Evaluation of Seasonal Impacts on Nitrifiers and Nitrification Performance of a Full-Scale Activated Sludge System

This work is submitted in complete fulfilment for the degree of Doctor of Philosophy (Biotechnology) in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences at the Durban University of Technology, Durban, South Africa

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(BSc. (Hons): Microbiology; MSc: Environmental Control and Management)

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DECLARATION

"I declare that the thesis herewith submitted for the degree Doctor of Philosophy: Biotechnology at the Durban University of Technology is my original work and has not been previously submitted for a degree at any other institution of higher education, and that its only prior publication was in the form of conference papers, book chapter and/or journal articles. I further declare that all the sources cited or quoted are acknowledged and indicated by means of a comprehensive list of references."

Oluyemi Olatunji Awolusi

I hereby approve the final submission of the following thesis.

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ABSTRACT

Seasonal nitrification breakdown is a major problem in wastewater treatment plants which makes it difficult for the plant operators to meet discharge limits. The present study focused on understanding the seasonal impact of environmental and operational parameters on nitrifiers and nitrification, in a biological nutrient removal wastewater treatment works situated in the midlands of KwaZulu Natal.

Composite sludge samples (from the aeration tank), influent and effluent water samples were collected twice a month for 237 days. A combination of fluorescent in-situ hybridization, polymerase chain reaction (PCR)-clone library, quantitative polymerase chain reaction (qPCR) were employed for characterizing and quantifying the dominant nitrifiers in the plant. In order to have more insight into the activated sludge community structure, pyrosequencing was used in profiling the amoA locus of ammonia oxidizing bacteria (AOB) community whilst Illumina sequencing was used in characterising the plant's total bacterial community. The nonlinear effect of operating parameters and environmental conditions on nitrification was also investigated using an adaptive neuro-fuzzy inference system (ANFIS), Pearson's correlation coefficient and quadratic models.

The plant operated with higher MLSS of 6157±783 mg/L during the first phase (winter) whilst it was 4728±1282 mg/L in summer. The temperature recorded in the aeration tanks ranged from 14.2^{o}C to 25.1^{o}C during the period. The average ammonia removal during winter was 60.0±18% whereas it was 83±13% during summer and this was found to correlate with temperature ($r = 0.7671$; $P = 0.0008$). A significant correlation was also found between the AOB (amoA gene) copy numbers and temperature in the reactors ($\alpha = 0.05$; $P=0.05$), with the lowest AOB abundance recorded during winter. Sanger sequencing analysis indicated that the dominant nitrifiers were *Nitrosomonas* spp. *Nitrobacter* spp. and *Nitrospira* spp. Pyrosequencing revealed significant differences in the AOB population which was 6 times higher during summer compared to winter. The AOB sequences related to

uncultured bacterium and uncultured AOB also showed an increase of 133% and 360% respectively when the season changed from winter to summer. This study suggests that vast population of novel, ecologically significant AOB species, which remain unexploited, still inhabit the complex activated sludge communities. Based on ANFIS model, AOB increased during summer season, when temperature was 1.4-fold higher than winter (*r* 0.517, *p* 0.048), and HRT decreased by 31% as a result of rainfall (*r* -0.741, *p* 0.002). Food: microorganism ratio (F/M) and HRT formed the optimal combination of two inputs affecting the plant's specific nitrification ($q_{N)}$, and their quadratic equation showed r^2-value of 0.50.

This study has significantly contributed towards understanding the complex relationship between the microbial population dynamics, wastewater composition and nitrification performance in a full-scale treatment plant situated in the subtropical region. This is the first study applying ANFIS technique to describe the nitrification performance at a full-scale WWTP, subjected to dynamic operational parameters. The study also demonstrated the successful application of ANFIS for determining and ranking the impact of various operating parameters on plant's nitrification performance, which could not be achieved by the conventional spearman correlation due to the non-linearity of the interactions during wastewater treatment. Moreover, this study also represents the first-time amoA gene targeted pyrosequencing of AOB in a full-scale activated sludge is being done.

Keywords: amoA; Nitrifiers; NGS; Illumina; Pyrosequencing; Adaptive neuro-fuzzy inference system; SAOR; SNFR; qPCR; activated sludge

DEDICATION

This thesis is dedicated to all who have contributed positively to my building in life and most importantly, to those who constituted themselves as stumbling blocks on my path in one way or the other. You are all important and necessary part of my motivation in my journey to success.

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TABLE OF CONTENTS

This thesis is a compilation of different manuscripts, where each chapter is an individual entity; therefore, certain repetitions are unavoidable across chapters. Moreover, Chapter Six has been published as it is and retains the Microbial Ecology Journal format with results and discussion written together.

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ABBREVIATIONS

ALR : Ammonia loading rate (kg COD/m3.d)

amoA : Ammonia monooxygenase

Anammox : Anaerobic ammonium oxidation

ANFIS : Adaptive neuro-fuzzy inference system

AOA : Ammonia oxidizing archaea

AOB : Ammonia oxidizing bacteria

BLAST : Basic Local Alignment Search Tool

BOD : Biological oxygen demand

C: N : Carbon: Nitrogen

CARD-FISH : Catalysed Reporter Deposition Fluorescence In Situ Hybridization

CAS : Conventional activated sewage sludge system

CO_2 : Carbon dioxide

COD : Chemical oxygen demand

C_q : Threshold cycle

Cr(VI) : Hexavalent Chromium

DAPI : 4′-6-diamino-2-phenylindole

DGGE : Denaturing gradient gel electrophoresis

DNA : Deoxyribonucleic acid

DNTPs : Deoxy-nucleotide triphosphates

DO : Dissolved oxygen

EDTA : Ethylenediaminetetra-acetate

F/M : Food to microorganism ratio

FISH : Fluorescent in-situ hybridization

FISH-MAR : Fluorescent in situ hybridization- Microautoradiography

HAO : Hydroxylamine oxidoreductase

HRT : Hydraulic retention time

IPTG : Isopropyl-β-d-thiogalactopyranoside

LB : Luria-Bertani

MBR : Membrane bioreactor

MCRT : Mean cell residence time

MLSS : Mixed liquor suspended solids

MPN : Most probable number

NCBI : Centre for the Biotechnology Information

ng : Nanogram

NGS : Next generation sequencing

NH_2OH : Hydroxylamine

NH_3 : Ammonia

NO_2^- : Nitrite

NO_3^- : Nitrate

NOB : Nitrite oxidizing bacteria

NOR : Nitrite oxide

OD : Optical density

ORL : Organic loading rate (kg COD/m3.d)

OUT : Operational taxonomic unit

PacBio : Pacific Biosciences

PBS : Phosphate saline buffer

PCR : Polymerase chain reaction

PCR-DGGE : Polymerase chain reaction–denaturing gradient gel electrophoresis

PGM : Personal Genome Machine

PVP : Polyvinyl pyrrolidone

qN : Specific nitrification rate

q-PCR : Quantitative real-time

rDNA : Ribosomal Deoxyribonucleic acid

RFLP : Restriction fragment length polymorphism

$R_{nitrification}$: Rate of plant's nitrification

rRNA : Ribosomal ribonucleic acid

SAOR : Specific ammonium oxidizing rate

SBS : Sequencing-by-synthesis

SDS : Sodium dodecyl sulfate

SNFR : Specific nitrate forming rate

SNOR : Specific nitrite oxidizing rate

SRT : Sludge retention time

TBE : Tris-boric acid-ethylene

TE : Tris-EDTA

TGGE : Temperature gradient gel electrophoresis

TN : Total nitrogen

T-RFLP : Terminal restriction fragment length polymorphism

Tris-HCl : Tris hydrochloride

UQ : Ubiquinone

WWTP : Wastewater treatment plant

X-gal : β-galactosidase substrate

Qin : Influent flowrate (L^3/ T)

PREFACE

The following publications has emanated from this study:

(a) Journal Articles

1) **Awolusi, O. O.,** Kumari, S. K. S., Bux, F. 2015. Ecophysiology of nitrifying communities in membrane bioreactors. International Journal of Environmental Science and Technology 12: 747-762

2) **Awolusi, O. O.,** M. Nasr, Kumari, S. K. S., Bux, F. (2016) Artificial intelligence for the evaluation of operational parameters influencing nitrification and nitrifiers in an activated sludge process. Microbial Ecology DOI: 10.1007/s00248-016-0739-3

3) **Awolusi, O. O.,** M. Nasr, Kumari, S. K. S., Bux, F. Principal component analysis for interaction of wastewater characteristics and nitrifiers at a full-scale activated sludge plant. Environmental Science and Pollution Research (submitted)

(b) Book Chapter

1) Gokal, J., **Awolusi, O. O.,** Enitan, A. M., Kumari, S. K. S., and Bux, F. 2015. Molecular characterization and quantification of microbial communities in wastewater treatment systems. In: Shukla, P. eds. Microbial biotechnology: an interdisciplinary approach. Taylor & Francis (ISBN: 978-1-4987-5677-8) (In press)

Conference Papers

1) **Awolusi O. O.,** Kumari S.K., Bux F. Investigation of nitrogen converters in municipal wastewater treatment plant. Water Institute of Southern Africa (Water - The ultimate constraint) biennial conference, the International Convention Centre, Durban, 15th - 19th May 2016.

2) **Awolusi O. O.,** Kumari S. K., Bux F. 2015. Seasonal impact on nitrification potential of activated sludge treating municipal wastewater. Paper presented at 4th Young Water Professional (South Africa) Biennial Conference and 1st Africa-wide YWP Conference, CSIR International Convention Centre, Pretoria, South Africa, 16th – 18th November 2015 (Oral presentation).

3) **Awolusi, O. O.,** Enitan, A. M., Kumari, S. K. S., and Bux, F. 2015. Nitrification efficiency and community structure of municipal activated sewage sludge. Paper presented at 17th International Conference on Biotechnology, Bioengineering and Bioprocess Engineering, Rome, Italy, September 17 - 18, 2015 (Oral presentation).

4) **Awolusi O. O.,** Kumari S.K., Bux F. 2014. Characterization and quantification of nitrifying community in activated sludge system treating municipal wastewater. Paper presented at Water Institute of Southern Africa (WISA) 2014 (Water Innovations) biennial conference, Mbombela Stadium, Nelspruit, South Africa (Oral presentation).

CHAPTER ONE: INTRODUCTION

Historically, the epidemics in London between 1831 and 1866, which resulted from water pollution, necessitated the requirement of specific regulations on wastewater treatment and discharge. This in turn prompted the construction and operation of wastewater treatment plants (WWTPs) and the eventual development of advanced wastewater treatment technologies (Glicksman and Batzel, 2010; Sciampacone, 2013).The untreated wastewater is usually laden with high content of pollutants (organic and inorganic) and pathogens. Discharging this untreated wastewater into water bodies usually results in disruption of the normal ecological functioning of aquatic ecosystems and widespread waterborne diseases (Holeton *et al.*, 2011; Uan *et al.*, 2013). In aquatic environments, the inorganic nitrogen (NH_3 and NO_2^-) and phosphates at elevated levels (>0.05 mg/L) have been implicated to stimulate eutrophication (WEF, 2009; Chuai *et al.*, 2012). Even at low concentrations (<0.2 mg/L), the unionized ammonia has been reported to be acutely toxic to fish (Yang *et al.*, 2010; Chen *et al.*, 2012). As a result, national governments around the globe have environmental agencies saddled with the responsibility of enforcing compliance with effluent discharge limits which includes dissolved organic carbon (biological or chemical oxygen demand) as well as nitrogen compounds and phosphates (Holeton *et al.*, 2011).

Although there are biological and chemical ways of treating wastewater, the former is usually the preferred choice as it is more environmentally friendly (Akpor and Muchie, 2010). Activated sludge, membrane bioreactors (MBRs), trickling filters, up-flow anaerobic sludge blanket reactors, lagoons and artificial wetlands are the most commonly used biological treatment processes for both industrial and domestic wastewaters (Akpor and Muchie, 2010; Heffernan *et al.*, 2011). Biological wastewater treatment harnesses the ability of the microorganisms to metabolize the pollutants in

wastewater for synthesizing their cells' building blocks. These bioreactors are configured and operated in a way to select and enrich the specialized microbial consortium that are required for nutrient removal (Lopez-Vazques, 2009). The bioprocesses through which nutrients are transformed and removed within these engineered systems depend on structure and concentration of the mixed microbial community with nitrifiers occupying an important ecological niche within it (Graham *et al.*, 2007; Ozdemir *et al.*, 2011).

Nitrification as a process is essential for nitrogen removal in biological nutrient removing (BNR) systems (You and Lin, 2008; Ward, 2013). Nitrifying bacteria form an important part of this ecological system and are responsible for ammonia biotransformation and removal through nitrification. Nitrification process involves the bioconversion of ammonia into nitrite by either ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) and subsequent conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) (Bae *et al.*, 2013; Ward, 2013). Nitrifiers are 'vulnerably delicate', with characteristic slow growth rate (Wei *et al.*, 2013). Nitrification, like any other process that hinges on microbial physiology, is subject to failure whenever there is a shift in operational conditions or in the presence of toxic compounds (Ducey *et al.*, 2010). This shift may result in the loss of key microbial populations which need to be replenished in order for the system to recover its functional ability (Kim *et al.*, 2011).

Nitrification process in BNR systems is usually limited by the nitrifiers' physiological activities (Zhang *et al.*, 2011a; Dogan *et al.*, 2014; Lotti *et al.*, 2014). Monitoring the nitrifiers, especially ammonia oxidizing bacteria (AOB) in wastewater treatment systems, is therefore important in order to prevent nitrification failure (Siripong and Rittmann, 2007). According to Gentile *et al.* (2007), it is important to understand how the microbial communities correlates to system stability in a bid to design functionally reliable wastewater treatment systems. The nitrifiers could serve as indicator parameter for preventing washout of important functional groups in wastewater

treatment facility. Various studies have reported nitrification depletion or loss due to seasonal temperature variations especially during winter (Caballero, 2011; Flores-Alsina *et al.*, 2011; Yao *et al.*, 2013). It is therefore important to understand the role of nitrifiers' diversity and abundance in such functional fluctuations. Majority of the studies however, have been carried out in regions with extremely low winter average temperatures (temperate regions) contrary to the sub-tropical temperature regime experienced in some parts of the Southern hemisphere.

Seasonal fluctuations and failures in nutrients removal due to the operational and environmental changes have been widely reported in engineered wastewater treatment systems (Wang *et al.*, 2012). Among these, seasonal temperature variation has been implicated as a major cause of breakdown in ammonia removal efficiency and nitrification (Vieno *et al.*, 2005; Wan *et al.*, 2011) and with climate change experienced globally these impact could be further magnified. Majority of the studies have been conducted over short period of time. What we knew about the organisms involved in nitrification is changing with some novel detection tools (Sorokin *et al.*, 2012; Stahl and de la Torre, 2012). There have been reports by different authors (Limpiyakorn *et al.*, 2011; Mussmann *et al.*, 2011; Zhang *et al.*, 2011b) on the probable involvement of AOA in wastewater nitrification which indicate our inconclusive knowledge of wastewater microbiology. However, there is still need to establish if AOA are ubiquitous in all engineered systems in order to establish a holistic knowledge of nitrogen biotransformation and AOA contribution in wastewater treatment.

Understanding the microbial community structure of activated sludge has never been an easy task. Culture-dependent methods have been relied upon solely in the past to study microorganisms present in the natural environment including wastewater ecosystems. Only a few bacterial species were thought to be involved in the process based on these laboratory culture techniques, whereas in reality, a great diversity of organisms have been involved and are nonculturable. The advent of molecular

techniques has brought about a better understanding of the structure and functions of microbial communities including nitrifiers in wastewater treatment systems. In recent past, the nitrifying community structure of activated sludge systems have been investigated using different techniques including; quinone profiling, (a chemotaxonomic method) (Li *et al.*, 2006), restriction fragment length polymorphism (RFLP) (Gao and Tao, 2012), fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) (Yu *et al.*, 2011), clone library (Yao *et al.*, 2013), terminal restriction fragment length polymorphism (T-RFLP) (Whang *et al.*, 2012), quantitative real-time PCR (qPCR) (Cho *et al.*, 2014). However, these techniques have well documented limitations that often reduce their ability to unravel the complete microbial community structure of the activated sludge (Awolusi *et al.*, 2015). Next-generation sequencing which has the capacity of performing about several hundreds of thousands of reactions simultaneously is gaining ground in studies involving understanding the microbial community structure of wastewater treatment systems (Liu *et al.*, 2012; Ye and Zhang, 2013). It has the advantage of revealing the microbial diversity with more accuracy and details.

South Africa is a sub-tropic region with warm temperate weather conditions. Wastewater treatment plants in the KwaZulu-Natal have a history of nitrification failure especially during the winter. This study therefore, investigated the nitrification efficiency and nitrifying community in one of the full-scale municipal wastewater treatment plants situated midlands of KwaZulu-Natal province, over two seasons (summer and winter). The plant's nitrification efficiency was investigated in correlation to diversity and abundance of the different nitrifying populations. A combination of FISH, PCR-clone library, quantitative polymerase chain reaction (qPCR) and next generation sequencing (pyrosequencing and Illumina) was employed in understanding the microbial community structure of the plant. The effect of operating parameters and environmental conditions on nitrification and nitrifiers was also investigated using an adaptive neuro-fuzzy inference system (ANFIS), Pearson's correlation coefficient and quadratic models. To the best of our knowledge this is the first time ANFIS, rather than the typical simple correlation approach would be used

for investigating nitrification. The amoA-locus-targeted pyrosequencing was also employed to study AOB diversity for the first time.

1.1 AIMS AND OBJECTIVES OF THE STUDY

1.1.1 Aim

The aim of this study was to monitor the seasonal changes in population profiles of chemolithoautotrophic nitrifying bacteria in a full-scale municipal activated sludge, using advanced molecular techniques and correlate this to the plant's performance.

1.1.2 Specific objectives

The specific objectives are to:

- characterize the dominant nitrifers in activated sludge system using conventional molecular methods
- evaluate the seasonal variation in the microbial community structure of the activated sludge using the next-generation sequencing approach
- quantify the dominant nitrifying bacterial populations using qPCR and determine their nitrification performance
- evaluate the influence of operational and environmental parameters on nitrification and nitrifiers in the activated sludge using artificial intelligence (AI)

CHAPTER TWO: LITERATURE REVIEW

2.1 NITRIFICATION

Nitrification involves the biological conversion of ammonia/ammonium to nitrite by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea followed by the conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) (Bae *et al.*, 2013; Mukherjee, 2013). In a wastewater treatment process, most of the organic nitrogen contained in raw sewage in the form of urea and faecal material will be converted to ammonia by hydrolysis through anaerobic processes while travelling through the sewer pipes (Daims and Wagner, 2010). Various authors have noted that nitrification can be carried out by organisms other than bacteria such as protozoa, algae and fungi, however, at a very low level rate (1,000–10,000 times less than the rates associated with bacteria) (Gerardi, 2002; Nicol and Schleper, 2006).

Nitrifying bacteria are aerobes and chemolithoautotrophs, obtaining their energy by oxidation of either NH_3 or NO_2^- (Daims and Wagner, 2010; Jin *et al.*, 2010). Certain bacteria called anammox (anaerobic ammonium oxidation) are capable of oxidizing NH_3 under anaerobic conditions. These organisms use NH_4^+as their energy source and NO_2^-as electron acceptor with the production of hydrazine in an intermediate process (Xiao *et al.*, 2013). The AOB uses the enzyme ammonia monooxygenase (amoA) to catalyse the oxidation of ammonia to hydroxylamine (NH_2OH) (Bahadoorsingh, 2010), and the enzyme hydroxylamine oxidoreductase converts it to NO_2^- (Canfield *et al.*, 2010). The NOB further oxidizes NO_2^- to NO_3^- using nitrite oxidoreductase enzyme (Bahadoorsingh, 2010) (Fig. 2.1). Although all nitrifiers are known to be slow growers, NOB has a lower specific growth rate than the AOB (Daims and Wagner, 2010). Thus, a successful nitrification process requires a balance in the linked activity of these two nitrifying bacterial groups involved. It has been reported that factors such as high ammonia concentration and low dissolved oxygen (DO) level can result in the disruption of the equilibrium between these two nitrification steps, resulting in

significant reduction in the activities of nitrite oxidizers which can lead to toxic nitrite build-up and a subsequent failure of nitrification process (Mbakwe *et al.*, 2013). According to Graham *et al.* (2007), there is a delicate and vulnerable AOB–NOB mutualism present, which makes the process prone to chaotic behaviour and incomprehensible failure at times.

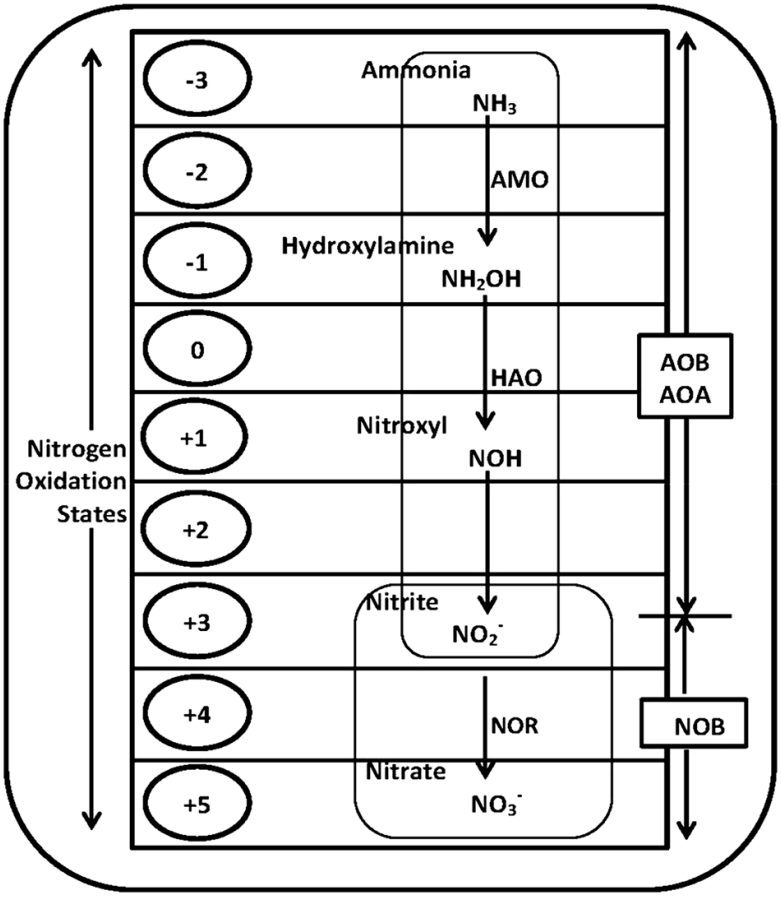

Fig. 2.1: The nitrification pathway incorporating the AOA (Awolusi *et al.*, 2015): ammonia oxidizing archaea; AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase; NOR: nitrite oxide

The nitrification process in wastewater is therefore limited by the AOB and NOB population densities and their physiological activities in different types of wastewater treatment plants (WWTP), including conventional activated sludge treatment systems and membrane bioreactor (MBR) (Graham *et al.*, 2007; Huang *et al.*, 2010a; Zhang *et al.*, 2011b). In both cases, the nitrification efficiency seems to be controlled by the prevalent operational and environmental conditions (Manser *et al.*, 2005; Bahadoorsingh, 2010). Various authors have reported 96–99 and 92–98 % ammonia–nitrogen and chemical oxygen demand (COD) removals in treatment systems investigated (Liang *et al.*, 2010; Yu *et al.*, 2010; Ozdemir *et al.*, 2011). Specific ammonia oxidization rate and nitrite oxidization rates were also reported to vary with no particular pattern during wastewater treatment (Liang *et al.*, 2010; Yu *et al.*, 2010; Ozdemir *et al.*, 2011).

2.2 NITRIFYING COMMUNITY STRUCTURE

2.2.1 Ammonia oxidizing bacteria and Nitrite oxidizing bacteria

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

The 16S rDNA sequences revealed that these two groups of nitrifying bacteria (AOB and NOB) are phylogenetically distinct (Daims and Wagner, 2010). All ammonia oxidizers can be classified in the β-subclass of *Proteobacteria* with the exception of

Nitrosococcus, which belongs to a distinct branch of the γ-subclass. The NOB can be found within the α- and γ-subclasses of *Proteobacteria*, with the exceptions of *Nitrospira*, which has its own distinct phylum (Duan *et al.*, 2013), and Nitrospina, which belongs to the δ-subclass of *Proteobacteria* (Zeng *et al.*, 2012) (Fig. 2.2). Due to their low specific growth rate and sensitivity to stress from environmental and operational factors, their population and physiological activities can limit the rate of biotransformation of nitrogen in many WWTPs. Although the dominance of *Nitrospira* (K-strategist) over *Nitrobacter* (r–Strategist) in activated sludge has been widely reported (Yapsakli *et al.*, 2011; Ye *et al.*, 2011); however, earlier observations by Nogueira and Melo (2006), and Wagner *et al.* (2002) were contrary. These authors noted that there is usually an irreversible prevalence of *Nitrobacter spp*. over *Nitrospira spp*. in plants after spike in nitrite concentration even after subsequent reduction in nitrite concentration. *Nitrobacter* spp. usually exhibit inhibitory effect on the growth of *Nitrospira* once it dominates (Nogueira and Melo, 2006). Fukushima *et al.* (2013) also reported that *Nitrobacter* spp. though a weak competitor compared to *Nitrospira* under low nitrite concentration, however it can be selected over *Nitrospira spp*. in plants with low inorganic carbon in addition to low nitrite concentration.

The recognized diversity of NOB has been curiously low and they have been known to belong to two bacterial phyla. Sorokin *et al.* (2012) expanded our knowledge of NOB with the discovery of *Nitrolancetus hollandicus* isolated from a nitrifying reactor. This organism belongs to the popular phylum *Chloroflexi* not previously known to contain any nitrifying organism. This organism is distinguished from all other NOB being Gram positive with an unusual membrane lipids consisting 1, 2-diols. It is thermo-tolerant over a broad range of 25-63^{o}C. It has low affinity for nitrite (Ks ≈ 1 mM). It grows on nitrite and CO_2 with ability to use formate as a source of energy and carbon. It has the genes for CO_2 fixation via Calvin cycle and nitrite oxidoreductase similar to proteobacterial NOB.

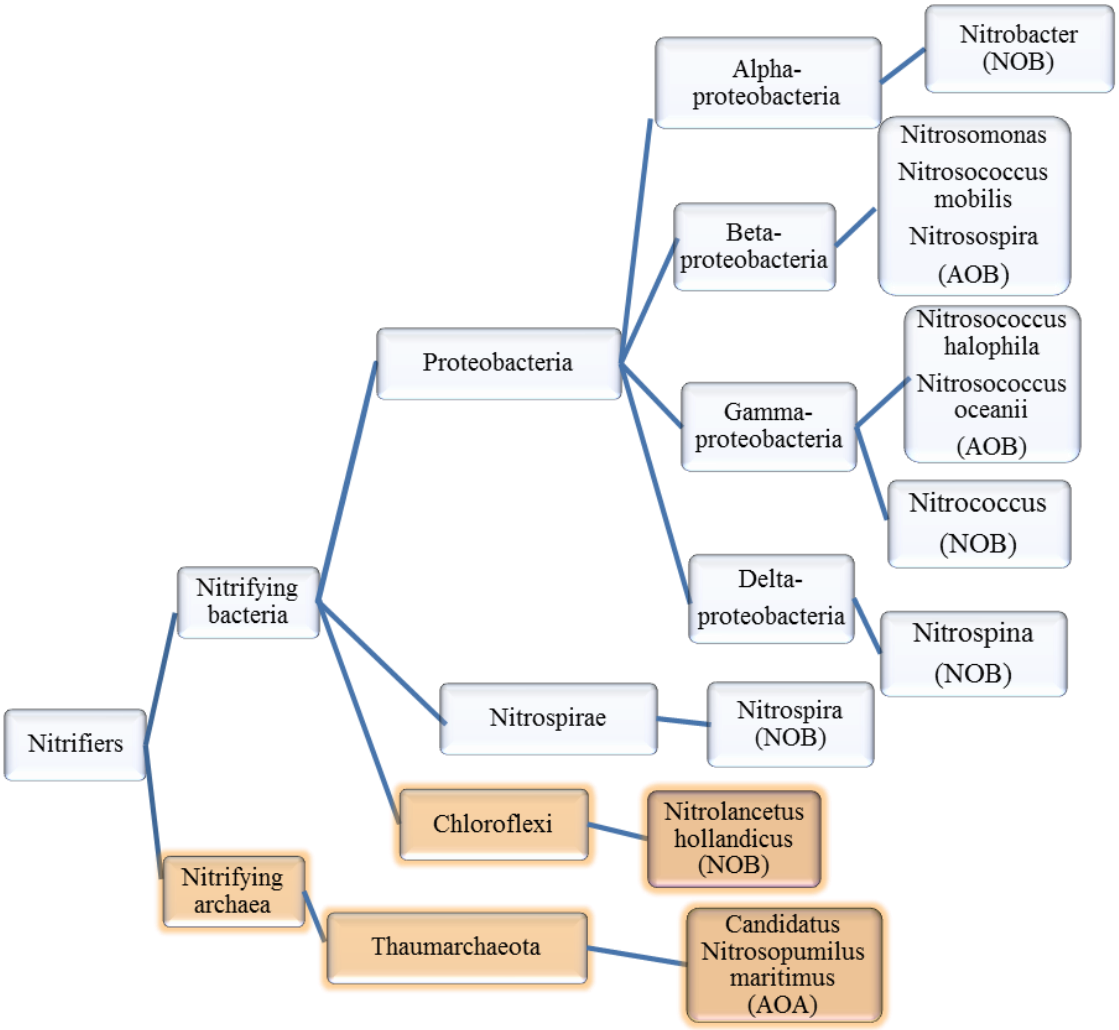

Fig. 2.2: Schematic representation of the nitrifying community in wastewater [adapted from (Awolusi *et al.*, 2015)]

2.2.2 Ammonia oxidizing archaea

The developments in molecular biology techniques have helped us to understand the diversity, distribution and abundance of possible functional archaea in engineered systems such as WWTPs (Hatzenpichler, 2012). In recent times, the contribution of archaea to nitrification in WWTPs is being acknowledged by various researchers;

however, very little information is available on the physiology and activity of ammonia-oxidizing archaea in the environment. Among the different AOA, *Candidatus Nitrosopumilus maritimus* was the first member of the group to be isolated (Stahl and de la Torre, 2012). This species is reported to have the same growth and cell production rates as those of AOB and is capable of using ammonia as the sole energy source for growth (You *et al.*, 2009).

AOA can tolerate environment with oxygen concentration over the range of <3.1 µM–0.2 mM; however, the environment with low oxygen may select them in contrast to AOB (Limpiyakorn *et al.*, 2011). The AOB uses the Calvin cycle to fix their carbon, whereas the AOA rely on the 4-hydroxybutyrate pathway or citric acid cycle (Hatzenpichler, 2012). The AOA was previously grouped under phylum *Crenarchaeota* (Jin *et al.*, 2010; Zhang *et al.*, 2011a) but have recently been reclassified under phylum *Thaumarchaeota* (Stahl and de la Torre, 2012). Ozdemir *et al.* (2011) in a study on nitrification, reported the presence of a small fraction of AOA among other nitrifiers.

An observation of interest about AOA is that they are less sensitive to plant operational conditions (e.g. DO and ammonia loading) compared to AOB (Jin *et al.*, 2010; Sonthiphand and Limpiyakorn, 2010). The fact that in natural environments AOA are associated with significant role in nitrification contrary to what is currently known about engineered systems leaves the question of whether the optimum conditions for their ecological functioning have been adequately understood or replicated in such systems. If the assertion by some authors that AOA are tolerant to fluctuations in operational conditions (Jin *et al.*, 2010; Sonthiphand and Limpiyakorn, 2010) is valid, then it may be desirable to harness its potential for a stable and efficient wastewater treatment. There would be need for the development of engineered systems that can adequately replicate the conditions which support AOA's optimal growth as obtained in the natural environment. The role and efficiency of AOA in nitrification remains unclear, since their isolation and cultivation is still largely unsuccessful. The archaeal

membrane lipids (isoprenoid glycerol dialkyl glycerol tetraethers) have been suggested as a bio-indicator for investigating the ecophysiology of AOA in wastewater treatment bioreactors. These lipids have been observed to correlate with archaeal amoA gene copies (You *et al.*, 2009). Recently, AOA that were deficient in both carbon fixation and NH_3 oxidation abilities despite possessing amoA gene were reported (Mussmann *et al.*, 2011; Stahl and de la Torre, 2012). This indicates that AOA importance in nitrification cannot be determined based on mere presence or abundance of arch-amoA gene. Various authors observed that the AOA are able to utilize amino acids as a carbon source akin to heterotrophs (You *et al.*, 2009; Bouskill *et al.*, 2011). Recently, studies based on radiocarbon and genomic analyses suggest that the AOA either include both heterotrophs and autotrophs, or they are single population of mixotrophs (Bouskill *et al.*, 2011).

2.3 FACTORS AFFECTING NITRIFIERS AND NITRIFICATION

Nitrification, like any other process that hinges on microbial physiology, is subject to failure whenever there is a shift in operational conditions or in the presence of inhibitory substances (Ducey *et al.*, 2010). Thus, efficient nitrification in wastewater treatment plants depends on a combination of environmental and operational parameters which include pH, temperature (Gerardi, 2002; Kim *et al.*, 2011), DO, sludge retention time (SRT), hydraulic retention time (HRT), substrate concentration (Bae *et al.*, 2002; Liu *et al.*, 2010a) and presence of inhibitory or toxic substances (Cecen *et al.*, 2010).

2.3.1 Environmental factors

2.3.1.1 pH and alkalinity

Traditionally, efficient nitrification has been reported at a pH ranging from 7.5 to 8.5 (Sajuni *et al.*, 2010; Fulweiler *et al.*, 2011). A higher pH value of 8–9 was reported to favour elevated nitrite accumulation, thereby affecting the optimal nitrification process

(Bae *et al.*, 2002). A study conducted by Bae *et al.* (2002) on activated sludge plants reported an increase in the specific ammonium oxidizing rate (SAOR) when the pH increased from 7 to 8; however, the rate dropped as the pH reached 9 and yielded the lowest activity at pH 10 (Bae *et al.*, 2002). In the same experiment, the specific nitrite oxidizing rate (SNOR), on the other hand, correlated with an increase in pH from 7 to 9, but was decreased at pH 10. Similarly, He *et al.* (2009) in their study on nitrification, noted that pH plays a major role in NH_3 and total nitrogen (TN) removal in reactor. When the influent pH was acidic (approximately 4.8), the NH_3 removal rate was 56 %, whereas that of TN was 45 %. An increased removal of NH_3 up to 99 % was observed, while that of TN rose to 91 % when the pH was neutral (7.2), and a decrease to 75 % (NH_3) and 60 % (TN) was noted when the pH increased to about 9.7. These findings by He *et al.* (2009) also indicate that efficient nitrification falls within pH range of 7.5–8.5.

2.3.1.2 Temperature

Temperature typically has a significant effect on nitrifiers and their nitrification efficiency (Ducey *et al.*, 2010). According to earlier studies, the optimal temperature range for nitrifiers was observed to be between 15 and 30 ^{o}C (Chandra and Sathasivan, 2011). Colliver and Stephenson (2000) reported that most nitrifiers will grow optimally in a temperature range of 25–30^{o}C. A study conducted by Huang *et al.* (2010b) showed that the optimal temperature regime that supported *Nitrobacter* growth was between 24 and 25 ^{o}C, whereas higher range of 29–30 ^{o}C favoured *Nitrospira*. The activity of the AOB is generally faster than that of NOB because of their different activation energy, which is between 72 and 60 kJ mol^{-1} for AOB, whereas it is from 43 to 47 kJ mol^{-1} for NOB (Hulle, 2005). In a reactor treating synthetic wastewater, Kim *et al.* (2008) found that when the temperature increased from 20 to 30 ^{o}C, oxidation of ammonia proceeded from 0.253 to 1.33 g N/g VSS d (5.3-fold increase) whereas nitrite oxidation was just by a multiple of 2.6 times (0.45–1.18 g N/g VSS d), thereby indicating a high correlation of temperature with ammonia

oxidation. These findings therefore indicate that the influence of environmental conditions on different nitrifying populations (AOB and NOB) varies.

Recent reports on activity of nitrifiers at very low temperature and DO level indicate that nitrifiers are capable of adapting to extreme conditions such as low temperature. Zhang *et al.* (2011a) observed that *Nitrosospira* spp. thrives at lower temperatures (4–10 ^{o}C) compared to other nitrosifyer bacteria. Ducey *et al.* (2010) reported a high nitrification rate of 11.2 mg N/g MLVSS/h at low temperatures (5 ^{o}C) by an acclimatized nitrifying community, which is far above the optimum 1.71–2.0 mg NO_3-N/gVSS-h reported by Fan *et al.* (2000) and Kornboonraksa *et al.* (2009). These observations show that nitrifiers are capable of adaptation in response to environmental and operational conditions; however, a sudden shift in environmental factors would certainly affect the nitrifiers' activity. Earlier research efforts also documented the effect of temperature on the nitrifying populations' diversity. Caballero (2011) observed in an activated sludge a higher diversity of AOB during winter compared to summer. Wang (2013) reported significant seasonal changes in AOB community structures and abundances in a wetland. He noted AOB community structures were significantly different between winter and summer with winter having a higher diversity (Wang, 2013). Similarly Miller (2011), observed a higher diversity of AOB in a pond during winter whereas the AOB amoA gene copy number was higher during summer.

2.3.2 Plant Operational conditions

2.3.2.1 Dissolved oxygen

A DO concentration of between 3 and 4 mg O_2 L^{-1} has been described as optimum for AOB and NOB growth (Hulle, 2005). However, Sarioglua *et al.* (2009) observed a higher nitrogen removal of about 85–95 % in WWTP treating domestic wastewater when the DO level was maintained at low level (1.5 mg O2 L^{-1}). Niche-specific adaptation to DO concentration has been observed within the NOB, with *Nitrospira*

demonstrating a negative correlation to DO concentrations ($r = -0.46$, $P<0.01$), whereas *Nitrobacter* exhibited a positive correlation ($r = 0.38$, $P<0.01$) (Huang *et al.*, 2010a). *Nitrobacter* population was also found to increase in winter (low temperatures) and high DO levels (Huang *et al.*, 2010b). This shows that nitrifiers can be highly specialized; exhibiting niche-specific adaptation in response to environmental and operational conditions. The microbial ecology of nitrifiers reveals that *Nitrospira* thrives optimally in an environment with a combination of low nitrite and oxygen levels, whereas *Nitrobacter* requires an environment with elevated levels of nitrite and oxygen. This makes them k- and r-strategists, respectively, based on r–k selection theory (Bahadoorsingh, 2010). Studies on the relationship between NOB (*Nitrospira* and *Nitrobacter*) populations and their sensitivity to environmental/operating factors that favours good nitrification (under high DO and limited NH_3 conditions) are necessary to understand their effect on plant performance (Huang *et al.*, 2010b). Liu (2012) noted that NOB exhibited a significant O_2 affinity under prolonged low DO concentrations (0.16–0.37 mg/l) which in turn made them a better competitor for O_2 as their abundance increased comparably to AOB. According to Liu and Wang (2013), under extended period of low dissolved oxygen concentrations ($\leq$ 0.5 mg/L) the endogenous decay of both ammonia/nitrite oxidizing bacteria was retarded. This resulted in increased biomass density which nullified some low DO impact on nitrification. He also reported near complete nitrification with 0.16–0.37 mg/l DO range. Under extended low DO period NOB demonstrated significant increase in O_2 affinity which in turn made them a better O_2 competitor.

2.3.2.2 Sludge retention time (SRT) and Hydraulic retention time (HRT)

It has been reported that longer SRT could impact the biological activities negatively, including nitrification rate (Yu *et al.*, 2010). A study conducted by Yu *et al.* (2010), observed a negative correlation between SRT and the nitrifier activities, i.e. for both specific ammonia oxidizing rate (SAOR) and specific nitrate formation rate (SNFR). The reactor operated at a shorter SRT of 30 days showed a higher SAOR and SNFR (0.22 kg NH_4^+-N/kg MLSS/day and 0.13 kg NO_3-N/kg MLSS/day, respectively),

compared to the system operated for a longer SRT of 90 days (0.12–0.14 kg NH_4^+-N/kg MLSS/day and 0.068–0.042 kg NO_3-N/kg MLSS/day, SAOR and SNFR, respectively). This reflects earlier reports which also indicated a negative correlation of SRT to SAOR/SNFR in wastewater treatment (Li *et al.*, 2006). Similarly, Huang *et al.* (2001) reported that SRT has no significant influence on the biological activity when the reactor was operated at SRT of less than 40 days. Cicek *et al.* (2001) reported that when a pilot-scale bioreactor was operated at an increased SRT up till 30 days, there was no significant effect on nitrification. The impact of hydraulic retention time (HRT) on nitrification efficiency of wastewater treatment systems has also been reported. Li *et al.* (2013) observed that a decrease in HRT from 30 to 5 h resulted in an increase in specific ammonium-oxidizing and nitrate-forming rates. Additionally, the study indicated that the decrease in HRT led to a reduction of AOB population density, whereas the NOB, especially the fast growing *Nitrobacter* spp., increased significantly.

2.3.2.3 Substrate concentration and Food to microorganism (F/M) ratio

Research findings have shown that nitrifiers get inhibited by free ammonia and unionized nitrous acid (Gil and Choi, 2001; WEF, 2011; Mousavi *et al.*, 2014). Increased accumulation of NH_3 in bio-treatment systems occurs whenever toxicant or any inhibitory factor disrupts the nitrifiers' functional ability. This increased NH_3-N level often gets to inhibitive level, which can result in loss of nitrification that can last for several days to months. Concentration of NH_3-N above a threshold of 200 mg/L has been reported to inhibit nitrification efficiency (Mordorski, 1987; Kim and Kim, 2003). Optimizing the carbon: nitrogen (C: N) ratio is also essential for efficient nitrogen removal in waste treatment systems. A low C: N ratio favours nitrification, whereas a higher ratio supports the heterotrophs (Fu *et al.*, 2010). In a study of membrane-aerated biofilm reactor, nitrification efficiency of 93 % was achieved at C: N ratio 5; however, at C: N ratio of 6, increased heterotrophic bacteria growth was observed with resultant inhibition of nitrifiers (Liu *et al.*, 2010b). According to Fu *et*

al. (2010) also, it was observed that AOB and NOB showed negative correlation with C: N ratio.

The available carbon substrate for the unit mass of microorganism (known as F/M ratio) can impact nitrification. According to Wu *et al.* (2014), F/M ratio between 0.2 and 0.4 proved to be optimum for nitrification, whilst the inhibitory effect of higher F/M ratio on the nitrifiers noted.

2.3.2.4 Inhibitory substances

When toxic substances inhibit the nitrifier population, their cell growth and the ammonia oxidation are affected. Strong toxicity will cause nitrification to be impaired due to the disappearance of the nitrifiers (Kim and Kim, 2003). According to Caballero (2011) there are many compounds that can exert inhibitory effect on nitrification which include; metals, amines, proteins, tannins, phenols, alcohols, carbamates, benzene and un-ionized ammonia at certain concentrations. Various authors have reported that metal toxicants inhibits AOB populations and ammonium oxidation whereas it has little or no effect on NOB and nitrite oxidation (Hu *et al.*, 2002; Kelly *et al.*, 2004 ; Hawkins *et al.*, 2008; Zhang *et al.*, 2014). In activated sludge treating wastewater containing Cr(VI), the AOB were found to be more sensitive than NOB to Cr(VI). However, AOB recovery was rapid both in activity and quantity compared to NOB. In another study involving cyanide toxicity, Kim and Kim (2003) observed that the free cyanide has high toxicity on nitrifiers whereas complex cyanide has comparably low toxicity effect.

2.3.2.4 Predators

Predators grazing can strongly impaired nitrification in wastewater treatment processes since the plant's nitrification efficiency is determined by the nitrifying bacteria abundance in the system and their specific activity. Lee and Welander (1994)

noted significant correlation between predators (protozoa and metazoan) abundance and nitrification efficiency of a nitrifying plant in their study. They also report that vigorous grazing of these predators on the bacterial population can hamper bio-transformations that are critical for process performance. According to different studies (Carvalho *et al.*, 2006; Shi *et al.*, 2010; Filali *et al.*, 2012), due to nutrient availability, AOB are usually found at surface of the flocs and granules in activated sludge whereas the NOB are located deeper. This can give rise to a lowering of AOB population abundance which is important for the first rate limiting process of nitrification.

2.4 ASSESSING NITRIFIERS DIVERSITY AND ABUNDANCE

Only a few bacterial species, were thought to be involved in the process, based on the laboratory culture techniques, whereas in reality, a great diversity of the organisms involved are nonculturable. The advent of molecular techniques has brought about a better understanding of the structure and functions of microbial communities including nitrifiers in wastewater treatment systems. These, however, still have limitations when applied in full-scale WWTPs. Summary of these techniques used for detecting nitrifiers from wastewater ecosystem is presented in Tables 2.1 and 2.2. As shown in the Tables (2.1 and 2.2), FISH and PCR-DGGE has been extensively used for nitrifiers' biodiversity, whilst qPCR and FISH among the most widely used techniques for their quantification. Some advantages and disadvantages of these commonly used techniques are highlighted below.

2.4.1 Most probable number (MPN)

The MPN involves samples being incubated in a mineral medium selective for nitrifiers. This method usually involves bias because the synthetic medium and laboratory conditions cannot truly reproduce the complex ecological interactions which apply in the activated sludge environment (Hirooka *et al.*, 2009; Xia *et al.*, 2010a; Ayanda and Akinsoji, 2011). The cells are sometimes bound within the

complex matrix called floc, and some microbes interdepend on others for their metabolic activities, which can only be achieved in a complex ecosystem such as the activated sludge system (Ducey *et al.*, 2010). In effect negligible diversity and amount of the nitrifiers are enumerated using the MPN (Xia *et al.*, 2010a; Ayanda and Akinsoji, 2011). Li *et al.* (2006) investigated nitrifiers' population dynamics using MPN and FISH, and they observed that FISH correlated more with the specific nitrification rate analysis than the MPN.

2.4.2 Quinone profiling

Quinone profiling, a chemotaxonomic method, is used for microbial community structure analysis from environmental samples (Kurisu *et al.*, 2002). This technique is based on the presence of specific respiratory quinone as an indicator of a particular bacterial population. However, since some bacterial groups that are phyletically different share similar quinone groups, this technique is inadequate for analysis beyond the phylum level (Kurisu *et al.*, 2002). In a study by Li *et al.* (2006), ubiquinones belonging to UQ-8 (β-Proteobacteria), UQ-9 (α-Proteobacteria) and UQ-10 (γ-Proteobacteria) were recovered. However, the authors noted the difficulty in reconciling the NOB with the quinone profiles. The entire analysis based on the quinone profiling is characterized by assumptions and would require more specific techniques to complement it. Thus, the species specificity was a limiting factor for this technique when applying to complex environmental samples.

2.4.3 Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization, a widely used molecular method, involves the binding of fluorescent oligonucleotide probes to ribosomal ribonucleic acid (rRNA) (Fig. 2.3) (Nielsen, 2009; Junier *et al.*, 2010; Xia *et al.*, 2010b; Yu *et al.*, 2011). This method can be employed for both identification and quantification of specific bacterial groups directly from the environment even up to the species level (Li *et al.*, 2006). However, the major limitations of this technique include the lack of availability of

probes, inefficient cell permeability, inadequate or difference in ribosome content which can lead to low signal intensity, loop and hairpin formation of rRNA structure, as well as rRNA-protein interactions which hinders hybridization, auto-fluorescence and non-specific bindings (Nielsen, 2009; Ge *et al.*, 2015).

Table 2.1: Summary of techniques that have been employed for biodiversity studies on nitrifiers

Type of reactor	Sample type	Method of analysis*	Population detected	Reference
Lab Scale	Synthetic Wastewater	FISH	*Nitrospira sp., Nitrosomonas sp., Nitrobacter sp*.	(Li *et al.*, 2006)
Lab Scale	Synthetic Wastewater	Quinone profiling	α, β, and γ *Proteobacteria*	(Li *et al.*, 2006)
Full-scale	Municipal sewage	FISH	AOB and *Nitrobacter sp.*	(Yu *et al.*, 2011)
Full-scale	Municipal sewage	PCR-DGGE	*Nitrosomonas sp.,*	(Yu *et al.*, 2011)
Full-scale	Municipal sewage	RFLP	*Nitrosomonas sp.,*	(Yu *et al.*, 2011)
Lab Scale	Synthetic Wastewater	FISH	β-*Proteobacteria* (*Nitrobacter*)	(Yu *et al.*, 2010)
Lab Scale	Synthetic Wastewater	PCR-DGGE	*Nitrosomonas sp.; Nitrosospira sp.; Nitrospira sp*.	(Yu *et al.*, 2010)
Lab Scale	Synthetic Wastewater	RFLP	*Nitrosomonas sp.; Nitrosospira sp.*	(Yu *et al.*, 2010)
Lab Scale	Synthetic Wastewater	FISH	β-*Proteobacteria; Nitrobacter sp*.	(Zhang *et al.*, 2009b)
Lab Scale	Synthetic Wastewater	T-RFLP	*Nitrosomonas sp.; Nitrospira sp.; Nitrobacter sp.*	(Liang *et al.*, 2010)
Full Scale	Industrial wastewater	Microarrays	*Nitrosomonas sp.*	(Kelly *et al.*, 2005)
Full Scale	Municipal wastewater	Microarrays	*Nitrospira sp.*	(Siripong *et al.*, 2006)

*FISH: Fluorescent in-situ hybridization, PCR-DGGE: Polymerase chain reaction–denaturing gradient gel electrophoresis; RFLP: Restriction fragment length polymorphism, T-RFLP: Terminal restriction fragment length polymorphism, MBR: Membrane bioreactor

Table 2.2: Summary of techniques that have been employed for quantitative studies on nitrifiers

Type of Reactor	Influent	Method of analysis	Estimated population	Reference
Lab Scale	Synthetic Wastewater	FISH	AOB (% among total bacteria) ≈ 23 – 57%; *Nitrosomonas sp*. (% among AOB) ≈ 50 - 90%	(Li *et al.*, 2006)
Lab Scale	Synthetic Wastewater	MPN	AOB ≈ 10^7 - 10^9 l^{-1}; NOB ≈ 10^5 - 10^8 l^{-1}	(Li *et al.*, 2006)
Full-scale	Municipal sewage	FISH	AOB≈ 1.9 - 4.5%; NOB≈ 0.9 – 2.8%	(Yu *et al.*, 2011)
Pilot Scale	Domestic Wastewater	q-PCR	amoA AOB≈ 1.15 – 4.05%; *Nitrobacter*≈0.04-1.17%; *Nitrospira*≈8.23-13.01%; amoA AOA≈0.05-0.09%	(Ozdemir *et al.*, 2011)
Lab Scale	Synthetic Wastewater	MPN	AOB ≈1.5 x 10^7 - 3.4 x 10^7 cells g^{-1} MLSS; NOB ≈ 2.7 x 10^4- 1.4 x 10^7cells g^{-1} MLSS	(Yu *et al.*, 2010)
Lab Scale	Synthetic Wastewater	q-PCR	AOB ≈10^8 cells/l	(Liang *et al.*, 2010)

FISH: Fluorescent in-situ hybridization, MPN: Most probable number, q-PCR: Quantitative real-time PCR, MBR: Membrane bioreactor, AOB: Ammonia oxidizing bacteria, NOB: Nitrite oxidizing bacteria, MLSS: Mixed liquor suspended solids, amoA: Ammonia monooxygenase

Unlike the fast-growing microorganisms, the cellular rRNA content of anammox and β-*Proteobacterial* ammonia oxidizers do not really reflect the physiological activity of these organisms, especially during starvation and inhibition periods (Schmid *et al.*, 2005). Thus, correlation of the nitrifier population to its physiological activity can be biased (Schmid *et al.*, 2005). Witzig *et al.* (2002) observed that due to the low food-to-microorganisms conditions in membrane bioreactor with resultant low rRNA molecules for the organisms, less than half of the population were detectable by FISH whereas 80 % in activated sludge. However, few researchers have reported a direct correlation between nitrifier population and specific ammonium and nitrite oxidation rate using FISH probes (Yu *et al.*, 2011). Yu *et al.* (2011) in a study using FISH observed a direct correlation between the nitrifier population and the specific ammonium and nitrite utilization rate. In spite of all the above-mentioned limitations, FISH is still considered important as it provides information about the presence, relative abundance, morphology and spatial distribution of microorganisms in its natural habitat. Due to these disadvantages, researchers have come up with new ideas for its improvement, viz catalysed reporter deposition–FISH, microautoradiography combined with FISH, FISH–confocal scanning laser microscope, and combinatorial labelling and spectral imaging–FISH (Egli *et al.*, 2003; Daims *et al.*, 2006; Valm *et al.*, 2012).

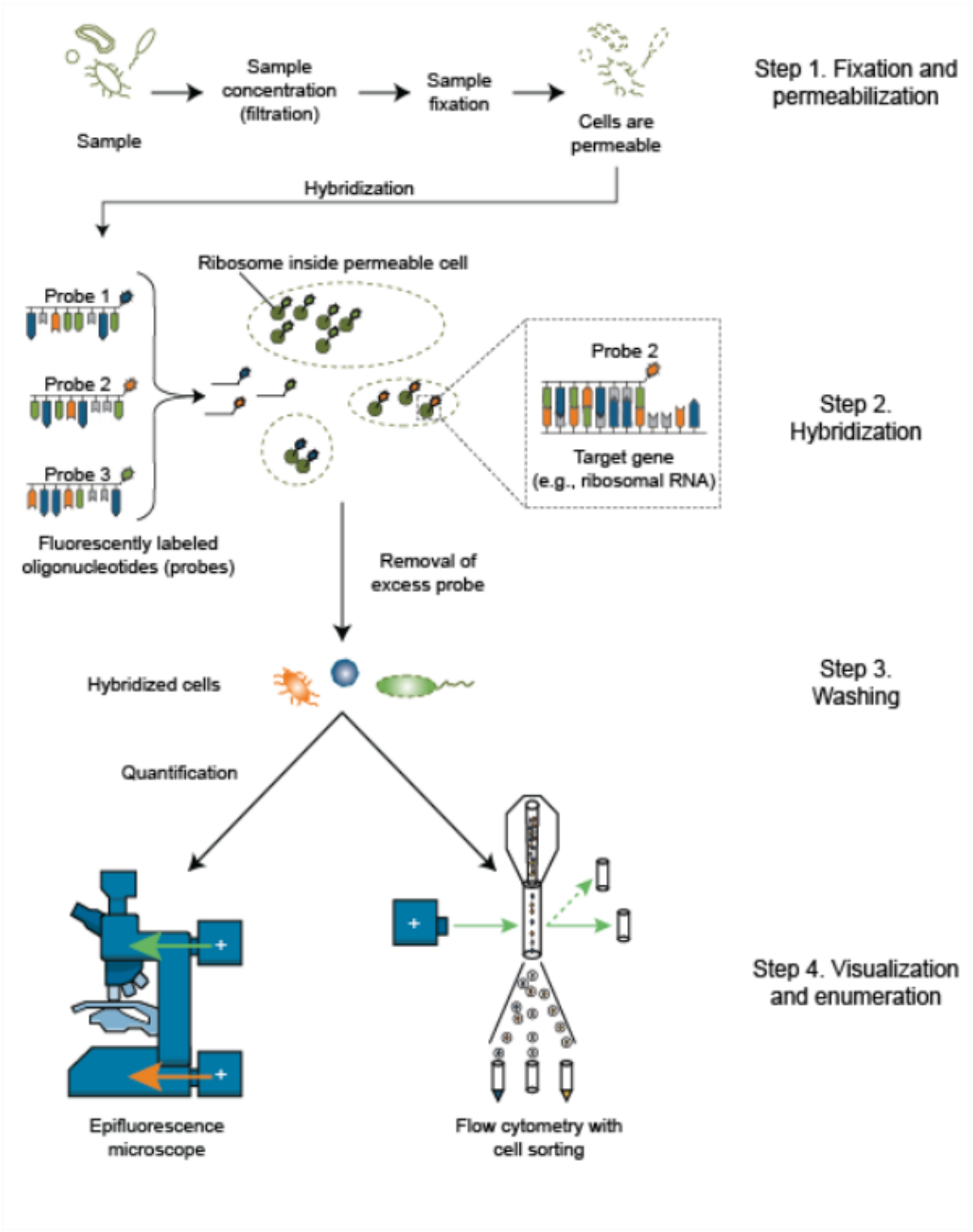

Fig. 2.3: Diagrammatic description of Fluorescent in situ hybridization (ITRC, 2013)

2.4.4 Microarray

Some other less frequently applied techniques for wastewater samples include microarray and most probable number (MPN). Microarray is a multiplex technique that harnesses the characteristics of DNA or RNA to bind to their complementary sequences (Gilbride *et al.*, 2006). Siripong *et al.* (2006) in a study on WWTPs noted that the microarray technique was able to confirm the presence of nitrifiers; however, due to insufficient fluorescence intensity, it failed to differentiate adequately between matched and mismatched sequence. This indicates a significant shortcoming of this technique. Kelly *et al.* (2005) investigated nitrifiers in samples from a wastewater treatment facility and observed that the microarray technique could detect nitrifiers directly without any need for complementary PCR amplification. However, they observed that other methods, especially T-RFLP, were sensitive enough to confirm the presence of more diversity of nitrifiers (*Nitrospira* sp. and *Nitrobacter* sp.) apart from only *Nitrosomonas* sp. that microarray could detect (Kelly *et al.*, 2005).

2.4.5 Polymerase chain reaction (PCR) – based techniques

Polymerase chain reaction (PCR) is a fundamental, routinely used non-culture based technique of analysing activated sludge community structure and function. The 16S rRNA gene is usually targeted when determining the overall microbial diversity in activated sludge (Fukushima, 2010), whilst genes such as polyphosphate kinase (ppk); nitrite reductase (nirK and nirS); ammonia monooxygenase (amoA) and nitrate reductase (narG) among others target functional level (Fukushima, 2010; Kim *et al.*, 2011). The bacterial 16S rRNA molecule is about 1.5 kb size. When partially or wholly sequenced, it has sufficient conserved and variable nucleotide regions with reliable phylogenetic information (Amann *et al.*, 1995; Clarridge, 2004; Ramdhani, 2012). Hence, the 16S rRNA targeted primers are usually employed to obtain amplicons from genomic DNA extracted from activated sludge. Some of the widely used PCR-based techniques are discussed below.

2.4.5.1 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis, a PCR-based method, is a common method of choice by researchers and is based on generating a genetic profile or ‘‘fingerprint’’ of the microbial community of complex environmental samples (Li *et al.*, 2006; You *et al.*, 2009). The species richness of the microbial community being examined is revealed by the different base pair sequences in the amplicons (Gao and Tao, 2012). This method has been employed extensively by researchers to evaluate the microbial community composition of different wastewater treatment samples (Boon *et al.*, 2002; Xia *et al.*, 2010b; Zhang *et al.*, 2010) or ‘‘shifts’’ in microbial community composition over time (Zhang *et al.*, 2009a; Wan *et al.*, 2011; Yu *et al.*, 2011). Yu *et al.* (2011) using a combination of PCR-DGGE and clone library analysis established *Nitrosomonas* spp. as the dominant AOB. This technique has also been used successfully by researchers to study the shift in the dominance of different species of nitrifiers (Yu *et al.*, 2011).

The sensitivity of this method is high, and its main advantage is that the individual DNA bands, or fragments from the gel can be excised and phylogenetically analysed. However, since the DGGE analysis can only be performed for shorter PCR amplicons (≤500 bp), the sequences of the bands obtained from a gel correspond to only short fragments of DNA (200–500 bp), and thus, the phylogenetic relations are less constantly established using DGGE bands (Sanz and Kochling, 2007; Gao and Tao, 2012). Analysis from DGGE technique can also be influenced adversely by the following limitations: the difficulty of DNA extraction and PCR amplification, depending on the nature of the samples, the variations in DNA copy number after PCR, depending on the abundance of the specific microorganisms and the intensity of the band obtained on a DGGE gel (Sanz and Kochling, 2007; Gao and Tao, 2012). The nonspecific amplification of the PCR primers and the presence of duplex molecules of DNA can also introduce error into the results obtained by this method (Guler, 2006; Li *et al.*, 2006).

2.4.5.2 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is one of the techniques which are being used by the researchers to monitor the microbial shift based on the restriction banding pattern. The technique involves cleavage of terminally labelled PCR-amplified gene by the restriction enzymes (Sanz and Kochling, 2007; Gao and Tao, 2012). The technique can be employed to investigate the shift in both the spatial and temporal microbial community composition from a given natural or engineered ecosystem (Yang *et al.*, 2011). It is a highly sensitive technique and can be used for semi-quantitative analysis of microbial populations in a particular microbial ecological system as an alternative to PCR-DGGE (Liu *et al.*, 2010b). The fingerprints from T-RFLP are usually inadequate for identification of individual taxonomic units (Yang *et al.*, 2011). Nonetheless, it is possible to sequence and identify the dominant organisms via comparison of the fragments generated with a sequence from a public database or a related clone library (Yang *et al.*, 2011). However, like any other PCR-based techniques, the biases related to DNA isolation steps and amplification also can affect the accuracy of this method (Sanz and Kochling, 2007). Liang *et al.* (2010) used the T-RFLP technique successfully to investigate the difference in nitrifier population from two different reactors.

2.4.6 Quantitative real-time PCR (qPCR)

The quantitative real-time PCR (qPCR) is the most commonly used and accepted technique in the recent years to quantify microbes from natural and engineered environments. This technique can be used to quantify the particular gene copies of target organisms from a complex environment using species-specific primers. It is an efficient and rapid technique regarded as more sensitive than FISH (Haarman and Knol, 2005; Fukushima, 2010). However, according to Zhang *et al.* (2009a), the application of either AOB 16S rDNA or the functional gene amoA for the analysis usually has their different shortcomings of false positives and false negatives, respectively. A combination of the two assays is therefore usually a way of overcoming and compensating for the disadvantages when applied to AOB detection and

quantification. Using qPCR, Ozdemir *et al.* (2011) investigated nitrifiers and found that the NOB (*Nitrospira* sp.) population was 5–10 times higher than that of AOB in the WWTP. Kim *et al.* (2011) and Cho *et al.* (2014) in different studies investigated nitrifying communities of activated sludge and noted that *Nitrobacter* spp. had higher population densities compared to *Nitrospira* spp.

2.4.7 Next generation sequencing approaches

The traditional Sanger/dideoxy sequencing approach to process complex environmental samples has shown to be grossly inadequate, due to the hundreds or thousands of important sequences that go unnoticed when employing this method (Shokralla *et al.*, 2012). Due to thousands of potential DNA templates usually present in wastewater samples, there is a strong need for a technique that is capable of simultaneous detection of diverse microbial communities in different DNA templates (Morozova and Marra, 2008; Shokralla *et al.*, 2012). The Sanger or dideoxy sequencing method, though useful in its own right, is limited in the quantity of targets that can be sampled because of the read length limitations, purity requirements and expense involved (Mardis, 2008). Contrarily, the Next generation sequencing (NGS) approach offers a speedy, relatively inexpensive alternative with a vastly improved amount of data production. This allows for the investigation of microbial ecology on a larger scale and with more detail than was possible with previously used sequencing technology (Ju and Zhang, 2015a).

Next generation sequencing offers the advantage of direct sequencing from environmental samples without a prior cloning step in a bacterial host as in the traditional Sanger approach. Undoubtedly, NGS has revolutionized environmental metagenomic research, with stiff competition between manufacturers for ever improving platforms and technologies allowing for a variety of NGS options at an ever decreasing cost. The 454/Roche FLX system and Illumina/Solexa Genome Analyzer NGS platforms have been widely used in deciphering the community structure of

activated sludge (Ju *et al.*, 2014; Wang *et al.*, 2014; Yang *et al.*, 2014; Ju and Zhang, 2015a; Keshri *et al.*, 2015).

2.4.7.1 The 454/Roche FLX system

This employs an approach termed as pyrosequencing and it is one of the most widely used NGS for wastewater study. Pyrosequencing is a real-time DNA sequencing technique that monitors DNA synthesis through a series of linked enzymatic processes (Fig. 2.4) (Ronaghi, 2001; Shokralla *et al.*, 2012). Unlike other alternatives, it does not require cloning, gel electrophoresis, size separation, labelled oligonucleotides or labelled primers (Ju and Zhang, 2015a). The technique can be employed for detection, identifying and typing bacteria (Choi and Liu, 2014; Sekar *et al.*, 2014).

The maiden application of pyrosequencing wastewater was to investigate the plasmid metagenome and the antimicrobial resistance pattern of the activated sludge (Hu *et al.*, 2012). Ye *et al.* (2011) noted that the traditional molecular techniques do not give a complete profile of the community structure present in the wastewater; however, pyrosequencing has the potential of a truer estimation and more detail reflection of such communities. In a study of nitrifying communities in WWTPs, Ye *et al.* (2011) identified *Nitrosomonas* spp., *Nitrospira* spp., *Nitrosospira* spp., *Nitrosococcus* spp. and *Nitrobacter* spp. by using pyrosequencing technology. They noted that apart from *Nitrosomonas* spp. and *Nitrospira* spp., other nitrifiers did not have significant contribution in the nitrification process (Ye *et al.*, 2011). Zhang *et al.* (2011a) in a study, observed an incongruity in the results when nitrifying communities in different wastewater bioreactors were analysed using quantitative PCR and pyrosequencing. Majority of the nitrifiers identified with high-thoroughput pyrosequencing were related to *Nitrosomonas* spp. (Zhang *et al.*, 2011a). The abundance and diversity of AOB and NOB were equally investigated in tannery sludge samples by Wang *et al.* (2014). This technique has also been successfully applied in studying the microbial

community shift in different wastewater treatment systems different authors (Zhu *et al.*, 2013; Choi and Liu, 2014; Hai *et al.*, 2014).

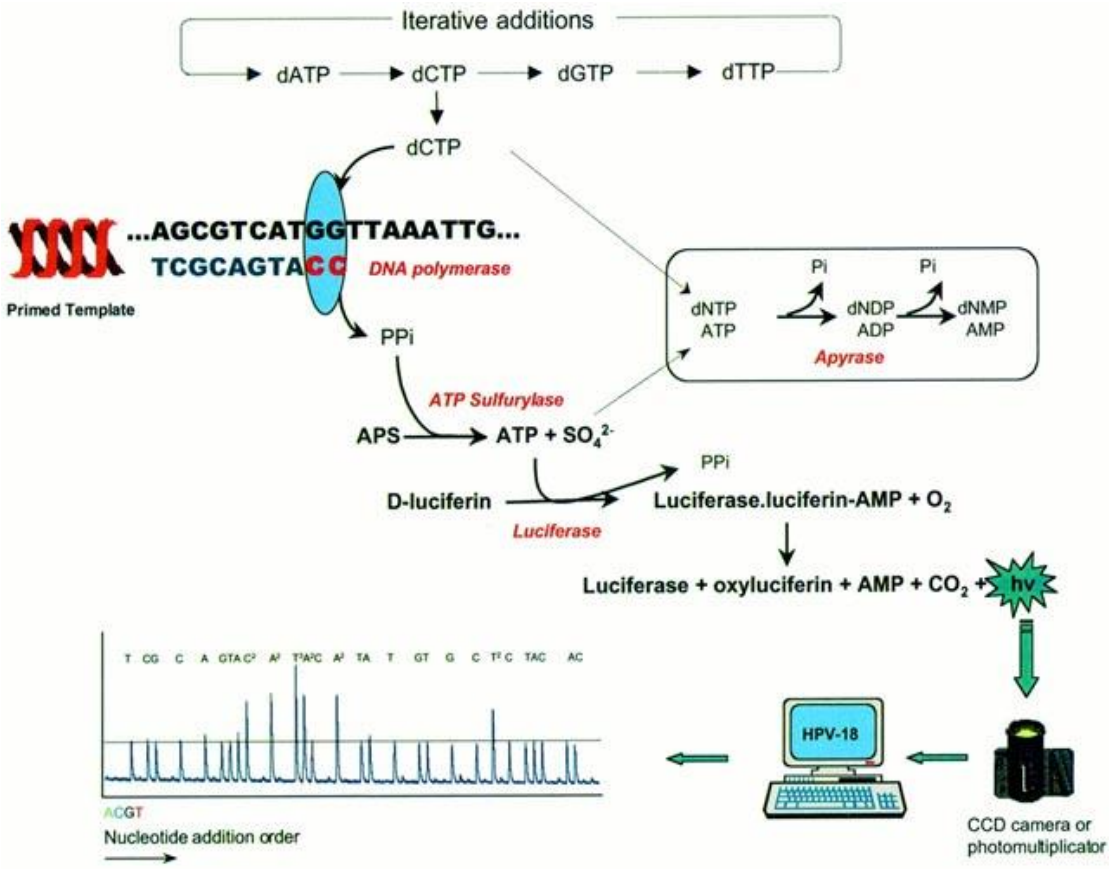

Fig. 2.4: Schematic diagram of pyrosequencing System (Gharizadeh *et al.*, 2001)

2.4.7.2 Illumina/Solexa Genome Analyzer

It is based on "DNA clusters" or "DNA colonies", which involves the amplification of DNA and primer that have been attached on a flow cell. Each cluster contains approximately one million copies of the original fragment which is enough to indicate bases incorporated at signal intensity adequate for detection during sequencing (Mardis, 2008). The Illumina system adopts the sequencing-by-synthesis (SBS) technology in which DNA polymerase and the four nucleotides are added at the same time to the flow-cell channels for incorporation into the oligo-primed cluster fragments

(Fig. 2.5) (Mardis, 2008; Liu *et al.*, 2012). This SBS technology uses an exclusive reversible terminator-based method to detect single base as they are incorporated into DNA template strands and non-incorporated nucleotides are washed away. The fluorescently labelled nucleotides images are captured by the camera. The fluorescent dye cum terminal 3' blocker is chemically removed for the next cycle of incorporation to begin (Quail *et al.*, 2012). Unlike pyrosequencing, the DNA chains are elongated one nucleotide at a time and image capturing can be done at a delayed time, giving room for very large arrays of DNA colonies to be captured by successive images taken from a single camera (Parmar *et al.*, 2014). Illumina platform is more effective at sequencing homopolymeric regions than pyrosequencing, however, it yields shorter sequence reads and the accuracy is still comparable to or superior to that of pyrosequencing (Varshney *et al.*, 2009; Ju and Zhang, 2015a). Ju *et al.* (2014) employed Illumina in investigating the seasonal dynamics of activated sludge over a period of 4 years. A combination of Illumina and pyrosequencing have been used by Sorokin *et al.* (2012) for genomic study of NOB and an entirely novel nitrifier named *Nitrolancetus hollandicus.*

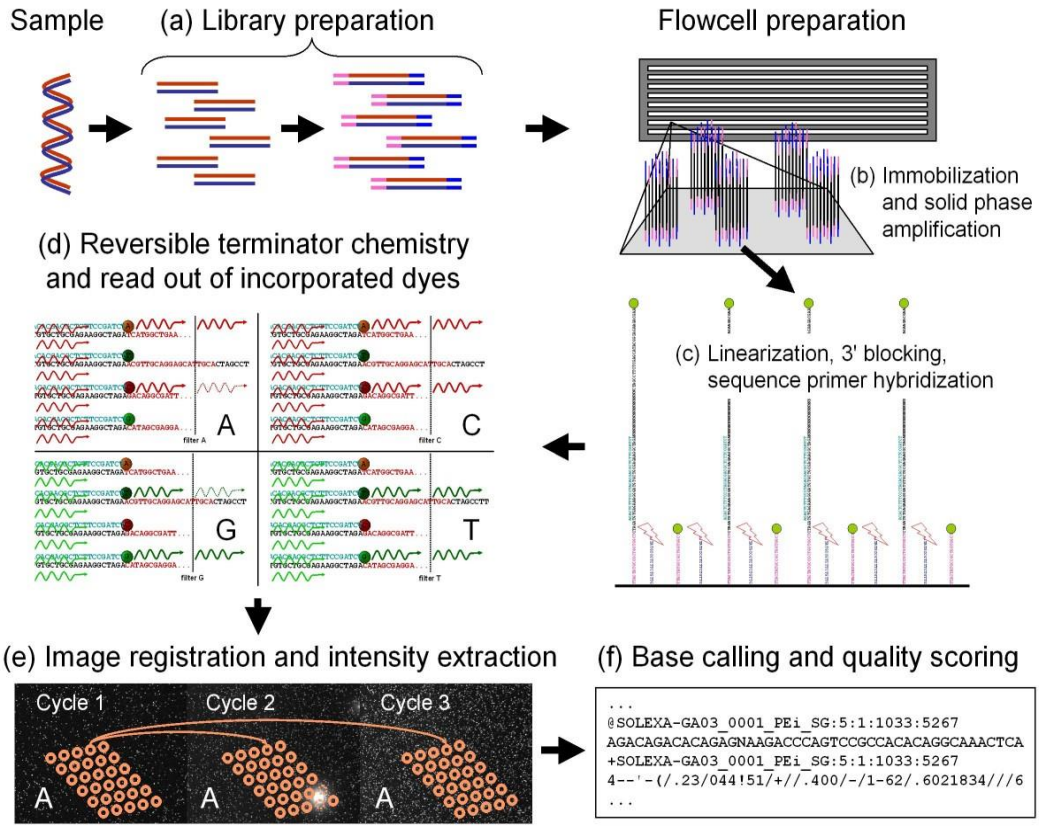

Fig. 2.5: Schematic diagram of Illumina sample preparation and sequencing (Kircher *et al.*, 2011)

According to Xia *et al.* (2010a), knowledge of microbial community structure of wastewater treatment bioreactors is still insufficient, the emergence of molecular techniques notwithstanding. Hai *et al.* (2014) noted that there is still a lack of knowledge of temporal dynamics of microbial community of activated sludge. In this study, a combination of Pyrosequencing and Illumina sequencing techniques were employed to investigate the total bacterial and AOB communities involved in the full scale municipal nitrifying plant over two different seasons. The wastewater characteristics and operational parameters were also monitored.

2.5 STATISTICAL ANALYSIS AND MODELLING IN WASTEWATER TREATMENT

Statistical measures such as correlation, covariance and simple regression have been used in capturing how two data series move together or relate over time (Damodaran, 2011). Many studies have applied simple correlation or cross-correlational approaches in capturing the relationships or interdependence between the various operational parameters and plant efficiencies in WWTPs (Kornboonraksa and Lee, 2009; Huang *et al.*, 2010a; Dong and Reddy, 2012; Wang *et al.*, 2014). However, due to simultaneous dependence on several factors, relationship between microbial groups in WWTPs is usually nonlinear (Klemetti, 2010). Hence, modelling the existing complex relationships in the full-scale WWTPs needs advanced nonlinear modelling tool (Kim *et al.*, 2011). A robust, nonlinear system known as artificial intelligence (AI) can depict the existing interactions between WWTP operational parameters as well as their correlation with the simulation output (Lu *et al.*, 2012; Alambeigi *et al.*, 2015). AI integrates artificial neural network (ANN), fuzzy inference system (FIS), and adaptive neuro fuzzy interference system (ANFIS). ANN is capable of modelling nonlinear systems effectively and have been used widely in engineering (Lu *et al.*, 2012; Alambeigi *et al.*, 2015; Nasr *et al.*, 2015a). FIS allows a logical data-driven modelling approach and has the ability to establish qualitative interdependency among variables (Pramanik and Panda, 2009; Nguyen and Sugeno, 2012). ANFIS is a neuro-fuzzy system that has the potential to capture the benefits of both ANN and FIS in a single framework which is capable of handling complex and nonlinear relationships among several parameters (Azar, 2010; Azar, 2011; Nasr *et al.*, 2015a).

2.6 RESEARCH OUTPUT

(a) Journal Articles

1) Awolusi, O. O., Kumari, S. K. S., Bux, F. 2015. Ecophysiology of nitrifying communities in membrane bioreactors. *International Journal of Environmental Science and Technology* 12: 747-762

(b) Book Chapter

1) Gokal, J., Awolusi, O. O., Enitan, A. M., Kumari, S. K. S., and Bux, F. 2015. Molecular characterization and quantification of microbial communities in wastewater treatment systems. In: Shukla, P. eds. *Microbial biotechnology: an interdisciplinary approach*. Taylor & Francis (In press)

CHAPTER THREE: CHARACTERIZATION OF THE DOMINANT NITRIFERS IN ACTIVATED SLUDGE SYSTEM USING CONVENTIONAL MOLECULAR METHODS

3.1 INTRODUCTION

Optimum and efficient nitrification hinges on a better understanding of the structure and dynamics of the nitrifying community structure within the wastewater treatment systems (Xia *et al.*, 2008; Hu *et al.*, 2012). Nitrification involves two sequential steps where ammonia (NH_3) is oxidized in turn into nitrite (NO_2^-) and nitrate (NO_3^-). A consortium of AOB and AOA are usually involved in the first rate limiting ammonia-oxidizing step in nitrification i.e. oxidation of ammonia to nitrite which is subsequently oxidized to nitrate by the NOB (Ramond *et al.*, 2015). Activated sludge system is unique in terms of its microbial community structure which plays an important role in the stability and efficiency of the plants (Eschenhagen *et al.*, 2003; Zhang *et al.*, 2012b). The advent of molecular techniques have revealed that only less than 15% of the microbial community in the activated sludge can be cultured (Wagner *et al.*, 1993). However, most often than not, the success of the molecular method employed depends largely on the integrity of the genomic DNA extracted. A representative and accurate bacterial diversity profiling requires nucleic acid extraction method without bias (Rajendhran and Gunasekaran, 2008).

Nucleic acids extraction method involves direct or indirect lysis procedures (Bourrain *et al.*, 1999). The direct lysis procedure involves the disruption of cells within the environmental sample matrix and subsequent purification of the DNA, whereas indirect method entails separating the cell from the sample matrix prior to lysis and nucleic acid extraction (Rajendhran and Gunasekaran, 2008). However, the indirect approach is usually not efficient as compared to the direct approach (Roh *et al.*, 2006). It involves an initial cation-exchange resin separation of the cell from the sample matrix and subsequent centrifugation (Bourrain *et al.*, 1999). Various non-culture

based techniques have also been employed in studying the microbial community structure of the activated sludge. Fluorescent in situ hybridization (FISH), is a quick and widely used molecular method, which involves the binding of fluorescent oligonucleotide probes to ribosomal ribonucleic acid (Awolusi *et al.*, 2015). It provides information about the presence, abundance, morphology and spatial distribution of microorganisms in its natural habitat. Different researchers have used FISH technique in understanding the nitrifiers present in wastewater treatment plants (Yu *et al.*, 2010; Yu *et al.*, 2011; Valm *et al.*, 2012). In this chapter, the aim was to ascertain the most suitable DNA extraction method for the activated sludge; and characterize the dominant nitrifers (AOA, AOB and NOB) in the activated sludge system, using conventional molecular methods including FISH, PCR and phylogenetic analysis.

3.2 MATERIALS AND METHODS

3.2.1 Plant description

The full-scale WWTP selected for this study is situated in the midlands of KwaZulu-Natal province, South Africa. The plant receives a discharge of 82880 ± 20832 m^3/d (average dry weather flow), including 90% domestic and 10% industrial wastewaters. The plant was designed based on the criteria of a modified Johannesburg (JHB) configuration, which offered anaerobic, anoxic, and aerobic biological processes (Daims and Wagner, 2010). As shown in Fig. 3.1, the effluent from primary settling tank is distributed to the pre-anoxic and anaerobic tanks. The pre-anoxic basin is enriched with return activated sludge from the bottom of a final settler, whilst effluent from anaerobic tank is discharged into the anoxic unit. An internal recycle is pumped from the last part of aerobic units to the anoxic zone. The mixed liquor, containing activated sludge, flows from the aerobic zone to a secondary settler, where it is separated under a quiescent condition into treated wastewater and return activated sludge. Wastewater characteristics and operational parameters for the plant are shown in Table 3.1.

3.2.2 Sample collection

Composite sludge samples (from the aeration tank), influent and effluent water samples were collected fortnightly for a period of 237 days (May - July 2012 and November 2012 - March 2013). Sterile sampling bottles were used in collecting the samples and all samples were maintained at 4^{o}C while in transit to the lab. Temperature, dissolved oxygen (DO) concentrations and pH measurements were done using a portable YSI meter (YSI 556 Multiprobe System). The plant operational parameters were obtained from the plant operators.

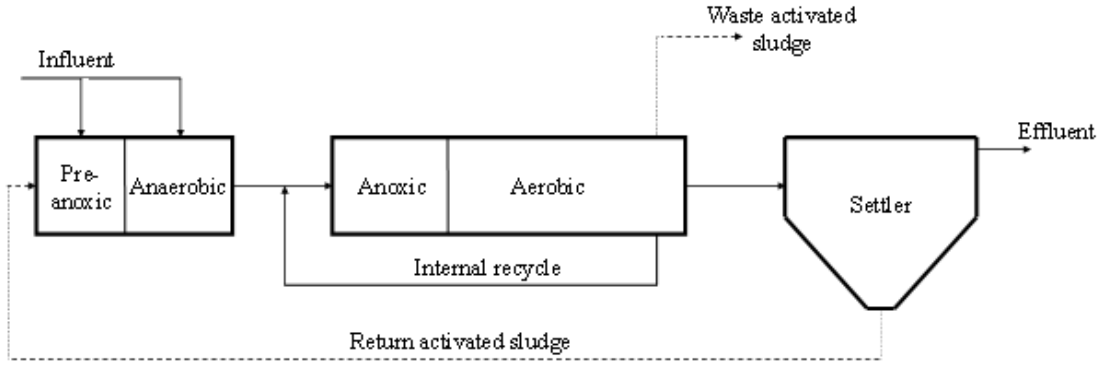

Fig. 3.1: Schematic diagram of the full-scale biological treatment process under study

Table 3.1: Average (winter and summer) wastewater characteristics and operational parameters of the selected plant as observed during the study period

Parameter	Phase-1	Phase-2
	Winter	Summer
Rainfall (mm)	26.0 ± 18.6	116.8 ± 32.0
Temperature	16.5 ± 2.1	22.8 ± 2.7
pH	7.3 ± 0.2	7.2 ± 0.1
DO (mg/l)	0.6 ± 0.3	0.6 ± 0.1
MLSS (mg/l)	6157 ± 783.3	4728 ± 1282.0
Chemical oxygen demand (mg/l)	1156 ± 976.1	684.7 ± 258.9
Ammonia (mg/l)	31.6 ± 6.0	24.4 ± 4.5
Flow Rate (ML/Day)	62.0 ± 2.2	96.8 ± 14.5
HRT (h)	6.3 ± 0.2	4.3 ± 1.0
OLR (kg-COD/m^3.d)	4.0 ± 1.1	4.5 ± 1.8
ALR (g-NH_4/m^3.d)	121 ± 22.0	144 ± 29.0
F/M (g-COD/g-MLSS.d)	0.6 ± 0.1	0.9 ± 0.3
COD removal (%)	97.5 ± 1.3	94.1 ± 2.6
Ammonia Removal (%)	60.0 ± 18.0	83.0 ± 13.0

COD: chemical oxygen demand; HRT: hydraulic retention time; OLR: organic loading rate; ALR: ammonia loading rate; F/M: food to micro-organisms ratio

3.2.3 Florescent *in-situ* Hybridization (FISH)

FISH analysis was performed for initial, rapid screening and characterization of the nitrifying community according to the modified protocols (Amann *et al.*, 1995; Fuchs *et al.*, 2007).

3.2.3.1 Cell fixation and pre-treatment

Sludge sample (1 ml) was centrifuged, then washed with 1X PBS and the resulting pellet was fixed in a 3:1 (v/v) mixture of paraformaldehyde solution and 1X PBS (PBS: 130 mM sodium chloride, 10mM sodium phosphate buffer [pH 7.2]). This above mixture was incubated overnight at 4^{o}C and afterwards span down by centrifugation and the pellet washed with 1X PBS. The fixed pellet was resuspended in equal volumes of fresh 1X PBS–absolute ethanol mixture; this was stored at -20^{o}C until used for hybridization. Before the hybridization step, the fixed samples were washed and resuspended in 1 ml of fresh 1X PBS. The resulting mixture was pre-treated according to the optimized wattage (8 watts) earlier reported (Ramdhani, 2012), using an Ultrasonic Liquid Processor (Misonix XL-2000). The floc dispersion was optimized at the optimum wattage (8 Watts) for 6, 8 and 10 min to ensure the best result. The mixture was further diluted with 0.5 ml sterile deionized water (Amann *et al.*, 1995; Fuchs *et al.*, 2007).

3.2.3.2 Oligonucleotide probe selection

The oligonucleotide probes (Table 3.2) targeting the commonly found nitrifying groups in the activated sludge (Betaproteobacterial AOB, Genus *Nitrospira* and *Nitrobacter* spp.) were used (Bassin *et al.*, 2012; Li *et al.*, 2013; Lienen *et al.*, 2014). The 5$^{'}$ end of the oligonucleotide probes used were labelled with CAL Fluor Red 590 fluorescence dye (Inqaba Biotechnical Industries (Pty), South Africa).

3.2.3.3 Whole cell hybridization

The Teflon-coated slides were washed in alcohol (1% HCl in 70% ethanol). Coplin jar was filled with 100 ml of diluted poly-L-lysin (0.01%, Sigma-Aldrich, Germany) at room temperature and the slides were allowed to stand for 5 min in coplin jar containing poly-L-lysin. Slides were then drained and dried at 60^{o}C for 1 hour. About ten microliter volume of the pre-treated fix sample was spotted on the Teflon-coated slide and dried at 46^{o}C for about 10 min. The specimen on the slide was dehydrated sequentially in 50, 80, and 100% ethanol for 3 min each (Amann, 1995). Fresh hybridization buffer was prepared by mixing 5 M NaCl, 1 M Tris-HCl (pH 8), Formamide (Table 3.2) and 10% SDS. One microliter of FISH probe and 9 µl of hybridization buffer were added onto the spotted well. The slides were then placed into a hybridization tube (50 ml falcon tube, protected from light) containing filter paper soaked with hybridization buffer. This was incubated overnight at 46^{o}C. After hybridization, the slide was placed in hybridization tube containing pre-warmed (at 48^{o}C) wash buffer (1 M Tris/HCl, 10 % SDS, 0.5 M EDTA and 5 M NaCl [Table 3.2]), and was incubated in hybridization oven for 1 hour at 48^{o}C. The slides were then washed with sterile deionized water and air-dried. Furthermore, 8 µl volume of 4′-6-diamino-2-phenylindole (DAPI) was added to the slide and left for 10 min. The slide was washed with 1X PBS and allowed to air-dry in the dark. The slides were thereafter mounted using anti-fading Vectashield solution (Vector Laboratories, Burlingame, CA).

Table 3.2: rRNA – targeted oligonucleotide probes used and their specificity

Probe name	Target	Sequence (5′ – 3′)	FA[1] (%)/NaCl (µl)	Reference
Nso1225	Betaproteobacterial AOB	CGCCATTGTATTACGTGTGA	35/700	(Bassin *et al.*, 2012)
NIT3	Genus *Nitrobacter*	CCTGTGCTCCATGCTCCG	40/460	(Lienen *et al.*, 2014)
Ntspa662	Genus *Nitrospira*	GGAATTCCGCGCTCCTCT	35/700	(Li *et al.*, 2013)

(1) FA = Formamide concentration

3.2.3.4 Image analysis

The hybridized slides were examined using a Zeiss Axio-Lab HB050/AC microscope (Carl Zeiss, Germany) equipped with a HBO 50 W Hg vapour lamp, with appropriate filter sets, specific for TAMRA and FAM using ×100 Plan Apochromat Objective. Images were captured using the Zeiss AxioCam MRC camera and analysis was carried out using Zeiss Axio vision Release 4.8 imaging software.

3.2.4 Genomic DNA extraction

Genomic DNA extraction forms a critical and important step for almost all molecular based analysis, as the extracted DNA becomes the precursor upon which the success of the analysis hinges. Genomic DNA was extracted from sludge samples (aerobic samples) collected during the winter and summer seasons. To ensure the integrity of the genomic DNA used in this study, three extraction methods (enzymatic, freeze-thaw and sonication) based on different lysis treatment, including enzymatic and physical techniques were optimized in this study.

The enzymatic method was carried out using the protocol described by Purkhold *et al.* (2000). Activated sludge sample (2 ml) was centrifuged at 5000x g for 5 min and the pellet was resuspended in 725 µl DNA extraction buffer (Tris-HCl [pH 8; 100 mM], EDTA [pH 8; 100 mM]. Fifty microliter volume of enzyme mix A (lysozyme [0.0142 mg/ µl]; lipase [0.0223 mg/ µl]; pectinase [0.017 mg/ µl]; β-Glucuronidase [0.011 mg/ µl]) was added to the resuspended pellet above, mixed gently by inversion and then incubated at 37°C for 30 min. Furthermore, 50 µl of enzyme mix B [Proteinase K (0.01 mg/µl); Protease (0.01 mg/µl); Pronase (0.04 mg/µl)] was added to the same tube in step above, gently mixed by inversion and then incubated at 37°C for 30 min. Afterwards, 75 µl volume of 20% SDS was added to the incubated mixture above and further incubated at 65°C for 2 hrs. The resultant mixture above was incubated again at 65°C for 20 min after 600 µl of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and then mixed by inversion. The tube above was then vortexed and centrifuged at 10,000 x g for 10 min at room temperature and the aqueous phase was transferred into a fresh Eppendorf tube.

With regards to the aqueous phase obtained, 100 µg/ ml of RNase A was added to a final concentration of 10 µg/ml and incubated at 37°C for 30 min. The sample was then extracted with 1 volume chlorofom: isoamyl alcohol (24:1), mixed by inversion, and centrifuged at 10,000x g for 10 min at room temperature. The aqueous phase was then transferred into a fresh Eppendorf tube. DNA was precipitated by adding 0.6 volume of isopropanol to the tube and incubated at room temperature for 1 hr. The DNA was collected by centrifugation (10,000 x g at 4°C for 20 min) and the supernatant discarded. The DNA pellet was washed with 2.5 volume cold 70% ethanol. The tube was inverted to air-dry pellets for about 10 min and pellet was resuspended in TE buffer. The quality and quantity of the extracted DNA was ascertained with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The extracted genomic DNA was stored at –20^{o}C until further analysis.

The freeze–thaw method was done according to modified Briese (2002) protocol. For this, two millilitre volume of the sludge sample was centrifuged at 7000 rpm for 5 minutes at 4^{o}C. The pellet was washed with 1X PBS twice. The pellet was resuspended in 750 µl volume of the lysis buffer (plus 0.2% each of PVP and β-Mercaptoethanol) and incubated at 65^{o}C for two hours. The mixture was then frozen-thawed (5 minutes in ice-ethanol slurry and 3 minutes in water-bath at 65^{o}C) for 5 times. To this, equal volume of Tris-saturated Phenol-Chloroform-Isoamyl alcohol (25:24:1) was added, vortexed and centrifuged for 2 minutes at 12000 rpm (4°C). The supernatant was transferred to a fresh tube and equal volume of Chloroform was added, vortexed and centrifuged 2 minutes at 12000 rpm 4°C. The supernatant was transferred into a fresh tube and 0.6 volume of Isopropyl alcohol was added. Precipitation was carried out at –20^{o}C for 1 hour. The DNA was pelletized at 12000 rpm (4°C) for 20 minutes. The pellet was washed (12000 rpm 4°C) with 1 ml cold 70% ethanol and air dried for 15 minutes at room temperature. The air dried DNA was thereafter dissolved in TE buffer and stored at -20^{o}C until further analysis. The procedure for the second mechanical lysis method which involved sonication was similar to the freeze-thaw method except that the freeze thaw step in ice-ethanol slurry/hot water bath was replaced with sonication using an Ultrasonic Liquid Processor (Misonix XL-2000). The pellet was sonicated for 15 minutes at maximal intensity according to the optimized method by Lemarchand *et al.* (2005), thereafter the DNA purification was carried out as described above.

3.2.5 Spectrophotometric analysis

A total of six replicates were used in evaluating the quality and quantity of the different extraction protocols with the aid of NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA) at 260/230 and 260/280 nm spectrophotometric absorbance ratios. Three genomic DNA extraction protocols that were based on three different lysis methods viz: enzymatic, freeze-thaw and sonication were optimized in this study. One microliter volume of genomic DNA sample was used for the analysis and the resulting

DNA was diluted to lower concentration (1-10 ng/µl) using molecular grade water for further analysis.

3.2.6 Polymerase chain reaction

The DNA was amplified using Veriti TM 96-well Thermal Cycler (Applied Biosystems, USA) and the thermocycling conditions are as shown in Table 3.3. The PCR was performed in a total reaction volume of 50 µl containing 10 ng of DNA template. The final concentrations of the different components in the reaction mix were 200 µM of DNTPs, 1.5 mM of MgCl (Taq buffer with initial MgCl concentration of 20 mM), 2.5 U of *Taq* DNA polymerase (Thermo Scientific, Lithuania) and 0.5 µM of each primer (Table 3.3). The extracted genomic DNA and PCR amplicons were subjected to agarose gel electrophoresis with 1% (wt/vol) agarose gel. The electrophoresis was carried out and optimized at 80 volts for 60 minutes and the gel was visualized under UV light using Vacutec gel documentation system (Vacutec, South Africa).

3.2.7 Cloning, sequencing and phylogenetic analysis

The PCR amplicons with the expected size were purified using QIAquick PCR purification kit (Qiagen, USA) following the manufacturer's instruction. The cloning was carried out using the InsTAclone PCR Cloning Kit (Thermo Scientific). Competent cells were prepared from *Escherichia coli* (DH5α). During PCR amplification, the *Taq* DNA polymerase enzyme adds one 3'-adenine overhang to the two terminals of the PCR product. This unique structure of the amplicon makes it suitable for cloning of amplicons with 3'-adenine overhang into the vector cloning directly. These overhangs at the insertion site also prevent the restoration of circularity to a vector.

Table 3.3: Primers and the optimized amplification conditions

Target	Primer	Initial Denaturation		Cycles	PCR conditions						Final elongation		Reference
					Denaturation		Annealing		Elongation				
		ºC	Min		ºC	S	ºC	S	ºC	S	ºC	Min	
AOB amoA	amoA-1F/ amoA-2R	95	2	35	94	45	55	45	72	45	72	7	(Jin *et al.*, 2011)
Nitrospira 16S rDNA	NSR1113F/NSR 1264R	94	5	40	94	30	65	30	72	30	72	15	(Dionisi *et al.*, 2002)
Nitrobacter 16S rDNA	FGPS872/FGPS1269	95	10	35	95	60	50	60	72	60	72	7	(Cebron and Garnier, 2005)
Archaea 16S rDNA	Arc622f/Arc915r	94	5	40	94	60	53	60	72	60	72	5	(Chang *et al.*, 2001)
AOA amoA	Arch-amoAF/Arch-amoAR	95	5	30	94	45	53	60	72	60	72	15	(Francis *et al.*, 2005)

3.2.7.1 Ligation

The purified PCR product was ligated to pTZ57R/T vector according to the manufacturer's instruction (Thermo Scientific, InsTAclone PCR Cloning Kit). The ligation mixture was prepared by adding 3 µl volume of vector pTZ57R/T, 6 µl 5X ligation buffer, 5 µl purified amplicon or supercoiled pTZ57R DNA as control DNA , 1 µl T4 DNA ligase and DNase/RNase free water to make up to 30 µl. The mixture was vortexed briefly and incubated overnight at 4ºC for maximum number of transformants.

3.2.7.1 Competent cell preparation and transformation

LB broth (2 ml) was seeded with a single colony of freshly streaked *E. coli* DH 5α and incubated at 37º C and 200 rpm overnight in a shaking incubator. On the day of transformation, 1.5 ml of pre-warmed (37º C) LB broth was inoculated with 150 µl volume of *E. coli* DH 5α overnight culture and incubated at 37º C for 30 minutes in a shaker. The resulting bacterial cells were centrifuged at 10000 x g for 1 minute and the cells were resuspended in 300 µl of T-solution and incubated on ice for 5 min. The cells were then centrifuged for 1 minute at 10000 x g, resuspended in 120 µl of T-solution and kept on ice for 5 minutes. In a 2 ml Eppendorf tube, 2.5 µl volume of the prepared ligation mixture (containing the vector DNA) was incubated on ice for 2 minutes, fifty microliter volume of the prepared cells were mixed with the DNA in the Eppendorf tube and incubated on ice for 5 minutes. This mixture was plated quickly plated on pre-warmed LB agar containing 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)/ isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated overnight at 37ºC (Sambrook and Russell, 2001).

3.2.7.2 Recombinant clones selection

Identification of the recombinant clones was carried out using the method previously described by Padmanabhan *et al.* (2011). This is based on the alpha complementation,

where cells containing vector with an insert may be identified using blue/white selection. In order to carry out the blue/white screening, a vector (pTZ57R/T) having both ampicillin resistance (Ap^R) gene and β-galactosidase (*lac*Z) enzyme which cleaves lactose into glucose and galactose was used. Recombinant plasmid was transformed into *E. coli* and white colonies that grew on LB plates containing antibiotics (ampicillin), X-Gal, and IPTG were picked for colony PCR amplification. The *E. coli* cells without the vector could not grow on the LB-ampicillin agar due to antibiotic toxicity. The plasmid pTZ57R/T and the DNA having the gene of interest (amplicon) were both split with the restriction enzyme. White clones were randomly selected on LB antibiotic agar plates containing X-gal and IPTG stock solutions and positive clones were confirmed by colony PCR using appropriate primer-sets and resolved on agarose gel for further confirmation of plasmids containing the targeted inserts.

3.2.7.3 Recombinant clone analysis using colony PCR

Colony PCR was carried out in order to confirm the positive transformants. The concentration of each component of the PCR mix was according to Degrange and Bardin (1995) protocol earlier described except for the template DNA prepared differently. Single white colony to be verified was picked from the agar plate randomly using sterile pipette tip and suspended into the PCR mix. Colony PCR was conducted using the appropriate primer sets as listed in Table 3 and amplification conditions.

3.2.7.4 Sequencing and phylogenetic analysis

The purified colony PCR amplicons were submitted to commercial lab (Inqaba Biotechnical Industries (Pty), South Africa) for sequencing and analysis. The obtained sequences were edited using Finch TV software. Clones that were 97% similar were assembled into single operational taxonomic unit (OUT), with their representative nucleotide sequences used for further analysis. The sequences obtained were checked against the National Centre for the Biotechnology Information (NCBI) GenBank

database using the Basic Local Alignment Search Tool (BLAST) for their phylogenetic affiliations. A combination of nucleotide sequences from this study and those obtained from NCBI database were aligned with CLUSTALX implemented in BioEdit (Hall, 1999). The aligned sequences were exported into MEGA6 (Tamura *et al.*, 2013) where matrices of evolutionary distances were computed. Phylogenetic trees were then constructed and checked by bootstrap analysis (based on 1,000 replicates) (Tamura *et al.*, 2011).

3.2.8 Nucleotide sequence accession numbers

The GenBank accession numbers for the nucleotide sequences of the clones isolated in this study are KP337415-KP337452.

3.3 RESULTS

3.3.1 Comparison of DNA extraction methods

Three different extraction methods namely; enzymatic, sonication and freeze-thaw were compared. The result obtained from samples A, B and C comparing these three methods are shown below (Table 3.4 and Fig. 3.2). Samples A, B and C were sludge samples taken on different days. Using the three extraction methods, DNA was extracted from replicates of each sample A, B and C. The integrity and shearing of each extracted DNA was examined visibly via electrophoresis on an agarose gel (1% [w/v]). Genomic DNA with purity within the acceptable range was successfully extracted with the 3 methods, but with different DNA concentration yields. The extraction method involving sonication yielded DNA with significant shearing in all the 3 samples A, B and C (Fig. 3.2). Furthermore, variability was observed in terms of DNA concentration obtained from the different extraction techniques, with the enzymatic method yielding highest concentrations (Table 3.4). Variability in terms of DNA concentration was also observed among replicates of the same sample (Table 3.4).

Table 3.4: Comparison of DNA extraction methods based on concentration and purity

Samples	Enzymatic method			Sonication method			Freeze-thaw method		
	A260/280	A260/230	Concentration (ng/µl)	A260/280	A260/230	Concentration (ng/µl)	A260/280	A260/230	Concentration (ng/µl)
A	2.06±0.20	1.69±0.10	799.60±14.70	1.81±0.10	2.20±0.40	482.80±19.40	1.87±0.10	2.20±0.50	352.79±19.40
B	2.21±0.10	1.74±0.10	775.20±15.50	1.83±0.11	2.52±0.20	403.90±11.30	1.85±0.10	2.31±0.21	433.93±13.10
C	2.18±0.10	1.59±0.02	802.90±37.31	1.81±0.10	2.11±0.11	521.00±15.30	1.83±0.11	1.95±0.10	370.00±17.30

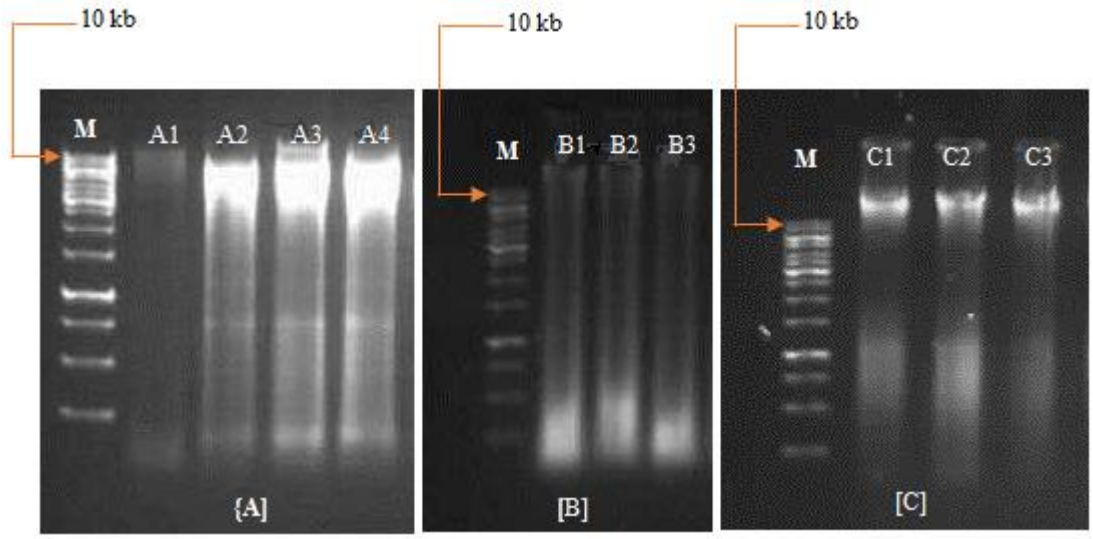

Fig. 3.2: Agarose gel depicting genomic DNA isolated from sludge samples. Plates [A], [B], and [C] depict extracted DNA using enzymatic, sonication and freeze-thaw treatments respectively. Lanes M = 1 kb DNA ladder; lanes A2 – A4 shows genomic DNA extracted from samples A, B and C respectively using enzymatic method; lanes B1– B3 shows genomic DNA from samples A, B and C respectively using sonication; and lanes C1 – C3 shows extracted DNA from samples A, B and C respectively using freeze-thaw method.

3.3.2 Detection of nitrifiers using FISH

Preliminary detection and identification of the nitrifiers was carried out using FISH probes targeting AOB and NOB (Table 3.2). The Betaproteobacterial AOB, *Nitrospira* spp. and *Nitrobacter* spp. were detected all through the sampling period. The FISH micrographs of the samples hybridized with CAL Fluor Red 590 positive with AOB, *Nitrospira* spp. and *Nitrobacter* spp. are shown in Fig.3.3.

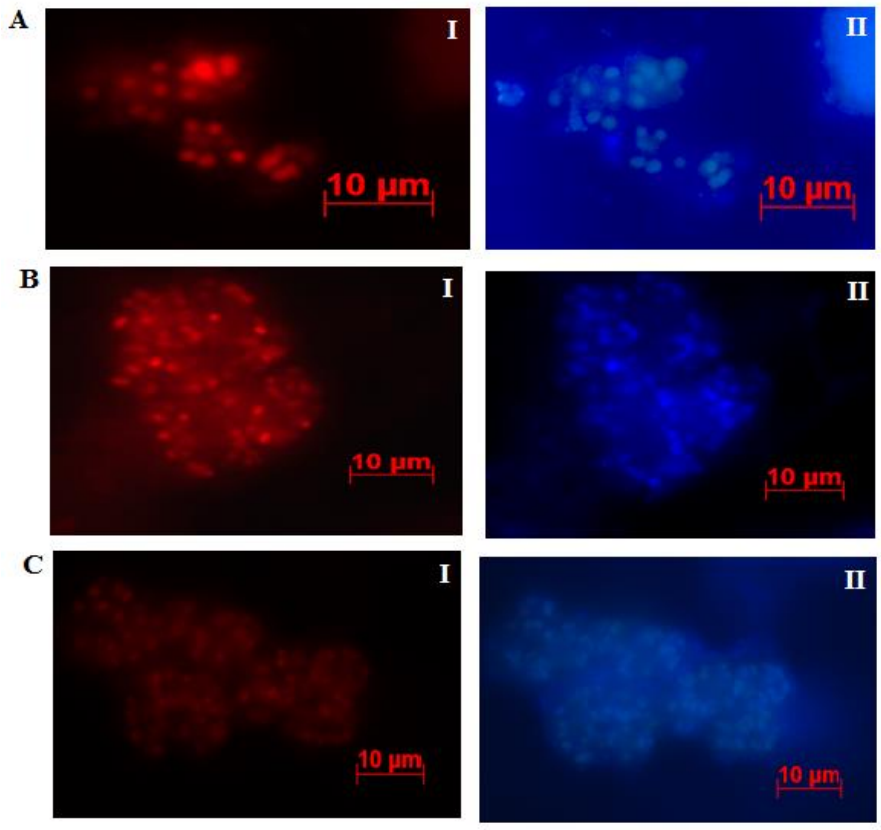

Fig. 3.3: Micrograph of hybridized samples. Nitrifiers on each plate are shown as follows: A(I) Micrograph of AOB hybridised by Cal Fluor Red 590 labelled AOB oligonucleotide probe (NSO 1225); A(II) corresponding image showing AOB stained with DAPI (blue); B(I) Micrograph of *Nitrobacter* hybridised with Cal Fluor Red 590 labelled NIT3 oligonucleotide probe; B(II) corresponding image showing *Nitrobacter* stained with DAPI (blue); C(I) Micrograph of *Nitrospira* hybridised with Cal Fluor Red 590 labelled Ntspa 662 oligonucleotide probe C(II) corresponding image showing *Nitrospira* stained with DAPI (blue).

3.3.3 Detection of the dominant nitrifiers using PCR

Using primers set amoA1F/amoA2R with the optimized PCR conditions (Table 3.3) resulted in specific amplicons at the expected base pair length (490 bp) (Fig 3.4). The primer sets NSR 1113F/NSR 1264R and FGPS872/FGPS1269 targeting the *Nitrospira* spp. and *Nitrobacter* spp. respectively were used to detect the NOB in the plant.

Successful amplification of *Nitrospira* spp. and *Nitrobacter* spp. were confirmed with amplicons yielding band size of 151 bp and 497 bp respectively (Fig 3.5). For the archaea, primer sets targeting the archaea amoA and 16S rDNA primer sets were used according to the conditions stated in Table 3.3. There was no successful archaea amplification during this study even after the annealing temperatures was optimized over a range (50 – 58^{o}C).

Fig. 3.4: Primer specificity: Agarose gel showing PCR products for AOB at 490-bp. Lane 1 denotes the 100-bp DNA ladder, whilst lanes 2 – 14 indicates resulting bands from using amoA1F/amoA2R

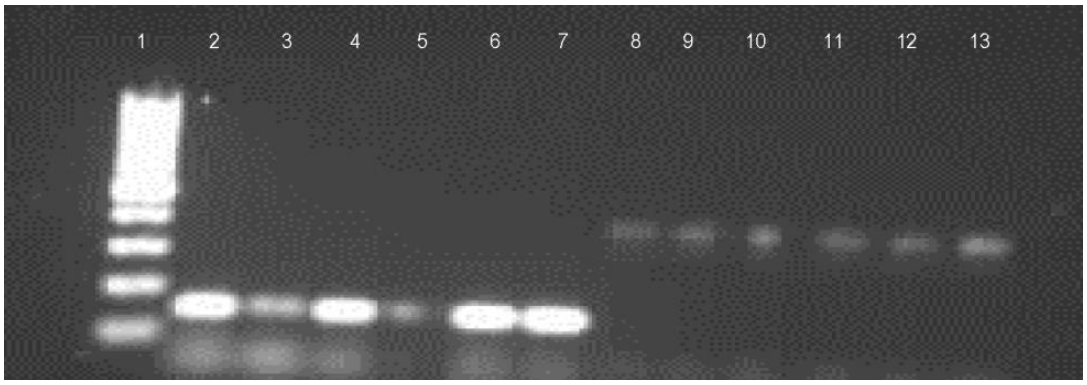

Fig. 3.5: Primer specificity: Agarose gel showing PCR products for *Nitrospira* spp. at 151 bp and *Nitrobacter* spp. at 397-bp. Lane 1 denotes the 100-bp DNA ladder, whilst lanes 2–7 indicates resulting bands from using NSR 1113F/NSR 1264R and lanes 8–13 depicting resultant bands from using FGPS872/FGPS1269

3.3.4 Cloning and analysis

Successful transformation was confirmed with the growth of white and blue colonies on IPTG/X-Gal-LB agar plates after overnight incubation (Fig. 3.6). The blue colonies represent the cells transformed with non-recombinant plasmids, whereas the colonies formed by recombinant cells appeared white. The PCR amplification from the recombinant clones (white colonies on IPTG/X-Gal-LB agar plate) yielded amplicons with expected base pair size on agarose gel after electrophoresis (Fig. 3.7 and 3.8). These were later sent for sequencing at Inqaba Biotechnical Industries (Pty), South Africa.

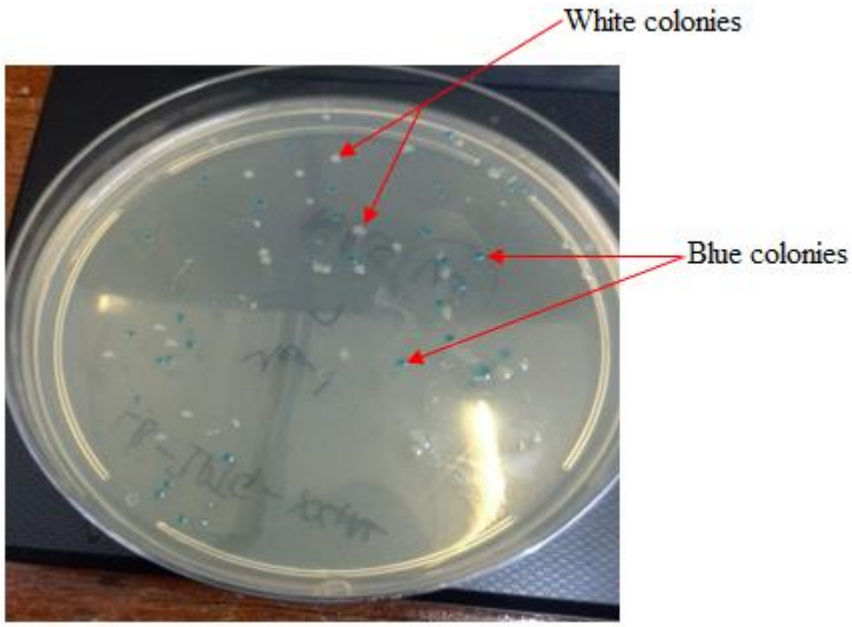

Fig. 3.6: Cloned nitrifiers PCR product on IPTG/X-Gal-LB agar plates. Overnight *E. coli* DH5α colonies incubated at 37ºC after transformation with pTZ57R/T plasmid vector. White and blue colonies were observed on LB agar containing IPTG and X-Gal

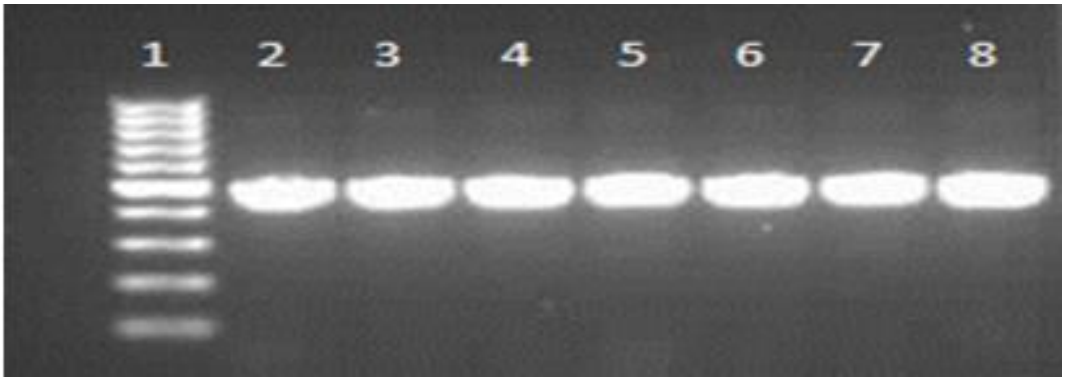

Fig. 3.7: Agarose gel image of PCR product from colony PCR products of amoA amplicons (~490 bp) obtained after transformation (Lanes 2 – 8). Lane 1 = 100 bp ladder. Colony PCR for amoA.

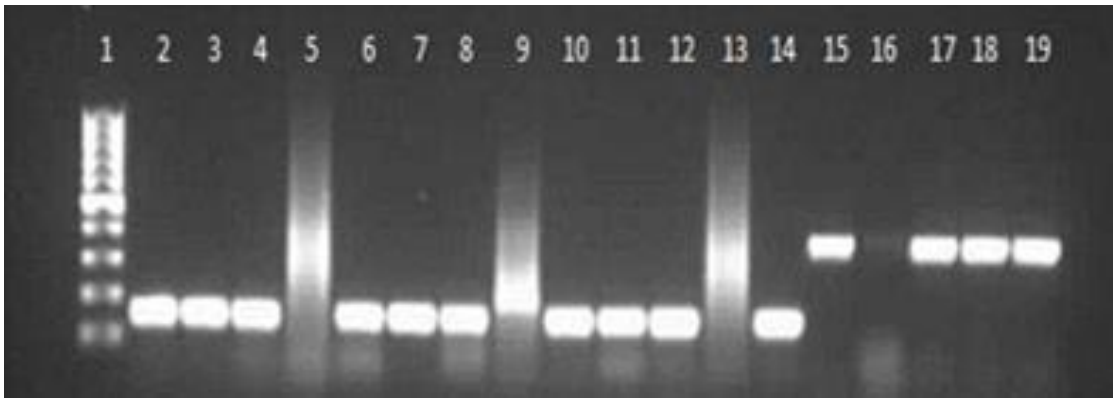

Fig. 3.8: Agarose gel image of PCR product from colony-PCR products of *Nitrospira* and *Nitrobacter* amplicons (~151 bp [Lanes 2 – 4, 6 – 8, 10 – 12, 14] and 397 bp [Lanes 15; 17 – 19] respectively) obtained after transformation. Lane 1 = 100 bp ladder.

3.3.5 Phylogenetic analysis

The sequences obtained were analysed using the NCBI BLAST programme. The clones were grouped into the same operational taxonomic unit (OTU) based on 97% sequence similarity and their representative sequences were employed for further analysis. The dominant AOB sequences during the study were related to uncultured ammonia oxidizing bacterium, uncultured *Nitrosomonadaceae* bacterium, *Nitrosomonas* sp. and uncultured bacterium (Fig. 3.9). The comparative sequences analysis for *Nitrobacter* clones revealed 95 – 97% relatedness to uncultured alpha proteobacteria, uncultured bacterium, uncultured *Nitrobacter* sp., and uncultured *Bradyrhizobium* sp. (Fig. 3.10). The partial sequences obtained from *Nitrospira* clones revealed 98 – 100% similarity to uncultured bacterium, *Candidatus Nitrospira defluvii*, uncultured *Nitrospira* sp. and uncultured *Nitrospirae* bacterium (Fig. 3.11). The partial sequences obtained were used in constructing the phylogenetic tree and also deposited with GenBank to obtain accession numbers (KP337415-KP337452).

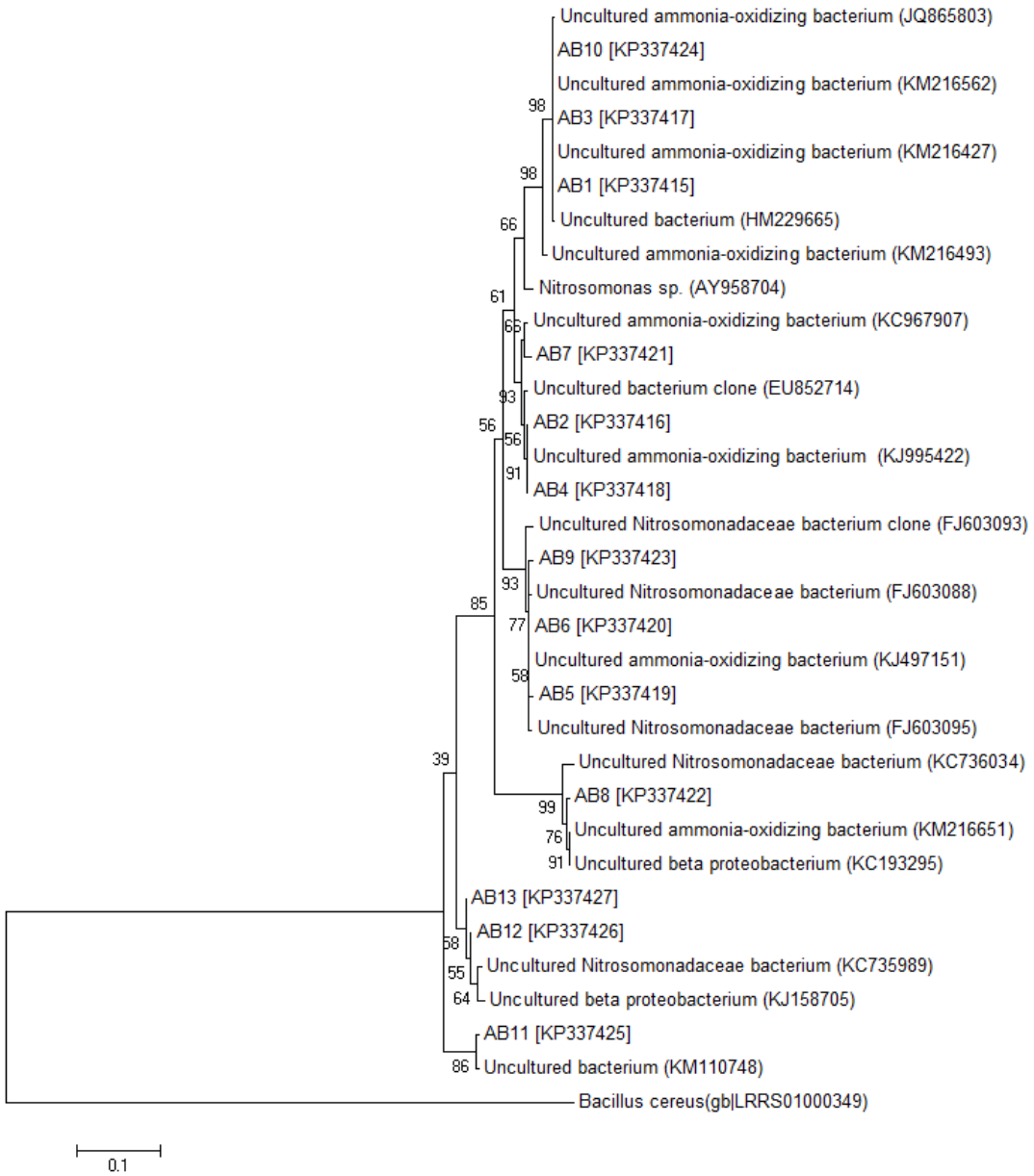

Fig. 3.9: Phylogenetic tree of the bacterial amoA gene sequences recovered from municipal activated sludge. A neighbour-joining tree was constructed from the resulting alignment with MEGA 6, and the bootstrap values were based on 1000 replicates. The scale bar represents 0.02 change per site. The sequence of *Bacillus cereus* was used as an out group.

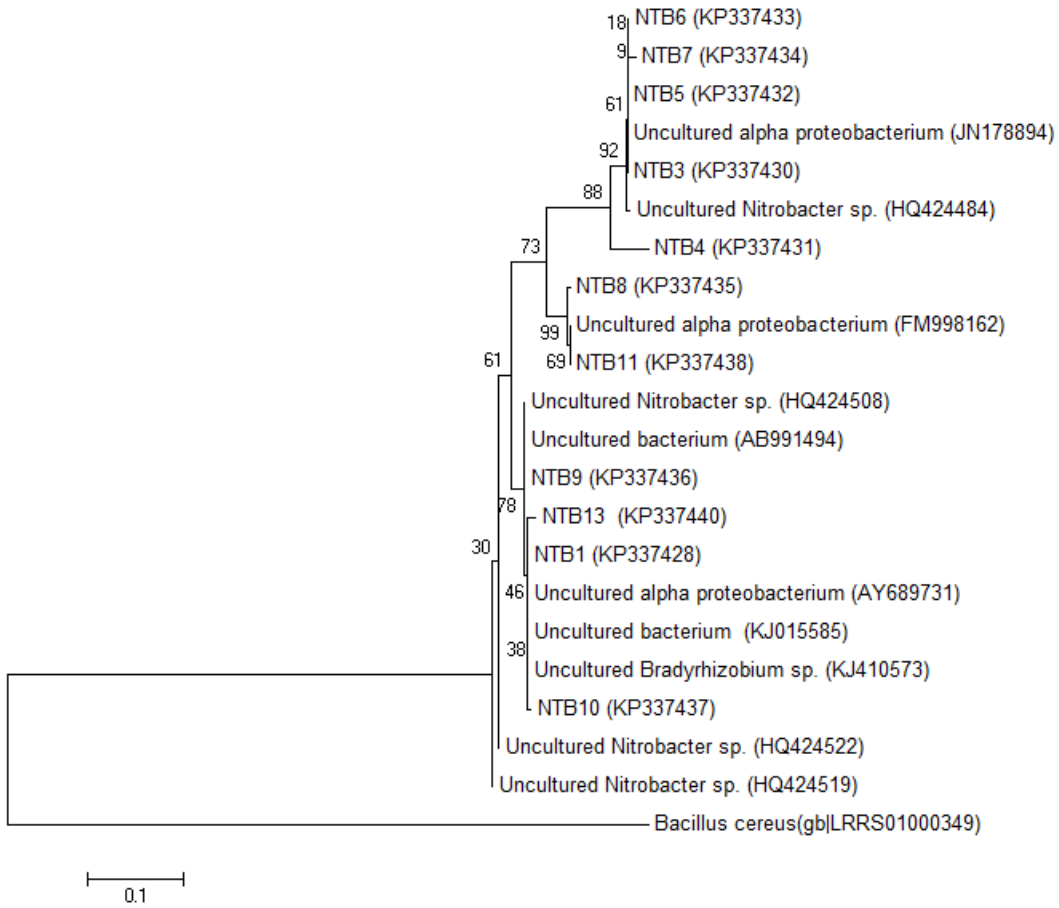

Fig. 3.10: Phylogenetic neighbour-joining tree based on partial 16S rRNA genes of *Nitrobacter* species. A neighbour-joining tree was constructed from the resulting alignment with MEGA 6, and the bootstrap values were based on 1000 replicates. The scale bar represents 0.01 change per site. The sequence of *Bacillus cereus* was used as an out group.

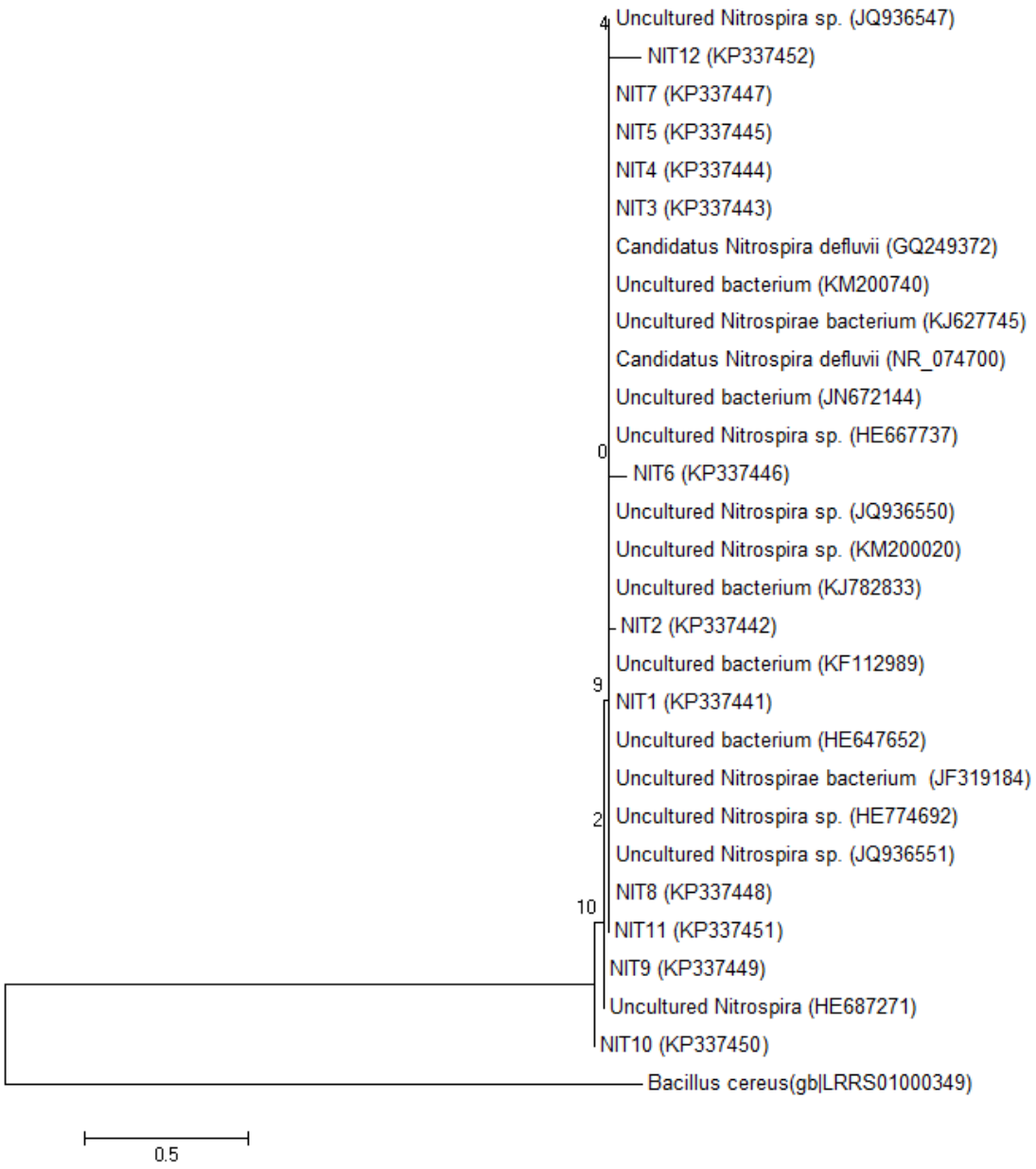

Fig. 3.11: Phylogenetic tree of *Nitrospira* species in municipal activated sludge. The scale bar represents 1% estimated sequence divergence. A neighbour-joining tree was constructed from the resulting alignment with MEGA 6, and the bootstrap values were based on 1000 replicates. The scale bar represents 0.01 change per site. The sequence of *Bacillus cereus* was used as an out group.

3.4 DISCUSSION

The success of molecular analyses of environmental samples is largely dependent on the purity, yield, and molecular weight of the extracted genomic DNA (Shan *et al.*, 2008). Isolation of high molecular weight DNA allows the characterization of large regions of the genomes (Rajendhran and Gunasekaran, 2008). In this study, the efficiency of the different DNA extraction methods was evaluated since cell lysis is extremely critical. Although there are different studies that have reported optimal DNA extraction methods from sludge, however, lysis method with the highest DNA yield for different sludge samples varies with specific sludge sample (Bourrain *et al.*, 1999). Due to variation in microbial compositions and floc morphology different activated sludge samples required specific cell lysis method adapted to them (Rajendhran and Gunasekaran, 2008). Optimization of the DNA extraction method is necessary in order to have efficient and representative release of nucleic acids from complex community such as activated sludge (Bourrain *et al.*, 1999). In this study, the enzymatic method yielded highest nucleic acid concentration (775.20 – 802.90 ng/µl) followed by sonication (403.90 – 521.00 ng/µl) and freeze-thaw (352.79 – 433.93 ng/µl). However, the mechanical cell lysis involving sonication proved to be harsh, with resultant genomic DNA having severe shearing. Sonication has been reported an effective technique for dispersing sludge sample aggregates during DNA extraction (Picard *et al.*, 1992; Lemarchand *et al.*, 2005). However, according to earlier studies, high genomic DNA shearing could result when employing this treatment, since it is capable of disrupting the DNA molecules (Lemarchand *et al.*, 2005; McIlroy *et al.*, 2009). According to Rajendhran and Gunasekaran (2008), when comparing mechanical lysis procedure, sonication is more vigorous than freeze-thaw method (thermal-shock) resulting in more DNA degradation as evident in this present study (Fig. 3.2).

The physical integrity of the genomic DNA extracted is very important as excessive shearing can inhibit the amplification of large gene regions or result in PCR artefacts (chimeras) (McIlroy *et al.*, 2009). There was little shearing effect observed in genomic DNA isolated with both freeze-thaw and enzymatic methods (Fig. 3.2), which makes

the obtained nucleic acid useful for the downstream analysis. McIlroy *et al.* (2009) noted that it was impossible to have a total cell lysis for DNA extraction from activated sludge without unacceptable shearing of the DNA, hence, a compromise must be made between total cell lysis and shearing. Both freeze-thaw and enzymatic methods yielded genomic DNA with good purity that was close to 1.8 at 260/280 nm absorbance ratio (Table 3.4). As indicated in Table 3.4, the sonication and freeze-thaw extraction methods yielded DNA with 260/230 absorbance ratio within the range for pure DNA (sonication: 2.11 - 2.52; Freeze-thaw: 1.95-2.20), however, it was relatively lower for enzymatic method (1.59 - 1.74). According to the Beer-Lambert law, there is a direct correlation between absorbance and concentration. Although the nucleic acids absorbs at many wavelengths, however their peak absorbance of UV light occurs at 260 nm. The 280 nm absorbance is measured because this is typically where proteins and phenolic compounds have a strong absorbance. Many organic compounds have strong absorbance at around 225 nm. In addition to phenol, TRIzol, and chaotropic salts, the peptide bonds in proteins absorb light between 200 and 230 nm (UCR, 2016; Thermoscientific, n.d.).

The A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample. The aromatic proteins have a strong UV absorbance at 280 nm. For pure DNA, A260/280 ratio should be approximately 1.8±0.1 (Lemarchand *et al.*, 2005; Singka *et al.*, 2012). A lower ratio indicates the sample is protein contaminated which affect downstream applications involving the nucleic acid (UCR, 2016; Thermoscientific, n.d.). The A260/230 ratio indicates the presence of organic contaminants including phenol, TRIzol, chaotropic salts and other aromatic compounds. The 260/230 ratio for pure DNA samples is between 2.0 and 2.2 (UCR, 2016; Thermoscientific, n.d.). However, according to earlier study by Lemarchand *et al.* (2005), DNA extracted from environmental samples with OD 260/280 range between 1.46 and 1.79 are pure enough for PCR without any inhibition. The freeze-thaw method gave genomic DNA of higher purity but lower DNA yield when compared to the enzymatic method. Ferrera *et al.* (2010) earlier observed that phenol–chloroform extraction after enzymatic lysis resulted in better DNA yields and

detectable diversity when compared to freeze-thaw method. The reduced concentration associated with the freeze-thaw procedure when compared to enzymatic lysis could be due to lower cell wall lysis efficiency achieved with the freeze-thaw treatment (Lemarchand *et al.*, 2005). Bourrain *et al.* (1999) also noted a lower DNA yield resulting from freeze-thaw (thermal shock) procedure when compared to enzymatic treatment of the sludge sample. Rajendhran and Gunasekaran (2008) noted freeze-thaw method is usually more efficient for lysing gram-positive bacterial cells. The quality of the extracted DNA with freeze-thaw method was sufficient for PCR amplification of 16S rRNA and amoA gene of bacterial nitrifiers without inhibition. Hence, freeze-thaw method was adopted throughout this study.

FISH was used as a quick screening method for identifying the nitrifiers in the samples. Nielsen (2009) earlier reported inefficient cell permeability as one of the many limits of the FISH technique. Ramdhani *et al.* (2013) reported optimum condition for dispersal of flocs obtained from nitrifying municipal activated sludge as 8 watts for 8 min. However, in this study when sonication was carried out at 8 watts for 8 min, the floc dispersion was not effective. Efficient floc dispersion was only recorded when sonication time was increased from 8 min (at 8 watts) to 10 min. The result indicated that sonication at 8 watts for 10 min was optimum for this particular sludge sample, and it further demonstrated the need for FISH pre-treatment optimization for sludge samples (Ramdhani *et al.*, 2010). The presence of the Betaproteobacterial AOB, *Nitrospira* spp. and *Nitrobacter* spp. (Fig. 3.3) were detected in abundance throughout the sampling period in the sludge samples using the optimized protocol (Table 3.2). This result is similar to various studies that have reported successful identification of nitrifiers in activated sludge using Nso1225 (AOB), NIT3 (*Nitrobacter* spp.) and Ntspa662 (*Nitrospira* spp.) FISH oligonucleotide probes (Egli *et al.*, 2003; Li *et al.*, 2006; Shi *et al.*, 2010).

The importance of molecular techniques in understanding the microbial community structure of activated sludge has been documented by different authors (Gao and Tao,

2011). However, the need for complementary use of these molecular techniques in order to obtain a better assessment of microbial diversity has been noted, since they all have both advantages and pitfalls (Rastogi and Sani, 2011). In this study, the FISH analysis was complemented by PCR, sequencing and phylogenetic analysis for identification purpose. All the nitrifier primers used in this study (Table 3.3) produced positive amplicons from the sludge samples, except the AOA that could not be successfully amplified using primer set amoAF/Arch-amoAR. Although, some earlier studies have reported AOA (using the same primer set) to be involved in ammonia biotransformation in engineered systems (Ozdemir *et al.*, 2011; Hatzenpichler, 2012), however, this organism was not identified to be involved in nitrification all through this study. This could suggest that AOA was not important in the nitrification process of this plant and may not be ubiquitous in all engineered wastewater treatment systems.

The phylogenetic analysis showed that the AOB sequences in this study were related to uncultured ammonia oxidizing bacterium, uncultured *Nitrosomonadaceae* bacterium, *Nitrosomonas* sp. and amoA gene of the uncultured bacterium (Fig. 3.9). This finding indicated similarity to previous report in which the dominant AOB in eight different activated sludge systems were closely related to *Nitrosomonas* spp. (Wang *et al.*, 2010). Siripong and Rittmann (2007) likewise reported the dominance of *Nitrosomonas* spp. in seven different WWTPs studied. The AOB in this study could not be identified beyond the genus level and there was no evidence of high species richness among them based on the results obtained (Fig. 3.9). Wang *et al.* (2012) also reported, the phylogenetic analysis of cloned amoA genes that indicated all the dominant AOB in the WWTP studied to be closely related to *Nitrosomonas* spp. It has been reported that *Nitrosomonas* spp. has a relatively high Ks for ammonia and this makes them frequently found in environments with moderately high ammonia concentrations (Wang *et al.*, 2012). An average ALR of 121 ± 22.0 - 144 ± 29.0 g-NH_4/m^3.d recorded in this plant could be one of the reasons for *Nitrosomonas* spp. dominance. Another possible reason for the low AOB diversity observed in this present study could be as a result of the industrial component of the municipal influent being received in this plant. Wang *et al.* (2010) earlier reported that WWTPs receiving

mixed domestic and industrial influent usually have low AOB diversity. Studies have also shown that activated sludge systems can possibly select for preponderance of single bacterial population or highly diverse bacterial populations can exist together (Rowan *et al.*, 2003; Ding *et al.*, 2011). Ramdhani (2012) reported variations in the abundance and nitrifying community structures of different South African's WWTPs. Identifying the microbes responsible for pollutants biotransformation and removal can reveal some information about the properties of the activated sludge (Cydzik-Kwiatkowska *et al.*, 2012). The prevailing factors in different wastewater treatment plants are complex and this usually results in high variability in microbial structure, which differs from plants to plants (Ahmed *et al.*, 2007; Calderon *et al.*, 2012; Fukushima *et al.*, 2013).

The *Nitrobacter* clones isolated in this study showed more affiliation to uncultured alpha proteobacterium, uncultured bacterium, uncultured *Nitrobacter* sp. and uncultured *Bradyrhizobium* sp. (Fig 3.10) whereas the *Nitrospira* clones were closely related to uncultured bacterium, *Candidatus Nitrospir*a defluvii, uncultured *Nitrospira* sp. and uncultured *Nitrospirae* bacterium (Fig 3.11). *Nitrospira defluvii* was first isolated in pure culture from activated sludge treating municipal wastewater by Spieck *et al.* (2006). *Nitrobacter spp. and Nitrospira* spp. have been commonly reported in different WWTP receiving municipal influent by many authors (Ramdhani, 2012; Ramdhani *et al.*, 2013; Fujitani *et al.*, 2014). *Nitrobacter* and *Nitrospira* have been reported as the key NOB that coexist in different activated sludge systems (Kim and Kim, 2006; Ramdhani, 2012; Hoang *et al.*, 2014). Other AOB species (*Nitrosolobus, Nitrosovibrio, Nitrosospira* and *Nitrosococcus*) were not identified in this plant. This indicates that the nitrifiers' community of the plant was not highly diverse. Various studies have reported the existence of *Nitrosomonas, Nitrosospira* and *Nitrosococcus* as the AOB in nitrifying wastewater treatment plants (Guler, 2006; Nielsen *et al.*, 2010; Duan *et al.*, 2013).

3.5 CONCLUSIONS

- The study revealed that DNA extraction method involving freeze-thaw yielded DNA with the best purity and minimal shearing. Sonication method resulted in significant degradation of the DNA, hence it was not suitable for this sample.
- The phylogenetic analysis of the dominant nitrifying populations in the plant revealed similarity in species richness over the seasons. Perhaps this is because the PCR-clone libraries of environmental samples could be limited in resolution; hence there is need for next generation sequencing which can give a better detail in terms of microbial diversity in environmental samples over time and space.
- This municipal reactor has its nitrifying community made up of *Nitrosomonas* spp. *Nitrobacter* spp. and *Nitrospira* spp. This indicates that the species richness among AOB in the plant was not high. Hence, the community did not exhibit substantial congeneric homotaxis, which can impart high functional redundancy on them.
- This study revealed that AOA was not part of the dominant nitrifiers in this plant. Hence, it was not an important player in ammonia biotransformation in this WWTP and may not be ubiquitous in all nitrifying WWTPs.

3.6 RESEARCH OUTPUTS

a) Conference papers

1. **Awolusi O. O.,** Kumari S.K., Bux F. Investigation of nitrogen converters in municipal wastewater treatment plant. Water Institute of Southern Africa (Water - The ultimate constraint) biennial conference, the International Convention Centre, Durban, 15th - 19th May 2016.

2. **Awolusi O. O.,** Kumari S.K., Bux F. Characterization and quantification of nitrifying community in activated sludge system treating municipal wastewater. Paper presented at Water Institute of Southern Africa (WISA) 2014 (Water Innovations) biennial conference, Mbombela Stadium, Nelspruit, South Africa (Oral presentation).

CHAPTER FOUR: SEASONAL VARIATION IN COMMUNITY STRUCTURE OF AEROBIC NITRIFYING ACTIVATED SLUDGE: THE NEXT-GENERATION SEQUENCING APPROACH

4.1 INTRODUCTION

Understanding the complex microbial community in WWTPs is important in designing functionally stable and effective treatment systems. The advent of molecular techniques has revealed the inadequacies of traditional microbiological methods of identifying and quantifying microbes in wastewater (Xia *et al.*, 2010a). The different Sanger-sequencing based molecular approaches including; polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism (T-RFLP), temperature-gradient gel electrophoresis (TGGE) and cloning have been successfully used in profiling the high microbial diversity harboured in WWTPs (McMahon *et al.*, 2009; Gomez-Silvan *et al.*, 2010). Fluorescent in situ hybridization among others have also been used in microbial diversity profiling of engineered wastewater treatment environments with some degrees of success (Awolusi *et al.*, 2015). More recently, it has been shown that the traditional Sanger sequencing is yet, grossly underestimating the communities of the complex environmental samples due to the hundreds or thousands of important sequences that go unnoticed when employing this method (Shokralla *et al.*, 2012). A major shortcoming of this technique is that it requires in vivo amplification of DNA fragments in bacterial hosts (cloning) prior to sequencing. Cloning is labour-intensive, tediously long and subject to bacterial host bias (Morozova and Marra, 2008).

Due to the thousands of template DNA usually present in wastewater samples there is need for technique that is capable of simultaneous reading from different DNA templates (Shokralla *et al.*, 2012). The dideoxy or Sanger sequencing, due to the expenses involved, can limit the depth of diversity that could be sampled (Mardis, 2008). Moreover, the diversity of clones being selected for subsequent dideoxy

sequencing is highly subjective and limited by human bias. Contrarily, the Next generation sequencing (NGS) approach offers a speedy, and extensive data production with the opportunity of investigating the microbial ecology on a larger scale and with more details (Ju and Zhang, 2015a). Next generation sequencing offers the advantage of direct sequencing from environmental samples without prior cloning into a bacterial host before sequencing, as obtained in the traditional Sanger approach. The NGS has revolutionized the genomic and metagenomic research with different platforms being commercially available. In this chapter, variation in community structure of the aerobic nitrifying activated sludge over the winter and summer was evaluated using the combination of pyrosequencing and Illumina sequencing techniques.

4.2 MATERIALS AND METHODS

4.2.1 Wastewater treatment plant operation

Wastewater characteristics and operational parameters for the selected plant in this study are shown in Table 3.1.

4.2.2 DNA extraction

Genomic DNA was extracted from sludge samples (aeration basin) taken over the winter and summer seasons using the freeze-thaw method as described in section 3.2.4.

4.2.3 Seasonal analysis of community structure in the activated sludge

4.2.3.1 Sample preparation and DNA extraction

The total bacterial community diversity was investigated using Illumina Genome Analyser whilst the diversity of AOB group was assessed using high-throughput pyrosequencing approach. Samples taken between the 1st and 78th day represented the winter period (May - July 2012) whilst the samples from 79th through 237th day

(November 2012 - March 2013) represented summer. The extracted DNA samples for the winter months were pooled together in equimolar quantities to make up the winter template DNA sample, whilst the same was done for the summer months. These resulted in two separate template DNA samples that used for the Illumina and pyrosequencing analysis. The detail freeze-thaw DNA extraction protocol is shown in Section 3.2.4.

4.2.3.2 Sequencing and Analysis

The pooled DNA (winter and summer samples) were subjected to high-throughput sequencing using the Illumina MiSeq platform at Inqaba Biotechnical Industries (Pty), South Africa, using the universal bacterial fusion primer sets targeting the hypervariable V3-V4 region of the 16S rRNA with adapter primers attached to its 3′ end (Wang *et al.*, 2014). The obtained reads were trimmed and filtered where only q30 (i.e. high quality) reads and a minimum length of 50 bp (after trimming) were selected (Dogan *et al.*, 2014). Using the genomic CLC software, the selected reads were aligned and this was compared to known 16-18S rRNA gene tag database (E-value cut-off at 0.005) using BLASTn programme (Sekar *et al.*, 2014). Dissimilarity cut-off of 0.03 and 0.20 was used to cluster the cleaned (selected) reads into operational taxonomic units. Taxonomic classification into domain, phylum, order, class, families and genus was performed using a set of confidence threshold based on OTU diversity and reads (OTU abundance) (Keshri *et al.*, 2015). The percentage or relative taxonomic abundance of individual taxon within the community was computed by comparing the number of sequences assigned to a specific taxon with the total number of sequences obtained from the sample. The raw Illumina file (fastq) has been deposited into NCBI sequence Read Archive.

The amoA locus of the AOB in the activated sludge was amplified using the primer set: amoA-1F/amoA-2R. The PCR was performed in a total reaction volume of 50 µl containing 10 ng of DNA template. The final concentrations of the different

components in the reaction mix (200 µM of DNTPs, 1.5 mM of MgCl [Taq buffer with initial MgCl concentration of 20 mM], 2.5 U of Taq DNA polymerase [Thermo Scientific, Lithuania] and 0.5 µM of each primer) were according to modified protocols from Degrange and Bardin (1995). The PCR products were purified and end-repaired. The amoA amplicons generated from the PCR were sent to Inqaba Biotechnical Industries (Pty), South Africa, where the composition of the amplicon of amoA targeted locus was then determined by pyrosequencing. The barcodes for multiplexing were incorporated between the forward primers (amoA-1F) and the 454-adpter sequence for pyrosequencing using Roche 454 FLX Titanium sequencing platform (Roche, USA) (Schloss *et al.*, 2009). After sequencing, the unique tags obtained were aligned with the 16S rRNA database with the aid of the BLASTN programme (Sekar *et al.*, 2014). The tag redundancy was eliminated and sequences were assigned into OTU based on similarities of greater than 90%. With the aid of MEGA6 software, representative sequences were aligned using the ClustalW programme. The neighbour-joining method was employed for the phylogenetic analysis (Tamura *et al.*, 2013). The raw pyrosequencing .sff file has been deposited into the NCBI sequence Read Archive.

4.2.4 Short-read archive accession numbers

The raw reads for the pyrosequencing and Illumina have been deposited into NCBI Sequence Read Archive under the accession numbers SRP053412 and SRP058452 respectively.

4.3 RESULTS

4.3.1 The total microbial community diversity as revealed by Illumina sequencing analysis

The total bacterial community diversity in summer and winter was investigated using Illumina (Miseq) sequencing platform. Seasonal variation was observed in the species

richness of the plant's microbial community. The summer sample was higher in diversity compared to the winter samples (Figs. 4.1 – 4.4). In total, 9 and 12, bacterial phyla apart from the unclassified sequences were identified for the winter and summer seasons respectively. *Verrucomicrobia, Firmicutes, Bacteroidetes, Chloroflexi, Planctomycetes, Actinobacteria, Proteobacteria* and *Acidobacteria* sequences were common in both samples. Representatives of *Spirochaetes, Fusobacteria* and *Fibrobacteres* were present in abundance in summer sample (Fig. 4.1). *Actinobacteria* (29.4%) was the most abundant during winter, whilst majority of the sequences from the summer sample were related to *Proteobacteria* (13.2%). The majority of sequences from both seasons could not be classified even at the phylum level [50.6% (winter) and 66.5% (summer)]. Substantial variations in proportions of the phyla (i.e. composition) were observed over the seasons (Fig. 4.1). During winter, *Proteobacteria* (9.5%) was the second predominant category followed by *Planctomycetes* (2.7%), *Firmicutes* (2.5 %), *Bacteroidetes* (1.8 %), *Verrucomicrobia* (1.5 %), *Chloroflexi* (1.3 %), *Acidobacteria* (0.5 %) and *Euryarchaeota* (0.3 %). *Spirochaetes, Fibrobacteres* and *Fusobacteria* were not identified during this season. However, in contrast, during summer *Verrucomicrobia* (5.6 %) were found to be the second most dominant phyla followed by *Planctomycetes* (4.4 %), *Actinobacteria* (3.4 %), *Firmicutes* and *Bacteroidetes* were both fourth most dominant (2.0 %), followed by *Acidobacteria* (1.0 %), *Spirochaetes* (0.7 %), *Fibrobacteres* (0.5 %) and *Fusobacteria* (0.2 %).

The class *Actinobacteria* accounted for 29.9 % during winter, whilst it was 3.4 % in summer (Fig. 4.2). *Flavobacteria, Deltaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Acidobacteria, Chloroflexi, Clostridia, Gammaproteobacteria, Bacteroidetes,* and *Planctomycetacia* were found during both seasons (Fig. 4.2). *Methanobacteria* and *Bacilli* were exclusively found during winter whilst *Sphingobacteria, Gemmatimonadetes, Spirochaetes, Fusobacteria, Verrucomicrobiae, Fibrobacteres* and *Chlamydiae* where found only during summer (Fig. 4.12). The sequence reads were classified taxonomically which showed high diversity at lower taxonomic levels with 20 orders and 26 families identified during winter whilst it was 27 orders and 35 families during summer (Figs. 4.3 and 4.4). The

Mycobacteriaceae, *Planctomycetaceae* and *Verrucomicrobiaceae* were the most dominant families among the sequences classified during the two seasons sampled (winter and summer).

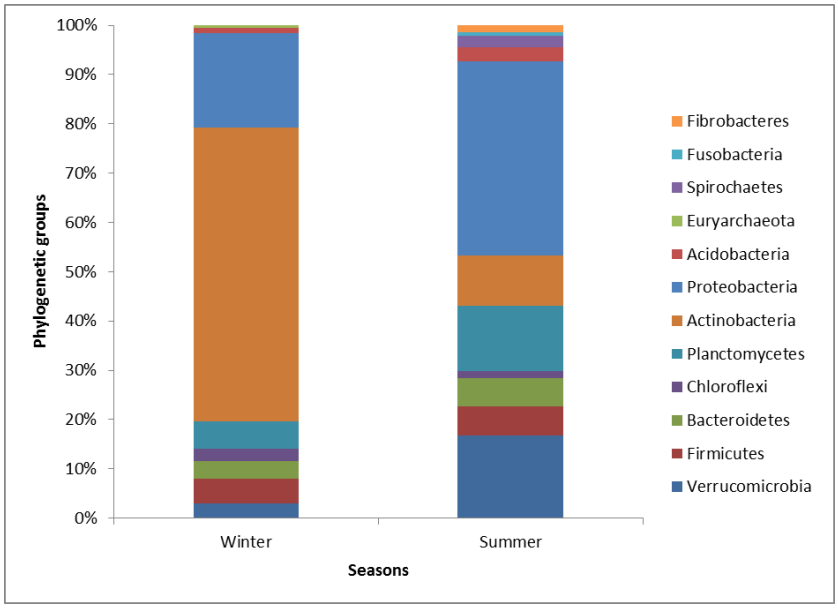

Fig. 4.1: Taxonomic distribution of different bacterial phylogenetic groups during summer and winter in the reactor at Phylum level. The percentages of the phylogenetically classified sequences are plotted on the y-axis.

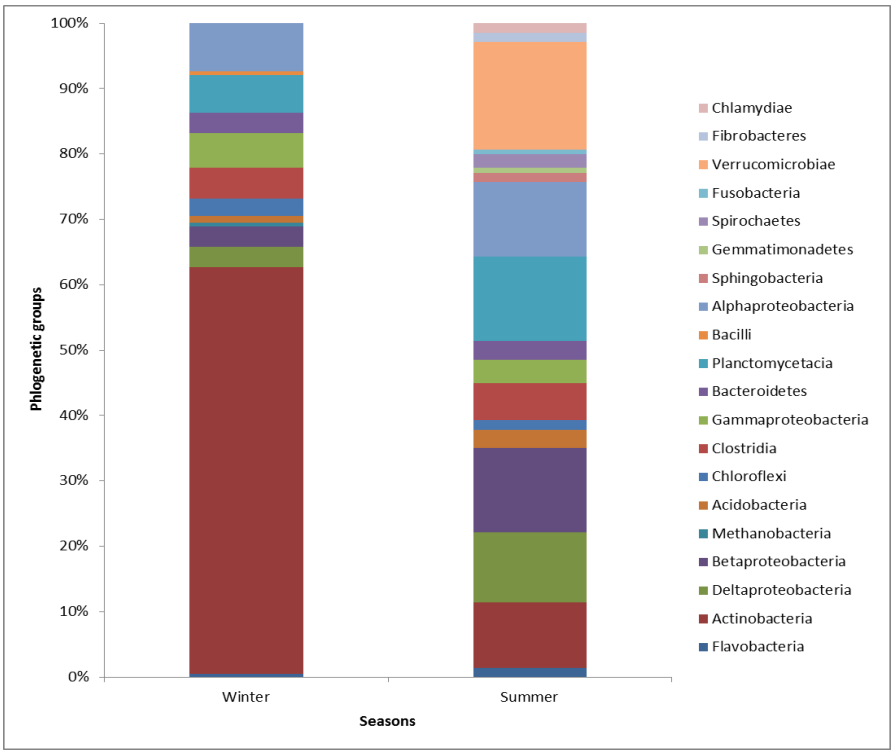

Fig. 4.2: Taxonomic distribution of different bacterial phylogenetic groups during summer and winter in the reactor at the Class level. The percentages of the phylogenetically classified sequences are plotted on the y-axis.

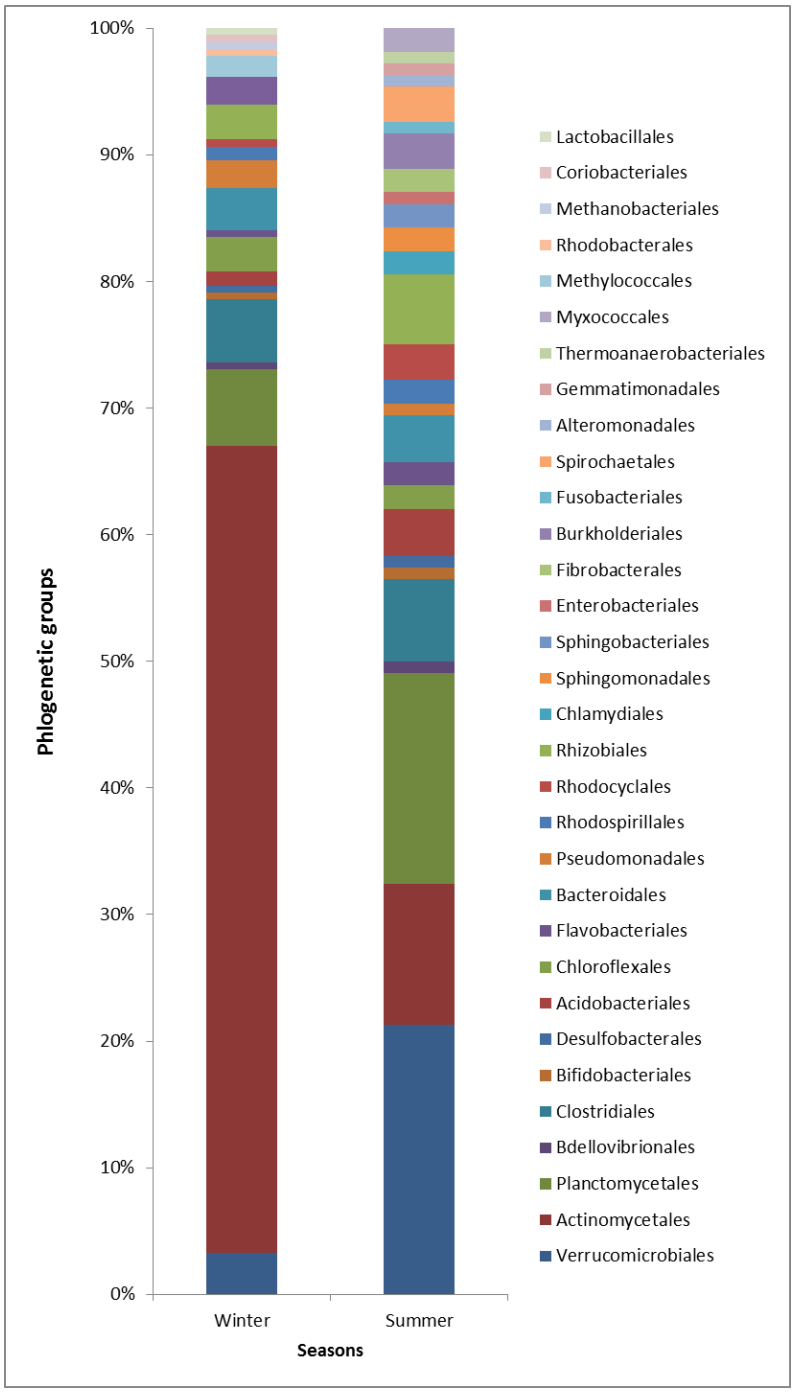

Fig. 4.3: Taxonomic distribution of different bacterial phylogenetic groups during summer and winter in the reactor at Order level. The percentages of the phylogenetically classified sequences are plotted on the y-axis.

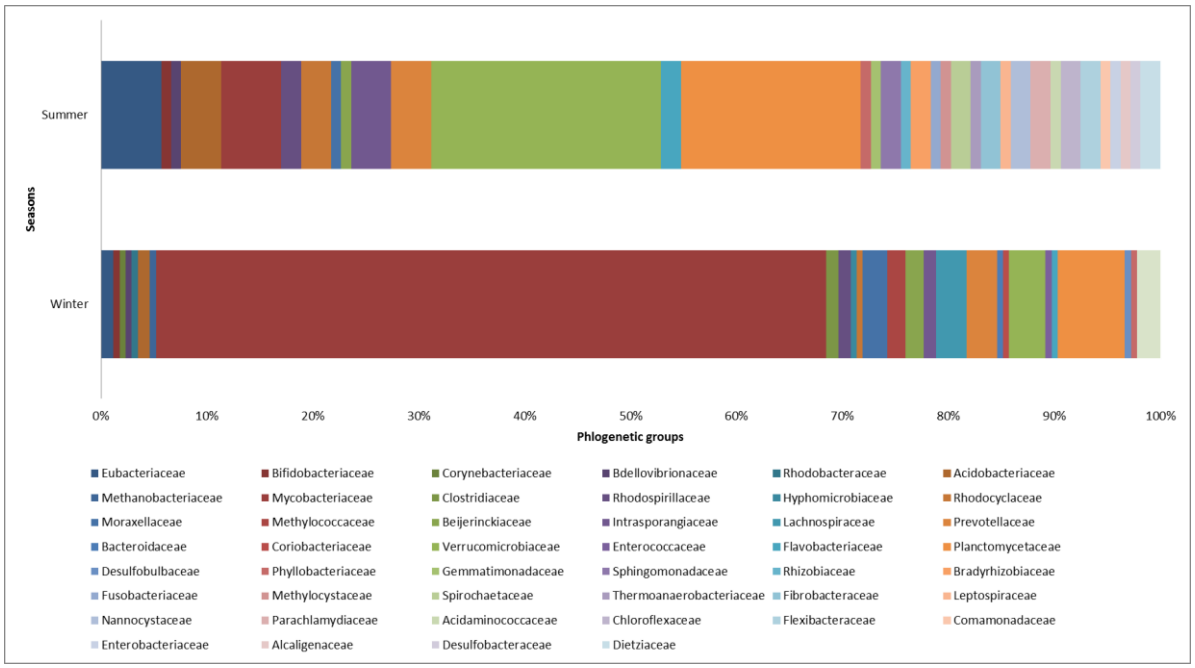

Fig. 4.4: Taxonomic distribution of different bacterial phylogenetic groups during summer and winter in the reactor at the Family level. The percentages of the phylogenetically classified sequences are plotted on the y-axis.

The rank abundance plot which is used in NGS analysis to represents the species richness (number of species) and evenness (relative abundance of species) (Kim *et al.*, 2013; Keshri *et al.*, 2015) was constructed with the abundance of the uncultured sequences during the study (Fig 4.5a and b). During winter, the uncultured bacterium was the most abundant followed by *Methylocella silvestris*, uncultured *Chloroflexales*, *Tetrasphaera australiensis*, *Mesorhizobium* sp., and *Hyphomicrobium* sp. These groups accounted for 92 % of the total sequences. The six most abundant groups during summer were uncultured bacterium, uncultured *Betaproteobacteria*, *Knoellia subterranean*, uncultured *planctomycete*, *Afipia genosp*, uncultured *verrucomicrobia*, which covered 82 % of total sequences. In total, about 0.04 % of the sequences could not be classified up to genus level during the winter whilst it was only 0.03 % during summer. The analysis based on diversity and relative abundance of different species indicated a strong difference among the bacterial communities at different seasons in the plant (Fig 4.5a and b). It was observed that the plant harboured diverse microbial

communities over the different seasons and only a small microbial community overlap was observed during the winter and summer. About 30 sequences were found in common to both seasons whilst there were 33 other sequences that were found in the winter samples which did not exist in summer and 49 others that were found only during summer.

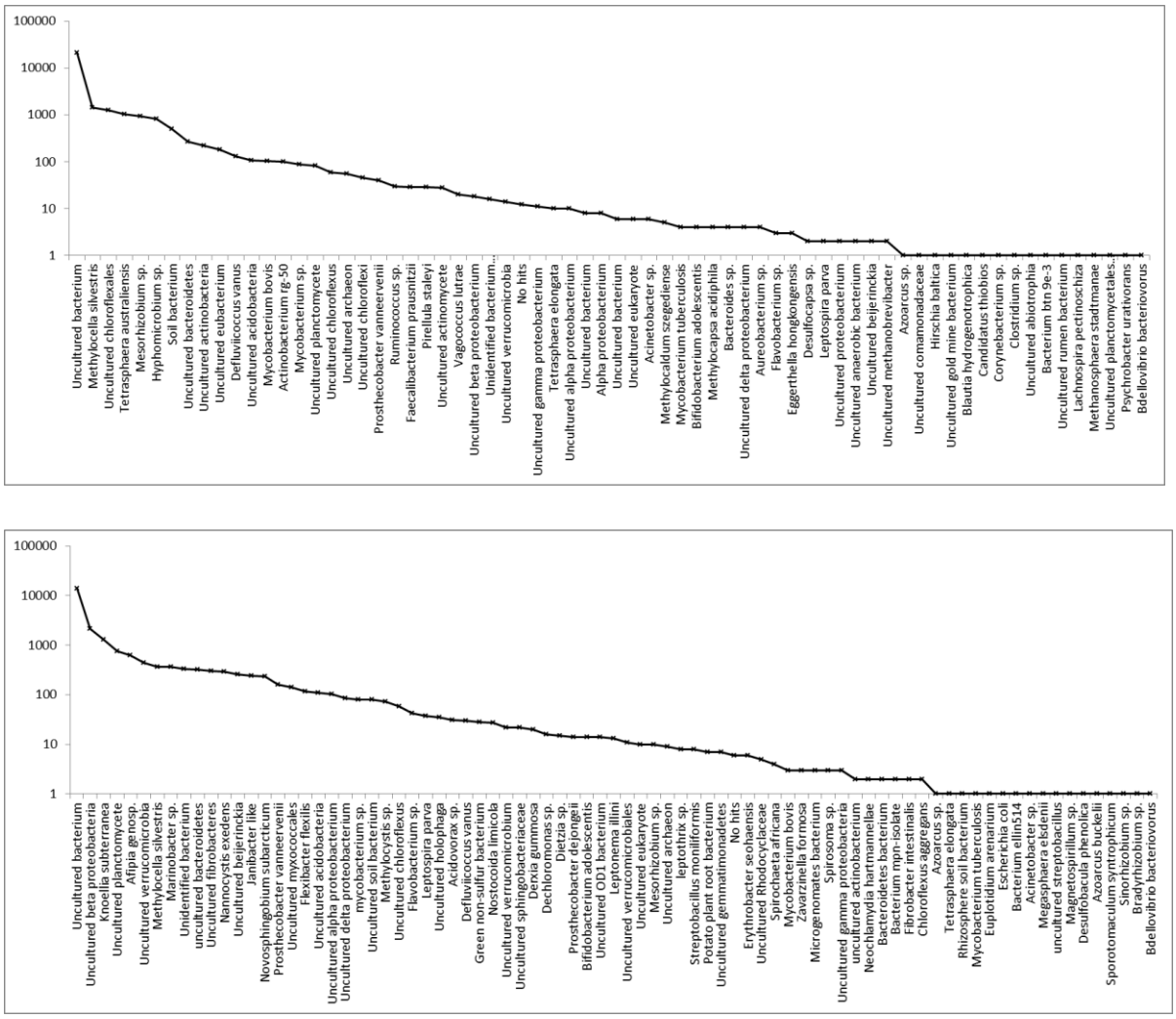

Fig. 4.5: Rank abundance plot: (a) Phase 1 (winter); and (b) Phase 2 (summer). The plots show the taxonomic abundances ordered with the most abundant ranked first and plotted at the leftmost side and the least abundant ranked last and plotted toward the right. The y-axis plots the abundances of annotations in each taxonomic group in log scale

4.3.2 Ammonia oxidizing bacteria profiling based on amoA gene using high throughput pyrosequencing

Ammonia oxidation has been identified as the rate limiting step in nitrification hence, the diversity of the AOB in this system was examined to understand their role in nitrification efficiency. The AOB diversity for the different seasons was revealed by 454-pyrosequencing using the amoA-1F and amoA-2R primer sets (with multiplex barcodes inserted between the forward primers and the 454 adapter sequence). With the aid of MEGA6 software, representative sequences were aligned using the ClustalW programme and the neighbour-joining method was employed for the phylogenetic analysis (Figs. 4.6 – 4.7). A total record of 212 and 1192 effective sequences were obtained from the winter and summer samples respectively. After comparing them with the NCBI database, substantial percentages of the read from either sample (72 % in winter and 78 % in summer samples) returned no hits and could not be assigned to any phylum. (Fig. 4.8).

The identified AOB populations during study include the uncultured ammonia oxidizing bacteria, uncultured bacterium and *Nitrosomonas oligotropha*. Among these identified AOB populations, the uncultured ammonia oxidizing bacteria was the most dominant throughout the study with 97% and 95% during summer and winter seasons respectively, whilst the uncultured bacterium was 2% and 5% during the summer and winter respectively. The *Nitrosomonas oligotropha* was about 1% of the AOB population during the summer season, however, it was not detected during the winter (Fig. 4.8). The AOB diversity was 6 times higher during summer than winter (Fig. 4.8) when a higher NH_3 removal rate and temperature were recorded (Table 3.1). The AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer (Fig. 4.8).

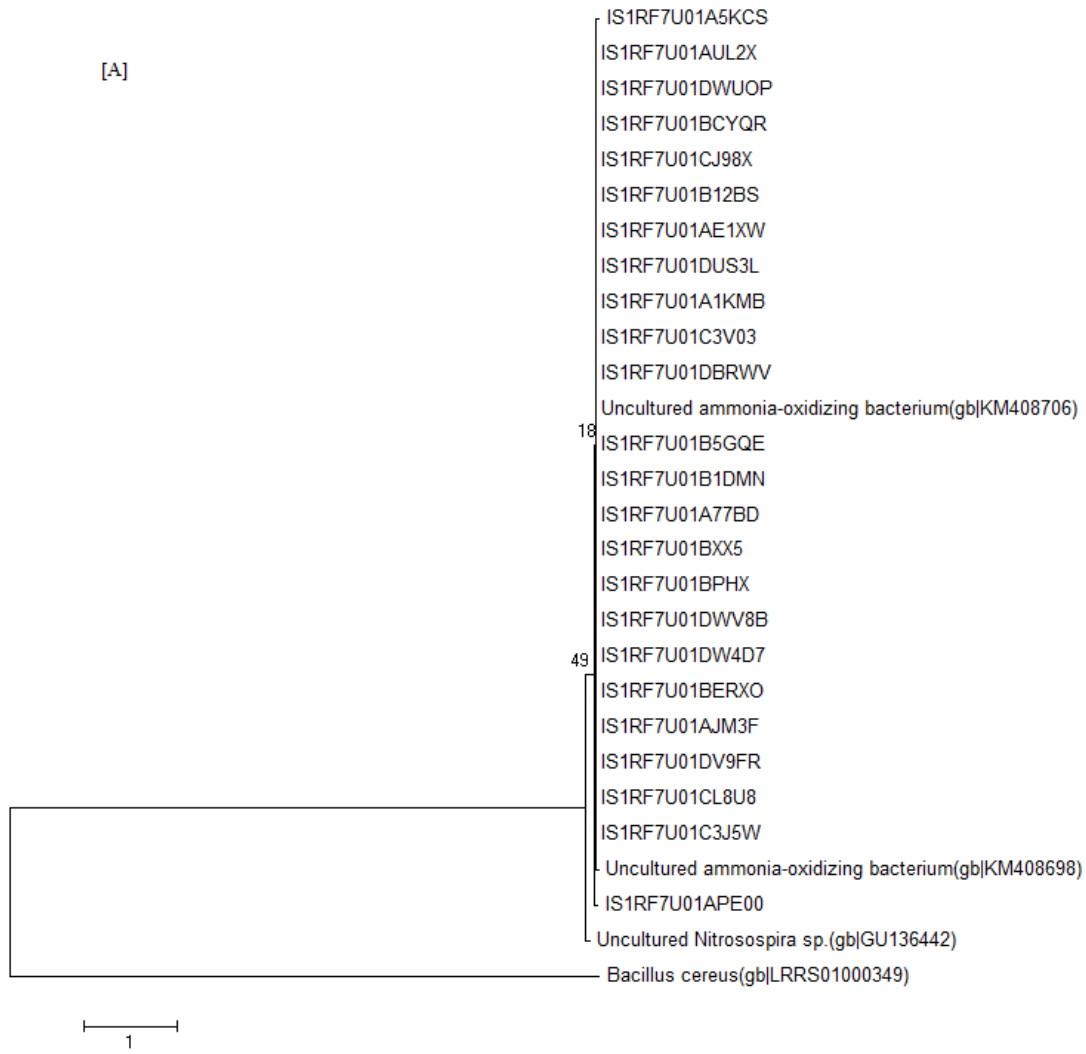

Fig. 4.6: Phylogenetic tree of some selected ammonia oxidizing bacteria OTUs based on amoA gene locus pyrosequencing reads using BLASTN and MEGAN for samples collected during summer

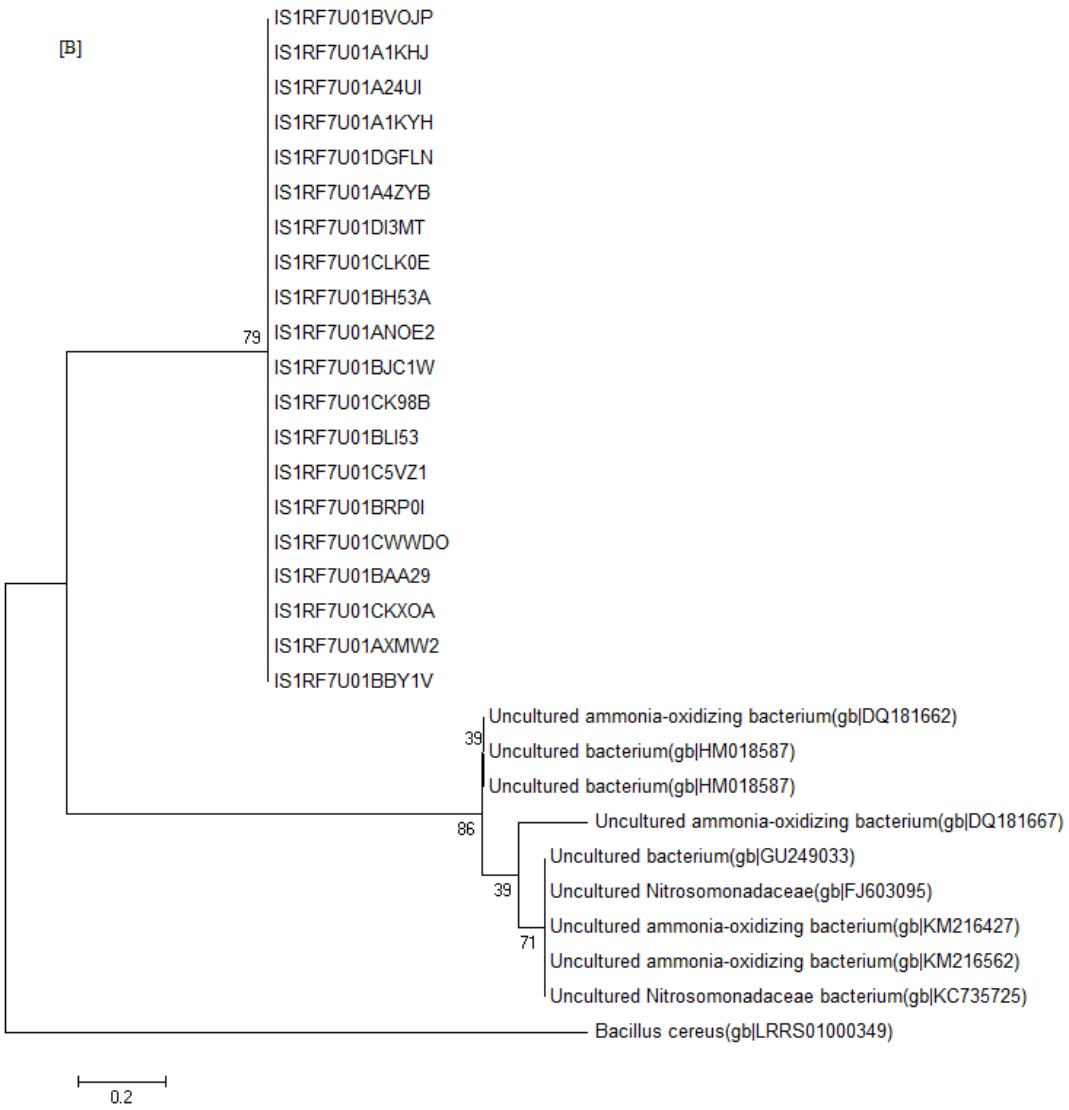

Fig. 4.7: Phylogenetic tree of some selected ammonia oxidizing bacteria OTUs based on amoA gene locus pyrosequencing reads using BLASTN and MEGAN for samples collected during winter

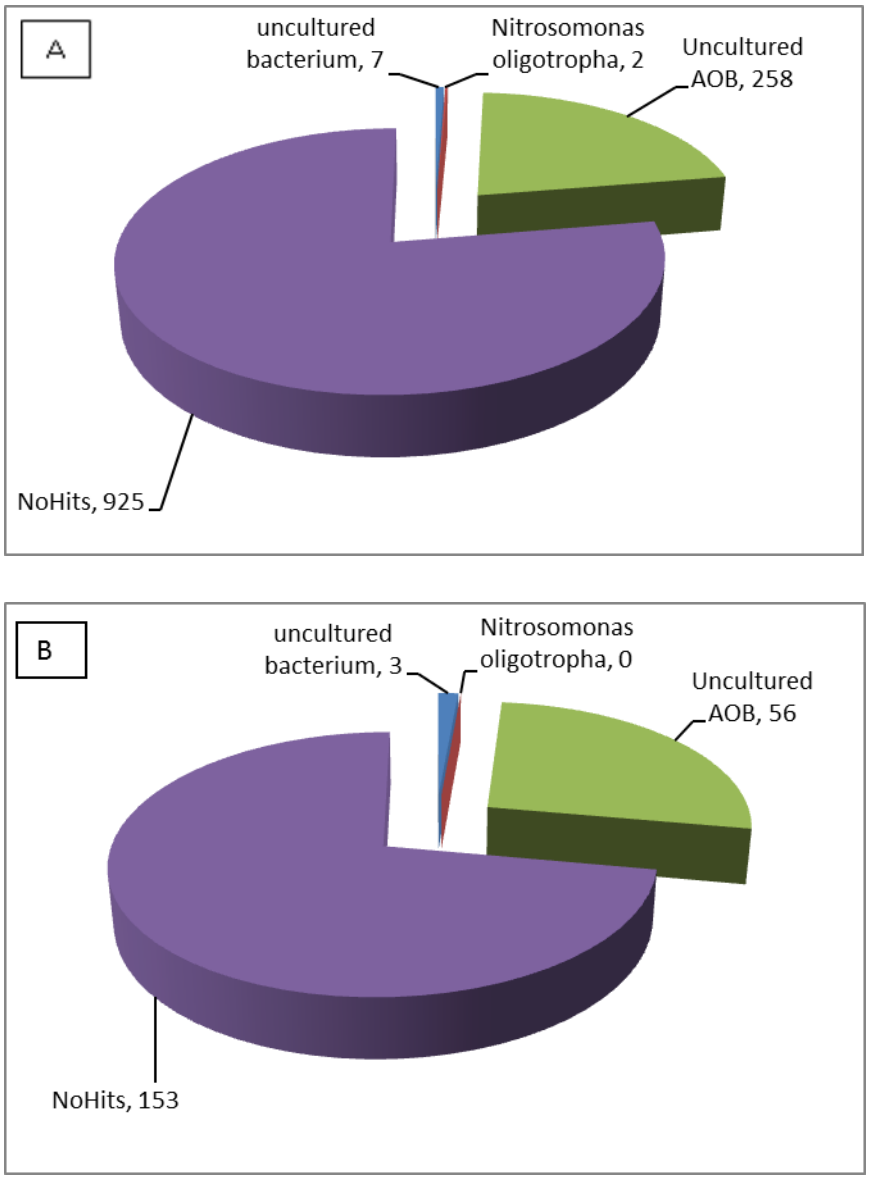

Fig. 4.8: (a) AOB diversity as revealed by pyrosequencing during summer, (b) AOB diversity as revealed by pyrosequencing during winter

4.4 DISCUSSION

The seasonal population dynamics of nitrifying and total bacterial communities of the full-scale municipal bioreactor was investigated. The physicochemical analysis during winter and summer revealed significant difference in operating conditions for the investigated seasons (Table 3.1). Higher organic and ammonia loading rates, solid

retention time, MLSS, COD, were noticed during winter (Phase 1), whereas temperature and influent flow rates during the phase 2 (summer) were higher for the plant (Table 3.1). According to Cydzik-Kwiatkowska *et al.* (2012), identifying the microbes responsible for pollutants biotransformation and removal reveals a lot of information regarding the properties of the activated sludge. Previous studies by Keshri *et al.* (2015) and Imarla *et al.* (2006) showed a high correlation between the microbial community structure and physicochemical parameters as noted by previous studies. The importance of the molecular techniques in understanding the microbial community structure of activated sludge has been documented by different authors (Gilbride *et al.*, 2006; Gao and Tao, 2011; Rastogi and Sani, 2011). Generally, the traditional molecular techniques such as ribosomal intergenic spacer analysis (RISA), terminal restriction fragment length polymorphism (t-RFLP), denaturing gradient gel electrophoresis (DGGE), 16S rRNA clone libraries and FISH are known to be effective for microbial community characterization, however, they are inefficient for full spectrum or in-depth taxa detection in highly complex communities (Xia *et al.*, 2010a; Rastogi and Sani, 2011; Shokralla *et al.*, 2012). On the other hand, next-generation sequencing approach (including Illumina and pyrosequencing) provides sufficient sequencing depth to cover the complex microbial communities (Zhang *et al.*, 2012b; Keshri *et al.*, 2015).

In this study, the Illumina sequencing technique was employed in profiling the microbial communities of the activated sludge system treating municipal wastewater at different seasons. The community structure as revealed by Illumina indicated that during summer, a season characterized with warmer temperature had more species richness when compared to the winter. Researchers have shown that temperature has an influence on microbial community compositions (Cydzik-Kwiatkowska *et al.*, 2012). On the contrary, earlier findings by Ju *et al.* (2014) indicated that there was higher bacterial species richness in the activated sludge studied during winter as compared to summer. However, geographical comparisons is difficult since environmental conditions and operating parameters differ substantially across regions. Activated sludge systems can either select for preponderance of single bacterial

population or highly diverse bacterial populations can exist together simultaneously (Ramdhani, 2012). It was also observed that despite overall higher microbial diversity obtained during summer; certain microbial populations (*Tetrasphaera spp*. and *Ruminococcus spp*.) had higher abundance during winter when compared to summer. This was similar to earlier finding by Ju *et al.* (2014) that reported higher abundance of *Tetrasphaera spp*. and *Ruminococcus spp*. during winter compared to summer. However in this present study, genus *Bifidobacterium* had a lower abundance in winter contrary to earlier observations by Ju *et al.* (2014). This result indicates that knowledge of activated sludge metagenomics is still incomplete and that individual plant may select for their unique microbial community compositions based on the prevailing operational conditions of the plant (Miura *et al.*, 2007; Ramdhani, 2012; Ramdhani *et al.*, 2013; Ju and Zhang, 2015b). The plant had significant variation in the influent wastewater compositions, prevailing operational and environmental parameter (Table 3.1) which correlated with variations in the microbial community structure of the reactor. This variation in microbial diversity affects the plant's seasonal nitrification performance as earlier reported (Rowan *et al.*, 2003; Miura *et al.*, 2007). The highest percentage ammonia removal efficiency was recorded during summer when the greatest AOB diversity was found in the reactor (Table 3.1). It indicates that AOB diversity could be a contributory factor to efficient ammonia removal in activated sludge. However, the significant variation in the total bacterial community diversity of the plant had no significant effect on the carbon removal of the plant. The average COD removal of the plant across the two seasons investigated did not indicate significant variation (Table 3.1).

Based on the pyrosequencing analysis, the higher AOB diversity was observed in summer when there was a comparatively higher ammonia removal efficiency (Table 3.1). The *Nitrosomonas oligotropha* was only detected in the summer sample which indicate a possible higher diversity during summer compared to the winter period (Fig 4.8). Furthermore, the AOB diversity was 6 times higher during summer than winter when a higher NH_3 removal rate and temperature were recorded. Using pyrosequencing, Zhang *et al.* (2015) also observed higher AOB diversity in summer

compared to winter when monitoring three different WWTP. Niu *et al.* (2016) also reported a significant decrease in bacterial amoA genes copy numbers during winter, in a water purification plant. In this study, the AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer. *Nitrosomonas oligotropha*-like sequence that were detected from summer samples (1%) were absent in winter samples (Fig 4.8). Earlier research efforts have documented similar trend in different environment. Temperature has a major influence on AOB diversity and population structure with lower species richness correlating to lower temperatures (Urakawa *et al.*, 2008). Similarly, Ju *et al.* (2014) observed a higher abundance of *Nitrosomonas* in activated sludge during summer. Faulwetter *et al.* (2013) also reported seasonal impact on AOB community structure in constructed wetland with higher diversity during summer compared to winter. A higher diversity of amoA gene was also recorded during summer in the tidal wetland investigated by Zheng *et al.* (2013).

Overall, the summer period harboured larger AOB diversity compared to winter as revealed in this study. A significant proportion of the effective sequences (72 % in winter and 78 % in summer sample) termed "no hits" could not be successfully classified into any known bacterial 16S rRNA sequences since they showed no similarity to the available sequences in NCBI database. This suggests yet unidentified populations and vast unexploited AOB diversity. Yang *et al.* (2011) reported that unassigned or unclassified bacteria usually consists higher proportion in activated sludge compared to other environments such as soil, because activated sludge have a more complex microbial communities. Shi *et al.* (2013) also reported the occurrence of unclassified bacteria sequences in chlorination and clear water tanks. They noted that these unclassified OTUs from the different samples were closely clustered in the phylogenetic tree. There is need for more study in order to identify these ecologically significant diversity of novel AOB species which makes up the complex activated sludge communities.

4.5 CONCLUSIONS

- Pyrosequencing reveals higher diversity of AOB in the reactor during summer that was characterized by higher temperature. Furthermore, *N. oligotropha* was only identified during summer. This indicates that higher temperature elicited increased AOB diversity.
- The AOB diversity was 6 times higher during summer than winter when a higher NH_3 removal rate and temperature were recorded. The AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer. This suggests that higher AOB diversity resulted in increased nitrification in activated sludge.
- Despite the high variability in seasonal diversity of total bacteria in the plant, the carbon removal efficiency of the plant was not affected as it remains stable across the two seasons.
- Our finding suggests that vast diversity of novel, ecologically significant AOB species, which remain unexploited still inhabit the complex activated sludge communities
- Future research should target characterization of the nitrifying populations in wastewater as pyrosequencing revealed a large percentage of the microbial community that did not match any of the know sequences on the existing database.

4.6 RESEARCH OUTPUT

1) **Awolusi O. O.,** Kumari S.K., Bux F. Investigation of nitrogen converters in municipal wastewater treatment plant. Water Institute of Southern Africa (Water - The ultimate constraint) biennial conference, the International Convention Centre, Durban, 15th - 19th May 2016 (Oral presentation).

2) **Awolusi O. O.,** Kumari S.K., Bux F. 2014. Characterization and quantification of nitrifying community in activated sludge system treating municipal wastewater. Paper presented at Water Institute of Southern Africa (WISA) 2014 (Water Innovations) biennial conference, Mbombela Stadium, Nelspruit, South Africa (Oral presentation).

CHAPTER FIVE: IMPACT OF ENVIRONMENTAL AND OPERATIONAL PARAMETERS ON NITRIFYING COMMUNITY AND PLANT PERFORMANCE

5.1 INTRODUCTION

The activated sludge has been largely successful in treating municipal wastewater; however, it is sensitive to operational or environmental variations, as well as toxic loading (Kim *et al.*, 2011). There are a number of physiochemical and operational factors that affect the success of nitrification in WWTPs. It has been reported that factors such as high ammonia concentration and low dissolved oxygen (DO) level can result in the disruption of the equilibrium between the two nitrification steps, resulting in significant reduction in the activities of nitrite oxidizers which can lead to toxic nitrite build-up and a subsequent failure of nitrification process (Mbakwe *et al.*, 2013). The nitrifying populations are also sensitive to variability in pH, temperature, alkalinity, NH_3^-N and NO_2^-concentrations, the presence of inhibitory or toxic substances which often lead to failure of the system (Kim *et al.*, 2011). Nitrification could also be impacted by influent variability, organic loading, Sludge retention time (SRT) and Food/microorganism ratio (F/M ratio). A balance between the two linked ammonia and nitrite oxidation steps is required for nitrification to proceed efficiently.

Nitrification efficiency of a WWTP can be determined by directly monitoring the nitrogen species biotransformation (plant's) in the wastewater treatment plants. Also the biomass from the WWTP can be used in laboratory batch experiments to estimate the nitrification capacity of activated sludge in the full-scale plant (Yu *et al.*, 2011). Generally, this laboratory batch estimation is done in terms of the specific ammonia oxidizing rate (SAOR) and specific nitrite formation rate (SNFR) (Yu *et al.*, 2011; Li *et al.*, 2013). SAOR and SNFR are usually estimated from the rates of decrease in NH_4^+–N concentration and increase in NO_3^-–N concentration over time. These have

been used in determining the activities of activated sludge biomass in oxidizing ammonia and nitrite respectively (Yu *et al.*, 2010; Yu *et al.*, 2011; Li *et al.*, 2013).

In a quest for rational design of functionally stable biological wastewater treatment systems there is need to understand the basic relationships between microbial community structure, its dynamics and functional stability (Gentile *et al.*, 2007). Also there is need to study and understand the effects of various environmental factors on the two bacterial groups and on the overall nitrification in order to know how best to operate the reactor for maximum nitrification and stability. The aim of this chapter was to quantify the dominant nitrifying bacterial populations using qPCR and determine their nitrification performance. The cross-correlational relationship existing between the nitrifier populations (copy numbers) and the plant's nitrification performance was also determined.

5.2 MATERIALS AND METHODS

5.2.1 Wastewater treatment plant operation and samples

The configuration, wastewater characteristics and operational parameters for the plant under investigation are shown in Table 3.1. The detailed sampling procedure for this study is also shown in section 3.2.2.

5.2.2 Process monitoring and chemical analysis

The collected samples were filtered with MN 85/90 filter papers (Macherey – Nagel, Germany) and the supernatant were analysed for the nitrogen species (NH_4^+ −N, NO_2^- −N, NO_3^- −N) using Thermo Scientific™ Gallery™ Automated Photometric Analyser (Vantaa, Finland) (Appendix 1). Samples for chemical oxygen demand (COD) measurement were first digested according to standard method 5220D (APHA, 1998) – closed reflux colorimetric method – in the microwave digester (Milestone Start D, Sorisole, Italy) at 150^{o}C for 1 h in COD vials containing the digestion solution (Appendix 2). The COD concentration in the resulting digest was measured using

Gallery™ Automated Photometric Analyser. Temperature, dissolved oxygen (DO) concentrations and pH measurements were done on-site using a portable YSI meter (YSI 556 Multiprobe System). The mixed liquor suspended solids (MLSS) was also measured using standard methods (APHA, 1998).

5.2.3 Calculating nitrification rate of the wastewater treatment plant

Nitrification rates in the plant were calculated based on the parameters monitored in the plant and the information supplied by the plant operators (Surmacz-Górska, 2000; Ramdhani *et al.*, 2013) in this study. The nitrification rate was calculated according to the equation below:

$$R_{nitrification} = \frac{Qin[NH_4^+ - N]_{in} - [NH_4^+ - N]_{out})}{V_{reactors}[MLSS]_{reactors}}$$

Where:

$R_{nitrification}$ = rate of plant's nitrification

Qin = influent flowrate (L^3/ T)

$[MLSS]_{reactors}$ = Mixed liquor suspended solid of the reactor (M/L^3) and

$V_{reactors}$ = reactor working volume (L^3).

5.2.4 Batch experiment for specific nitrification rate determination

Specific nitrification rate which includes specific ammonium oxidization rate (SAOR) and specific nitrate formation rate (SNFR) were measured on sampling days in the laboratory according to the method previously described (Zhang *et al.*, 2009b). Batch experiments were performed to evaluate the variations of nitrification activities of activated sludge (AOB, and NOB) in the bioreactor. Sludge sample (100 ml) from the aerobic tank was centrifuged at 8,000 rpm for 10 min and then washed with buffer medium to remove the background concentrations of nitrogen. The biomass (pellet)

was then transferred to a 250-ml Erlenmeyer flask and suspended with 100 ml of mineral medium. This was performed in a shaking incubator at 30°C. Samples were taken at an interval of 30 min to analyse the concentrations of NH_4^+-N and NO_2^--N. The SAOR and SNFR was determined by monitoring the decreased rate of NH_4^+-N concentration and NO_2^--N concentration versus time, respectively.

5.2.5 DNA extraction

Genomic DNA was extracted from sludge samples (aerobic samples) as described in section 3.2.4. The quality and quantity of the extracted DNA was ascertained with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The extracted genomic DNA was stored at –20°C until further use.

5.2.6 Standard curve preparation for quantitative real-time PCR analysis

Individual standard curves were prepared for different species of nitrifiers using purified 16S rRNA gene fragments (target DNA) obtained from PCR-amplified *Nitrobacter* spp. (FGPS872f and FGPS1269r), *Nitrospira* spp. (NSR1113F and NSR1264R) and AOB (amoA-1F and amoA-2R) respectively (Lienen *et al.*, 2014). The concentrations (µg/µl) of the purified DNA (purified 16S rRNA gene fragments) used as templates for the standard were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). This was used in calculating their copy numbers, which was based on their molecular weight and Avogadro's number (Trivedi *et al.*, 2009). The formula used in estimating the copy number is as shown below:

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

The average weight of a base pair (bp) is 650 Daltons and Avogadro's number is 6.022×10^{23}. The Ten-fold serial dilutions of the target DNA were prepared from 10^8 to 10^2 copy numbers.

5.2.7 Quantitative real-time PCR (qPCR) analysis

The real time PCR quantification was carried out with the primer sets targeting different species of nitrifiers (Table 5.1) according to the modified method described by Steinberg and Regan (2009). A Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) was employed for the qPCR. The qPCR reaction mixture was made up of 8 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Pty Ltd, USA), 1 µl of each primer (final concentration of 0.4 µM), 4 µl of template DNA (final concentration of 1 ng), and molecular grade water to a final volume of 20 µl. A modified Steinberg and Regan (2009) amplification protocol was employed (Table 5.2). For each experimental set up, appropriate negative controls containing no genomic DNA were subjected to the same amplification condition. The specificity of each qPCR assay was confirmed by using both melting curve analysis and agarose gel electrophoresis.

Table 5.1: Primers used and their specificity

Primers	**Sequence** (5′ → 3′)	**Target**	**Reference**
amoA-1F	GGGGTTTCTACTGGTGGT	Ammonia Monooxygenase	(Yu *et al.*, 2010)
amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
FGPS872	CTAAAACTCAAAGGAATTGA	*Nitrobacter*	(Ozdemir *et al.*, 2011)
FGPS1269	TTTTTTGAGATTTGCTAG		
NSR1113F	CCTGCTTTCAGTTGCTACCG	*Nitrospira*	(Wang *et al.*, 2011)
NSR1264R	GTTTGCAGCGCTTTGTACCG		

Table 5.2: Optimized real-time PCR protocols for quantifying nitrifiers

Real-time PCR step	Quantitative real-time PCR conditions using the primers		
	AOB	*Nitrospira* spp.	*Nitrobacter* spp.
	amoA-1F/amoA-2R	NSR1113F/NSR1264R	FGPS872/FGPS1269
1. Initial activation	3:30 min at 95°C	3:30 min at 95°C	3:30 min at 95°C
2. Denaturation	0:30 min at 95°C	0:30 min at 95°C	0:30 min at 95°C
3. Annealing	0:30 min at 54°C	0:30 min at 65°C	0:30 min at 52°C
4. Extension	0:30 min at 72°C	0:30 min at 72°C	0:30 min at 72°C
5. Read fluorescence	Read	Read	Read
6. Go to step 2 for	40 times	40 times	40 times
7. Melt curve	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s	55 to 65°C, increment of 0.5°C every 50 s
8. Read fluorescence	Read	Read	Read

In each assay, a plot of the threshold cycle (C_q) against the logarithmic starting quantity value of every 16S rRNA gene fragment (target DNA) was made and the standard curve with linear range having regression analysis correlation co-efficient (R^2) value that is greater than 0.98 was considered suitable (Kumari *et al.*, 2009; Yapsakli *et al.*, 2011). In addition, each qPCR assay with standard curve having suitable efficiency (90 to 110 %), and slope ranging from –3.58 to –3.1 only were considered. In order to quantify the 16S rRNA gene concentration of each nitrifier in the unknown DNA sample, the C_q values of each sample was interpolated into the respective standard curve.

5.2.8 Statistical Analysis

GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) was used in carrying out Pearson correlation, one-way analysis of variance and unpaired t-test. Microsoft Excel 2010 was used in calculating standard deviation.

5.3 RESULTS

5.3.1 Process performance and operational conditions

A full-scale system treating municipal wastewater was monitored for 237 days. The sampling regime was divided into two: phase 1 (winter) which lasted from the 1st day up till the 78th day; and phase 2 (summer) which was from 79th to 237th day. The average precipitation of the study site, influent wastewater characteristics and operational parameters measured in the plant during the study period is shown in Table 3.1. The temperature recorded in the aeration tanks ranged from 14.2^{o}C to 25.1^{o}C (Fig. 5.1a). The average temperature during summer and winter seasons were 22.8±1.5 and 16.5±2.0 ^{o}C respectively. The aeration tanks had fifteen surface aerators for sludge mixing. Simultaneous breakdown of two to three aerators was frequently observed during the sampling at the plant. The average DO concentration in the aeration tank during the summer and winter were 0.60±0.10 and 0.66±0.40 mg/l respectively (Fig. 5.1a).

The influent COD load varied during the sampling period with an overall average of 999.3±694 mg/L. The lowest influent COD (377 mg/l) load was recorded during summer, whereas the highest COD load of 1340 mg/l was observed during winter (Fig. 5.1b). The plant showed an efficient COD removal rate during the two seasons (95.44±2.754%). The average mixed liquor suspended solids (MLSS) of 6157±783 mg/l was recorded during winter whereas it was 5070±1172 in summer (Fig. 5.2a). The SRT of the plant was lower during summer (average of 22 days) as compared to the winter period (average of 25 days). The pH in the reactor over the entire sampling

period (winter and summer seasons) was relatively stable with a mean of 7.3±0.2 pH units (Table 3.1).

The average influent ammonia concentrations during the first 78th days (winter) was 31.69±6.04 mg/L whilst it was 24.47±4.58 mg/l during the summer. The effluent NH_4^+–N concentrations were 3.40 and 13.24 mg/l summer and winter respectively (Fig 5.2b). The influent and effluent nitrite concentrations were always lower than 1 mg/l during winter and summer periods. The nitrogen removal performance of the plant in terms of ammonia is shown in Fig 5.2. There was a significant variation in ammonia removal efficiency across the two seasons. The average ammonia removal during winter was 60.0±18.0% whereas it was 83.0±13.5% during summer and this was found to correlate with temperature ($r = 0.7671$; $P = 0.0008$). The ammonia removal also demonstrated significant correlation with the AOB population (copies/l) ($r = 0.55$; $P = 0.03$).

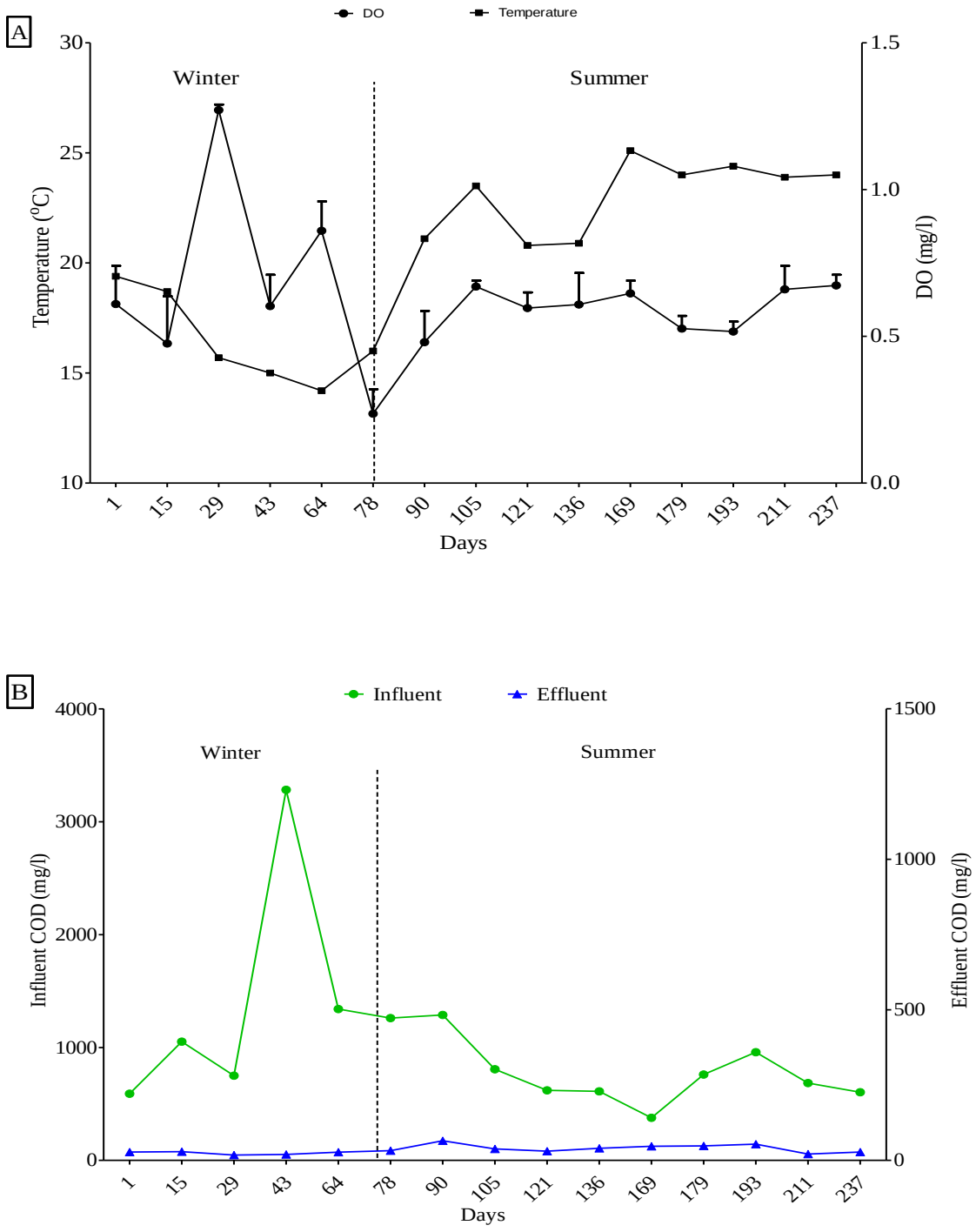

Fig. 5.1: (a) Temperature variation in the reactor during the study and dissolved oxygen concentration within the reactor during the study (b) Wastewater quality indicating COD concentrations in the influent and effluent

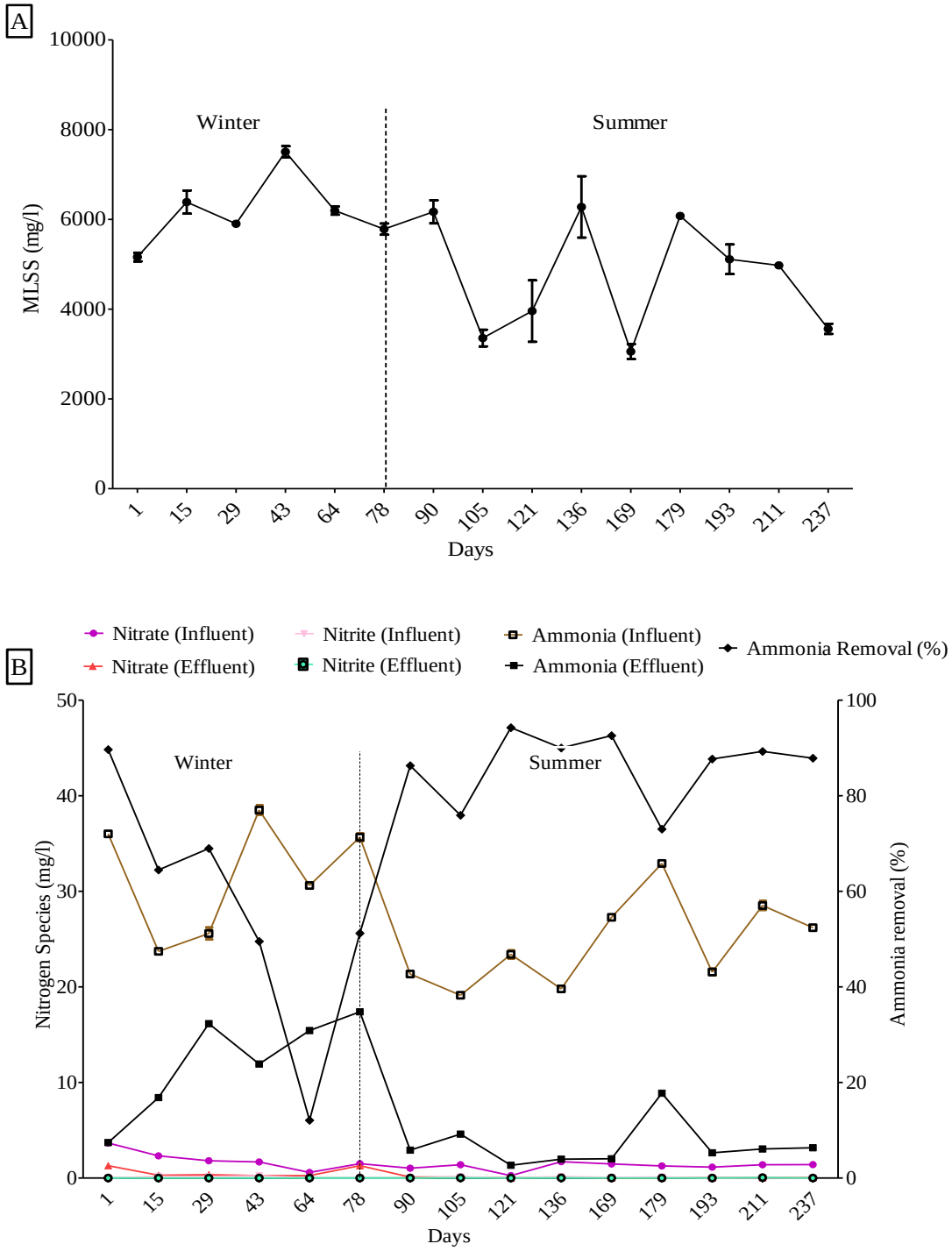

Fig. 5.2: (a) Variation in mixed liquor suspended solid concentration during the study. (b) Measured nitrogen species concentrations of the plant during the study and ammonia removal efficiency

5.3.2 Nitrification rate

In this study, the plant's highest nitrification rate was observed during the summer with an average of 0.085±0.020 g $N\text{-}NH_4^+$ g $MLSS^{-1}$ d^{-1}; whereas the lowest average rate of 0.063±0.054 g $N\text{-}NH_4^+$ g $MLSS^{-1}$ d^{-1} was recorded during winter (Fig. 5.3). Nitrification rate of the plant ranged from 0.022 to 0.071 g $N\text{-}NH_4^+$ g $MLSS^{-1}$ d^{-1} during winter whilst it was between 0.035 and 0.138 g $N\text{-}NH_4^+$ g $MLSS^{-1}$ d^{-1} during summer. The percentage ammonia removal (Fig. 5.2) and the calculated nitrification rate of the plant (Fig. 5.3) showed a significant correlation (r = 0.74; P = 0.003). The nitrification rates of the plant during the period investigated is shown in Fig 5.3. At the peak of winter, when the lowest temperature (Fig. 5.1) of 14ºC was recorded on day 64, nitrification rate was 0.022 $N\text{-}NH_4^+$ g $MLSS^{-1}$ d^{-1} (Fig. 3) which was the lowest recorded during the study.

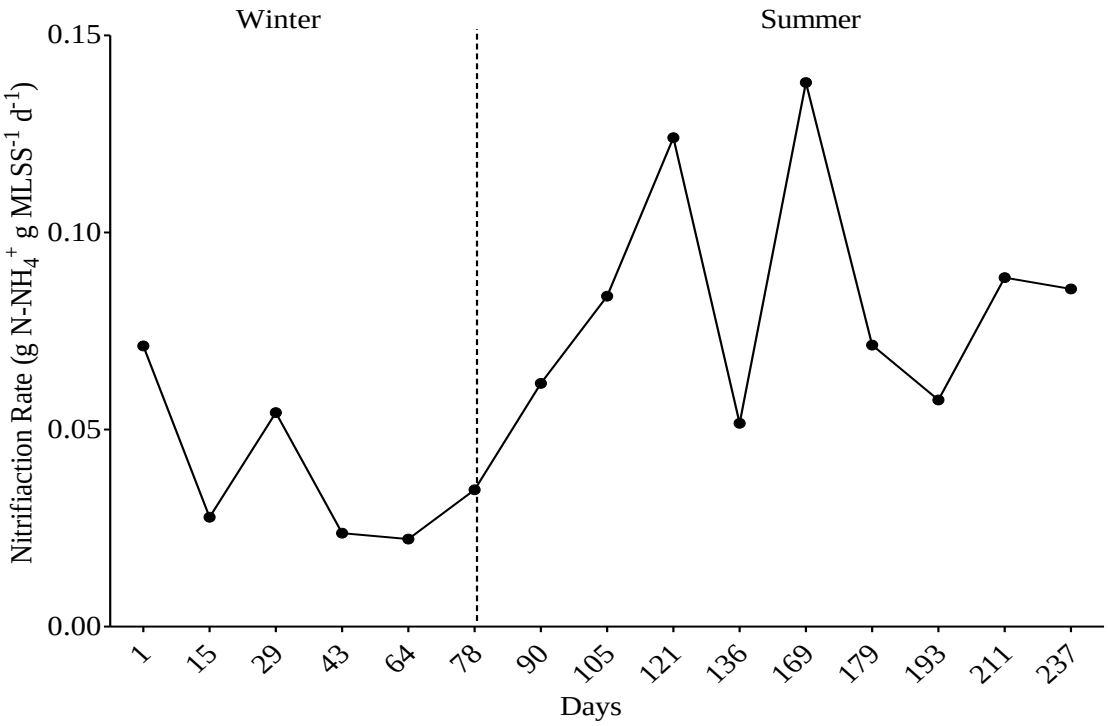

Fig. 5.3: The nitrification rates measured in the aeration tanks during the period investigated

5.3.3 Specific nitrification rate determination by batch experiment

The specific nitrification rate of the biomass in terms of specific ammonium oxidization rate (SAOR) and specific nitrate formation rate (SNFR) were determined in a laboratory batch experiment (Fig. 5.4). The SAOR obtained ranged between 0.010 and 0.148 g N g^{-1} MLSS d^{-1} whilst SNFR was from 0.1700 to 0.6310 g N g^{-1} MLSS d^{-1}. The highest values of both SAOR and SNFR recorded during the batch experiment were 0.1488 and 0.6310 g N g^{-1} MLSS d^{-1} respectively. These values were obtained from the biomass taken during summer period (day 88). The estimated SNFR from the batch experiment was higher than the SAOR all through the experiment. A significant correlation was observed between the plant's nitrification rate and SAOR ($r = 0.65$; $P = 0.04$). The SNFR ($r = 0.70$; $P = 0.02$) also had a significant correlation with the plant's nitrification rate.

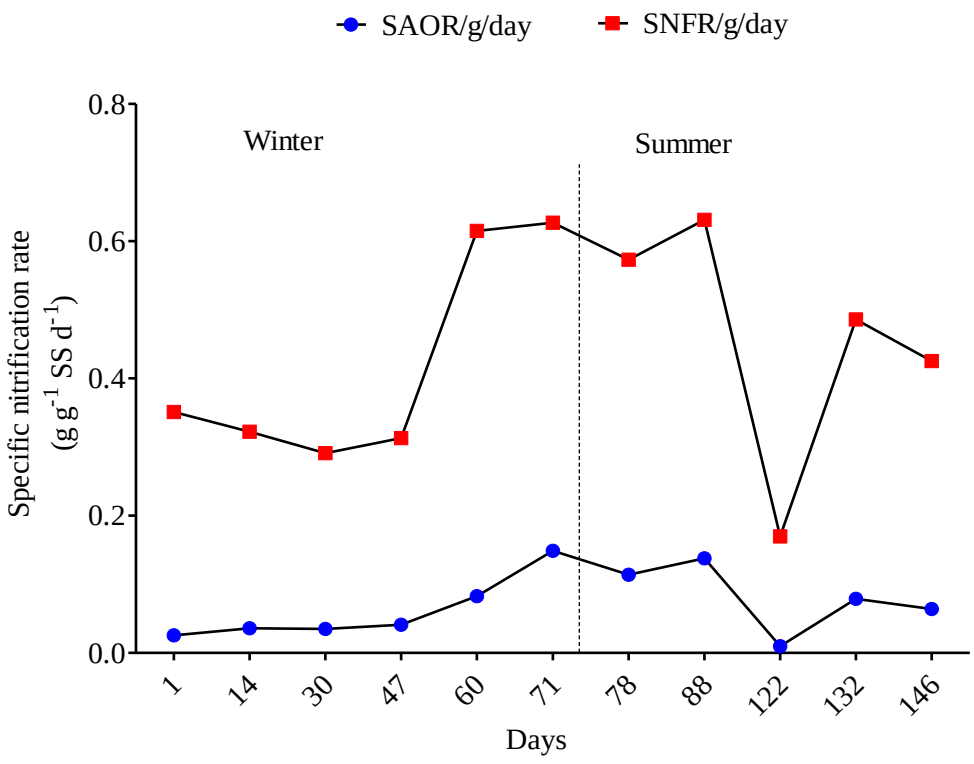

Fig. 5.4: Time-course profiles of the specific ammonium oxidizing rate (SAOR) and specific nitrate forming rate (SNFR) of the plant

5.3.4 Quantification of AOB and NOB

Parameters for the qPCR standard curve, obtained after optimization is shown in Table 5.3 below. The qPCR efficiencies were between 90 and 110 % and the standard curves were linear over six order of magnitude (R^2>0.99) (Fig. 5.5). The AOB population abundance was quantified using the primer set targeting the amoA (ammonia monooxygenase) gene locus whereas *Nitrospira* and *Nitrobacter* 16S rDNA were targeted for the NOB (Table 5.1). The AOB abundance was within the range of 1.6 x 10^7 – 1.7 x 10^9 copies/l. The *Nitrospira* and *Nitrobacter* spp. were found to be 2.4 x 10^8 – 3.8 x 10^9 copies/l and 9.3 x 10^9 – 1.4 x 10^{11} copies/l respectively. The *Nitrobacter* spp. abundance was highest during the study, having about 2 orders of magnitude above the AOB throughout the study. The *Nitrobacter* spp. was the dominant NOB throughout this study. The changes in AOB, *Nitrospira* spp. and *Nitrobacter* spp. abundance across the study period is shown in Fig. 5.6 below. A significant correlation was found between the AOB (amoA gene) copy numbers and temperature in the reactors (α= 0.05; P=0.0498). The lowest AOB abundance was recorded during the winter, whereas there was no correlation observed between the NOB and temperature. The NOB population remained almost stable with slight shifts throughout the sampling period (Fig. 5.6).

Table 5.3: Description of qPCR standard curves parameters optimized for the analysis during this study

Parameter	Target		
	AOB	*Nitrobacter* spp.	*Nitrospira* spp.
Efficiency	102.5±2.1	92.75±1.63	107.3±1.9
Slope	-3.3±0.05	-3.5±0.05	-3.2±0.04
R^2 of Slope	0.998±0.001	0.99±0.01	0.998 ± 0.04
Intercept	39.8±2.2	35.0±0.59	37.2±0.14

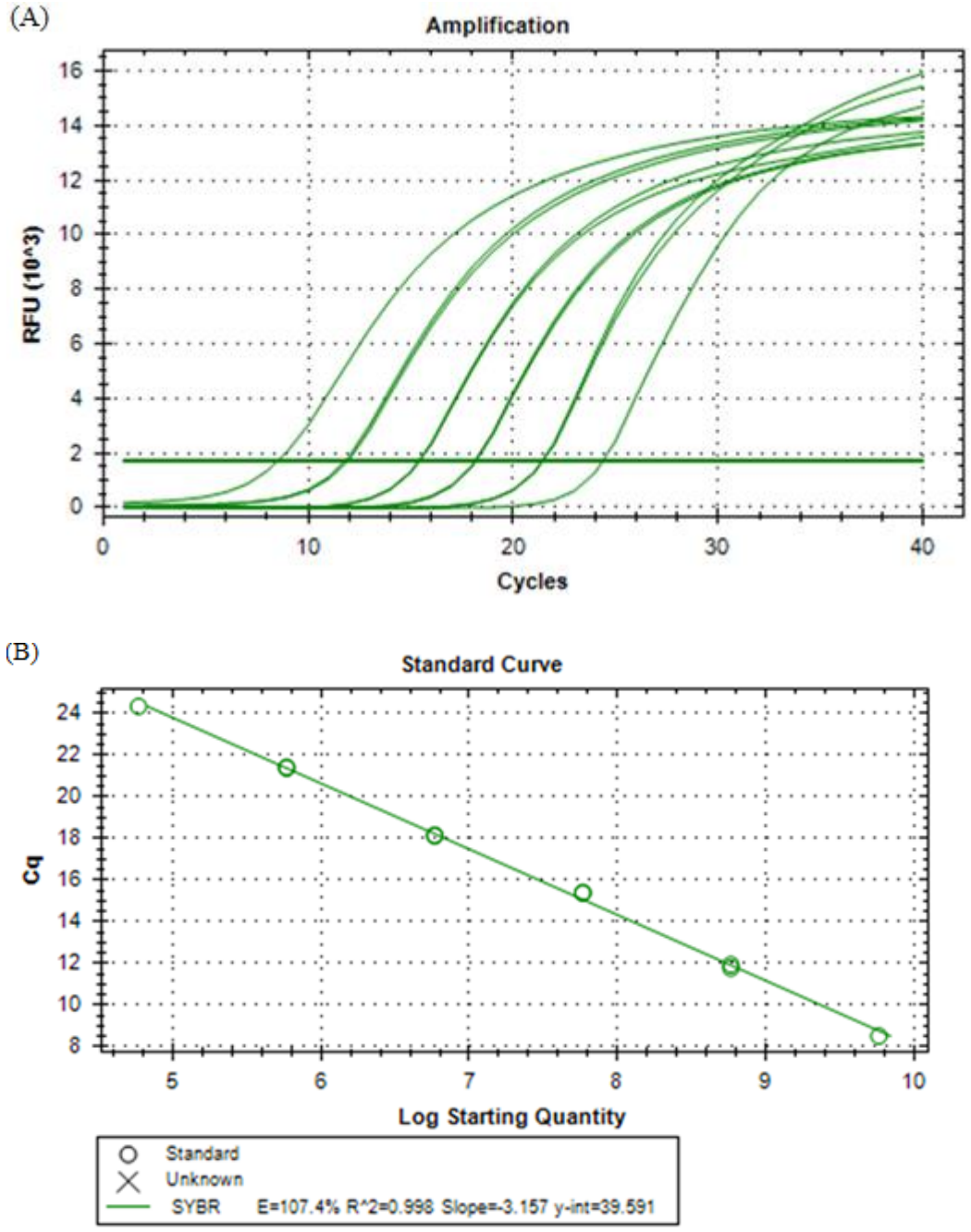

Fig. 5.5: Real-time PCR data for the purified DNA (*Nitrospira*) used in generating standard curve indicating linearity over six order of magnitude (R2>0.99) (a) The qPCR amplification curve (b) Standard curve.

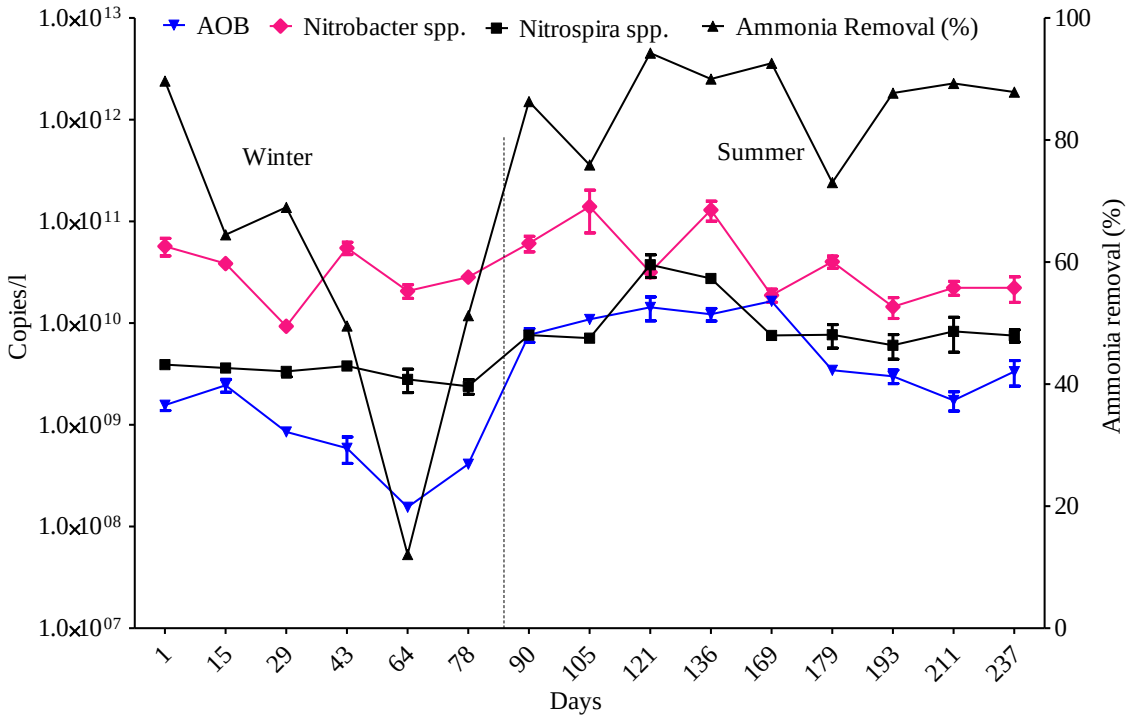

Fig. 5.6: qPCR temporal changes in AOB, *Nitrospira* spp. and *Nitrobacter* spp. during this study and ammonia removal rate of the plant

5.4 DISCUSSION

Seasonal temperature variation has been implicated as one of the environmental factors affecting nitrification rates in wastewater treatment facilities (Kim, 2013; Arévalo *et al.*, 2014). In this study, a significant seasonal variations in temperature (α= 0.05; P=< 0.0001) was observed in the reactor. The experimental site had its highest rainfall during its summer season as observed during the study (Table 3.1). The dilution effect of the rainfall contributed to lowering the plant's loading rates during summer (Table 3.1). There was a significant variation in the influent ammonia concentration of the plant. The average influent ammonia concentration was 24.5±4.6 mg/l during summer whilst it was 31.7±6.1 mg/l in winter. The highest NH_3 concentration in the effluent (17.4 mg/l) was observed during the winter period when the temperature was 16.0ºC (Fig 5.2). The lowest ammonia removal of 12.1% and AOB abundance (1.6 x 10^8 ±

1.3×10^7 copies/l) were observed when the lowest temperature (14.2^{o}C) was recorded (Figs. 5.2 and 5.6). Similarly, Kim *et al.* (2008) earlier noted a significant correlation between temperature and ammonia oxidation. In this study, a reduction in the average NH_3 removal efficiency of the plant from 83.0±13.0% during summer (day 1 – 78) to 60.0±18% during winter (day 79 – 237) was observed. This was also found to correlate significantly with seasonal temperature fluctuation (α= 0.05; P=0.0008). However, neither temperature nor NOB population density had correlation with nitrite oxidation rate of the plant. The non-correlational relationship observed between NOB and the plant's operational parameters could be because of the cross-correlation approach that was employed. It has been noted that several parameters interact simultaneously to influence the biological process under the uncontrolled environment which prevails in the full-scale WWTP (Kim *et al.*, 2011). Hence, there is need for an advanced nonlinear modelling tool in order to be able to determine some of the relationships that exists in the WWTP.

The highest percentage ammonia-nitrogen removal (83 ± 13%) and nitrification rate (0.138 N-NH_4^+ g $MLSS^{-1}$ d^{-1}) were recorded at the plant during summer. The SAOR was found to be lower than the SNFR throughout the study period with the average values of 0.07036 and 0.4368 g N g^{-1} MLSS d^{-1} respectively. This is in accordance with earlier findings in an aerobic batch experiment, where a lower SAOR was obtained in comparison to SNFR (Fujita *et al.*, 2010). Furthermore, it was observed that the SAOR and SNFR obtained had no proportional relationship with ammonia and nitrite-oxidation measured in their experiment (Fujita *et al.*, 2010). However, in the current study, both SAOR and SNFR obtained showed significant correlation with the plant's nitrification rate. Moreover, the specific nitrification (SNFR and SAOR) results obtained in this study reflects the population densities (higher NOB copy numbers compared to AOB) of the nitrifiers obtained using qPCR. This demonstrates that specific nitrification rate determined in the laboratory batch experiment, can serve as an indicator of the plant's nitrifying community and performance. Yu *et al.* (2011) reported similar finding in which the SAOR and SNFR obtained were related to the AOB: NOB ratio in a batch experiment.

The quantitative PCR results revealed the constant dominance of *Nitrobacter* spp. among the nitrifier's community and the NOB was clearly more abundant than AOB throughout the study. Due to the limiting oxygen levels observed (Table 5.3), there was a possibility of nitrite-loop and hence an increased NOB population. The average copy number of AOB to NOB ratio varied from 0.11 (summer) to 0.02 (winter) during the period investigated (Fig. 5.6), which was lower than the theoretical ratio of 2.0 reported for good nitrification (Winkler *et al.*, 2012). However, low AOB/NOB ratios of 0.2 and 0.3 was recorded by Winkler *et al.* (2015) in an investigation of a lab-scale and pilot-scale aerobic granular sludge. It was observed that an increase in temperature and/or a decrease in DO level led to an elevated NOB/AOB ratio (Winkler *et al.*, 2015). Seasonal temperature was observed to have positive correlation with the AOB gene copy number ($r = 0.5$; $P = 0.05$) whereas NOB was not affected by the temperature shifts (Fig. 5.6). This result is consistent with earlier findings, which revealed that AOB abundance had correlation with the wastewater effluent quality; with AOB population decrease resulting in nitrification rate reduction (LaPara and Ghosh, 2006; Zhang *et al.*, 2009a).

Under low DO level, denitrifiers can carry out incomplete nitrate reduction which can serve as additional nitrite source for NOB and partially uncouple their growth from AOB, thereby resulting in their elevated population density (Winkler *et al.*, 2012). Liu (2012) noted that NOB exhibited a significant O_2 affinity under prolonged low DO concentrations (0.16 - 0.37 mg/l) which in turn made them a better competitor for O_2 as their abundance increased comparably to AOB. Another possible reason for the higher population load of NOB observed in this study could be due to the fact that under extended period of low dissolved oxygen concentrations (<= 0.5 mg/l) the endogenous decay of both ammonia/nitrite oxidizing bacteria would be retarded (Liu and Wang, 2013). According to Liu and Wang (2013), this reduced endogenous decay would result in increased biomass density which would nullify some low DO impact on nitrification. They also reported near complete nitrification with 0.16 - 0.37 mg/l DO range. Furthermore, they noted that under extended low DO period, NOB demonstrated significant increase in O_2 affinity which in turn made them a better O_2

competitor than AOB. However, it is important to note that these results were based on laboratory experiments, unlike the different scenario in this study, involving a full-scale plant where more than one factor influence each other.

The dominance of *Nitrospira* (K-strategist) over *Nitrobacter* (r – Strategist) in activated sludge has been previously reported (Yapsakli *et al.*, 2011; Ye *et al.*, 2011), however in this study a contrary observation was noted. The *Nitrobacter*/*Nitrospira* ratio was 5.4 and 10.2 during summer and winter respectively. This could possibly be explained based on the earlier observations of Nogueira and Melo (2006); and Wagner *et al.* (2002) in which irreversible prevalence of *Nitrobacter* spp. over *Nitrospira* spp. in WWTP after a spike in nitrite concentration and even after subsequent reduction in nitrite concentration was recorded. *Nitrobacter* usually exhibit inhibitory effect on the growth of *Nitrospira* once it dominates. Fukushima *et al.* (2013) also reported that *Nitrobacter* spp., though a weak competitor compared to *Nitrospira* under low nitrite concentration, can be selected over *Nitrospira* spp. in plants with low inorganic carbon in addition to low nitrite concentration. The plant studied had low AOB population density which could result in lowered rate of ammonia conversion to nitrite. This could probably give a selective advantage to *Nitrobacter* spp. as observed in this study. Furthermore, Winkler *et al.* (2015) observed an unprecedentedly high *Nitrobacter*/*Nitrospira* ratios in different aerobic reactor. They observed an increase in the ratio from 1.5 to 3.5 when the temperature increased from 10^{o}C to 30^{o}C.

5.5 CONCLUSIONS

- The seasonal dynamics of nitrifying community in full-scale municipal bioreactor was investigated and there was a significant difference in the nitrification efficiency of the plant during the two seasons monitored with a two-fold increase in nitrification observed during summer compared to winter.
- The ammonia removal efficiency of the plant had a significant linear correlation with the AOB population density of the plant. However, no significant correlation could be

established between NOB and operational parameters, which may be due to the cross-correlational approach used. Hence, there is need for the application of a nonlinear modelling approach such as ANFIS, in order to determine the relationship.

- There was a significant correlation between the nitrification rates of the plant and the batch specific nitrification rate (in terms of SAOR) determined in the laboratory experiment. Hence, the specific ammonia oxidation rate (SAOR) can be used as indication of nitrifying population densities and nitrification performance of wastewater treatment plants.

5.6 RESEARCH OUTPUT

a) Journal Articles

1) **Awolusi, O. O.,** M. Nasr, Kumari, S. K. S., Bux, F. Principal component analysis for interaction of wastewater characteristics and nitrifiers at a full-scale activated sludge plant. Environmental Science and Pollution Research (Submitted)

b) Conference Papers

1) **Awolusi O. O.,** Kumari S. K., Bux F. 2015. Seasonal impact on nitrification potential of activated sludge treating municipal wastewater. Paper presented at 4th Young Water Professional (South Africa) Biennial Conference and 1st Africa-wide YWP Conference, CSIR International Convention Centre, Pretoria, South Africa, 16th – 18th November 2015 (Oral presentation).

2) **Awolusi, O. O.,** Enitan, A. M., Kumari, S. K. S., and Bux, F. 2015. Nitrification efficiency and community structure of municipal activated sewage sludge. Paper presented at 17th International Conference on Biotechnology, Bioengineering and Bioprocess Engineering, Rome, Italy, September 17 - 18, 2015 (Oral presentation).

3) **Awolusi O. O.,** Kumari S.K., Bux F. 2014. Characterization and quantification of nitrifying community in activated sludge system treating municipal wastewater. Paper presented at Water Institute of Southern Africa (WISA) 2014 (Water Innovations) biennial conference, Mbombela Stadium, Nelspruit, South Africa (Oral presentation).

CHAPTER SIX: APPLICATION OF ARTIFICIAL INTELLIGENCE FOR EVALUATING OPERATIONAL PARAMETERS INFLUENCING NITRIFICATION AND NITRIFIERS IN AN ACTIVATED SLUDGE PROCESS

6.1 INTRODUCTION

Harnessing chemolithotrophic nitrifiers' ability to remove ammonia from wastewater, is one of the primary tasks in protecting water resources from pollution discharges (Wang *et al.*, 2012). However, these nitrifying bacteria are highly sensitive to changes in environmental parameters and plant operational conditions, such as pH, temperature, dissolved oxygen (DO) level, organic loading rate (OLR), ammonia loading rate (ALR) and hydraulic retention time (HRT) (Hu *et al.*, 2009). Neutral to slightly basic pH range (7.5 to 8.5) has been reported as optimum for efficient nitrification (Fulweiler *et al.*, 2011). According to an earlier finding, at neutral pH, 99% of ammonia was removed, however, this dropped to about 75% at a basic pH of 9.7 whilst acidic pH (4.8) resulted in an impaired removal efficiency of 56% (Hu *et al.*, 2009). Although it was reported that nitrification would proceed at a temperature range of 20°C to 37°C (Stark, 1996), nonetheless niche differentiation exists among the members of NOB group, with *Nitrobacter* having preference for relatively low temperatures (24 – 25°C) whist *Nitrospira* thrives at higher temperatures (29 – 30°C) (Huang *et al.*, 2010a). Kim *et al.* (2011) demonstrated that, raising temperature from 20 to 30^{o}C resulted in a 5.3-fold increase in ammonia oxidation, and a 2.6-fold increase in nitrite oxidation.

Lower nitrification performance has been observed at higher organic loading due to the competition for DO between total bacteria and nitrifying organisms (autotrophic bacteria) in wastewater treatment system (Wu *et al.*, 2013). Huang *et al.* (2010a) demonstrated that higher DO concentrations were more suitable for *Nitrobacter* growth, whilst *Nitrospira* was selectively enriched when DO concentrations were less than 1.0 mg/L. The available carbon substrate for the unit mass of microorganism

(known as F/M ratio) can also impact nitrification. A study by Wu *et al.* (2013) suggested that high F/M ratio should be avoided to minimize its negative impact on nitrification, and it indicated that F/M between 0.2 and 0.4 was the optimum range for nitrification. The influence of HRT on nitrification efficiency was also observed when HRT decreased from 30 to 5 h with a resultant increase in specific ammonium-oxidizing and nitrate-forming rates (Li *et al.*, 2013). Additionally, the decrease in HRT led to a reduction of AOB population density, whereas the NOB, especially the fast growing *Nitrobacter* spp., increased significantly (Li *et al.*, 2013).

Modelling of a full-scale wastewater treatment plant (WWTP), operated under an uncontrolled environment, requires advanced nonlinear modelling tools to simulate the complex relationships between inputs and outputs (Kim *et al.*, 2011). According to Klemetti (2010), due to simultaneous dependence on different factors, competition between microbial groups are usually nonlinear. Artificial intelligence (AI) is an example of a nonlinear system that is capable of depicting the interactive influence between variables as well as their correlation with the simulation output (Nasr *et al.*, 2015b). AI incorporates artificial neural network (ANN), fuzzy inference system (FIS) and adaptive-neuro fuzzy inference system (ANFIS). ANN has the ability to model nonlinear systems efficiently, owing to their high accuracy, adequacy and quite promising applications in engineering (Nasr *et al.*, 2012). FIS allows a logical data-driven modelling approach, which uses "if–then" rules and logical operators to establish qualitative relationships among the variables (Nasr *et al.*, 2014).

ANFIS is a neuro-fuzzy system that has the potential to capture the benefits of both ANN and FIS in a single framework (Nasr *et al.*, 2015b). Moreover, ANFIS can handle complex and highly nonlinear relationships between several parameters, without the difficult task of dealing with deterministic non-linear mathematics (Nasr *et al.*, 2015a). Modelling based on ANFIS needs a little knowledge about the process to track given input/output data. In this context, it is expected that the effect of system operation on nitrification process could be studied using ANFIS. To the best of our knowledge, this

is the first study applying ANFIS technique to describe the nitrification performance at a full-scale wastewater treatment plant subjected to dynamic operational parameters. In this chapter, the focus was to evaluate the influence of operational and environmental parameters, on nitrification and the different nitrifiers' population abundance in the plant, using an advanced nonlinear modelling tool (ANFIS). ANFIS results were further validated with cross-correlation coefficients and quadratic models.

6.2 MATERIALS AND METHODS

6.2.1 Plant description

The full-scale WWTP under study is situated midlands of KwaZulu-Natal province, South Africa. The plant description has been given previously (Section 3.2.1 and Fig. 3.1).

6.2.2 Sampling protocol

The WWTP was monitored for a period of 237 days; i.e. from May to July 2012 and from November 2012 to March 2013. The sampling protocol is as described in Section 3.2.2.

6.2.3 DNA extraction and real-time quantitative PCR (qPCR) amplification

The freeze-thaw DNA extraction procedure employed in this study has been previously described in Section 3.2.4. The qPCR was carried out as described in chapter 4. For the quantitative real-time PCR, the primers already described in Table 4.1 were used. The optimized protocols used for quantifying the nitrifiers are shown in Table 4.2. To confirm amplification of the correct product, the amplicons from qPCR were electrophoresed in 1.2% (wt/vol) agarose gel for the presence of the expected gene product sizes. The qPCR standard curve parameters used for the analysis are listed in Table 4.3.

6.2.4 Analytical analysis

Concentrations of inorganic nitrogen species and COD were estimated using standard methods (APHA, 1998). Temperature, DO and pH measurements were carried out using the YSI 556 MPS (Multiprobe System). The full physicochemical analysis is as previously described in Section 5.2.2.

6.2.4.1 Calculations

Operational conditions such as HRT, OLR, ALR and F/M ratio were calculated according to Tchobanoglous *et al.* (2003) as follows (**Eqs. 1 – 4**):

$$HRT = \frac{V}{Q} \quad \text{Eq. 1}$$

$$OLR = \frac{Q \times COD}{V} \quad \text{Eq. 2}$$

$$ALR = \frac{Q \times N - NH_4^+}{V} \quad \text{Eq. 3}$$

$$F/M = \frac{Q \times COD}{MLSS \times V} \quad \text{Eq. 4}$$

Where, HRT: hydraulic retention time; V: reactor volume; Q: flow rate; OLR: organic loading rate; COD: chemical oxygen demand; ALR: ammonia loading rate; N-NH_4^+: ammonia nitrogen; F/M: food-to-microorganisms ratio; MLSS: mixed liquor suspended solids.

6.2.5 Adaptive neuro fuzzy inference system (ANFIS)

6.2.5.1 Architecture of ANFIS

To present the ANFIS architecture, two fuzzy "if–then" rules based on a first-order Takagi–Sugeno fuzzy model were considered:

Rule-1: If (x is A_1) and (y is B_1) then ($f_1 = p_1x + q_1y + r_1$)

Rule-2: If (x is A_2) and (y is B_2) then ($f_2 = p_2x + q_2y + r_2$)

Where x and y are the inputs, A_i and B_i are the fuzzy sets, f_i are the outputs within the fuzzy region specified by the fuzzy rule; p_i, q_i and r_i are the design parameters determined during the training process.

As shown in Fig. 6.1, the ANFIS architecture to implement these two rules has a total of five layers, in which a circle indicates a fixed node, whereas a square indicates an adaptive node. The functioning of each layer can be described as follows (Jang, 1993):

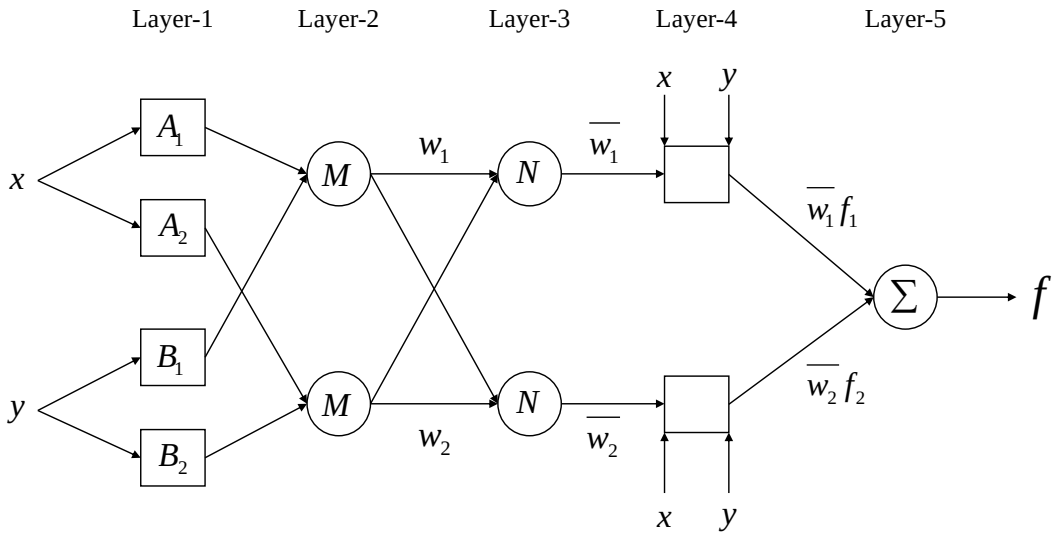

Fig. 6.1: Typical first-order Sugeno ANFIS architecture

Layer-1 (Input node): Parameters in this layer referred to "premise parameters". Every single node generates a fuzzy membership grade of linguistic label. The membership functions (MFs) of A_i and B_{i-2} are given by **Eq. 5** and **Eq. 6**, respectively.

$$O_i^1 = \mu_{A_i}(x) \qquad i = 1, 2 \qquad \text{(Eq. 5)}$$

$$O_i^1 = \mu_{B_{i-2}}(y) \qquad i = 3, 4 \qquad \text{(Eq. 6)}$$

Where x (or y) is the input to node i, and A_i (or B_{i-2}) is the linguistic label (small, large, etc.) related to this node. If the bell-shaped MF is generalized, $\mu_{A_i}(x)$ is given by **Eq. 7**.

$$\mu_{A_i}(x) = \frac{1}{1 + \left\{ \left(\frac{x - c_i}{a_i} \right)^2 \right\}^{b_i}} \qquad \text{(Eq. 7)}$$

Where a_i, b_i and c_i are the MF parameters, governing the bell-shaped functions accordingly.

Layer-2 (Rule nodes): In the second layer, the nodes are labelled with M, indicating that they perform as a simple multiplier. The AND/OR operator is used to get one output that represents the antecedent of the fuzzy "if–then" rule. The outputs of this layer are defined as firing strengths of the rules. Each node analyses the firing strength by cross multiplying all the incoming signals (**Eq. 8**).

$$O_i^2 = w_i = \mu_{A_i}(x)\mu_{B_i}(y) \qquad i = 1, 2 \qquad \text{(Eq. 8)}$$

Layer-3 (Average nodes): In the third layer, the nodes are labelled with N, demonstrating that they play a normalization role to the firing strengths from the previous layer. Thus, outputs of this layer are called "normalized firing strengths". As shown in **Eq. 9**, the i^{th} node calculates the ratio of the i^{th} rules firing strength to the sum of all rules' firing strengths.

$$O_i^3 = \overline{w_i} = \frac{w_i}{w_1 + w_2} \qquad i = 1, 2 \qquad \text{(Eq. 9)}$$

Layer-4 (Consequent nodes): In this layer, every node *i* is an adaptive node with a node function. The output of each node is the product of the normalized firing strength and a first order polynomial (for a first-order Sugeno model). Hence, the outputs of this layer are expressed by **Eq. 10**.

$$O_i^4 = \overline{w_i} f_i = \overline{w_i}\left(p_i x + q_i y + r_i\right) \qquad i = 1, 2 \qquad \text{(Eq. 10)}$$

Where $\overline{w_i}$ is the output of layer-3, and $\{p_i, q_i, r_i\}$ are consequent parameters, pertaining to the first order polynomial.

Layer-5 (Output node): In the fifth layer, there is only one single fixed node labeled with $\sum$. The single node computes the overall output as the summation of all incoming signals. Thus, the overall output of the model is given by **Eq. 11** as follows:

$$O_i^5 = \sum_{i=1}^{2} \overline{w_i} f_i = \frac{\left(\sum_{i=1}^{2} w_i f_i\right)}{w_1 + w_2} \qquad \text{(Eq. 11)}$$

6.2.5.2 Application of ANFIS

The function *exhsrch* in MATLAB was used to select the set of inputs that considerably impact the nitrification activity. Theoretically, *exhsrch* builds an ANFIS model for each combination and trains it for one epoch, sequentially reports the performance achieved. ANFIS uses a hybrid learning algorithm to tune the parameters of a Sugeno-type fuzzy inference system (Azar, 2011). The algorithm uses a combination of the least-squares and back-propagation gradient descent methods to model a training data set (Jang, 1993). The dataset is randomly classified into training (70%) and checking (30%) arguments. The training process stops if the designated epoch number is reached or the error goal is achieved, whichever comes first. The checking data are used for testing the generalization capability of the ANFIS, and monitor how well the model predicts the corresponding dataset output values. Moreover, ANFIS validates models using a checking data set to test for overfitting of the training data. Recently, this technique has been successfully implemented in the field of wastewater treatment technology (Fawzy *et al.*, 2015; Nasr *et al.*, 2015a).

The results and discussion in this chapter was combine into a single section as it appears in the already published article (Appendix Four: Microbial Ecology, DOI 10.1007/s00248-016-0739-3). Hence, it does not follow the separate "results" and "discussion" format used in other chapters.

6.3 RESULTS AND DISCUSSION

6.3.1 Environmental conditions and system performance

The winter season (1^{st} – 78^{th} d) experienced little or no rainfall resulting in an average influent flow rate of 61,990 ± 2,172 m^3 d^{-1}, whilst it increased to 93,062 ± 18,106 m^3 d^{-1} during the summer (79^{th} – 237^{th} day). The influent COD during the summer was 1.3-fold lower than in the winter due to the dilution effect of increased rainfall (Table 6.1). The OLR increased during summer with increasing flow rate, despite the lower

COD, and reached 4.5 ± 1.8 kg COD m^{-3} d^{-1}. The deviations in OLRs could be attributed to the variation in the type of influent as a result of commercial and industrial activities occurring around the treatment plant. The F/M ratio was 0.6 ± 0.1 d^{-1} in the winter and increased by 48% during the summer (Table. 6.1). There is no ideal F/M ratio that can work for all activated sludge treatment systems. Previous studies indicate that the recommended range for F/M ratio in conventional, completely mixed and high rate activated sludge processes ranged between 0.2 – 0.4, 0.2 – 0.6 and 0.4 – 1.5 d^{-1}, respectively (Tchobanoglous and Burton, 1991).

Although the influent ammonia during summer was 1.2-fold lower than in winter, the respective ALR increased by 18.8% resulting from the summer rainfall (Table. 6.1). The ammonia removal efficiency improved from 60 ± 18 to 83 ± 13% although the ALR rose from 121 ± 22 to 144 ± 29 g $N\text{-}NH_4^+$ m^{-3} d^{-1} through winter to summer, respectively. The increase in ammonia removal efficiency and nitrification rate with ALR indicated that ammonia concentration was not the nitrification limiting factor in this plant. This suggested that other operational conditions had a greater influence on the nitrification process rather than ALR. Results from this study (60.0 ± 18.0 - 83.0± 13.0%) were lower than the 97 - 99.9% ammonia removal that was obtained by Campos *et al.* (1999) when the nitrifying activated sludge unit was subjected to high nitrogen loading rates (up to 7,500 g $N\text{-}NH_4^+$ m^{-3} d^{-1}).

The higher nitrification performance of the plant could be due to operation of the unit under a controlled environment, where the nitrifying activated sludge was fed with synthetic wastewater containing ammonia and other nutrient sources with regulated pH. The influent nitrate concentration during the study period varied between 0.25 and 3.68 mg/l with an average value of 1.51 ± 0.77 mg/l (Fig. 5.3; Chapter 5). Nitrate removal efficiencies exhibited 64.6 ± 27.6% and 88.0 ± 26.3% during winter and summer seasons, respectively. The concentration of effluent nitrate during the winter was 3.6-fold higher than summer. This might be an indication of the increased

NOB/AOB ratio during the winter that promotes nitrate accumulation and thus a higher effluent nitrate concentration in winter (Zhang *et al.*, 2012a; Mozumder *et al.*, 2013).

Table 6.1: Operational conditions and ANFIS model parameters of the full-scale wastewater treatment plant under study

Parameters	Phase 1 (Winter)	Phase 2 (Summer)
Rainfall (mm)	26.0 ± 18.6	116.8 ± 32.0
Temperature (°C)	16.5 ± 2.1	22.4 ± 2.7
HRT (h)	6.3 ± 0.2	4.3 ± 1.0
OLR (kg COD m^{-3} d^{-1})	4.0 ± 1.1	4.5 ± 1.8
ALR (g $N\text{-}NH_4^+$ m^{-3} d^{-1})	121 ± 22	144 ± 29
F/M (g COD g^{-1} MLSS d^{-1})	0.6 ± 0.1	0.9 ± 0.3
q_N (mg $N\text{-}NH_4^+$ g^{-1} MLSS d^{-1})	12.3 ± 6.1	26.6 ± 10.7
AOB (copies $\times 10^9$ l^{-1})	1.00 ± 0.86	7.35 ± 5.75
Nitrobacter spp. (copies $\times 10^9$ l^{-1})	34.8 ± 19.0	50.8 ± 46.1
Nitrospira spp. (copies $\times 10^9$ l^{-1})	3.32 ± 0.60	11.9 ± 11.2

The DO levels throughout the aeration tank varied between 0.24 and 1.27 mg/l, with an average value of 0.63 ± 0.22 mg/l. The DO concentration in our study was lower than the optimum value of 1.7 mg/l for a complete nitrification process (Ruiz *et al.*, 2003). The pH in the feed over the entire sampling period was relatively stable with an average value of 7.2 ± 0.1, which is close to the optimum for nitrifiers (7.5 – 8.0) (Ruiz *et al.*, 2003). Ruiz *et al.* (2003) reported that at the range of pH 6.45 – 8.95, as observed here, a complete nitrification to nitrate occurs, while at pH lower than 6.45 and higher than 8.95 would result in complete inhibition of nitrification.

6.3.2 Temporal changes in dominant nitrifiers' abundance ratios

The AOB abundance was within the range of $1.55 \times 10^8 – 1.65 \times 10^{10}$ copies/l MLSS, whereas the *Nitrobacter* spp. and *Nitrospira* spp. were found to be in the range of $9.32 \times 10^9 – 1.40 \times 10^{11}$ copies/l MLSS and $2.39 \times 10^9 – 3.76 \times 10^{10}$ copies/l MLSS, respectively (Fig. 5.6). The *Nitrobacter* spp. abundance was 29-fold higher than AOB throughout the study period showing a clear dominance of NOB in the selected WWTP (Fig. 6.2). The average AOB to NOB ratio varied from 0.03:1 (winter) to 0.15:1 (summer), which was lower than the theoretical ratio of 2:1 reported for good nitrification (Winkler *et al.*, 2012). Due to the prevailing limiting oxygen levels (0.63 ± 0.22 mg O_2 l^{-1}) in the reactor, there was a possibility of nitrite-loop. This phenomenon usually occurs when denitrifiers reduce nitrate to nitrite supplying additional nitrite for NOB, leading to a higher NOB/AOB ratio than theoretically expected (Winkler *et al.*, 2012). Additionally, Liu (2012) noted that under long-term low DO, the oxygen affinity of NOB increases significantly, which makes NOB a better competitor for oxygen compared to AOB. This high NOB/AOB ratio resulted in sub-optimal ammonia transformation in this study. Whereas earlier studies have recorded about 99% ammonia removal (Hu *et al.*, 2009), in this study the highest NH_3 removal that was recorded was $83 \pm 13\%$.

A higher *Nitrobacter*: *Nitrospira* ratio of 7.4: 1.0 was also recorded. This could possibly be explained based on the earlier observations of Wagner *et al.* (2002) and Nogueira and Melo (2006). They noted an irreversible prevalence of *Nitrobacter* spp. over *Nitrospira* spp. in WWTP after a history of spike in nitrite concentration, even after subsequent reduction in nitrite concentration. *Nitrobacter* usually exhibit inhibitory effect on the growth of *Nitrospira* once it dominates. Furthermore, Fukushima *et al.* (2013) reported that *Nitrobacter* spp. can be selected over *Nitrospira* spp. in plants with low inorganic carbon in addition to low nitrite concentration. However, there have been no studies on the distribution of *Nitrospira* and *Nitrobacter* in DO-limiting condition over an extended period of time at a full-scale WWTP. Despite several other reports that *Nitrospira* spp. often are the dominant NOB in

activated sludge systems (Whang *et al.*, 2009), the result from this study indicates that the knowledge about nitrifying bacteria populations at full-scale level still needs further investigations.

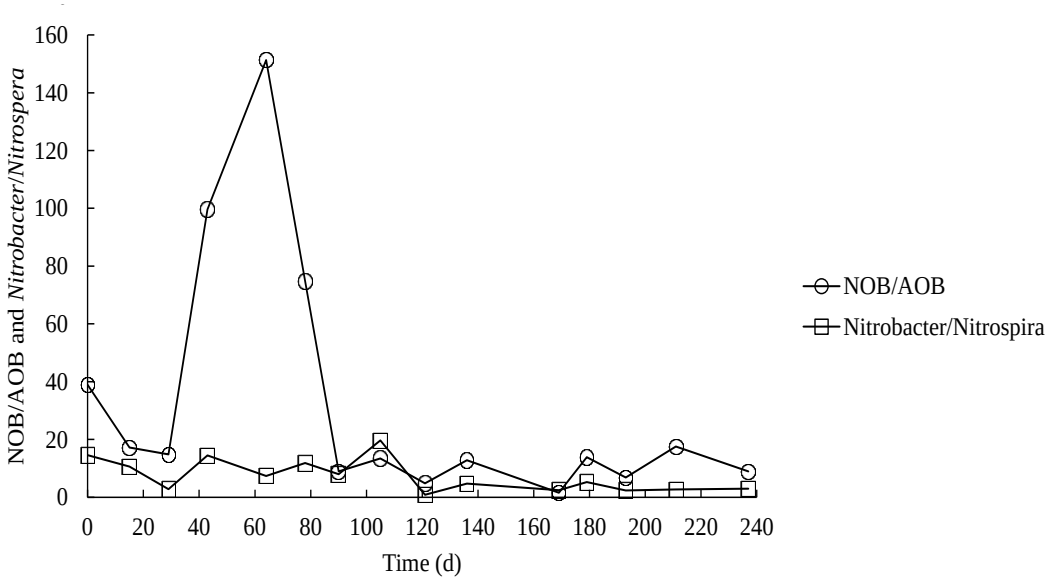

Fig. 6.2: Temporal changes in dominant nitrifiers' ratios in the full-scale wastewater treatment plant under study.

6.3.3 Effect of operational conditions on specific nitrification rate

In this investigation, it was observed that the efficiency and effectiveness of a nitrifying activated sludge system depended on several factors. The specific nitrification rate (q_N) showed a strong positive correlation to temperature (r 0.726, p 0.002). The q_N noticeably increased by 2.2-fold and exhibited 26.6 ± 10.7 mg $N\text{-}NH_4^+$ g^{-1} MLSS d^{-1} during the summer season when the temperature was elevated to 22.4 ± 2.7°C. The ANFIS model indicated that temperature exhibited the least error, demonstrating its relevance with respect to the q_N (Fig. 6.3a). The considerable impact of temperature on q_N could be attributed to the high seasonal variation observed during the monitored period. This observation was in agreement with previous studies, which stated that increasing temperature could enhance the rate of nitrification and nitrifier

growth (Tarre and Green, 2004). Xu *et al.* (2012) reported that growth rate of nitrifiers increased exponentially at a temperature range of 10 – 25°C, reaching a constant and optimal growth rate between 25 and 35°C, however, at 40°C the growth rate diminished drastically.

According to the ANFIS results in Fig. 6.3a, HRT has the second rank after temperature regarding the operational conditions affecting q_N. The current study witnessed a significant increase in q_N with a decrease in HRT (*r* -0.651, *p* 0.009). This result was in agreement with a study by Li *et al.* (2013), where q_N increased from 320 to 450 mg $N\text{-}NH_4^+$ g^{-1} MLSS d^{-1} (elevated by 41%) when the HRT decreased from 10 to 5 h, showing that the decline in HRT led to an enhancement in ammonia oxidation activity. On the contrary, other studies have reported that lower HRT results in increasing loading rates, which negatively affect the nitrifiers due to their competition with heterotrophic bacteria for substrates (oxygen and ammonia) (Nogueira *et al.*, 2002). In our study, the negative trend between q_N and HRT can be linked to the seasonal change, which was the key factor in the development of nitrification, since q_N increased to 26.6 ± 10.7 mg $N\text{-}NH_4^+$ g^{-1} MLSS d^{-1} in summer season when temperature increased to 22.4 ± 2.7ºC, while HRT declined to 4.3 ± 1.0 h.

In the current study, the q_N showed a significant positive correlation with ALR (*r* 0.571, *p* 0.026). However, it was found that high concentration of ammonia in the influent can negatively affect nitrification due to substrate inhibition by free ammonia (Kim *et al.*, 2006). This discrepancy could be attributed to the fact that the ALR of 120 - 140 g $N\text{-}NH_4^+$ m^{-3} d^{-1} observed in our study was still lower than the inhibitory limits reported in previous studies (Campos *et al.*, 1999; Kim *et al.*, 2006). For example, Campos *et al.* (1999) investigated the possibilities of obtaining a full ammonia oxidation at increasing ALR from 500 to 7,500 g $N\text{-}NH_4^+$ m^{-3} d^{-1}. Additionally, Kim *et al.* (2006) found that nitrification efficiency increased to 100% with $N\text{-}NH_4^+$ loading of 700 g m^{-3} d^{-1} at 18°C, and leachate was completely nitrified up to a load of 1,500 g $N\text{-}NH_4^+$ m^{-3} d^{-1} at 28°C.

Other operational conditions such as DO showed no significant effect on q_N (*r* -0.141, *p* 0.617). This observation was in agreement with a study by Kim *et al.* (2006), where the DO was not a limiting factor for nitrification. Additionally, the role of pH in our study was not significant when compared to other environmental conditions (*p* 0.332) due to its narrow range of variation. The pH range observed in this study (6.97 – 7.47) was within the optimal range for the metabolism and growth of autotrophic nitrifiers (Zhang *et al.*, 2012a).

Referring to the ANFIS model (Fig. 6.3a), the training and checking errors were comparable, which implies that no overfitting occurred. This means that selection of more than one input can be explored to re-build the ANFIS model. The plot in Fig. 6.3b showed all two input variable combinations and their influence on q_N.

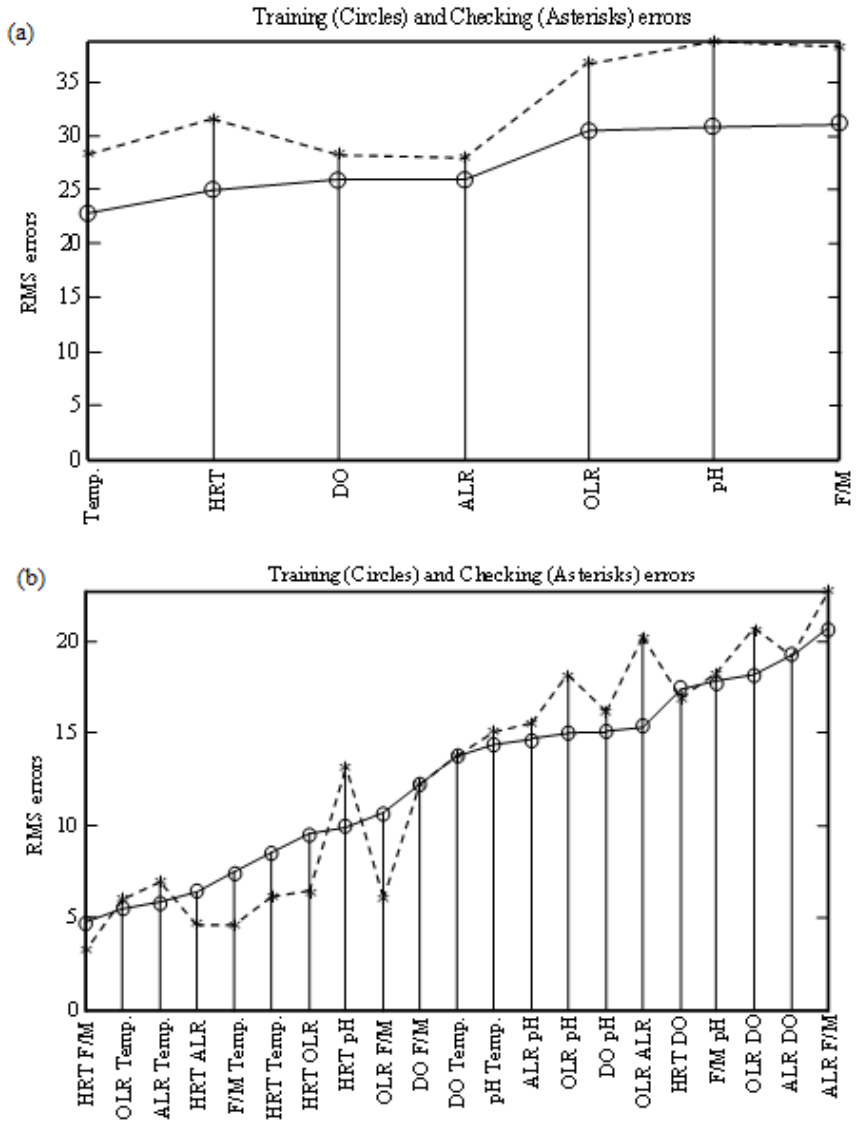

Fig. 6.3: Effect of operating conditions on q_N (The left-most input variable is the most relevance with respect to q_N). a) Every input variable's influence on q_N; b) All two input variable combinations and their influence on q_N.

It was found that HRT and F/M (the left-most input variable) formed the optimal combination of two input attributes. Additionally, it was observed that the minimal training and checking errors reduced significantly from that of the best 1-input model, indicating that the combination of HRT and F/M improved the prediction performance. A quadratic model was developed to confirm the ANFIS results by estimating the q_N over independent variables (HRT and F/M). The polynomial equation (**Eq. 12**),

including constant, linear, interaction, and squared terms, provided a determination of coefficient (r^2-value) with the experimental data of 0.50.

$$q_N = \mathrm{A} + \mathrm{B}(\mathrm{HRT}) + \mathrm{C}(\mathrm{F/M}) + D(\mathrm{HRT} \times \mathrm{F/M}) + \mathrm{E}(\mathrm{HRT})^2 + \mathrm{F}(\mathrm{F/M})^2 \qquad \text{(Eq. 12)}$$

A = -76.2257; B= 24.3626; C = 149.0787; D = -17.1593; E = -1.8146; F = -41.3636

Where, q_N in mg $N\text{-}NH_4^+$ g^{-1} MLSS d^{-1}; HRT in h; F/M in d^{-1}

6.3.4 Effect of operational conditions on AOB

The AOB was found to be HRT dependent with *r*-value of -0.741 (*p* 0.002). The high correlation between HRT and AOB was in accordance with the ANFIS results, where HRT exhibited the least training error among other operational conditions (Fig. 6.4a). It was found that AOB exhibited a negative correlation with the current range of HRT (4.3 – 6.3 h). Similar results were reported by Li *et al.* (2013) where an enhancement in AOB community was observed when HRT declined from 7 to 5 h. They also noted that AOB had a positive correlation when HRT became higher than 10 h (Li *et al.*, 2013). Our results showed a good correlation between AOB and temperature (*r* 0.517 and *p* 0.048), indicating that temperature had a positive impact on the AOB community. The positive correlation between temperature and AOB was previously illustrated by Park *et al.* (2008), who found that low temperature could not only decrease the attached biomass and activity of AOB, but also produced a change in the composition of the AOB species. According to ANFIS results, the two variables, HRT and temperature were the most relevant parameters with respect to AOB (Fig. 6.4a). Our results suggested that an increase in AOB during the summer season resulted from an increase in temperature in line with a decrease in HRT.

As noticed from the ANFIS model (Fig. 6.4b), interaction of HRT with other environmental factors provided a reliable assessment of the plant performance. This

might be due to the fact that the current study was based on full scale observations where several environmental parameters were interacting together in a dynamic manner. The influence of two environmental parameters indicated that the combination of HRT and temperature exhibited lower training error than either HRT or temperature by 64% and 70%, respectively. These results further confirm our hypothesis that the AOB dominated in summer season due to the impact of both HRT and temperature.

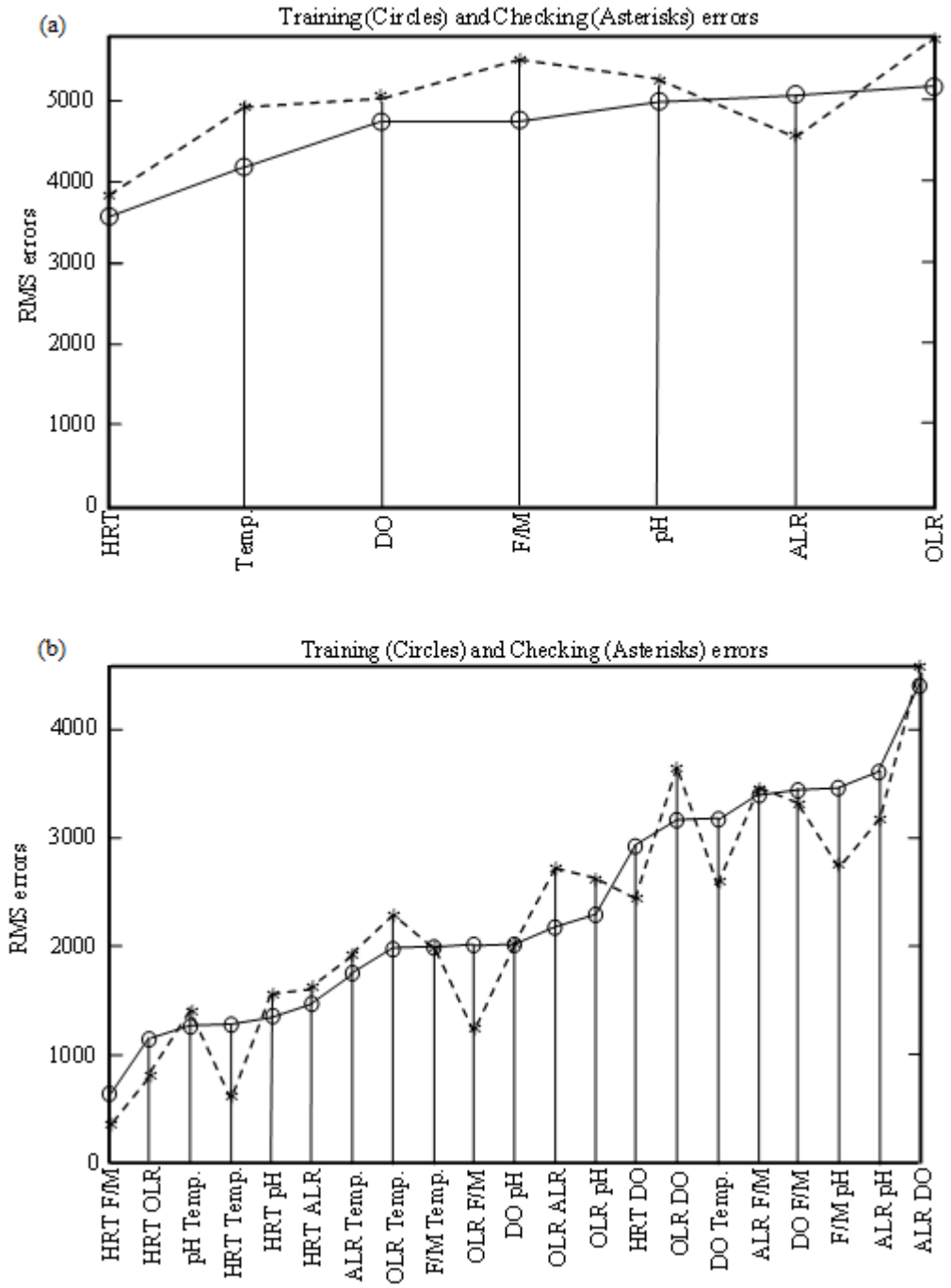

Fig. 6.4: Effect of operating conditions on AOB (The left-most input variable is the most relevance with respect to AOB). a) Every input variable's influence on AOB; b) All two input variable combinations and their influence on AOB.

Comparable to the q_N results, the ANFIS model indicated that the combination of HRT and F/M could be the most relevant input to the AOB (output) (Fig. 6.4b). Subsequently, HRT and F/M were employed in a polynomial function of degree 2 to determine their quadratic regression (**Eq. 13**). The estimated coefficient of determination showed r^2-value of 0.614.

$$AOB = \left(\mathrm{A} + \mathrm{B}(\mathrm{HRT}) + \mathrm{C}(\mathrm{F/M}) + D(\mathrm{HRT} \times \mathrm{F/M}) + \mathrm{E}(\mathrm{HRT})^2 + \mathrm{F}(\mathrm{F/M})^2\right) \times 10^{11} \quad \text{(Eq. 13)}$$

A = 1.0114; B= -0.2456; C = -0.6173; D = 0.0567; E = 0.0161; F = 0.1696

Where, AOB in copies l^{-1}; HRT in h; F/M in d^{-1}

6.3.5 Effect of operational conditions on NOB (*Nitrobacter* and *Nitrospira*)

The presence of NOB is necessary for wastewater treatment plants to achieve complete nitrification. In this investigation, *Nitrobacter* spp. indicated no significant correlation with the operational conditions ($p > 0.1$). This suggests that the abundance of *Nitrobacter* could tolerate seasonal and environmental variations. The ANFIS model indicated that F/M had the highest impact on *Nitrobacter* abundance (Fig. 6.5a). The strength of the relationship between the F/M and *Nitrobacter* spp. was further estimated by Pearson correlation coefficient which showed an *r*-value of 0.359 ($p > 0.1$). The importance of F/M was previously reported, where some organic compounds in the wastewater positively affected the activity of NOB (Kim *et al.*, 2006). The ANFIS model was also used to identify relationships between *Nitrobacter* spp. abundance and the combination of two operational parameters (Fig. 6.5b). The model indicated that F/M and temperature form the optimal combination of two input attributes. The polynomial equation of their quadratic interaction is presented in **Eq. 14** (r^2-value 0.49).

$$Nitrobacter = \left(\mathrm{A} + \mathrm{B}(\mathrm{F/M}) + \mathrm{C}(\mathrm{T}) + D(\mathrm{F/M} \times \mathrm{T}) + \mathrm{E}(\mathrm{F/M})^2 + \mathrm{F}(\mathrm{T})^2\right) \times 10^{11} \quad \textbf{(Eq. 14)}$$

A = -0.7143; B= -3.4550; C = 0.2579; D = -0.0050; E = 1.9650; F = -0.0062

Where, *Nitrobacter* in copies L^{-1}; F/M in d^{-1}; T in °C

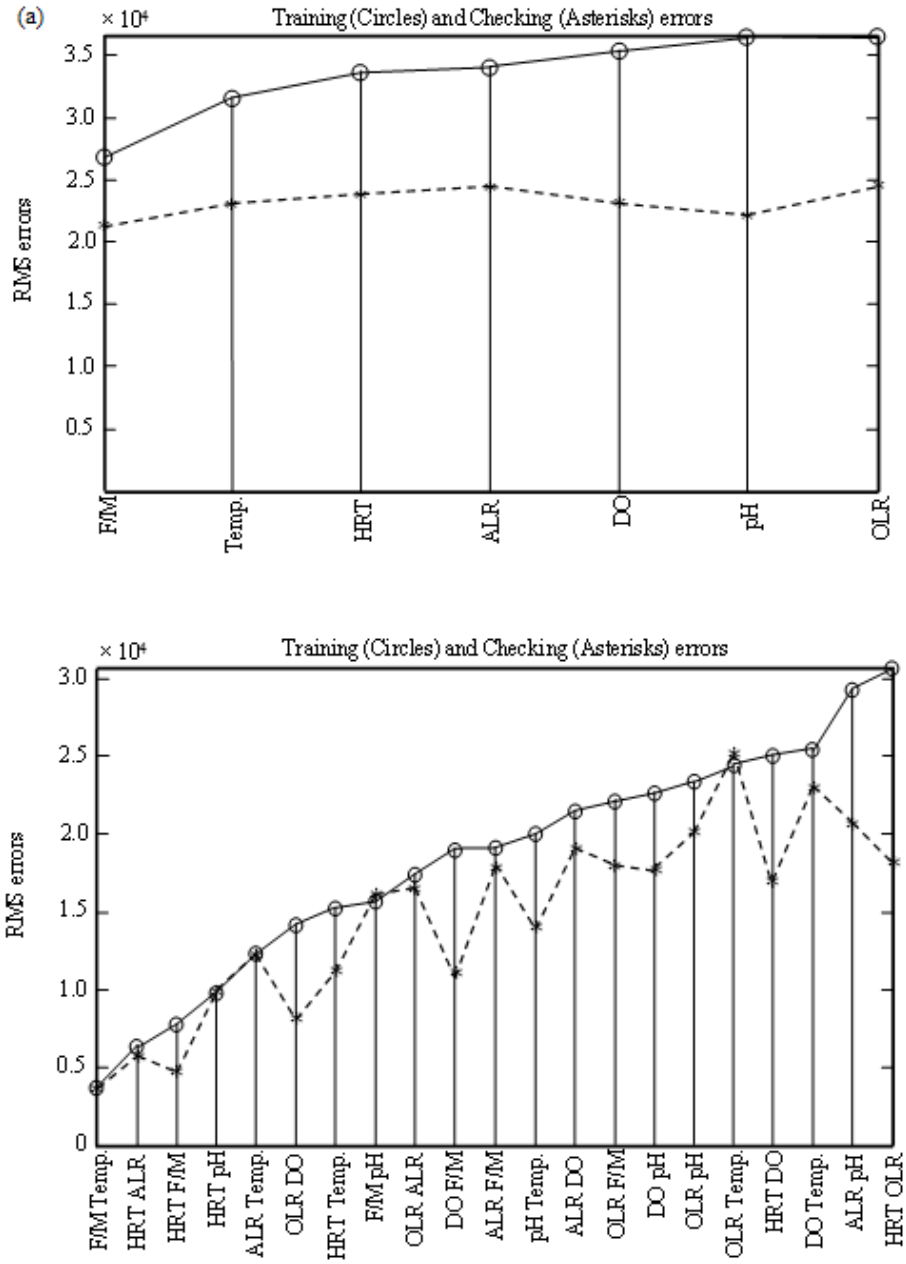

Fig. 6.5: Effect of operating conditions on *Nitrobacter* (The left-most input variable is the most relevance with respect to *Nitrobacter*). a) Every input variable's influence on *Nitrobacter*; b) All two input variable combinations and their influence on *Nitrobacter*.

Cross-correlation coefficients indicated that increase in *Nitrospira* spp. was significantly affected by a decline in HRT (*r* -0.627 and *p* 0.012). Similarly, the ANFIS model showed that the input HRT exhibited the least training error (Fig. 6.6a), which was in accordance with the *p* values. Additionally, HRT showed inverse correlation with *Nitrobacter* spp. (*r* -0.364 and $p > 0.1$). Similarly, Li *et al.* (2013) reported that a short HRT favoured the relative growth of NOBs, particularly the fast-growing *Nitrobacter* spp., in the conventional activated sludge system. As observed from the ANFIS results (Fig 6.6b), the combination of HRT and F/M exhibited a 4-fold lowering of the training error when compared to HRT individually. Therefore, the quadratic polynomial formula indicating the combinatory effect of HRT and F/M on *Nitrospira* can be presented by **Eq. 15** (r^2-value 0.716).

$$Nitrospira = \left(A + B(HRT) + C(F/M) + D(HRT \times F/M) + E(HRT)^2 + F(F/M)^2\right) \times 10^{11} \quad \text{Eq. 15}$$

A = 1.9980; B= -0.7133; C = 0.1599; D = -0.0062; E = 0.0636; F = -0.1640

Where, *Nitrospira* in copies l^{-1}; HRT in h; F/M in d^{-1}

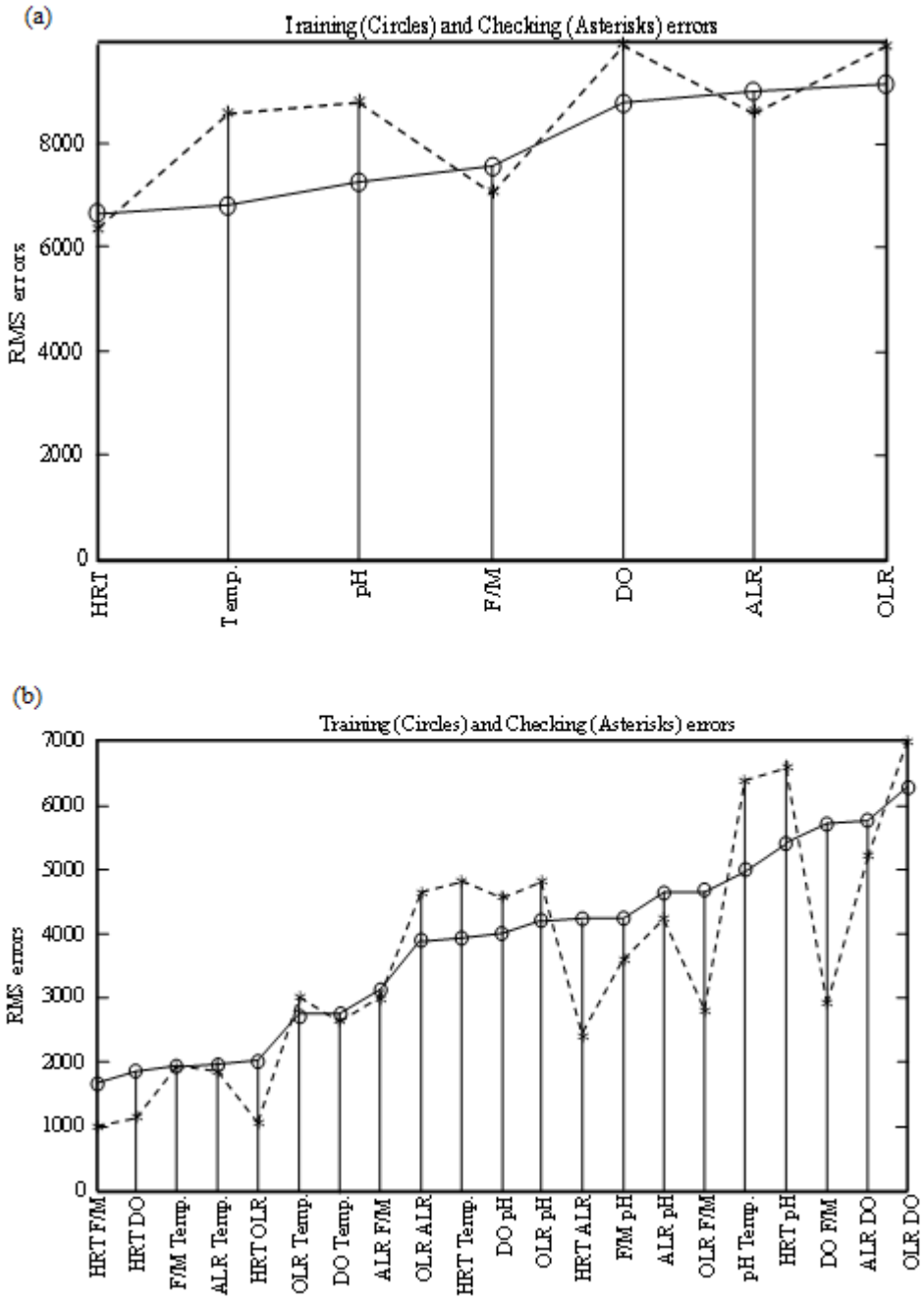

Fig. 6.6: Effect of operating conditions on *Nitrospira* (The left-most input variable is the most relevance with respect to *Nitrospira*). a) Every input variable's influence on *Nitrospira*; b) All two input variable combinations and their influence on *Nitrospira*.

In a similar study, Huang *et al.* (2010a) investigated the impact of environmental variables on *Nitrobacter* and *Nitrospira*. They observed that, *Nitrobacter* populations were negatively correlated to temperature (r -0.49 and $p < 0.001$), while the *Nitrospira* abundance showed a strong positive correlation to temperature (r 0.59 and $p < 0.0001$). Additionally, *Nitrobacter* populations were significantly and positively correlated to DO (r 0.38 and $p < 0.01$). However, *Nitrospira* abundance showed a significantly negative correlation to DO (r -0.46 and $p < 0.01$). Moreover, HRT showed a significant impact on *Nitrobacter* spp. (r 0.334 and $p < 0.05$). When comparing our findings with that of Huang *et al.* (2010a) both studies used the cross-correlation coefficients to determine the significant impact of operational parameters on *Nitrobacter* and *Nitrospira*. However, our study further applied artificial modelling technique in confirming the *r* results, as well as to determine the optimum combination of two input variables.

6.4 CONCLUSIONS

The AI approach succeeded in describing the effect of operating condition on nitrification process. Results from the ANFIS model were in accordance with Spearman's correlation coefficients, and it was concluded that:

- The q_N was noticeably increased by 2.2-fold and exhibited 26.6 ± 10.7 mg N-NH_4^+ g^{-1} MLSS d^{-1} when the temperature elevated from 16.5 ± 2.1 to 22.4 ± 2.7°C (r 0.726, p 0.002).
- The q_N was also significantly affected by these individual parameters: HRT (r -0.651, p 0.009) and ALR (r 0.571, p 0.026).
- HRT and F/M formed the optimal combination of two inputs affecting the q_N, and their quadratic equation showed r^2-value of 0.50.
- AOB increased in the summer season, when temperature was 1.4-fold higher than during winter (r 0.517, p 0.048), and HRT decreased by 31% as a result of rainfall (r -0.741, p 0.002).

- No single input had a significant effect on *Nitrobacter* spp., indicating that the abundance of *Nitrobacter* could tolerate seasonal and environmental variations once the population is established in the system.
- *Nitrospira* spp. increased by 3.6 times when HRT declined during the summer season (*r* -0.627, *p* 0.012).
- A polynomial function of 2nd degree for (HRT, F/M and AOB), (F/M, temperature and *Nitrobacter*) and (HRT, F/M and *Nitrospira*) showed r^2-values of 0.61, 0.49 and 0.72, respectively.

6.5 RESEARCH OUTPUTS

a) Journal article

1) **Awolusi, O. O.,** M. Nasr, Kumari, S. K. S., Bux, F. Artificial intelligence for the evaluation of operational parameters influencing nitrification and nitrifiers in an activated sludge process. Microbial Ecology DOI: 10.1007/s00248-016-0739-3 (In press)

CHAPTER SEVEN: GENERAL CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

This study examined the nitrifying community structure in a full-scale municipal wastewater treatment plant. The diversity and abundance of the different nitrifying populations in the plant was investigated, and their impact on nitrification efficiency was determined. A combination of FISH, PCR-clone library, quantitative polymerase chain reaction (qPCR) and next generation sequencing was applied. The effect of operating parameters and environmental conditions on nitrification was investigated. ANFIS, Pearson's correlation coefficient and quadratic models were used to determine and rank the plant's operational conditions that influenced the nitrification performance.

The research succeeded in establishing the following key findings:

- PCR-clone libraries of environmental samples was limited in taxonomic resolution, whereas pyrosequencing yielded a better detail in terms of microbial diversity in activated sludge.
- The species richness among AOB in the plant was not high. Hence, the community did not exhibit substantial congeneric homotaxis, which can impart high functional redundancy on them.
- Pyrosequencing reveals higher diversity of AOB in the reactor during summer that was characterized by higher temperature. Furthermore, *N. oligotropha* was only identified during summer. This indicates that higher temperature elicited increased AOB diversity.
- The AOB diversity was 6 times higher during summer than winter when a higher NH_3 removal rate and temperature were recorded. The AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133%

and 360% respectively when the season changed from winter to summer. This suggests that higher AOB diversity resulted in increased nitrification rate of the activated sludge.

- There was a significant difference in the nitrification efficiency of the plant during the two seasons monitored with a two-fold increase in nitrification observed during summer compared to winter.
- The cross-correlational approach could not establish any significant correlation between NOB population abundance and operational parameters except when ANFIS was used.
- There was a significant correlation between the nitrification rates of the plant and the batch specific nitrification rate (in terms of SAOR) determined in the laboratory experiment. Hence, the specific ammonia oxidation rate (SAOR) can be used as indication of nitrifying population densities and nitrification performance of wastewater treatment plants.
- The qN was noticeably increased by 2.2-fold and exhibited 26.6 ± 10.7 mg N-NH4+ g^{-1} MLSS d^{-1} when the temperature elevated from 16.5 ± 2.1 to 22.4 ± 2.7°C (r 0.726, p 0.002).
- Based on ANFIS, HRT and F/M formed the optimal combination of two inputs affecting the qN, and their quadratic equation showed r^2-value of 0.50.
- AOB increased in the summer season, when temperature was 1.4-fold higher than during winter (r 0.517, p 0.048), and HRT decreased by 31% as a result of rainfall (r -0.741, p 0.002).
- Using ANFIS, no single input had a significant effect on *Nitrobacter* spp., indicating that the abundance of *Nitrobacter* could tolerate seasonal and environmental variations once the population is established in the system.

SIGNIFICANCE AND NOVELTY OF THE RESEARCH FINDINGS

- This study can be considered as novel since it was the first time pyrosequencing was used for amoA locus profiling of AOB community composition in full scale wastewater plants.

- The findings from the current study have set the foundation for future application of these advanced molecular techniques in understanding the diversity and role of uncultured AOB in wastewater treatment
- This study suggests that vast population of novel, ecologically significant AOB species, which remain unexploited, still inhabit the complex activated sludge communities.
- The PCR yielded no positive amplification for AOA, which could suggests that archaea did not play an important role in the nitrification process of this WWTP and may not be ubiquitous in all nitrifying WWTPs.
- Furthermore, novelty was demonstrated in this study since earlier studies on nitrification used the cross-correlation coefficients to determine the significant impact of operational parameters on nitrifiers. This study in addition to cross-correlation, further applied artificial modelling technique in confirming the results, as well as to determine the optimum combination of two input variables.
- This study indicated that artificial intelligence could be used as a tool to elucidate the factors influencing nitrification process in full-scale wastewater treatment plants. It further indicates that ANFIS, is better than conventional Spearman correlation, since the latter could not (in some cases) detect correlation between microbial populations (NOB) and plant's operating parameters due to the non-linearity of the interactions in wastewater treatment.

RECOMMENDATIONS

- There is need for more research effort towards isolating and characterization of the nitrifying populations (AOB, AOA and NOB) in wastewater, as pyrosequencing revealed a large diversity of uncultured AOB and previously unidentified organisms.
- The operational conditions of the WWTP including HRT and loading rates should be manipulated in order to overcome the diminishing nitrification arising from low temperature during winter.

- The findings of the research would be workshopped with staff from Umgeni Water Board whom commissioned the research.

REFERENCE

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APPENDICES

APPENDIX ONE: Reagents preparation for thermo scientific™ gallery™ automated photometric analyzer (vantaa, finland)

A. TOTAL OXIDISED NITROGEN (TON)

Principle: Nitrate was reduced to nitrite by hydrazine under alkaline conditions. The total nitrite ions are then reacted with sulphanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions to form a pink azo-dye. The absorbance was measured at 540nm and was related to the TON concentration by means of a calibration curve.

Stock Solutions Reagent Preparation

Sodium Hydroxide (Reagent 1): 0.8g Sodium Hydroxide (NaOH) was dissolved in 100ml of distilled water. This solution was stable for 1 day.

Reductant (Reagent 2): 0.325g of hydrazine sulphate $N_2H_4.H_2SO_4$ was dissolved in 400ml distilled water. 0.75ml of stock copper sulphate solution and 5ml of zinc sulphate was adde and maked up to 500ml with distilled water. This solution was stable for 1 month.

Stock Solutions required for Reductant:

Copper Sulphate Solution: 0.78g $CuSO_4.5H_2O$ dissolved in 200ml of distilled water. This solution was stable for 1 month.

Zinc Sulphate Solution: 9.0g Zinc Sulphate was dissolved in 200ml distilled water. This solution was stable for 1 month.

Colour Reagent (Reagent 3): 50ml of concentrated Phosphoric acid (HP_3O_4) was carefully added to 500ml of distilled water. 5g of sulphanilamide was added and dissolved completely before adding 0.25g N-(1-naphthyl)-ethylenediamine

dihydrochloride. Then diluted to 1000ml with distilled water, stored in an amber bottle between 2 - 8°C. This solution was stable for 1 month.

TON Standard Solution: Dissolve 1.6306g of dried Potassium Nitrate (KNO_3) in 1000ml volumetric flask of distilled water. Stored between 2 - 8°C this solution was stable for 1 month.

Measurement: Concentration was measured using Thermo Gallery photometric analyser (Thermo Scientific, UK)

B) NITRITE (NO_2)

Principle: Diazotization of sulphanilamide by nitrite in the presence of Phosphoric acid, at 1.9 pH and the subsequent formation of an azo dye with N-1-naphthylethylenediamine (NEDD). The absorbance of this compound was measured spectrophotometrically at 520nm and was related to the nitrite by means of a calibration curve.

Stock Solutions Reagent Preparation

Colour Reagent: 50ml of concentrated Phosphoric acid (HP_3O_4) was carefully added to 500ml of distilled water. 5g of sulphanilamide was added and dissolve completely before adding 0.25g N-(1-naphthyl)-ethylenediamine dihydrochloride. Then, diluted to 1000ml with distilled water, stored in an amber bottle between 2-8°C. This solution was stable for 1 month.

Nitrite Standard Solution 100mg/l: 0.493g of dried sodium nitrite ($NaNO_2$) was dissolved in 1000ml distilled water.

Measurement: Concentration was measured using Thermo Gallery photometric analyser (Thermo Scientific, UK)

C) AMMONIA NITROGEN (NH_3–N).

Principle: Ammonia reacts with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. This reacts with salicylate ions in the presence of sodium nitroprusside at around pH 12.6 to form a blue compound. The absorbance of this compound was measured spectrophotometrically at wavelength 660nm and was related to the ammonia concentration by means of a calibration curve.

Stock Solutions Reagent Preparation

Sodium Salicylate Solution (Reagent 1): 65g of Sodium Salicylate [$C_7H_5O_3Na$] and 65g of tri-Sodium Citrate [$C_6H_5Na_3O_7.2H_2O$] was dissolved in 400ml ammonia free deionised water, the pH was adjusted to less 8.0 of necessary with 0.4% Nitric acid. 0.49g of Sodium Nitroprusside [$Na_2[Fe(CN)_5NO].2H_2O$] was added, dissolved and maked up to 500ml with ammonia free deionised water. This solution was stable for 1 month.

D.I.C Solution (Reagent 2): 16g Sodium Hydroxide [NaOH] was dissolved in 250ml ammonia free deionised water. Cooled and 1.0g of Sodium Dichloroisocyanurate [$Cl_2Na(NCO)_3.2H_2O$] as added, allowed to dissolve and make up to 500ml with ammonia free deionised water. This solution was stable for 1 month.

Ammonia Standard Solution – 1000mg/l as N: 3.819g of dried Ammonium Chloride (NH_4 Cl) was dissolved in 1000ml of ammonia free water. Stored between 2 - 8°C, the solution was stable for 1 month.

Measurement: Concentration was measured using Thermo Gallery photometric analyser (Thermo Scientific, UK)

APPENDIX TWO: Determination of total chemical oxygen demand (cod) in wastewater sample

Procedure

Water samples for analysis were collected in bottles from the sampling points of both full and pilot-scale UASB reactors. The closed reflux colorimetric method (APHA, 1998) with was used. The COD concentration in the wastewater was determined according to the standard method 5220D, microwave digestion (Milestone Start D, Sorisole, Italy) was first used to digest the samples at 150 °C for 1 h in COD vials containing the Digestion Solution (0–15,000 mg COD/L). Then, the concentration was measured using Aquakem Gallery discrete autoanalyser (Thermo Scientific, UK). Five millilitre sample was measured and transferred into a microwave Teflon tube. The Teflon tube was inserted into the microwave safety vessel. Three millilitre (3ml) of digestion solution (0.01667M, $K_2Cr_2O_7$) and 7 ml of sulphuric acid reagent were carefully added to the sample. The sample was carefully mixed, tightly capped and digested at 150^{o}C for 55 minutes using the microwave digester (Milestone Start D, Sorisole, Italy). Standard were prepared and blank reagent was also digested with the sample as control. After cooling, the solution was transferred into Gallery cuvettes for colorimetric analysis using the Thermo Gallery photometric analyser. Results were recorded in mg/L.

APPENDIX THREE: Fluorescent *in- situ* hybridisation

1 x Phosphate-Buffered saline (PBS)

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

All components were dissolved in distilled water, made up to 1000ml and pH was adjusted to 7.4 and autoclaved.

Paraformaldehyde (4%)

65 ml Distilled water

4g Paraformaldehyde

2M NaOH

Distilled water was heated to 60˚C. Paraformaldehyde was added. One drop 2M NaOH was added and stirred rapidly until solution clarified. This was removed from heat and 33 ml of 3 x PBS was added. The pH was adjusted to 7.2 with HCl, filtered and cooled cool down to 4˚C. This solution was made up fresh for each use.

Cell Fixation

1 x PBS

Paraformaldehyde

Ethanol

Procedure: The samples were washed twice with 1 x PBS. Three volumes 4% paraformaldehde was used with 1 volume of sample for Gram negative bacteria, whilst one volume of ethanol was used with one volume of sample for Gram positive bacteria. This was stored at 4˚C for 1-3 hrs. This was centrifuged and supernatants were discarded. Pellets were washed twice with 1 x PBS. Supernatant were discarded. To store, the pellets was resuspended in 1 x PBS. (50% of original sample volume) and absolute ethanol (50% of original sample volume). This was stored at -20˚C for further analysis.

Pre-treatment of Microscope Slides

Teflon-coated slides were cleaned by soaking in diluted Poly-L-Lysine solution (1:10-Sigma Diagnostics, USA) for 15 minutes at room temperature

Solutions:

(1:10) Poly – L – Lysine (Sigma diagnostics, U.S.A. Procedure no. P8920)

Allow solution to reach room temperature (18 - 26˚C) before use.

Dilute 1:10 with dH_2O.

Procedure:

- Clean slide surface by soaking in a warm soap solution for 1 h, rinse thoroughly with dH_2O and air dry.
- Immerse clean slides in diluted (1:10) Poly-L-Lysine solution for 10 min.
- Drain Poly-L-Lysine solution from slide and dry in an oven for one hour at 60˚C or at room temperature overnight.
- Store treated slide in a sealed container.

Cell dispersion using sonication

Introduction:

The sludge was compact and thick. In order to get to all cells in the sludge, it is often necessary to disperse the cells by sonication using a sonicator at low frequency, after paraformaldehyde fixation.

Procedure:

- Fill a 2 mL Eppendoff tube with 1 mL fixed sample.
- In order to prevent spillage of the sample, slightly cover the opening of the Eppendoff tube with the cover.
- Insert the probe so that the tip of the probe is approximately 1 cm above the base of the Eppendoff tube and sonicate 8 watts for 5 to 10 min for an ML =2500 - 3000

Immobilization of fixed microbial cells on treated microscope slides

Procedure:

- Dilute sonicated sample in ddH_2O
- Spot 10 µL of sonicated sample onto each well of a pre-treated microscope slide.
- Allow the spots to air dry
- Dehydrate the spots through successive passages through 50%, 80% and 100% v/v ethanol washes (lasting 3 min each).
- Subsequently slides can be stored dry at room temperature in a desiccators indefinitely.

Calculation of working probe dilution

Since 50 ng of probe is required per hybridization and 10 µL of hybridization solution is required per spot, a working concentration of 5 ng/µL is used.

M_1V_1 = M_2 V_2

(840 ng/µL)(1 µL) = (5ng/µL)(V_2)

V_2 = (840 ng/µL X 1 µL) / 5ng/µL

164 µL hyb buffer + 1 µL probe

Therefore 16 hybridizations can be performed using 10 µL of probe/hybridization solution (containing 50 ng of probe) per hybridization.

Hybridization Solution (pH 7.2; 20%)

5.26g NaCl

0.24g Tris

0.01 SDS

0.16g EDTA

20 ml fortunately*

All components were dissolved in 80 ml distilled water. Formamide was added and solutions were made up to a final volume of 100 ml.

*All chemical and quantities remained constant for all percentages of hybridization solutions. Only the formamide concentration changes as per the percentage required. E.g. 30% hybridisation solutions will require 30 ml formanide.

Wash Buffer

2.42g Tris

1.86g EDTA

0.1g SDS

12.56g 5M NaCl (20% wash buffer)

8.76g (25%)

6.55g (30%)

4.68g (35%)

3.27g (40%)

2.33g (45%)

NaCl was added to 1ml 1M Tris-HCl, 50µl 10% SDS, sterile distilled water and made up to 50 ml. Add 5M NaCl according to the breakdown above, 1 Ml of 1M Tris/HCl, 50 µl 10% SDS, sterile distilled water to make up to 50 ml.

5M NaCl stock solution

2150µl (20%)

1020 (30%)

700 (35%)

460 (40%)

300 (45%)

Oligonucleotide Probe Preparation

Probes were thawed and centrifuged at 3000 rpm for 1 minute. Probes were removed (10 µl) and placed in sterile Eppendorf tubes (stock). Stock probes were then further distributed (1 µl) into sterile Eppendorf tubes and stored at -20˚C until required.

Whole cell hybridisation

- Strips of fitter paper were soaked in the appropriate hybridisation solutions and placed in polypropylene chambers.
- The hybridization chamber were prepared by inserting filter paper in into 50ml falcon tube and pour the remaining buffer in the tube for it to soak, cover tube with foil to protect from light.
- Immediately slide were transferred into hybridisation chambers and incubated for overnight at 46˚C.
- Probe/buffer mixture (10 µl) were placed on each well.
- Slide were then placed in the pre-warmed chamber and incubated to allow hybridisation at 46˚C for 2 h.
- Slides from the oven were gently wash twice using pre-warmed (48°C) wash buffer and the slide were inserted into the remaining wash buffer and incubate at 48°C for 2 h.
- Slides were washed twice with ddH_2O and air dried. DAPI solution (10 µl) was placed over each well and allowed to stain for 10 minutes in the dark.
- Slides were subsequently wash with ddH_2O and air dried.
- Vectashield (1 drop) was added to each well, covered with a cover-slip and sealed with clear varnish around the edges to prevent movement of the cover-slip and escape of the Vectashield.

APPENDIX FOUR: Publications

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REVIEW

Ecophysiology of nitrifying communities in membrane bioreactors

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Abstract Membrane bioreactors (MBRs) are rapidly becoming the technology of choice over conventional activated sludge treatment systems due to their smaller footprint, reduced sludge production, rapid start-up of biological processes, complete removal of suspended solids and better effluent quality. The retention of sufficient amount of slow-growing nitrifiers makes it feasible for the MBRs to achieve strong tolerance against the shock loads with stable and highly efficient nitrogen removal. Various studies have focused on the ecophysiology of nitrifiers in MBRs as well as their distinctive operational parameters as well as their impact on the selection and activity of nitrifying community. Several techniques have been employed over the years to understand the nitrifying community and their interaction within the MBR system, which led to its modification from the initial design. This review focuses on the identification of optimal operational and environmental conditions for efficient nitrification in MBRs. The advantages and limitations of different techniques employed for investigating the nitrifying communities in MBRs are also emphasized.

Keywords Ammonia-oxidizing archaea · Proteobacteria · Nitrification · Ammonia-oxidizing bacteria · Nitrite-oxidizing bacteria · Activated sewage sludge

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Introduction

Wastewater in its raw and untreated form usually contains a heavy load of nutrients, chemicals and pathogens, which results in pollution and widespread waterborne diseases when discharged into the receiving aquatic environments (Holeton et al. 2011; Uan et al. 2013). Inorganic nitrogen (ammonia and nitrate) and phosphates at a higher level (>0.05 mg/L) may stimulate eutrophication (WEF 2009; Chuai et al. 2012). Even at low concentrations (<0.2 mg/L), the unionized ammonia has been reported to be acutely toxic to fish (Yang et al. 2010; Chen et al. 2012). Globally, there are national environmental agencies which regulate and oversee compliance with the effluent discharge limits which includes dissolved organic carbon (biological or chemical oxygen demand) as well as nitrogen compounds and phosphates (Holeton et al. 2011).

The epidemics in London between 1831 and 1866, which resulted from water pollution, necessitated the requirement of specific regulations on wastewater treatment and discharge. This in turn prompted the construction and operation of wastewater treatment plants (WWTPs) and the eventual development of advanced wastewater treatment technologies (Glicksman and Batzel 2010; Sciampacone 2013). Although there are biological and chemical WWTPs, the former are usually the preferred choice as they are more environmentally friendly (Akpor and Muchie 2010). Activated sludge, membrane bioreactors (MBRs), trickling filters, up-flow anaerobic sludge blanket reactors, lagoons and artificial wetlands are the most commonly used biological treatment processes for both industrial and domestic wastewaters (Akpor and Muchie 2010; Heffernan et al. 2011). However, the design and operation of treatment systems are constantly being improved for better efficiency and robustness. The current

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ENVIRONMENTAL MICROBIOLOGY

Artificial Intelligence for the Evaluation of Operational Parameters Influencing Nitrification and Nitrifiers in an Activated Sludge Process

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Abstract Nitrification at a full-scale activated sludge plant treating municipal wastewater was monitored over a period of 237 days. A combination of fluorescent in situ hybridization (FISH) and quantitative real-time polymerase chain reaction (qPCR) were used for identifying and quantifying the dominant nitrifiers in the plant. Adaptive neuro-fuzzy inference system (ANFIS), Pearson's correlation coefficient, and quadratic models were employed in evaluating the plant operational conditions that influence the nitrification performance. The ammonia-oxidizing bacteria (AOB) abundance was within the range of 1.55×10^{8} 1.65×10^{10} copies L^{-1}, while *Nitrobacter* spp. and *Nitrospira* spp. were 9.32×10^{9} 1.40×10^{11} copies L^{-1} and 2.39×10^{9} 3.76×10^{10} copies L^{-1}, respectively. Specific nitrification rate (q_N) was significantly affected by temperature (r 0.726, p 0.002), hydraulic retention time (HRT) (r 0.651, p 0.009), and ammonia loading rate (ALR) (r 0.571, p 0.026). Additionally, AOB was considerably influenced by HRT (r 0.741, p 0.002) and temperature (r 0.517, p 0.048), while HRT negatively impacted *Nitrospira* spp. (r 0.627, p 0.012). A quadratic combination of HRT and food-to-microorganism (F/M) ratio also impacted q_N (r^2 0.50), AOB (r^2 0.61), and *Nitrospira* spp. (r^2 0.72), while *Nitrobacter* spp. was considerably influenced by a polynomial function of F/M ratio and temperature (r^2 0.49). The study demonstrated that ANFIS could be used as a tool to describe the factors influencing nitrification process at full-scale wastewater treatment plants.

Keywords Adaptive neuro-fuzzy inference system · Ammonia-oxidizing bacteria · Nitrite-oxidizing bacteria · Operational parameters · Statistical tools

Introduction

Ammonia toxicity is one of the several forms of nitrogen pollution that exist in aquatic environments [1]. Ammonia can be harmful to aquatic life and contributes to eutrophication of water bodies [2]. Moreover, at sufficiently high levels, ammonia can create a large oxygen demand in receiving waters, where the total consumption of oxygen is 4.57 g O_2 g^{-1} N-NH_4^+ oxidized [3]. In this context, ammonia removal from wastewater is one of the primary tasks to protect water resources from pollution discharges. Biological nitrification-denitrification is the most commonly used process for removing nitrogen from wastewater [4]. During nitrification process, ammonia (NH_3) is converted to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB), while nitrite-oxidizing bacteria (NOB) convert the NO_2^- to nitrate (NO_3^-) [5]. In denitrification, NO_3^- is reduced to nitrogen gas (N_2) in a four-step process, in which NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O) are electron acceptors in energy generating reactions [6].

Nitrifying bacteria are highly sensitive to changes in environmental parameters and plant operational conditions, such as pH, temperature, dissolved oxygen (DO) level, organic loading rate (OLR), ammonia loading rate (ALR), and hydraulic retention time (HRT) [7]. Neutral to slightly basic pH range (7.5 to 8.5) has been reported as optimum for efficient nitrification [8]. According to an earlier finding, at neutral pH,

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