can be solved for effluent substrate concentration by introducing the values of  $U_{\max}$  and  $K_B$  values using the following equation:

$$S_e = S_i - \frac{U_{\max} S_i}{K_B + \left(Q \cdot \frac{S_i}{V}\right)} \quad (5.6)$$

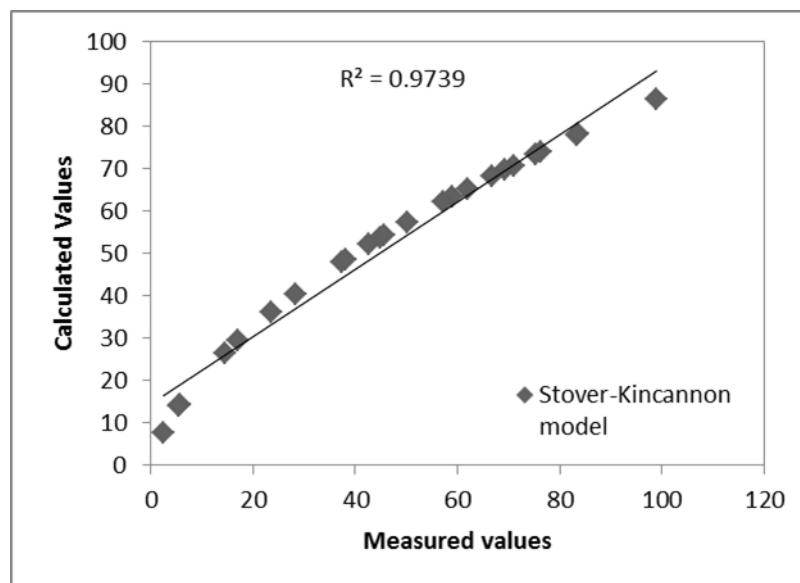
### 5.3.2: Model Kinetics

The nitrogen removal kinetics of the anammox reactor was determined using modified Stover-Kincannon model. Figure 5.5 shows the plot of  $V/[Q(S_i - S_e)]$ , the reciprocal of substrate removal rate against  $V/(QS_i)$ , the reciprocal of substrate loading rate. Saturation value constant  $K_B$  and maximum substrate removal rate  $U_{\max}$  were calculated from the slope and intercept of the line plotted in Figure 5.5 and determined to be 35.8 and 34  $\text{gNL}^{-1}\text{d}^{-1}$ , respectively. This implied that the anammox reactor had a maximum total nitrogen removal rate of 34  $\text{g N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ . These values were higher than those obtained in other studies (Gong et al., 2008; Jin & Zheng 2009; Ni, et al., 2010). The maximum total nitrogen removal rate from the experimental data was 5.8622  $\text{gN}\cdot\text{L}^{-1}\text{d}^{-1}$ . This value was much less than that of predicted values and only accounted for 17% of  $U_{\max}$ , indicating the nitrogen removal full capacity of the reactor had not been reached yet. The plot also gave correlation coefficient of  $R^2=0.7061$ , thus supporting the suitability of the modified Stover-Kincannon model.

The effluent nitrogen concentration was calculated using Equation. 5.6 after introducing the  $K_B$  and  $U_{\max}$  values in Equation. 5.3. The calculated values were compared with experimental data in order to validate the model. Figure 5.6 illustrates the comparison between predicted and experimental effluent concentrations. The linear relationship was obtained from the comparison. The linear relationship represented a good agreement between experimental and predicted total nitrogen effluent concentrations, and gave a high correlation coefficient ( $R^2=0.9739$ ). This indicated that the Stover-Kincannon model was suitable for nitrogen removal kinetics in sequencing batch reactor.



**Figure 5.5: Nitrogen removal model plot– modified Stover-Kincannon model**



**Figure 5.6: Validation of the modified Stover-Kincannon model**

#### 5.4: Summary

This study presents the achievability of successful start-up of the anammox process in a laboratory-scale sequencing batch reactor. The start-up duration of an anammox reactor with pre-enriched non-granular sludge was significantly reduced compared to the ones reported in

literature. The study showed that anammox non-granular culture had a great activity. A maximum total nitrogen removal rate of  $5.86 \text{ g.N.L}^{-1}\text{d}^{-1}$  was achieved. A maximum substrate removal rate of  $34 \text{ gN.L}^{-1}\text{d}^{-1}$  was also predicted. The model simulation matched the experimental data very well, proving the Stover-Kincannon model to be appropriate for nitrogen removal kinetics for the anammox process. The anammox bacteria that dominated the reactor were *Candidatus* Brocadia-like species. The presence of anammox bacteria in the reactor was considered to be responsible for nitrogen removal in the system.

## CHAPTER 6: GENETIC ENGINEERING STUDIES

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### 6.1: Introduction

The concern over nitrogen compounds as vital pollutants of wastewater is increasing due to the effects they have on the environment. Nitrogenous compounds play a critical role in eutrophication and oxygen depletion and are toxic to aquatic life (Paredes et al., 2007). Nitrogen compounds such as ammonium ( $\text{NH}_4^+$ ), nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) need to be removed from wastewater before being discharged to receiving water bodies in order to avoid adverse effects, such as algal blooms and hypoxic conditions in aquatic ecosystems.

For a long time, the biological process for nitrogen removal from wastewater has been the combination of nitrification-denitrification processes (Jeyanayagam, 2005; Kuenen & Robertson, 1994; Ward, 1996). However, the conventional method for nitrogen removal requires a significant amount of energy to generate aerobic conditions for bacterial nitrification, and also utilise organic carbon for removal of nitrate by bacterial denitrification (Chen et al., 2012; Kartal, et al., 2010;). These properties translate into high operational cost (Khin & Annachhatre, 2004) which is a cause for concern when treating nitrogen-rich wastewater. The latest process that is used as an alternative approach to conventional method is the anammox process. During the anammox process, ammonium is converted directly to nitrogen gas under anoxic conditions, with nitrite as an electron acceptor (Jetten et al., 1998; Kuenen, 2008; Mulder et al., 1995). The anammox process is autotrophic; it does not require the addition of a carbon source for denitrification (van de Graaf et al., 1996). Anammox is a one step process and translates into energy saving i.e. when the anammox process is combined with a preceding nitrification step, only part of the ammonium is nitrified to nitrite and the anammox process combines the residual ammonium with this nitrite to yield dinitrogen gas and less oxygen demand in the nitrification reactor (Strous et al., 1997). Anammox also produces less sludge due to less biomass yield. All the above mentioned advantages make the anammox process an appealing process for nitrogen removal and can save up to 90% of operational cost compared to conventional nitrogen treatment process

(Jetten et al., 2001). The worldwide establishment and sustainability of the anammox process has been hindered by the slow growth rates of anammox bacteria, combined with the inhibitory effects.

Various studies to overcome these limitations and to enhance the efficiency of the process have been carried out. (Chen et al., 2012) reported that the crucial approaches in anammox start-up and stable operation are to minimise the biomass washout and improve its retention rate. In order to minimise biomass washout, biofilm reactors were used (Fernández et al., 2008; Strous et al., 1997b). Furthermore, inhibition analysis studies were performed to optimise operational conditions in order to achieve high nitrogen performance (Dapena-Mora et al., 2007; Fernández et al., 2012; Tang et al., 2009).

However, little attention has been paid to genetic engineering technology as a means of improving the anammox process. In this study, an effort to improve the bacterial growth rate, efficiency and stability of the anammox process was made with the aid of genetic engineering technology. Therefore the aim of this study was to evaluate the use of genetically modified bacteria for nitrogen removal from nitrogen contaminated wastewaters.

## **6.2: Results and discussion**

### **6.2.1: Overview**

This study was undertaken to do the first step in the development of genetically engineered anammox bacteria for advance improvement of the anammox process. In this study, the hydrazine oxidoreductase (hzo) gene was inserted and expressed in *E. coli*. Hydrazine oxidoreductase is the key enzyme in the anammox process. It acts by catalysing the oxidation of hydrazine, which is the main intermediate in the anammox process, to dinitrogen (N<sub>2</sub>). By inserting the hydrazine oxidoreductase gene, we are providing *E. coli* with the properties of anammox bacteria. *E. coli* was chosen as the host due to many factors that are advantageous over anammox bacteria. Unlike native anammox bacteria, *E. coli* would not be affected by the diffusion of oxygen into the system, as it is a facultative anaerobe and would also not be affected by temperature fluctuation, as it can grow in a wide range of temperature (4 °C – 45 °C). Additionally, *E. coli* grows rapidly; therefore the growth rate of the bacteria would be improved

### 6.2.2: Codon optimisation for host engineering

The hydrazine oxidoreductase (hzo) gene was synthesised for maximal expression in *E. coli*. For high level expression, the target gene design was based on preferred codon usage of *E. coli*. Table 6. shows the frequently used codons in *E. coli*. For amino acids that can be encoded by more than one codon, the most frequently used codon in *E. coli* was used. The codon usage of *E. coli* was used to measure the Codon Adaptation Index (CAI) of the hzo gene, which was 0.97. Additionally, the GC content of the synthetic gene was kept within 40% - 60% by using codons containing AT and GC bases. The optimised gene was inserted into the pET15b expression vector and positioned downstream in the same frame with the sequence that encodes N-terminal His-tag fusion peptide. For the other DNA insert, the 6 His-tag was fused at the C-terminal end. Both DNA sequences were expressed in *E. coli* strains. The hzo genes to be cloned were optimised for expression in *E. coli*, as codon optimisation can significantly improve the expression level. These sequences of encoding hydrazine oxidoreductase were synthesised with codon preference of *E. coli* as reported by Young & Dong (2004) (Table 6.).

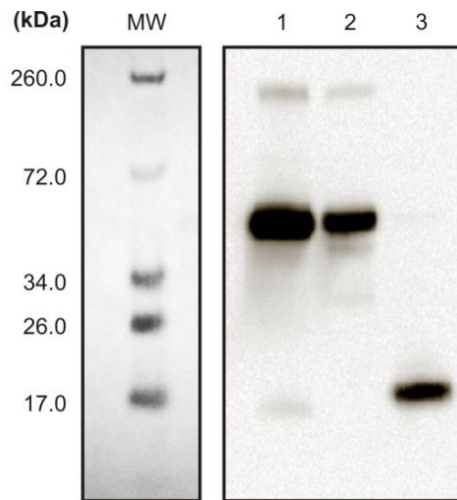
**Table 6.1: Codons frequently used in *E. coli***

AAA	Lys	ATC	Ile	CCG	Pro	GAT	Asp	TAC	Tyr
AAC	Asn	ATG	Met	CGT	Arg	GCA	Ala	TGG	Trp
ACC	Thr	CAG	Gln	CTG	Leu	GGT	Gly	TGC	Cys
AGC	Ser	CAT	His	GAA	Glu	GTG	Val	TTC	Phe

### 6.2.3: Expression of recombinant hzo proteins in *E. coli*

DNA expression cassettes encoding Hydrazine oxidoreductase fused to C- and N- 6 x Histidine tags were synthesised and cloned into a standard prokaryotic protein expression vector. These were then introduced into a standard *E. coli* strain (BL21) DE3 and used to affect the high level expression of each hzo construct. SDS-PAGE analysis revealed the presence of recombinant proteins with molecular weight of approximately 50 kDa each as major bands in the eluted fractions. Western blot analysis confirmed the expression of the

recombinant hzo proteins by reacting strongly with the mouse anti His-tag antibodies. HO-6His and 6His-HO were detected after induction with IPTG at 37°C for five hours (Figure 6.7, lanes 1 and 2). A positive expression control is also shown in Figure 6.7 (lane 3). Western blots demonstrated successful expression of hzo as fusion proteins with a His-tag at both the C- terminal and N- terminal in *E. coli* BL21 (DE3) under the control of T7 promoter. A high level of expression of about 5mg/L was observed.



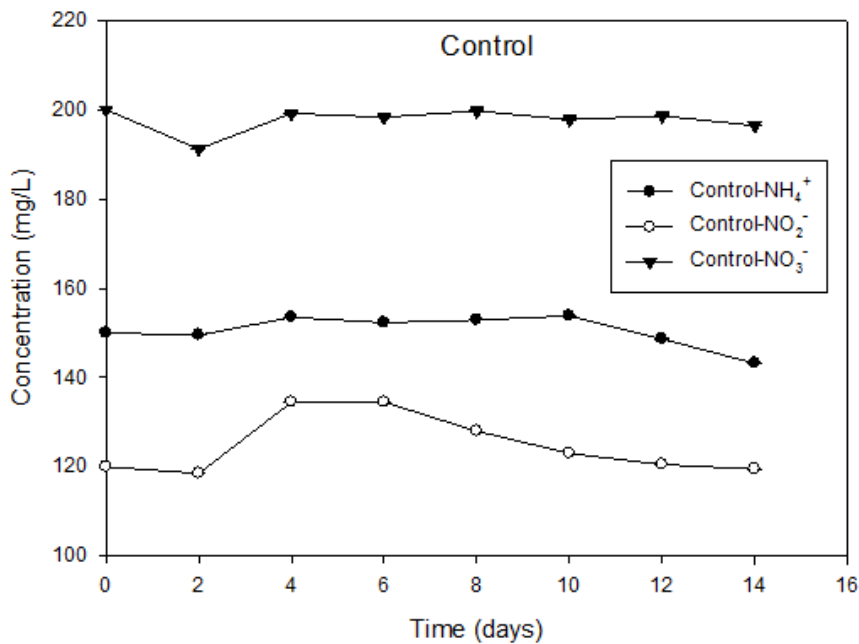
1. Hydrazine Oxidoreductase (C-terminal 6 X Histidine)
2. Hydrazine Oxidoreductase (N-terminal 6 X Histidine)
3. Positive Expression Control (6 X Histidine gp41)

**Figure 6.7: Western blot analysis of recombinant hzo products: First block; protein weight marker, Lane 1; recombinant HO-6 His, lane 2; recombinant 6 His-HO, induced with IPTG, Lane 3; Expression positive control- 6 x His tagged gp41 protein**

#### 6.2.4: Evaluation of nitrogen removal by transgenic *E. coli*

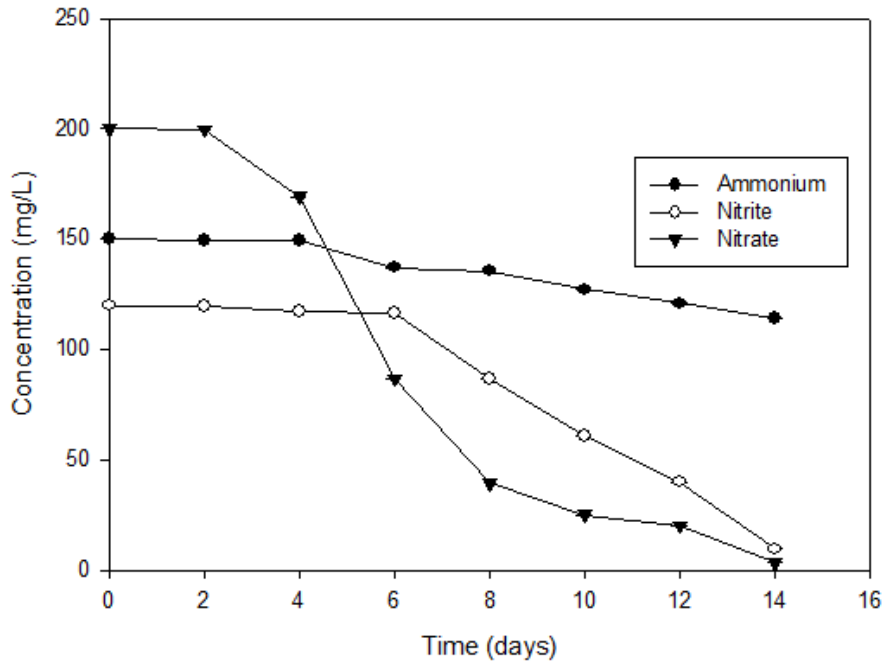
To evaluate the denitrogenation capability of the transgenic *E. coli* in conditions similar to those for anammox bacteria; it was inoculated on simulated wastewater containing nitrogen compounds i.e.  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ . Transgenic *E. coli* exhibited good performance for nitrogen removal from simulated wastewater. Nitrogen removal results are presented in Figure 6.. For the first six days, nitrogen removal was very slow, especially for ammonium and nitrite. Thereafter, the nitrogen removal of nitrate and nitrite improved and significant removal was observed (Figure 6.). Almost complete removal of nitrate and nitrite was achieved with the maximum removal efficiencies of 98% and 93% respectively (Figure 6. ).

However, ammonium was not removed significantly. During the entire experiment, the maximum ammonium removal efficiency was only 25%. On the other hand, no significant change was observed in the control of all the nitrogen compounds where transgenic *E. coli* was not added (Figure 6.8). These results revealed that the transgenic *E. coli* was able to remove nitrogen compounds in a short period of time as compared to the native anammox bacteria. After six days of incubation, nitrogen removal improved dramatically. Nitrate and nitrite were almost completely removed within 14 days of incubation, which is much less than previously reported in the anammox process. Thuan et al., 2004 reported that it took 200 days for anammox enrichment using synthetic medium while Yang et al., 2009 reported the period of 120 days to start up an anammox reactor using nitrifying sludge.

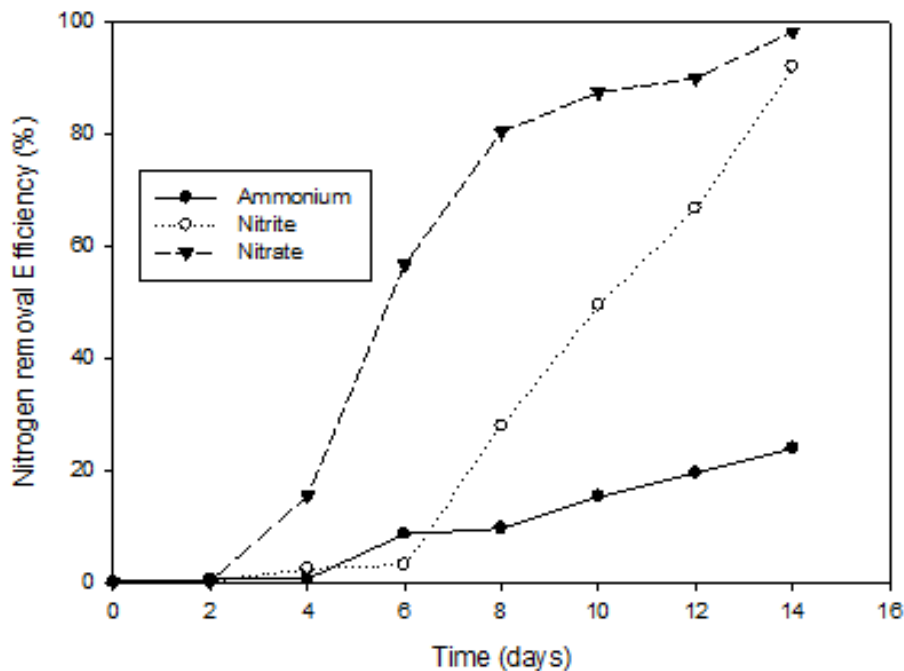


**Figure 6.8: Control represents the results for the reactor that was not inoculated with transgenic *E. coli***





**Figure 6.3: Nitrogen removal by transgenic *E. coli***



**Figure 6. 4: Efficiency of nitrogen removal by transgenic *E. coli***

Unexpectedly, transgenic *E. coli* also removed nitrate with the removal efficiency of 98%. In the anammox process, nitrate is accumulated. The nitrate removal made it possible for transgenic *E. coli* to remove the nitrogen compounds altogether and leaving no traces in the system, thereby demonstrating a great improvement of the process. However, ammonium was slowly removed. The slow ammonium removal could be associated with the absence of ammonium oxidising enzyme.

In the anammox process, nitrite is reduced to dinitrogen gas with ammonium as the electron donor. Anammox bacteria use a complex reaction mechanism comprising of hydrazine as a major intermediate. Hydrazine is in turn converted to dinitrogen gas by hydrazine oxidoreductase generating four reduction electrons that are believed to reduce nitrite to hydroxylamine (Van de Graaf et al., 1997). In the present study, the reaction mechanism utilised by transgenic *E. coli* to remove the nitrogen compounds is not well understood at this stage and is still yet to be determined. Although the reaction mechanism is still not clear, the consumption of nitrate and nitrite could be attributed to the fact that hzos are known to contain constituents that are important for oxidation of N-oxides (Klotz et al., 2008).

### 6.3: Summary

This study addresses the application of genetic engineering to improve wastewater treatment. The enzyme hydrazine oxidoreductase (hzo) is reported to be the key player in the anammox biochemical process. This study was aimed at producing the recombinant hzo using a bacterial expression system and determines its ability to remove nitrogenous compounds. The complete gene sequence encoding hzo was used as a template to synthesise the gene for cloning into the pET15b vector. Hzo was successfully ligated to the cloning vector and transformed into BL21 (DE3) competent *Escherichia coli* cells. The presence and orientation of the inserted gene was confirmed by enzyme restriction and sequencing, respectively. Optimal expression of recombinant hzo protein was achieved by induction at 37°C using a final concentration of 1 mM IPTG for five hours. A protein of ~50 kDa was detected on SDS-PAGE and confirmed by chemiluminescence on western blots using an antibody directed at the fusion tag. Expression of hydrazine oxidoreductase in *E. coli* resulted in nitrogen removal. The experiments revealed that the transgenic *E. coli* was able to remove nitrogen compounds in a shorter period of time compared to the native anammox bacteria. Our results, therefore, support the genetic engineering technology as a meaningful approach to improve the anammox process for nitrogen removal from wastewater. The results found in this study lay a foundation for further developments in genetic engineering to improve the anammox process.

# CHAPTER 7: MODELLING OF SEQUENCING BATCH REACTOR

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## 7.1: Introduction

Modelling biological processes is very important for appropriate control of full scale plants. A multi-variable control system is often used to govern all the biological and abiotic processes by differential-algebraic equations with dynamic state variables (Puyol et al., 2013). The biological processes under anaerobic conditions are usually rate-limiting; therefore the kinetics of the process need to be clarified so as to optimise the succeeding step of modelling and control (Puyol et al., 2013). There is a need for reliable predictions of kinetic parameters that can improve the understanding and the modelling of the anammox process in order to improve the design of the process. Biokinetic parameters, such as substrate affinities, maximum growth rate or maintenance need cannot be accurately evaluated as they are hindered by the mass transfer limitations within the anammox granule (Chu, et al, 2003; Harremoës, 1977). Therefore, transport limitations possibly result in the underestimation of the observed maximum specific growth rate ( $\mu_{\max}$ ) and overestimation of the intrinsic affinity constant ( $K_S$ ) (Lotti et al., 2014).

(Dapena-Mora et al., 2004b; Strous et al., 1998) studied anammox using a SBR. However, both studies adopted a granule based reactor design. Kinetic parameter values of interest, such as maximum growth rate and affinity constant for the substrates that are currently known and reported in literature (Strous et al., 1998; van der Star et al., 2008), may probably be suitable for granule based reactor designs and may result in analytical imprecision of other types of design (e.g. non-granular sludge design).

In this study, mathematical modelling describing anammox in a sequencing batch reactor using non-granular sludge was simulated and biokinetic parameters such as yield coefficient ( $Y$ ), decay coefficient ( $b$ ), maximum specific growth rate ( $\mu_m$ ) and affinity constant ( $K_s$ ) were predicted using AQUASIM software (Reichert, et al, 1998).

## 7.2: Model and Simulation

### 7.2.1: Biokinetic model theory

Mathematical modelling is a powerful tool to describe the comprehensive knowledge of the process, predicting behaviour, optimisation of the process, and process control. The best high level model developed for biological wastewater treatment processes is ASM1 (Henze et al., 1987).

ASM1 is an internationally accepted model for modelling the activated sludge in the urban wastewater treatment and includes various processes that take place in the activated sludge system which include the following processes:

- Aerobic growth of heterotrophic bacteria
- Anoxic growth of heterotrophic bacteria
- Aerobic growth of autotrophic bacteria
- Death and decay of heterotrophic bacteria
- Death and decay of autotrophic bacteria
- Hydrolysis of particulate organic matter
- Hydrolysis of particulate organic nitrogen
- Ammonification and dissolved organic nitrogen

These kinetic processes are expressed as follows:

Aerobic growth of heterotrophs

$$\rho_H = \mu_{max,H} \left( \frac{S_O}{K_{O,H} + S_O} \right) \left( \frac{S_S}{K_{S,H} + S_S} \right) \left( \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \right) \left( \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \right) X_H \quad (7.1)$$

Anoxic growth of heterotrophs

$$\rho_H = \mu_{max,H} \eta_g \left( \frac{K_{O,H}}{K_{O,H} + S_O} \right) \left( \frac{S_S}{K_{S,H} + S_S} \right) \left( \frac{S_{NO_3}}{K_{NO_3} + S_{NO_3}} \right) \left( \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \right) X_H \quad (7.2)$$

Aerobic growth of autotrophs

$$\rho_A = \mu_{max,A} \left( \frac{S_O}{K_{O,A} + S_O} \right) \left( \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \right) \left( \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \right) X_A \quad (7.3)$$

Decay of heterotrophs

$$\rho_{decay,H} = b_H X_H \quad (7.4)$$

Decay of autotrophs

$$\rho_{decay,A} = b_A X_A \quad (7.5)$$

Hydrolysis of particulate organic matter

$$k_h \frac{X_S/X_H}{K_X + X_S/X_H} \left( \frac{S_O}{K_{O,H} + S_O} + \eta_h \frac{K_{O,H}}{K_{O,H} + S_O} \frac{S_{NO_3}}{K_{NO_3} + S_{NO_3}} \right) X_H \quad (7.6)$$

Hydrolysis of particulate organic matter

$$k_h \frac{X_{ND}/X_H}{K_X + X_S/X_H} \left( \frac{S_O}{K_{O,H} + S_O} + \eta_h \frac{K_{O,H}}{K_{O,H} + S_O} \frac{S_{NO_3}}{K_{NO_3} + S_{NO_3}} \right) X_H \quad (7.7)$$

Ammonification and Dissolved organic nitrogen

$$k_{aN} S_{ND} X_H \quad (7.8)$$

The parameters used in the ASM1 model, their symbols, and their units are defined in Table 7.1.



**Table 7.1: Kinetic and Stiochiometric Parameters of ASM1**

Parameter Definition	Symbol	Units
<i>Stoichiometric parameter</i>		
Heterotrophic yield	$Y_H$	g COD/g COD
Autotrophic yield	$Y_A$	g COD/g COD
Nitrogen fraction in biomass	$i_{XB}$	g N/g COD
Nitrogen fraction in endogenous mass	$i_{XB}$	g N/g COD
Fraction of biomass leading to particulate material	$f_P$	
<i>Kinetic parameters of heterotrophs</i>		
Maximum specific growth rate	$\mu_{max,H}$	1/day
Substrate saturation constant	$K_{S,H}$	g COD/m <sup>3</sup>
Oxygen saturation constant	$K_{O,H}$	g O <sub>2</sub> /m <sup>3</sup>
Nitrate saturation constant	$K_{NO_3}$	g NO <sub>3</sub> -N/m <sup>3</sup>
decay rate coefficient	$b_H$	1/day
Decay rate	$\rho_H$	g COD/m <sup>3</sup> /day
Anoxic growth correction factor	$\eta_g$	
<i>Kinetic parameters of autotrophs</i>		
Maximum specific growth rate	$\mu_{max,A}$	1/day
Ammonium saturation constant	$K_{NH_4}$	g NH <sub>4</sub> -N/m <sup>3</sup>
Oxygen saturation constant	$K_{O,A}$	g O <sub>2</sub> /m <sup>3</sup>
Decay rate coefficient	$b_A$	1/day
Decay rate	$\rho_A$	g COD/m <sup>3</sup> /day
<i>Hydrolysis Parameters</i>		
Maximum specific hydrolysis rate	$k_h$	1/day
Anoxic hydrolysis correction factor	$\eta_h$	
Half saturation coefficient for hydrolysis of XS	$K_S$	
<i>Ammonification</i>		
Ammonification rate constant	$k_\alpha$	m <sup>3</sup> /g COD day

The ASM1 was later modified by extending it to fit a two-step nitrification and denitrification process as well as the anammox processes. In order to modify the ASM1, new groups of organisms were introduced to the model. ASM1 nitrifiers were then divided into ammonium oxidising organisms ( $X_{NH}$ ) and nitrite oxidising organisms ( $X_{NO}$ ), and their growth is represented as follows by the Monod terms Eq. 7.9 and Eq. 7.10:

Ammonium oxidising organisms

$$\rho_{NH} = \mu_{max,NH} \left( \frac{S_O}{K_{O,NH} + S_O} \right) \left( \frac{S_{NH}}{K_{NH,NO} + S_{NH}} \right) X_{NH} \quad (7.9)$$

Nitrite oxidising organisms

$$\rho_{NO} = \mu_{max,NO} \left( \frac{S_O}{K_{O,NO} + S_O} \right) \left( \frac{S_{NO_2}}{K_{NO_2,NO} + S_{NO_2}} \right) X_{NO} \quad (7.10)$$

To model the anammox process, the modified model comprises of anammox biomass ( $X_{AN}$ ), and Monod kinetics were used to describe the dependency of the growth rate of anammox on ammonium and nitrite according to Eq. 7.11:

$$\rho_{AN} = \mu_{max,AN} \left( \frac{K_{O,AN}}{K_{O,AN} + S_O} \right) \left( \frac{S_{NO_2}}{K_{NO_2,AN} + S_{NO_2}} \right) \left( \frac{S_{NH_4}}{K_{NH_4,AN} + S_{NH_4}} \right) X_{AN} \quad (7.11)$$

Notably, as much as nitrite is a substrate, it could also inhibit the anammox process (Dapena-Mora et al, 2004b, Strous et al, 1999). However, inhibition of anammox bacteria by nitrite was not included in this study because the concentrations of nitrite measured in the reactor were too low to cause inhibition.

In this study, the decay process of anammox is modelled according to the death-regeneration concept using Eq. 7.12. This death-regeneration approach used in this model is based on the fact that heterotrophic organisms were found to be active in autotrophic reactors without an



addition of external COD source, which can be explained by death-regeneration, and not by endogenous respiration, which was applied in ASM3.

$$\rho_{decay,AN} = b_{AN} X_{AN} \quad (7.12)$$

The kinetic and stoichiometric parameters for the anammox process in the developed model, their symbols, and their units are presented in Table 7.2, while the stoichiometric matrixes of the used in the model are listed in Table 7.3.

**Table 7.2: Kinetic and stoichiometric parameters used in anammox model**

Parameter Definition	Symbol	Units
Maximum growth rate of anammox	$\mu_{maxAN}$	day <sup>-1</sup>
Decay rate coefficient for anammox	$b_{AN}$	day <sup>-1</sup>
Ammonium affinity constant for anammox	$K_{NH_4}$	gNL <sup>-1</sup>
Nitrite affinity constant for anammox	$K_{NO_2AN}$	gNL <sup>-1</sup>
Yield coefficient for anammox growth	$Y_{AN}$	gCODg <sup>-1</sup> N

**Table 7.3: Stoichiometric matrix for the anammox model**

Process	Component							
	$X_S$ [gCOD m <sup>-3</sup> ]	$X_{AN}$ [gCOD m <sup>-3</sup> ]	$X_P$ [gCOD m <sup>-3</sup> ]	$S_{NO_2}$ [gN m <sup>-3</sup> ]	$S_{NO_3}$ [gN m <sup>-3</sup> ]	$S_{N_2}$ [gN m <sup>-3</sup> ]	$S_{NH_4^1}$ [gN m <sup>-3</sup> ]	$S_{ALK^2}$ [mol m <sup>-3</sup> ]
Growth of $X_{AN}$		1		$-1.52 - \frac{1}{Y_{AN}}$	1.52	$\frac{2}{Y_{AN}}$	$-i_{NXBA} - \frac{1}{Y_{AN}}$	$-\frac{i_{NXBA}}{14}$
Decay of $X_{AN}$	$1 - f_p$	-1	$f_p$				$i_{NXBA} - f_p i_{NXP}$	$\frac{1}{14}(i_{NXBA} - f_p i_{NXP})$

### 7.2.2: Model Simulation

A model describing the anammox process in a SBR was simulated in AQUASIM (Reichert, Ruchti & Simon, 1998). The anammox process was modelled using the modified ASM1 describing anammox. The soluble compounds that were considered were ammonium, nitrite and nitrate. The particulate compound considered was anammox biomass as the only active bacteria. The compound considered in the gas phase was the dinitrogen gas.

### 7.2.3: Model Calibration

Model calibration is an extremely significant step in the entire modelling procedure. It is the process of adjusting selected parameter values of the model in order for the results generated by the model, using these adjusted parameters, to corroborate with the experimental results. The selection of the most important parameters during calibration may increase the prediction power of the model (Cema et al., 2012). The number of stoichiometric and kinetic parameters coupled to anammox biomass ( $X_{AN}$ ) was incorporated to the developed model for simulation function.

In this study, the calibration approach was to fit the simulation results of the model on experimental data of the effluent substrates (i.e. ammonium and nitrite) that were measured during the operation of the reactor using calibrated parameters. Five parameters (i.e.  $K_{NO_2, AN}$ ,  $K_{NH_4, AN}$ ,  $\mu_{max, AN}$ ,  $b_{AN}$  and  $Y_{AN}$ ) were adjusted based on the influence they have on the experimental data and the effect they have on anammox bacteria.

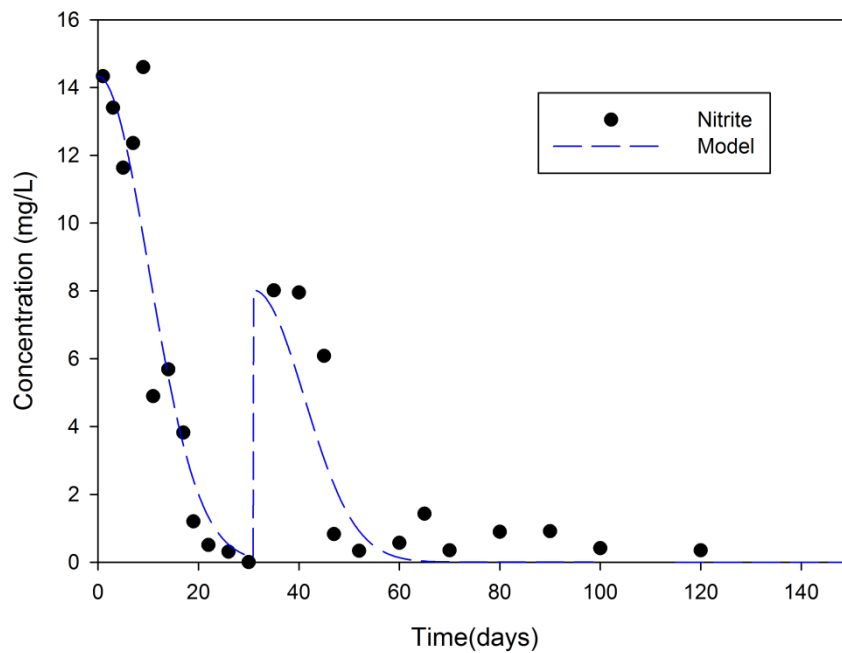
**Table 7.4: Parameter values adjusted during model calibration of anammox model**

Parameter	Unit	Calibrated value
$\mu_{max}$	day <sup>-1</sup>	0.32
$K_{NO_2, AN}$	mg.L <sup>-1</sup>	0.024
$K_{NH_4, AN}$	mg.L <sup>-1</sup>	0.054
$b_{AN}$	day <sup>-1</sup>	0.0009
$Y_{AN}$	gCOD.g <sup>-1</sup> N	0.230

Table 7.4 presents the parameter values that were adjusted during model calibration. The profiles of the calibration results showing the model predictions and experimental

measurements of the effluent nitrite and ammonium concentrations are illustrated in **Figure 7.1** and **Figure 7.2**, respectively.

As shown in **Figure 7.1** and **Figure 7.2**, the model and the calibrated parameters were able to appropriately simulate the distinction of the effluent substrate concentrations and the acceptable agreement between the measured and the simulated data was obtained.

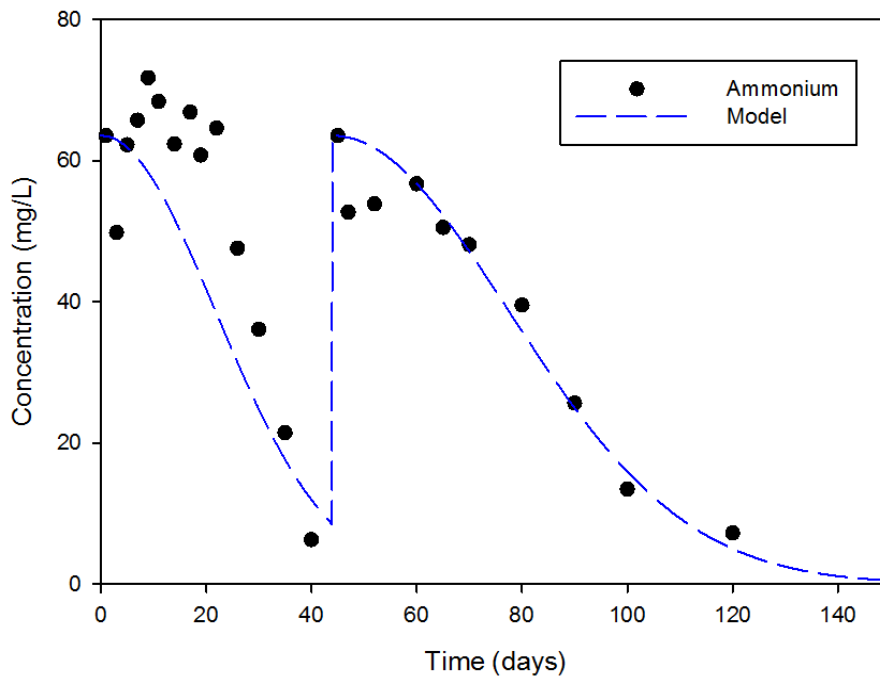


**Figure 7.1: The measured (●) and the calculated (---) nitrite concentrations in the effluent of the sequencing batch reactor**

#### 7.2.4: Substrate affinities

The half saturation or affinity constant is an important parameter for the construction of biological processes and for understanding the bacterial ecology. With regards to the affinity constants, the results of the present study showed that anammox had higher affinity for nitrite than ammonium, resulting in the  $K_s$  value for nitrite much lower than for ammonium (Table 7.1). These results are corresponding to the previously reported results by (Chen et al., 2011) who found the  $K_s$  values of granular anammox for ammonium and nitrite as 36.75 and 0.657 mg N/L, respectively. Correspondingly, (Zu, Zhang & Yan, 2008) reported the  $K_s$  values of ammonium and nitrite as 87.1 and 15.39 mg N.L<sup>-1</sup>, respectively for the anammox granular

sludge. In the present study, the affinity constant for both ammonium and nitrite were found to be 0.054 and 0.024 mg N.L<sup>-1</sup>, respectively.

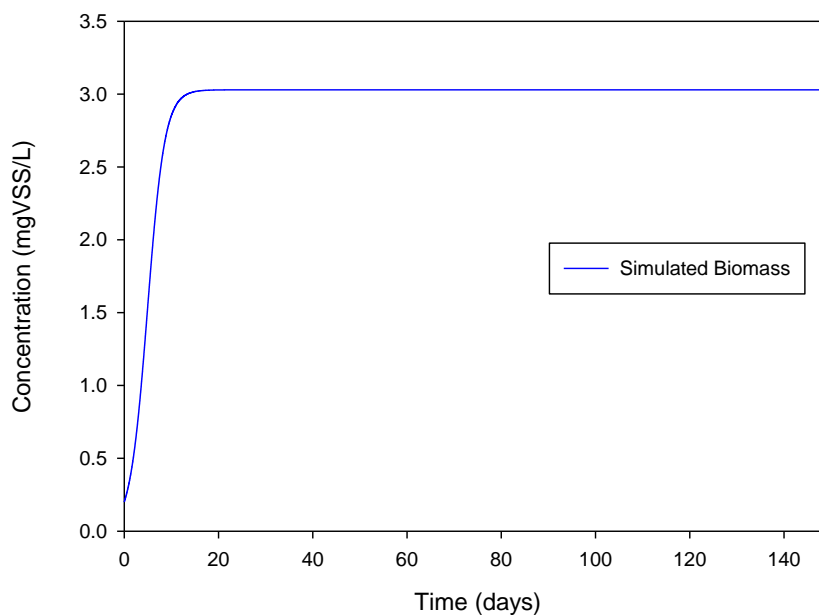


**Figure 7.2: The measured (●) and the calculated (---) ammonium concentrations in the effluent of the sequencing batch reactor**

Notably, although the results are all corresponding, the values for the granular anammox are extremely high compared to non-granular sludge reported in the present study. The higher values of  $K_s$  were attributed to diffusion or mass transfer limitations in granular systems. (Lotti et al., 2014) reported a direct link between high mass transfer limitations associated with bigger granular size and high half saturation/affinity constant. In addition, (Chen et al., 2011) also reported a stronger mass transfer resistance related to bigger anammox granular sludge, resulting in higher values of substrate affinity constant in their study. The  $K_s$  value with mass transfer resistance, mostly by granular sludge, is termed apparent affinity constant, whereas the one estimated by tiny free cells, such a non-granular sludge, is referred to as intrinsic affinity (Lotti et al., 2014). Therefore, in the present study, non-granular sludge was used and there was no mass transfer limitation and therefore affinities were considered intrinsic.

### 7.2.5: Growth rate and Biomass yield

In Figure 7.3, the growth of anammox bacteria is illustrated. The quantity of anammox biomass was initially low, and then rapid exponential growth, followed by a long term stationary phase, was observed. The cultures used in this study were pre-enriched using batch reactors before they were used to seed the sequencing batch reactor, consequently the bacteria were already growing when starting up the sequencing batch reactor. Therefore, the evolution seen in Figure 7.3 shows the stage at which the biomass was at (log phase) during the time of inoculation which is marked by the lack of a lag phase.



**Figure 7.3: Simulated biomass activity in the sequencing batch reactor**

With respect to the maximum growth rate and biomass yield, these parameters were calculated as  $0.32d^{-1}$  and  $0.230 \text{ gCOD} \cdot \text{g}^{-1} \text{ N}$ , respectively. There is a wide range of maximum growth values reported in literature. (van de Graaf et al., 1996) performed a mass balance during the period of highest substrate uptake rate on their laboratory-scale system and reported a  $\mu_{max, An}$  of  $0.001 \text{ h}^{-1}$ . (Strous et al., 1998) used the same mass balance approach with an enriched lab-scale system and estimated  $\mu_{max, An}$  to be  $0.0027 \text{ h}^{-1}$ . (Strous et al., 1998) attributed the difference in estimates to a higher degree of enrichment in their study than in

the previous study, or the inactivation of part of the population due to limited mass transfer in biomass granules. Furthermore, (Lotti et al., 2014) reported a  $\mu_{max,An}$  of 0.21 d<sup>-1</sup>. However, (Isaka et al., 2006) estimated  $\mu_{max,An}$  by coupling cell concentration, determined using fluorescent in situ hybridisation (FISH) with measured substrate uptake rate, to be 0.39d<sup>-1</sup>. This value was much higher than all the others reported.

### 7.3: Summary

The kinetic properties of a non-granular anammox enrichment culture was investigated in a sequencing batch reactor. A mathematical model describing the anammox process was developed. By using simulations in AQUASIM, the kinetics of anammox reaction was predicted. Kinetic parameters for anammox, such as the maximum specific growth rate ( $\mu_{max,An}$ ), the half-saturation constant ( $K_{An}$ ), the microbial growth yield ( $Y_{An}$ ), and the microorganism decay rate constant ( $b_{An}$ ), are presented. In this study, the maximum growth rate of the anammox enrichment culture was 0.32 d<sup>-1</sup>, which was higher than previously reported.

Estimated substrate affinities were corresponding to the previously reported parameters. However, in this study, the parameter values were improved due to the absence of mass transfer limitation. These simulation results indicated that non-granular sludge was suitable for nitrogen rich wastewaters. The anammox non-granular sludge exhibited outstanding anammox activity. Anammox non-granular sludge was proved to have remarkable substrate affinity.

## CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

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### 8.1: Summary and Conclusions

In this study, the start-up and the enrichment of anammox from wastewater treatment sludge samples in batch reactors were assessed. Anammox biomass could be enriched from the sludge samples that contained anammox biomass even in very low concentrations. Anammox was detected from three of the four sludge samples that were evaluated. Identification of anammox bacteria in batch reactors was done by PCR analysis. The study provided evidence for the existence of anammox bacteria in some of local wastewater treatment works. The study also provided the information of the possibility of the wide distribution of anammox with little diversity, as it was found that the habitats are dominated by only *Brocadia* species. The maximum growth rate of anammox bacteria was determined to be higher than previously reported in literature.

A sequencing batch reactor (SBR) was set up and operated for the removal of nitrogen compounds in wastewater. Samples of biomass from successfully enriched cultures were used as inoculum for the SBR. The systems worked effectively, achieving the total nitrogen removal rate of 93%. Samples of biomass obtained from the SBR were analysed for identification of the main bacterial populations present in the reactor. Next generation sequencing (NGS) was used for the identification of bacterial population. The reactor was found to be dominated by different *Bacillus* species with an abundance of 84%. Although the reason for the over-abundance of these bacterial species is not clear, it could be associated with the nature of the bacteria, as it is very resistant and able to survive in most environmental conditions. Although *Bacillus* species were dominating the reactor, they were not attributed to nitrogen removal in the system. However, the total of 5% anammox and planctomycetes bacteria was also seen in the reactor. All other species were available in insignificant amounts. For substrate removal kinetics, the saturation value constant  $K_B$  and maximum substrate removal rate  $\mu_{max}$  were determined to be 35.8 and 34 gNL<sup>-1</sup>d<sup>-1</sup> respectively. Generally the non-granular sludge culture showed the highest nitrogen removal rate.



With respect to genetic engineering technology, an effort to improve the anammox process with this technique was made. The major advantage of genetic engineering is that the required properties in an organism can be modified to suit the requirement. The expression of hydrazine oxidoreductase in *E. coli* resulted in nitrogen removal after inoculating simulated wastewater. Although the application of genetic engineering technology to improve anammox is still in its foundation level, it is an innovative technology that can speed up the anammox process, as well as improve the efficiency and stability of the process.

Based on the simulation results obtained during modelling, it can be concluded that a sequencing batch reactor with free cell suspension culture is best for investigating intrinsic properties of anammox bacteria. The acceptable match between the model and the experimental data indicates that the model has the appropriate prediction power.

The overall information obtained in this study provides useful input for improving the anammox process and contributes new and innovative information to the already existing knowledge on the anammox process.

## **8.2: Recommendations**

The study was a great success but there is still more room for improvement especially in the genetic engineering studies. The results found in this study lay a foundation for further developments in genetic engineering to improve the anammox process. The future research should include the following:

1. Inserting the gene that is responsible for ammonium oxidation in *E. coli* in order to improve the ammonium removal.
2. Determining the reaction mechanism utilized by transgenic *E. coli*.
3. To conduct the experiments with the actual wastewater to determine possible interactions with transgenic *E. coli*.

As the introduction of genetically modified organisms into the environment poses a risk of creating new and/or more virulent pathogens, safer alternatives are required. Therefore, the use of purified recombinant proteins would be advantageous over the use of the whole transgenic *E. coli*. This would make it easy for scaling up and integration into the existing

wastewater treatment system without the risk to harm the environment or disruption of biotic communities.

## CHAPTER 9: ENGINEERING SIGNIFICANCE

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Anaerobic Ammonium Oxidation (anammox) is a novel, autotrophic, and cost-effective alternative to the traditional biological nitrogen removal process. Anammox process has some remarkable properties such as low biomass yield, no need for aeration, and no addition of external carbon sources and these make it a favourable and sustainable technique for nitrogen removal. Regardless of these advantages, there is still uncertainty over implementing the technology at full-scale proven by very limited number of full scale anammox reactors worldwide. Although the anammox process unfolds new prospects for nitrogen removal from wastewater, the major hindrance for the implementation of anammox is the slow growth rate of anammox bacteria resulting in difficulty to utilise the process for practical wastewater treatments. Additionally anammox bacteria have not yet been isolated in pure cultures. In pursuit of the universal full practical application of anammox process, researchers focused on the enrichment of slowly growing anammox bacteria either by different methods such as biofilm or granulation, in various types of reactors.

This study addresses some of the anammox process limitations. Firstly, bio-kinetic parameters, such as substrate affinities and maximum growth rate that are currently known and reported in literature are based on granular anammox and are hindered by the mass transfer limitations within the anammox granule. This study provides a clear understanding of the modelling of non-granular anammox process for design purposes and provides accurate kinetics parameter values. The affinity constant for both ammonium and nitrite were estimated to be 0.054 and 0.024 mg N.L<sup>-1</sup>, respectively. The maximum growth rate parameter was calculated as 0.32d<sup>-1</sup>. All these parameters were improved due to the absence of mass transfer limitation. This suggests that there is great development for the future of the anammox process as the process limitation related to the intrinsic properties of bacteria has been overcome.

This study also highlights the attempt to overcome the limitation on the most alarming subject about the anammox process, i.e. very slow growth rate of anammox bacteria. The genetic engineering technology was utilised to overcome anammox slow growth rate. Insertion and expression of one of the key enzymes of anammox process in the *E. coli*

resulted in the fast and drastic removal of nitrogen compounds from wastewater. The results found in this study lay a foundation where more work is required to improve the bacterial growth rate and therefore fast start-up of the anammox. These results provide an optimistic perspective regarding the future of the anammox process relating to the process stability and sustainability.

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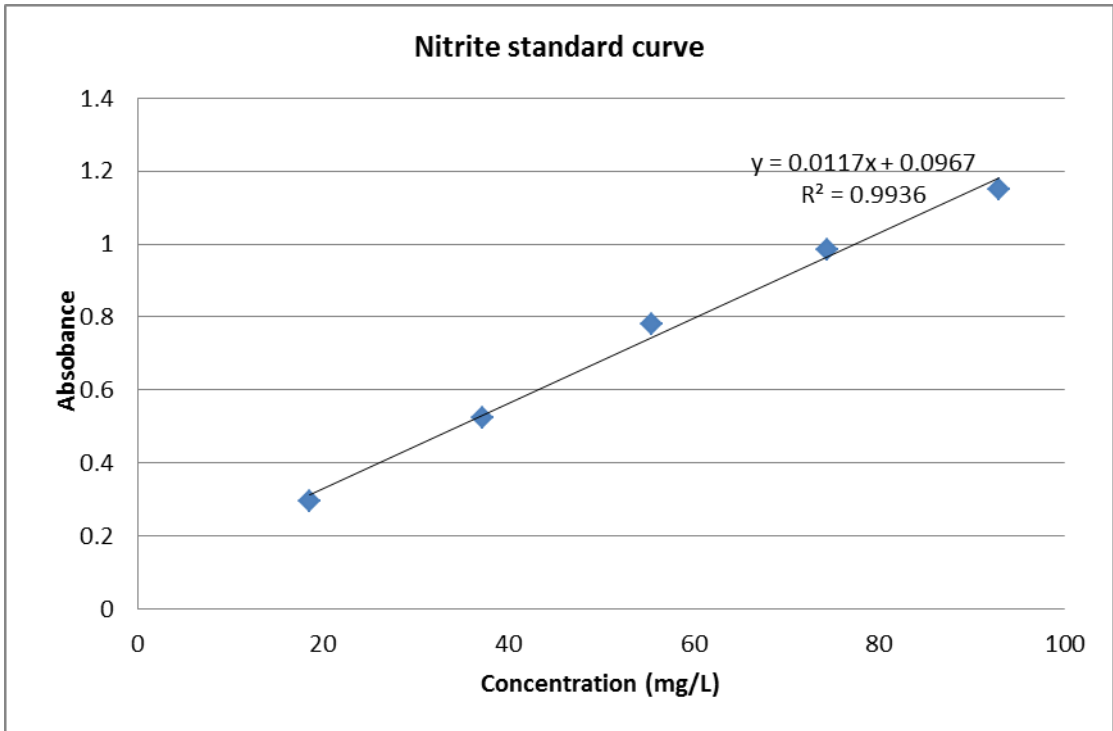
# APPENDICES

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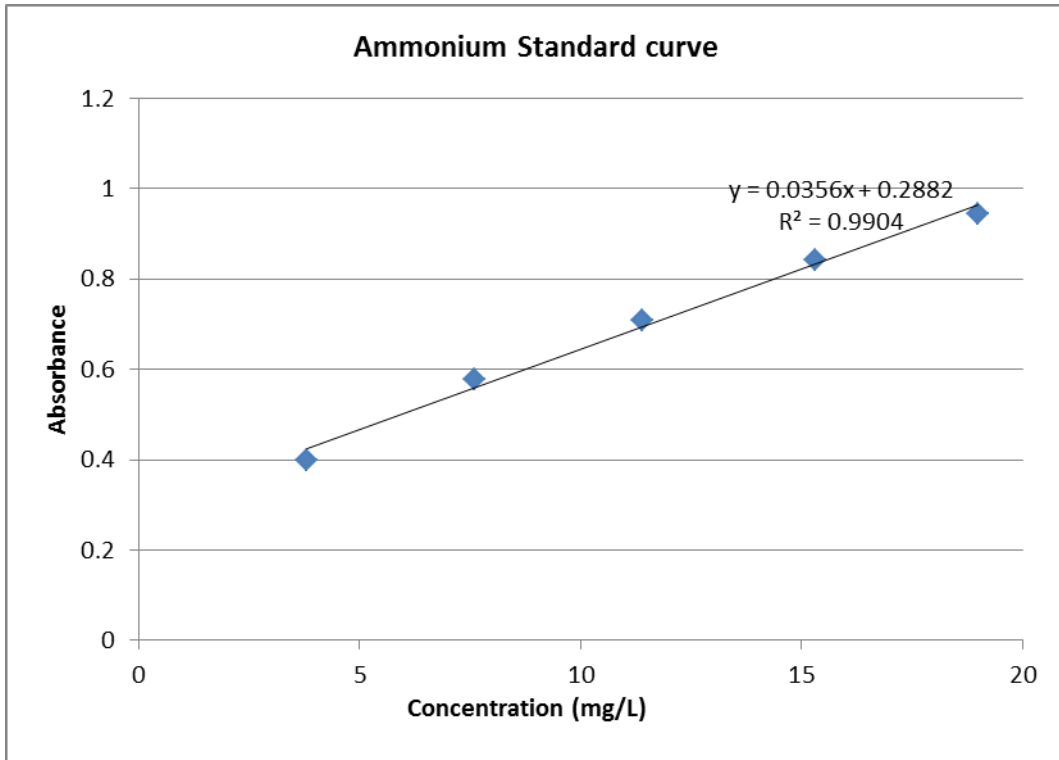
## APPENDIX A NITROGEN ANALYSIS STANDARD CURVES

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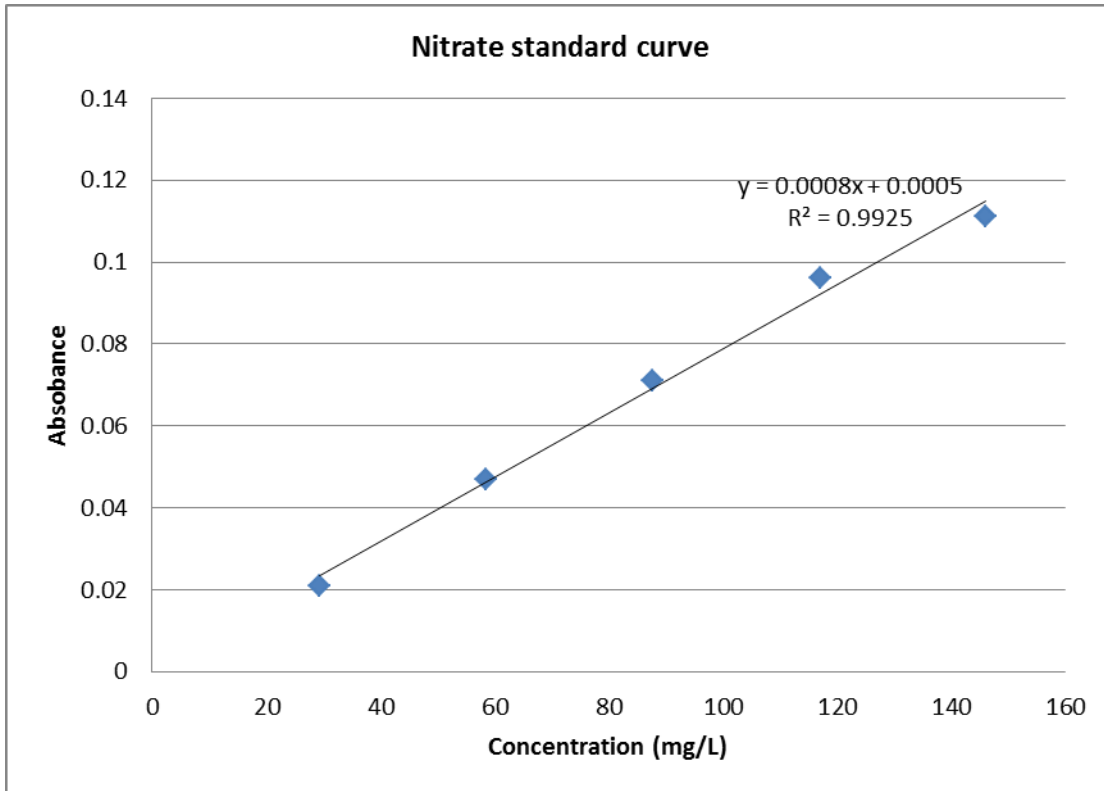
### Appendix A1



Appendix A2



Appendix A3



## APPENDIX B

### HYDRAZINE OXIDORECTASE GENETIC SEQUENCE

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Accession: HQ666192.1

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