Detection and Quantification of Nitrifying Bacteria from South African Biological Nutrient Removal Plants

This work is submitted in fulfilment for the requirements for the degree of Doctor of Technology: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology.

Nishani Ramdhani
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SUPERVISOR: Professor Faizal Bux

CO-SUPERVISOR: Dr S.K. Sheena Kumari

DECLARATION BY STUDENT

Detection and Quantification of Nitrifying Bacteria from South African Biological Nutrient Removal Plants

N. Ramdhani
2012

I declare that the thesis herewith submitted for the D Tech: Biotechnology at the Durban University of Technology has not been previously submitted for a degree at any other University.

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Nishani Ramdhani

I hereby approve the final submission of the following thesis.

_____	_____
Prof. F. Bux	Dr. S.K. Sheena Kumari
D. Tech (DUT)	PhD (Mangalore University)

This _____day_____ of 2012, at the Durban University of Technology.

ABSTRACT

Nitrification is a crucial step in biological nutrient removal (BNR) processes, mostly carried out by a group of nitrifying bacteria which includes ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). Nitrification failure has proven to be a common operational problem in full-scale wastewater treatment plants (WWTP) since nitrifying bacteria are very sensitive to sudden changes in environmental or plant operating conditions. The current investigation was carried out to advance our understanding of the distribution of nitrifying bacterial populations and their performance at three different BNR plants in KwaZulu-Natal, South Africa. The latest molecular techniques such as fluorescent *in situ* hybridisation (FISH)-confocal scanning laser microscopy (CSLM), polymerase chain reaction (PCR) and real-time quantitative PCR (Q-PCR) were applied to detect and quantify nitrifying bacteria. When using FISH to target the nitrifying population, it necessitated optimising pre-treatment protocols of the samples to improve accuracy during quantification. Sonication was found to be the superior method of dispersion based on the least disruption of nitrifier cell integrity, irrespective of the sludge type. The effect of plant configurations and wastewater characteristics on the distribution of the nitrifying bacterial population and subsequently on the nitrification performance was evaluated using FISH and PCR. FISH results revealed the dominance of *Nitrosomonas* (AOB), *Nitrobacter* (NOB) and *Nitrospira* (NOB) for all BNR plants. The 16S rRNA analysis of PCR products using genus-specific primers, revealed the presence of more than one species of the same group at these plants. *Nitrosomonas* spp. including *Nitrosomonas halophila*, *Nitrosomonas eutropha*, *Nitrosomonas europaea*, *Nitrosomonas aestuarii* and an unidentified *Nitrosomonas* spp. were found to dominate among the AOB and *Nitrobacter vulgaris, Nitrobacter alkalicus, Nitrobacter hamburgensis* and an unidentified *Nitrobacter* spp. were the dominant species for NOB. Among these species, *Nitrosomonas aestuarii*, *Nitrosomonas europaea*, *Nitrobacter hamburgensis* were detected only from the industrial wastewater samples. The efficiency of two commonly used techniques viz., FISH and Q-PCR for the detection of nitrifiers from WWTP were also studied and compared, specifically targeting *Nitrobacter* sp. Even though there were slight variations in the quantification results, changes in the *Nitrobacter* community at these plants were consistent for both FISH and Q-PCR results. Both techniques have their own limitations and advantages. This study has helped to add to the platform of understanding the distribution

and activity of nitrifying bacteria by correlating population dynamics with the operational parameters at full-scale level. The observations made in this study will assist researchers and engineers to minimise future nitrification failure at full-scale BNR plants. This study also confirmed the highly complex activities of wastewater treatment processes, which is dependant on a number of factors. Specific AOB or NOB predominant in wastewater rather suggests that the wastewater type and characteristics may contribute to significantly different microbial environments. Among the AOB, *Nitrosomonas* dominated at all BNR plants throughout the study period and for NOB both *Nitrobacter* and *Nitrospira* were found in significant numbers but their dominance varied across the plants. These dissimilar, distinct distribution patterns could be attributed to their environment which in turn impacted on the nitrification performance of the system. It was also noted that the co-existence of more than one group of these communities at the same plant could help the plant escape complete functional failures such as nitrification, due to sudden changes in temperature and substrate concentrations, as this function can be performed by different groups. Although it would have been meritorious to conduct a nitrogen balance in this study, this was not possible since the research focused on full-scale systems.

DEDICATION

This thesis is dedicated to the memory of my late beloved **father,** who taught me the true meaning of hard work, dedication, perseverance and whose words of inspiration and encouragement in pursuit of excellence, still linger on.

Thank you Dad for being my silent inspiration……..

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ABBREVIATIONS

Anammox	: Anaerobic ammonium oxidizers
AOB	: Ammonia oxidizing bacteria
BNR	: Biological Nutrient Plants
CARD-FISH	: Catalyzed Reporter Deposition Fluorescence In Situ Hybridization
CO_2	: Carbon Dioxide
CSLM	: Confocal Laser Scanning Microscopy
DGGE	: Denaturing Gradient Gel Electrophoresis
DNA	: Deoxyribonucleic Acid
DO	: Dissolved Oxygen
EBPR	: Enhanced Biological Phosphate Removal
FISH	: Fluorescent *in situ* hybridization
FISH-MAR	: Fluorescent *in situ* hybridization- Microautoradiography
GAO	: Glycogen Accumulating Organism
MLSS	: Mixed Liquor Suspended Solids
N_2	: Nitrogen gas
N_2O	: Nitrite oxide
NH_3^+	: Ammonia
NH_4^+	: Ammonium
NO	: Nitrous Oxide
NO_2^-	: Nitrite
NO_3^-	: Nitrate
NOB	: Nitrite Oxidizing Bacteria
O_2	: Oxygen
P	: Phosphorous

PAO	: Polyphosphate Accumulating Organisms
PCR	: Polymerase Chain Reaction
PHA	: Polyhydroxy Alkanoates
Q-PCR	: Real-Time Quantitative Polymerase Chain reaction
rDNA	: Ribosomal Deoxyribonucleic acid
RNA	: Ribonucleic Acid
rRNA	: Ribosomal ribonucleic acid
WWTP	: Wastewater Treatment Plant

PREFACE

Publications

- **N. Ramdhani**, S. Kumari and F. Bux. 2010. Impact of pre-treatments on nitrifying bacterial community analysis from wastewater using FISH and CSLM. *Journal of General and Applied Microbiology*, **56** (2): 101-106.

- **N. Ramdhani**, S.K Sheena Kumari and F. Bux. 2013. Distribution of *Nitrosomonas*-related AOB and *Nitrobacter*-related NOB in two full-scale Biological Nutrient Removal (BNR) plants. *Water Environment Research* (in press).

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- **N. Ramdhani**, S. Kumari, F. Bux. 2011. 2nd IWA Development Congress and Exhibition, Kuala Lumpur, Malaysia, 21-24 November 2011: "Microbial ecology of *Nitrosomonas*-related AOB and *Nitrobacter*-related NOB in three activated sludge plants treating different effluents"

- **N. Ramdhani**, S.K. Sheena Kumari, F. Bux. 2012. WISA - “water footprints”, Cape Town, South Africa, 6-9 May 2012: “Application of FISH vs. QPCR techniques for *in situ* detection and quantification of Ammonia-Oxidizing Bacteria (AOB) from two full-scale wastewater treatment plants”

- **N. Ramdhani**, S.K. Sheena Kumari, F. Bux. 2012. IWA World Water Congress and Exhibition, Busan, Korea, 16-21 September 2012: “Nitrification performance evaluation and quantification of dominant nitrifying bacterial communities at two full-scale domestic wastewater treatment plants in KwaZulu-Natal, South Africa”

- **N. Ramdhani**, S.K. Sheena Kumari, F. Bux. 2013. 5th International Conference, Microbial Ecology and Water Engineering, Ann Arbor, Michigan, United States, 7-10 July 2013: “Evaluation of Dominant Nitrifying Bacteria and their Nitrification Performance in Full-Scale BNR Plants”

CHAPTER ONE: INTRODUCTION

The most precious natural resource on our planet is undoubtedly water, comprising over 70% of the Earth's surface. Water is essential for everything to grow and prosper. Previously focus was placed on providing sufficient water to satisfy human demands, with little attention being given to the environment. The degree of industrialisation and urbanisation, standards of living, population size and agricultural activities are constantly on the increase. This has impacted immensely on the use and quality of available water sources. Current resources are limited which results in the need for present and future changes to urban water and wastewater treatment systems (Holtzhausen, 2002; El Kharraz *et al*., 2012). A far greater problem faces developing countries, like South Africa, which experience moderate to high stresses on our freshwater resources. There are a vast number of qualitative and quantitative inconsistencies in the water available to rural communities compared to that of the urban areas (Daigger, 2009). Thus, appropriate management strategies need to be implemented to optimise the use of water sources and ensure the efficient disposal of polluted water (Groom and Koundouri, 2011).

Globally, access to safe drinking water has improved substantially over the last few decades (Baroni *et al*., 2007; Eswarappa and Bhatia, 2013). In developing countries 90% of all wastewater still gets discharged, untreated, into local water bodies, which limits a safe water supply (UNEP International Environment, 2002; Breisha and Winter, 2010). The ever increasing water demand not only affects water bodies like rivers and lakes, but also degrades groundwater resources. Besides the nitrogen that is emitted into the atmosphere during the burning of fossil fuels, the two major sources of anthropogenic nitrogen released into the environment are from wastewater and fertilisers. The increasing population causes a higher wastewater deposition and in under-developed regions, results in untreated discharge into the environment.

Wastewater can be categorised as having an industrial or domestic composition. Industrial wastewater is that which has been contaminated in some way by industrial or commercial activities prior to its release into the environment or its reuse. Industrial waste can be obtained from agricultural, iron and steel industries, mines and quarries, food, chemical,

nuclear industries, etc. Domestic waste refers to that obtained primarily from residential waste. The major pollutants in wastewater comprise of acids, alkalis, organic and inorganic salts, bacteria, nutrients, suspended solids, toxic metals and metalloids which have either a direct and or an indirect impact on aquatic life (Makaya *et al*., 2007; Gadd, 2010; Singh *et al*., 2012). Among these pollutants, nitrogen ranks at the top of the list (Liu *et al*., 2012). Therefore, efficient elimination of nitrogen in modern biological nutrient removal (BNR) plants is crucial to reduce their discharge into the environment.

Increased anthropogenic nitrogen deposition have destabilised ecosystems globally by having unintended environmental consequences whereas elevated nitrite and nitrate levels in drinking water can have severe health consequences (Galloway *et al.*, 2008). Atmospheric reactions of emitted ammonia, nitric oxides and sulphur oxides result in fine particle formation (Davidson *et al*., 2012), which increase the risk of cardiovascular and pulmonary diseases (Neuberger *et al*., 2007). Additionally, ammonia and nitrite are highly toxic to aquatic life (Barik *et al.,* 2011). Nitrogen deposition effects would be far more drastic without the buffering capacity of nitrogen cycling microorganisms. However, to effectively monitor ecological changes a greater understanding of the mechanisms involved in the global nitrogen cycle is essential (Chapter 2; Figure 2.2) to understand the vast microbial diversity involved in nitrogen conversion (Hanke and Strous, 2010). During the last decade, groundbreaking findings in microbial ecology, such as bacteria capable of anaerobic ammonia oxidation (ANAMMOX); (Terada *et al*., 2011) or the existence of ammonia-oxidising archaea (AOA); (Biller *et al*., 2011), has proved that our knowledge regarding the nitrogen cycle and related microbial key players is still limited.

The effects of nitrogen discharges from wastewater treatment plants include ammonia toxicity to aquatic life which disrupts the ecological balance of water bodies, adverse public health effects and decreased suitability for reuse (Bernhard, 2010). In domestic wastewater, nitrogen is usually in the form of urea or protein, while in industrial wastewater it generally results in effluent from chemical industries. Total nitrogen is present in either one of two major components, the organic and inorganic forms (Aggarwal *et al*., 2012). Ammonia, nitrates and nitrites make up the total inorganic nitrogen. Nitrates are considered to be acutely toxic since they can be reduced in the stomach or by saliva to nitrites and hence induce

methemoglobinemia in infants (Hord *et al*., 2009). The World Health Organization (WHO) recommends a maximum limit for nitrite in drinking water of 50 mg/L.

A typical effluent discharge criterion for sensitive surface water allows a maximum limit of 1.5mg/L for NO_3/N (Nozaic and Freese, 2009). The ammonification of Total Kjeldahl Nitrogen (TKN) and subsequent nitrification and denitrification are key processes to reduce total nitrogen levels in treated effluents. Conventional activated sludge systems were originally designed to remove carbonaceous organic compounds and ammonia from wastewater. With increased community concerns for the long-term environmental hazards posed by eutrophication, wastewater treatment plants have been designed to remove both nitrogen and phosphorus microbiologically. BNR plants are those in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and biologically enhanced phosphorus removal (Tcobanoglous *et al*., 2003; Hu *et al*., 2012).

Prokaryotic microorganisms are reported to predominate in wastewater and are responsible for the biological activity. Previous studies confirm that chemo-organoheterotrophic bacteria are the major populations in activated sludge (Seviour and Nielsen, 2010). Culture dependent methods favour the growth and detection of Gamma-proteobacteria (Cui *et al*., 2012), whereas fluorescent *in situ* hybridisation (FISH) and clone library data using 16S rRNA sequence analyses suggest that a sub-group of the Beta-proteobacteria are among the dominant proteobacteria (Spring *et al*., 2004; Dang *et al*., 2010). Gamma-proteobacteria and Actinobacteria are generally well represented in both domestic and industrial wastes (Ghosh *et al*., 2010). The Actinobacteria often occur in high numbers, especially in some foaming plants and enhanced biological phosphorus removal (EBPR) processes (Seviour and McIlroy, 2008).

Delta-proteobacteria has been detected in many communities, and is represented mainly by the sulphate-reducing bacteria. Apart from the well-studied bacterial phyla such as *Proteobacteria* (Majed *et al*., 2012), *Actinobacteria* (Kawakoshi *et al*., 2012), *Firmicutes* (Kunisawa, 2010) and *Bacteroidetes* (Klindworth *et al*., 2012), as well as bacterial phyla such as *Chloroflexi* (Beer *et al*., 2004; Zhao *et al*., 2012), *Planctomycetes* (Ivanova and Dedysh, 2012); AOA, low G-C content (LGC) and high G-C content (HGC) bacteria have been detected.

In BNR systems, nitrification precedes denitrification during nitrogen removal. The microbes contributing to nitrification consist of two functional groups. The ammonia-oxidising bacteria (AOB) oxidise toxic ammonia to nitrite, which is then oxidised to the less toxic nitrate by the nitrite-oxidisng bacteria (NOB). Both functional groups are aerobic and chemolithoautotrophic (Huang *et al*., 2010). Consequently, all nitrifiers are slow growing and thus require a long sludge age which in turn causes the proliferation of slow growing filamentous bacteria. Nitrification failure is a common occurrence at BNR plants since nitrifiers are highly susceptible to environmnental changes (Ma *et al*., 2009). The distribution of nitrifiers seems to depend on the type of BNR plant and its operational conditions (Dytczak *et al*., 2008). Predominant AOB belong to one of three genera; *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira* and predominant NOB belong to one of three genera; *Nitrobacter*, *Nitrospira* or *Nitrospina*. The genus *Nitrobacter* and *Nitrospira* are considered to be the key NOB in biological wastewater treatment (Daims *et al*., 2001; Gieseke *et al*., 2003; Seviour *et al*., 2003).

Facultatively-anaerobic heterotrophic microbes facilitate the anaerobic process of denitrification. Many bacterial species have been isolated and includes members of the genera; *Alcaligenes, Bacillus, Hyphomicrobium, Methylobacterium, Parococcus, Pseudomonas* and others (Seviour and Nielsen, 2010). In EBPR plants, where phosphorus (P) and nitrogen (N) removal are combined, some polyphosphate-accumulating organisms (PAO) also act as denitrifiers. Activated sludge fed with acetate or methanol allows for the selection of different groups of denitrifiers such as *Comamonadaceae*, *Rhodocyclaceae*, *Methylophilus*, *Methylobacillus* and *Hyphimicrobium* (Ginige *et al*., 2005; Osaka *et al*., 2006; Tao *et al*., 2012).

A few studies have explored the possible occurrence of Archaea and ANAMMOX bacteria in activated sludge systems. It is reported that aerobic ammonia oxidation is performed by members of the AOB and AOA. These autotrophic microorganisms survive together with ANAMMOX bacteria in environments experiencing nitrogen input, and can work together to cycle N through the system (Hanke and Strous, 2010). Sediments of freshwater or marine systems provide an optimal environment for ANAMMOX bacteria (or denitrifiers) to survive in close association with AOB and AOA. These groups can be affected by a variety of environmental parameters (Bouskill *et al*., 2011). Methanogenic Archaea seems to occur in

activated sludge (Berger *et al*., 2012). PCR analyses suggest that archaeal ammonia-oxidisers may be commonly occurring nitrifying members (Chang, 2011).

Detection and quantification of these slow growing microorganisms from wastewater is a complex process. Traditionally, two approaches were used; standard light microscopy and conventional culture techniques (Jenkins *et al*., 2004), which resulted in several limitations. This included the possibility of polymorphism of certain microorganisms (Wagner *et al*., 1996) which complicates isolation and morphology-based identification. After the introduction of molecular techniques for community analyses it became obvious that only less than 16% of microorganisms could be isolated from activated sludge using standard cultivation methods and those colony-forming bacteria on the respective media are generally of minor numerical importance *in situ* (Burke *et al*., 2012). Therefore, these methods offer a biased and incomplete view on the bacterial diversity at BNR plants. On the other hand, direct cultivation of *in situ* important microorganisms is still a pre-requisite for their physiological and genetical analysis.

The 16S rDNA approach has become superior for cultivation-independent assessment of bacterial diversity in natural and engineered systems (Amann *et al*., 1995; Miqueletto *et al*., 2011). Current 16S rDNA databases contain more than 20,000 entries and thus provide a high-resolution framework for the assignment of those sequences found in 16S rDNA libraries from environmental diversity surveys (Hugenholtz *et al*., 2001; Brandt *et al*., 2012). The approach comprises DNA extraction, subsequent PCR amplification of the 16S rDNA gene using primers targeting conserved regions in the bacterial domain, cloning and sequencing. The sequences obtained are analysed together with adequate reference sequences to infer their phylogenetic affiliation. A meaningful phylogenetic analysis requires the use of almost full-length 16S rDNA sequences and different treeing methods to be applied to different data sets (Ludwig *et al*., 2004; Bik *et al*., 2012).

Quantitative based methods (FISH) provide culture-independent identification and quantification of bacterial populations and are based on 16S rRNA genes. This is exploited the most since the product is in ribosomes which occur in multiple copies in metabolically active cells of all prokaryotes. Thus fluorescently tagged probes targeting specific organisms can be designed. FISH and fluorescence microscopy permits *in situ* identification (Amann

and Fuchs, 2008). FISH can be used to target populations ranging from the whole taxonomic hierarchy. Many probes are now available to detect individual phylotypes e.g. nitrifying bacteria (Daims *et al*., 2001; Gieseke *et al*., 2001; Daims *et al*., 2006; Sanz and Köchling, 2007) as well as other important physiological groups of bacteria in activated sludge.

Recently, real-time quantitative Polymerase Chain Reaction (Q-PCR) (Smith *et al*., 2006; Smith and Osborn, 2009) and competitive PCR (cPCR); (Dionisi *et al*., 2002; Lyon and Wittwer, 2009) have been frequently used for the quantification of individual bacterial populations and copy numbers of functional genes of interest. Several nitrifying and denitrifying bacterial populations can be quantified using this approach (Limpiyakorn *et al*., 2007; Zielińska *et al*., 2012). Although nitrification is the innermost aerobic process of the nitrogen cycle, there is a limitation regarding the environmental purpose and occurrence of nitrifying bacteria. Due to their sluggish growth, nitrification is the principle bottleneck in many modern BNR plants, with ammonia oxidation taking place at a slower rate than nitrite oxidation. Therefore a greater understanding of autotrophic nitrifying bacteria is essential for the enhancement of overall treatment performance and control. This knowledge will be useful for both natural and constructed ecosystems.

Therefore this study focused on the analyses of autotrophic nitrifying bacterial communities from full-scale BNR plants using advanced molecular techniques, which are more rapid and reliable as compared to microbiological and biochemical methods. FISH analyses (allowed for identification and quantification); specific PCR amplification and Q-PCR (allowed for clarification, identification and quantification at species level); and cloning and sequencing of 16S rRNA genes (allowed for identification of dominant AOB and NOB populations) were applied during the investigative period. A study of this nature has not been conducted in South Africa to our knowledge, regarding autotrophic nitrifying bacteria and their levels of activity and dominance across five full-scale BNR plants, treating different influent waste streams. Automated confocal scanning laser microscopy (CSLM) combined with FISH has already been successfully applied to characterise floc arrangement and organisation (Schmid *et al*., 2003; Ramdhani *et al*., 2010) and for identifying and quantifying a range of bacterial populations in laboratory-scale systems. Q-PCR based methods also help to monitor functional groups in wastewater systems using certain functional genes e.g. amoA for monoxygense activity (Smith, 2005; Li and Gu, 2011).

The **aim** of this study was:

To quantify autotrophic nitrifying bacteria from full-scale BNR plants, using novel molecular techniques to determine their population profile and correlate this to plant operating parameters.

The **objectives** of this study were:

- Survey of full-scale BNR plants in KwaZulu-Natal for all plant operating conditions
- Quantification and evaluation of dominant nitrifying bacterial communities and their nitrification performance in full-scale BNR plants
- Elucidation of phylogenetic diversity of dominant nitrifying bacteria based on polymerase chain reaction (PCR), cloning and16S rDNA sequencing
- Comparative analysis of FISH and Q-PCR for the quantification of nitrifying bacteria

CHAPTER TWO: LITERATURE REVIEW

2.1 Wastewater Treatment

An understanding of the nature of wastewater is essential for the design of appropriate WWTP and the selection of effective treatment technologies. Wastewater predominantly originates from water usage by residences, commercial and industrial establishments, together with groundwater, surface water and storm water (Figure 2.1).

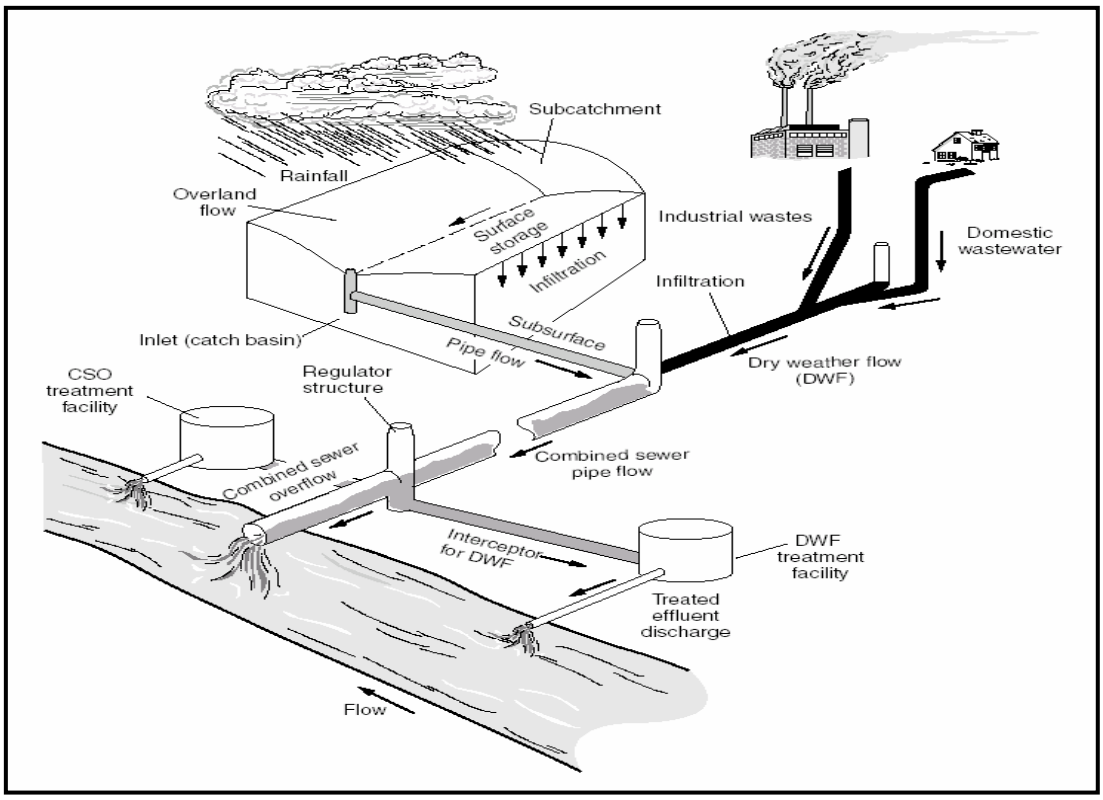


Figure 2.1: Sources of wastewater and treatment methods (Tchobanoglous *et al*., 2003)

The most common form of wastewater treatment is activated sludge which utilises a consortium of mixed microbial communities through an aerobic biological process to degrade wastewater components (LaPara *et al*., 2000; Leu *et al*., 2012). Insight regarding the importance of wastewater contaminants is critical and is listed in Table 2.1.

Table 2.1: Important contaminants in wastewater (Tchobanoglous *et al*., 2003)

CONTAMINANTS	IMPORTANCE
Suspended solids	Can result in sludge deposits and anaerobic conditions if untreated wastewater is discharged.
Biodegradable organics	Consists primarily of proteins, carbohydrates and fats and are commonly determined as biological oxygen demand (BOD) and chemical oxygen demand (COD). Their biological stabilisation can deplete oxygen resources and results in conditions detrimental to aquatic life, if discharged into receiving water bodies.
Pathogenic organisms	Present in wastewater; capable of transmitting infectious diseases.
Priority pollutants	Includes organic and inorganic compounds. These may be highly toxic, carcinogenic, mutagenic or teratogenic.
Refractory organics	Are known to resist conventional wastewater treatment and includes surfactants, phenols and agricultural pesticides.
Heavy metals	Is the result of commercial and industrial activities and must be removed from wastewater for reuse.
Dissolved inorganic constituents	Are often initially added to domestic wastewater and may require removal to allow for wastewater reuse. Includes calcium, sodium and sulphate.

To meet the requirements for acceptable disposal levels, wastewater needs to be treated via one of many processes. Conventional wastewater treatment is divided into three stages; primary, secondary and tertiary treatment.

2.1.1 Primary Treatment

This stage involves sediment removal by allowing the liquid portion of wastewater to pass through a series of screens to separate large objects from the wastwater. The water is thereafter allowed to settle to favour the precipitation of aluminium sulphate and ferrous sulphate. All remaining liquid is thereafter treated by the second phase and all the sediment is either incinerated or digested (Zara *et al*., 2012).

2.2.2 Secondary Treatment

This stage entails the stabilisation of organic materials and a reduction in the BOD. This is generally carried out by a community of microorganisms, aerobically. All organics are degraded by bacteria and incorporated into their cell material (Coppen, 2004; Zara *et al*., 2012).

2.1.3 Tertiary Treatment

This stage involves the removal of nitrates and phosphates which will prevent pollution of receiving water bodies and thus eutrophication. Once these nutrient levels are at acceptable levels to meet the relevant discharge standards, treated wastewater is discharged into receiving water bodies (Brodie *et al*., 2006; Narmadha and Kavitha, 2012). Tertiary treatment also includes the removal of pathogens by disinfection which involves the use of chlorine. The chlorine dosage depends upon the strength of the wastewater and other factors. Ozone and ultra violet (UV) irradiation can also be used for disinfection but these methods are not in common use.

2.2 The Activated Sludge Process

The activated sludge system represents a component of the largest biotechnology industry globally. Yet this process differs from most conventional biotechnological processes where pure cultures and controlled aerobic fermentations are required. Instead, activated sludge communities represent mixed populations which deal with a diversity of organic and inorganic compounds entering the system randomly. It is essential for activated sludge systems to be robust since they contain faecal material as well as domestic and industrial wastes, which carry a range of naturally occurring and xenobiotic organic compounds, including bioactive pharmaceuticals (Vieno *et al*., 2007) and surfactants. This would affect the community composition. Some represent possible sources of endocrine disrupting compounds (Vandenberg *et al*., 2012).

Bacterial populations from activated sludge degrade these compounds, but some are toxic and resist degradation (Chatterjee and Karlovsky, 2010). The most important factor for consideration in activated sludge systems is whether solely C-removal is included in the plant design (if only aerobic tanks present) or if denitrification or EBPR are also included

(anoxic/anaerobic tanks present). This would alter the structure of the population. To improve both efficiency and flexibility, the activated sludge process has undergone several changes regarding operational features and design. Conventional activated sludge systems were originally designed to remove carbonaceous organic compounds and ammonia. However WWTP are now designed to remove both nitrogen and phosphorus microbiologically due to increased community concerns regarding the long-term environmental hazards as a result of eutrophication (Akpor and Muchie, 2010).

Dissolved organic matter is transformed under aerobic conditions into additional microbial biomass and carbon dioxide. Once microbial growth is optimised under ideal conditions, the microorganisms aggregate to form settleable stable floc structures. Minerals present in the water may also be attached to the microbial biomass. Poorly formed flocs can present a network of filamentous microbes which will impede settling. When these biological processes occur during less than optimum conditions, particularly at lower oxygen levels or with a microbial community that is too young or too old, unsatisfactory floc formation and settling occur. This causes a massive development of filamentous bacteria, resulting in a bulking sludge (Ping *et al*., 2012).

The basic design for conventional BNR plants has barely changed except for the incorporation of more sophisticated instrumentation for *in situ* plant monitoring and computerised control of systems. Tanks with submerged diffusers or mechanical surface agitators which supply oxygen are still present as aerobic reactors. Mixed liquor effluent is still directed to a clarifier where sludge separation occurs. A portion of this sludge is recycled to allow for the inoculation of incoming raw waste (Tchobanoglous *et al*., 2003). These systems were originally established to remove carbonaceous material to enable the safe discharge of treated effluent into other water bodies. The design of WWTP were modified accordingly to encourage nitrification since ammonia is toxic compared to nitrate. Due to increased global concerns for the long term environmental hazards posed by eutrophication, BNR plants have been designed to remove both nitrogen (N) and phosphorus (P) microbiologically.

Nutrient removal systems have become increasingly popular as governments enforce strict rules with regard to effluent quality prior to discharge. Additional tanks provide differing

environmental conditions, especially in terms of oxygen availability. This in turn encourages the growth of particular physiological groups and has expanded performance capabilities of conventional plants to satisfy increased demands. Thus, the incorporation of anoxic zones with low dissolved oxygen levels but plentiful nitrate concentrations permits denitrification and nitrogen removal. Anaerobic zones which lack both nitrate and oxygen provide polyphosphate accumulating organisms (PAO) the selective advantage required for P-removal (Tchobanoglous *et al*., 2003).

2.3 Functional Microbial Populations in Activated Sludge

2.3.1 Bacteria and Archaea

There were many misconceptions that all activated sludge BNR plants are of similar configuration, treat similar wastes, and will thus have similar community compositions, regardless of their global geographical location. Bacterial communities of particular interest include those involved in P and N removal and those responsible for incidental bulking and foaming. It has been known for many years that activated sludge bacterial communities also contain a diverse group of faecal commensals and pathogens in relatively high numbers. Methanogenic archaea also occur in small numbers in activated sludge (Fredriksson *et al*., 2012).

2.3.2 Chemoorganoheterotrophic Bacteria

These are the major populations present in activated sludge and consist of Gamma-proteobacteria (Kampfer *et al*., 1996; Guo and Zhang, 2012), Beta-proteobacteria (Spring *et al*., 2004; Valentin-Vargas *et al*., 2012) and Alpha-proteobacteria (Neef *et al*., 1996; Guo and Zhang, 2012). *Actinobacteria* also often occur in high numbers in foaming plants and EBPR processes (Seviour and McIlroy, 2008). Delta-proteobacteria has also been detected and is represented by the sulphate-reducing bacteria (Hesham and Alamri, 2012). Large numbers of *Chloroflexi* (Gao and Tao, 2012), *Bacteroidetes* (Kong *et al*., 2007) and unusual *Planctomycetales* (Chouari *et al*., 2003) have also been found in conventional and EBPR plants. Activated sludge communities also represent a rich reservoir of new species including *Acinetobacter* (Carr *et al*., 2005; Zhang *et al*., 2006), *Acidovorax* (McMohan *et al*., 2008) and *Aeromonas* (Nsabimana *et al*., 2000; Angenent *et al*., 2006).

2.3.3 Denitrifying Bacteria

These are present in all BNR plants capable of N-removal but may be scarce at plants that only carry out nitrification (Ni *et al*., 2010). They can be recognised by their ability to assimilate and grow on respirable substrates such as acetate or methanol. Dominant denitrifying populations are unclear since populations vary between individual BNR plants with changes in influent and operational conditions (Hallin *et al*., 2005). *Aquaspirillum*, *Azoarcus*, and *Thaurea* species are often detected in plants designed for N-removal (Thomsen *et al*., 2004; Morgan-Sagastume *et al*., 2007). *Zoogloea* has been detected at industrial plants. It was also found that *Accumulibacter* PAO denitrify (Gonzalez-Gil and Holliger, 2011).

2.3.4 Polymer-degrading Bacteria

Organisms responsible for the enzymatic degradation of substrates are functionally important since most substrates are polymeric. Some protein hydrolysing populations reveal novel epiphytic members of *Saprospiraceae* in the *Bacteroidetes*, which were named *Candidatus* (Xia *et al*., 2008).

2.3.5 Iron Bacteria

Iron reduction by dissimilatory iron-reducing bacteria is well documented in activated sludge (Martins *et al*., 2012). Iron is critical for strong floc structure formation and biological and chemical P-removal pathways. Some bacteria that oxidise iron with nitrate as electron acceptors can be classified as iron-dependent denitrifying bacteria (Hedrich *et al*., 2011).

2.3.6 Sulfate Reducing Bacteria

These are present in activated sludge systems and are physiologically active during extended anaerobic periods (Yan *et al*., 2007). Since iron is present as well, the generated sulphide precipitates as black iron sulfide. About 0.5-8% of total bacteria are identified as sulfate-reducers. Dominant sulphate-reducing bacteria belong to either Desulfovibrionaceae or Desulfobacteriaceae (Sànchez-Andrea *et al*., 2012).

2.3.7 Polyhydroxy Alkanoate (PHA) Accumulating Organisms

These bacteria accumulate large amounts of intracellular PHA *in situ*, which facilitate their survival. These include PAO, GAO and some filamentous bacteria (Seviour *et al*., 2003; Majed *et al*., 2012).

2.3.8.1 Glycogen Accumulating Organisms (GAO)

These are probably ubiquitous in EBPR plants and are defined as organisms that possess the ability to assimilate substrates anaerobically for PHA synthesis. However, they are incapable of accumulating P aerobically. Other populations probably possess this anaerobic phenotype without accumulating glycogen aerobically. Unfortunately obtaining proof for an *in situ* ability to accumulate glycogen is difficult since it is not readily detected by staining (Majed *et al*., 2012). Several *Actinobacteria* accumulate glycogen (Seviour *et al*., 2003). Accepted EBPR GAO described thus far includes *Competibacter* members of Gamma-proteobacteria (Kong *et al*., 2006) and Alpha-proteobacteria, which are closely related to members of the genus *Defluviicoccus* (Wong *et al*., 2004).

2.3.8.2 Poly-phosphate Accumulating Organisms (PAO)

Acinetobacter was previously categorised as a favourite PAO candidate, however previous studies on EBPR plants globally reveal the importance of beta-proteobactetrial *Rhodocyclus*-related Candidatus '*Accumulibacter phosphatis*' (Seviour and McIlroy, 2008) in P removal. It can be noted that many different bacteria from activate sludge including bulking and foaming filamentous bacteria accumulate intracellular poly-P granules. Populations related to members of the genus *Tetrasphaera* (Kawakoshi *et al*., 2012) and possibly *Stenotrophomonas* (Ghosh *et al*., 2010) may eventually turn out to be important PAO.

2.3.9 Chemolithoautotrophic Bacteria

2.3.9.1 Nitrifying Organisms

Nitrification is a key process in N-removal and is carried out by the chemolithoautotrophic nitrifying bacteria. It involves a two-step process performed by two different groups of microorganisms. These groups are autotrophic and utilise CO_2 as the carbon source for biosynthesis and oxidation of nitrogen compounds as the energy source. The first reaction is

ammonia oxidation, where AOB oxidise ammonia to nitrite. This group of bacteria includes *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrosospira* sp. and *Nitrosolobus* sp. The second reaction is nitrite oxidation, where the nitrite is transformed to nitrate. This group of bacteria includes *Nitrobacter* sp., *Nitrospira* sp., *Nitrococcus* sp., and *Nitrocystis* sp. (Noophan *et al*., 2009).

Molecular methods have changed our view on important nitrifying populations in activated sludge and has revealed a level of phylogenetic diversity among AOB and NOB, which was previously unknown (Hatzenpichler *et al*., 2012). Predominant AOB in WWTP belong to one of three genera; *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira* and NOB belong to one of three genera; *Nitrobacter*, *Nitrospira* or *Nitrospina* (Daims *et al*., 1999; Gieseke *et al*., 2003). Nitrifiers differ substantially in their ecology with *Nitrobacter* considered to be an *r*-strategist or a slower growing K-strategist which has adapted to cope with the low nitrite levels in activated sludge (Nogueira and Melo, 2006). However, nitrifiers may co-exist in nitrifying plants (Siripong and Rittman, 2007). Differences in competitive abilities based on oxygen and ammonia affinities, have also been recorded among *Nitrosomonas* lineages (Lydmark *et al*., 2007) and may determine which phylotype succeeds at any particular plant.

2.4 The Nitrogen Cycle

Fritz Haber patented the process to "synthesise ammonia from its elements." We now live in a world highly dependent on, and transformed by man-made fixed nitrogen (Erisman *et al*., 2008). Ammonia is the starting product of several sectors in chemical industries. Most of the fixed nitrogen, however, is used to produce fertiliser in the forms of ammonium nitrate, calcium nitrate, ammonium bicarbonate and several mixtures of N and P-containing compounds. An increase in the world population has led to the large scale production of synthetic nitrogen fertiliser which supports at least half of the human population with an increased food supply (Galloway *et al.*, 2008).

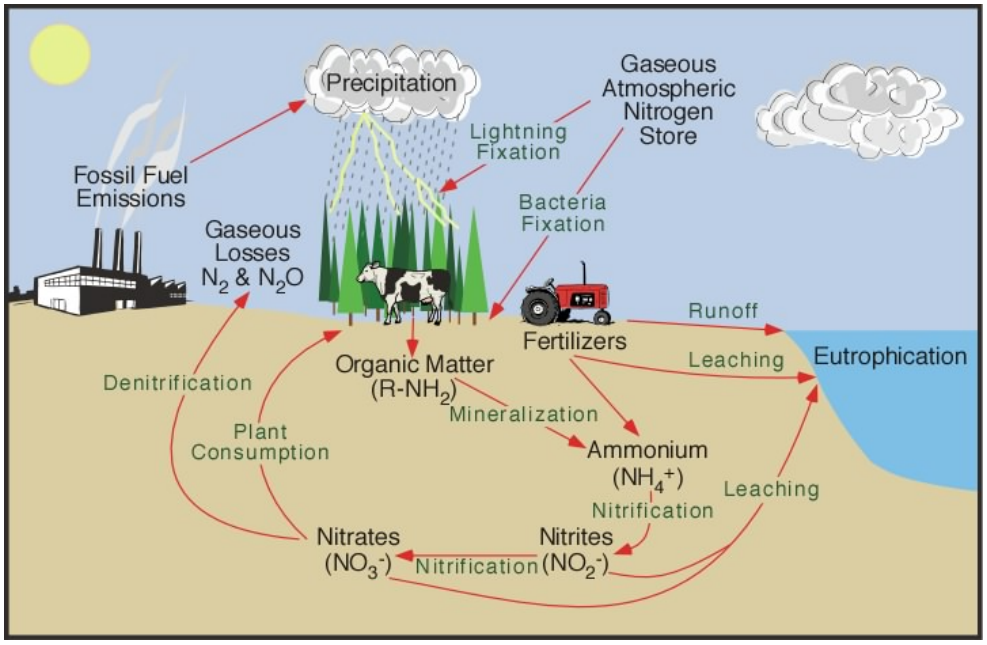


Figure 2.2: Nitrogen cycle in activated sludge (Pidwirny, 2006)

All life forms require nitrogen as an integral part of proteins and nucleic acids; therefore all living organisms are involved in the nitrogen cycle. Microorganisms, however, catalyse essential steps in the global biochemical nitrogen-cycle that other organisms are unable to accomplish. During microbial nitrogen fixation, gaseous di-nitrogen is reduced by the enzyme nitrogenase to produce ammonia and subsequently assimilated into cell material (Zhao *et al.,* 2012). Ammonia is also released during microbial decomposition of organic substances during a process called ammonification or mineralisation (Pathma and Sakthivel, 2012).

At a neutral pH most of the ammonia is available in the form of ammonium. One part of the released ammonium is recycled and converted to amino acids, whilst the remaining ammonia/ammonium can be catabolised either aerobically or anaerobically through two distinct microbial key processes in the nitrogen-cycle. Under aerobic conditions, microorganisms gain energy from oxidising ammonia to nitrite and subsequently nitrite to nitrate, through the process of nitrification. This is catalysed by two functional groups of chemolithotrophic prokaryotes: the AOB and AOA, and the NOB (Bock and Wagner, 2001; Off *et al.,* 2010). In anoxic habitats, however, ammonium can be catabolised by

physiologically specialised planctomycetes during a process called ANAMMOX (Fuchsman *et al*., 2012). This reaction couples ammonium oxidation to nitrite reduction with dinitrogen being the final product.

The gaseous product of the ANAMMOX process is released into the atmosphere. The nitrate produced through nitrification is either readily assimilated by plants and microorganisms or used as the respiratory electron acceptor under anaerobic conditions by facultatively anaerobic organisms (denitrification); (Vymazal, 2007). Denitrification is widespread among different bacterial genera and extends beyond bacteria to archaea and fungi. During denitrification nitrate is reduced to nitrite. This is followed by the subsequent reduction of nitrite to nitric oxide which serves as the terminal electron acceptor, regarding dioxygen, and is reduced to nitrous oxide, which is subsequently reduced to di-nitrogen. Hence, the denitrification and ANAMMOX processes return di-nitrogen to the atmosphere from terrestrial and aquatic habitats, thereby completing the nitrogen-cycle.

2.5 Wastewater Treatment and Nitrification

Nitrification is the successive conversion of ammonium to nitrite (nitritation) by the AOB and the subsequent oxidation of nitrite to nitrate (nitratation) by the NOB. These bacteria use ammonia or nitrite as an energy source and molecular oxygen as an electron acceptor, while carbon dioxide is used as a carbon source (Bernhard, 2010). However, the carbon dioxide must be reduced before it can form part of the cell mass. This reduction occurs through the oxidation of the nitrogen source of the organism concerned (Bellucci *et al*., 2011). *Nitrosomonas* spp. and *Nitrobacter* spp. are usually regarded as the respective typically dominating AOB and NOB in wastewater (Table 2.2), even though there have also been other nitrifying species such as *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio*, *Nitrospira*, *Nitrospina* and *Nitrococcus* (Wojnowska-Baryla *et al*., 2010).

Nitrification is a highly sensitive process that can be hindered in the presence of physical and chemical inhibitors that may cause this process to cease completely. The optimal conditions for nitrification in WWTP include a pH of 7.0-8.5 (Li *et al*., 2008). Sludge age and wastewater temperature are also important for adequate nitrogen removal. Dissolved oxygen also affects nitrification, depending on the diffusion rate of oxygen into bulk liquid and the

floc, the location of the nitrifiers and the microbial activity with the floc, nitrification rates can be affected by the dissolved oxygen available to the nitrifiers inside the floc.

The low-energy N-substrates and the energy consuming carbon dioxide fixation of the nitrifiers result in poor growth yields and low growth rates (Starkenburg *et al.*, 2008). In practice, the oxidation of ammonium proves to be the rate limiting step in the overall process. Critical factors that influence the nitrification process are dissolved oxygen concentration, pH, temperature, sludge age and inhibiting substances. Since their discovery more than a century ago lithotrophic bacterial nitrifiers have conventionally been classified as one family, the *Nitrobacteriaceae* (Nielsen *et al*., 2010). However, by comparative analysis of the small rRNA subunit gene sequences, the nitrifier classification was revised. Comparative 16S rRNA sequence analysis revealed that most known AOB and NOB belong to different subclasses of *Proteobacteria* (Koops *et al*., 2003). The only exception, however, are the NOB of the genus *Nitrospira* that form a distinct phylum within the domain *Bacteria* (Ehrich *et al*., 2010).

Table 2.2: List of nitrifying bacteria from activated sludge (Seviour and Nielsen, 2010)

Ammonia-oxidising Bacteria	Nitrite-oxidising Bacteria
Beta-proteobacteria	**Alpha-proteobacteria**
Nitrosomonas communis	*Rhodobacter capsulatus*
Nitrosomonas nitrosa	*Paracoccus denitrificans*
Nitrosomonas europaea	*Bradyrhizobium japonicum*
Nitrosomonas eutropha	*Nitrobacter sp.*
Nitrosomonas halophila	
Nitrosomonas cryotolerans	**Beta-proteobacteria**
Nitrosomonas marina	*Nitrotoga*
Nitrosomonas aestuarii	*Gallionella ferruginea*
Nitrosomonas ureae	Gamma-proteobacteria
Nitrosomonas oligotropha	*E.coli*
Nitrosococcus mobilis	*Thiobacillus ferrooxidans*
	Nitrosococcus mobilis
Gamma-proteobacteria	
Nitrosococcus oceani	**Delta-proteobacteria**
Nitrosococcus halophilus	*Nitrospina gracilis*
	Bdellovibrio bacteriovorus
	Phylum Nitrospirae
	Nitrospira defluvii
	Nitrospira moscoviensis
	Nitrospira marina
	Leptospirillum ferrooxidans
	Magnetobacterium bavaricum
	Thermodesulfovibrio islandicus

2.6 The Importance of Nitrogen Elimination from Wastewater

Nitrogen-containing wastewaters of domestic and industrial origin are continually discharged into the environment in large volumes without suitable treatment. This leads to extensive soil and water pollution. Suitable elimination of the pollutants from these effluents is essential and this is becoming increasingly important from an environmental and human health perspective in most developing countries.

Eutrophication which is associated with the discharge of wastewater containing nitrogen and phosphorus compounds into freshwater has become a severe water pollution problem in many countries (Molinuevo *et al*., 2009). Water quality has deteriorated and potential health hazards to humans or animals e.g. by toxic algal blooms have severe consequences. The presence of excess nitrogen in the environment has caused serious alterations of the natural nutrient cycle between soil, water and the atmosphere (Obaja *et al*., 2003). An excess

discharge of nitrate as a fertiliser is one of the most common water and groundwater pollutants and causes serious problems including cancer, blue-baby disease in infants and methaemoglobinaemia (Höring and Chapman, 2004). In recent years, a number of studies have focused on carbon, nitrogen and phosphate removal from domestic, agricultural and industrial wastewaters (Breisha, 2010; Aghayari and Darvishi, 2011; Jyothi *et al*., 2012).

The effluent of wastewater treatment plants contains high concentrations of inorganic nitrogen that may lead to eutrophication (de-Bashan *et al*., 2004). Effluents from septic tanks generally contain high concentrations of ammonia. Zeng *et al*. (2003) found that the ammonia concentration in domestic wastewater from one septic tank in China was 54-74 mg/L. The effluent of septic tanks is usually discharged to aerobic seepage fields, where ammonia and organic nitrogen are transformed to nitrate, which may be trickling into the groundwater. The concentration of nitrogen compounds in some industrial wastewaters is tremendously higher than what is found in agricultural and domestic wastewater. Thus, nitrate removal from these types of industrial wastewater is an inevitable step during treatment. Different industrial and agro-industrial wastewaters are reported to contain more than 200mg/L of nitrate-nitrogen (Peyton *et al*., 2001) and some contain even higher nitrate levels.

2.7.1 Biological Nutrient Removal (BNR) Plants Origin and Design

BNR plants are those in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and biologically enhanced phosphorus removal (Tcobanoglous *et al*., 2003). The addition of extra tanks provides different environmental conditions, especially in terms of oxygen availability, thus encouraging the growth of particular physiological groups. This has expended the performance capabilities of conventional activated sludge plants to satisfy these increased demands. Incorporation of an anoxic zone with low dissolved oxygen but plentiful nitrite (NO_3^-) permits denitrification and nitrogen (N) removal. An anaerobic zone lacking NO_3^- and oxygen are thought to provide the phosphate accumulating organisms with the selective advantage needed for phosphate (P) removal (Seviour *et al*., 2003; Oehmen *et al*., 2007).

Five stage Phoredox systems proposed by Barnard (1984) were the basis for a full scale EBPR plant where an anaerobic zone was added to the head of the plant to increase

phosphate removal. This comprised of anaerobic-anoxic1-aerobic1-anoxic2-aerobic2-clarifier tanks. There was sludge recycling from the aerobic to anoxic1 and from the clarifier to anaerobic tanks. The main disadvantage of this process was the presence of NO_3^- in the return activated sludge (RAS) to the anaerobic tank which may affect phosphate removal. This plant was re-designed to a three-stage-Phoredox process (anaerobic, aerobic and clarifier with sludge recycling only from the clarifier to anaerobic tank) to prevent NO_3^- entering into the anaerobic tank.

The Johannesburg process was developed from the Phoredox system, with the aim of overcoming the detrimental effects of NO_3^- during EBPR, by introducing a sludge denitrification vessel in the RAS line (Barnard, 1984). A further modification to the basic Phoredox system with the aim of avoiding problems of NO_3^- inhibiting P removal was that from the University of Cape Town (UCT), (Haas *et al*., 2000). Here the RAS first passes through the anoxic rather than the anaerobic zone and a mixed liquor recycle runs from the anoxic reactor to the anaerobic reactor (r cycle). This was designed to ensure that any NO_3^- in the RAS has fully denitrified before entering into the anaerobic tank. This was further re-designed into a Modified UCT process (MUCT) where the single anoxic zone is divided into two compartments, allowing for improved individual control of the mixed liquor and RAS streams (Oehmen *et al*., 2007). Most of the South African wastewater systems use long sludge ages (>20 days), which cause bulking and foaming due to the overgrowth of filamentous bacteria.

Besides the conventional nitrogen removal process, novel and economically feasible biological nitrogen elimination processes have been developed. This includes simultaneous nitrification and denitrification, anaerobic ammonium oxidation (ANAMMOX), completely autotrophic nitrogen removal over nitrite (CANON) and single reactor system for high activity ammonium removal over nitrite (SHARON). The ANAMMOX process involves denitrification with nitrite and ammonia as the electron donors. The ANAMMOX process requires fifty percent of partial nitrification as a preliminary step, whilst the oxidation of ammonium/nitrite is favoured by eliminating methanol and the anoxic time intervals in the reactor (Bernhard, 2010). The CANON process is the combination of partial nitrification and ANAMMOX in an aerated reactor. Nitrifying bacteria convert ammonia to nitrite whilst consuming oxygen to create the anoxic conditions needed during the ANAMMOX process

(Zhang *et al*., 2012). The SHARON process uses altered growth rates of ammonia-oxidisers and nitrite-oxidisers at temperatures greater than 26˚C. Aeration is responsible for supplying oxygen and to also strip carbon dioxide to keep the pH under control. Denitrification is undertaken during the SHARON process to reduce nitrite to nitrogen gas.

Every year new wastewater plant designs are continuously emerging based predominantly on engineer's perspectives. However, focus should be placed on improving existing plants, for enhanced plant performance (Hu *et al*., 2012). There is a need for understanding the microbiology and chemistry of wastewater treatment plants and monitoring of these systems should be more sensitive and rapid. A holistic approach to monitor the functioning of a wastewater treatment plant has become compulsory to resolve problems especially at BNR plants.

2.7.2 Biological Nitrogen Removal (BNR)

During BNR in wastewater, nitrogen is converted to nitrous oxide and nitric oxide. Autotrophic nitrification and heterotrophic denitrification were considered to be the origins of nitrous oxide and nitric oxide (Philippot *et al*., 2009). Nitric oxide can be easily oxidised to nitrogen dioxide through chemical oxidation with oxygen. Nitrogen dioxide could serve as an electron acceptor for *Nitrosomonas eutropha* to anaerobically oxidise ammonia, which was known as nitrogen dioxide-dependent ammonia oxidation. Nitric oxide was considered as a signal molecule during ammonia oxidation (Stahl and Torre, 2012). In the presence of oxygen, nitric oxide was chemically oxidised to nitrogen dioxide, and then participates in nitrogen dioxide-dependent ammonia oxidation. The technology of nitrogen removal from wastewater was separated into physical, chemical and biological treatment. BNR is attained by the combination of nitrification and denitrification in advanced wastewater treatment systems (Figure 2.3).

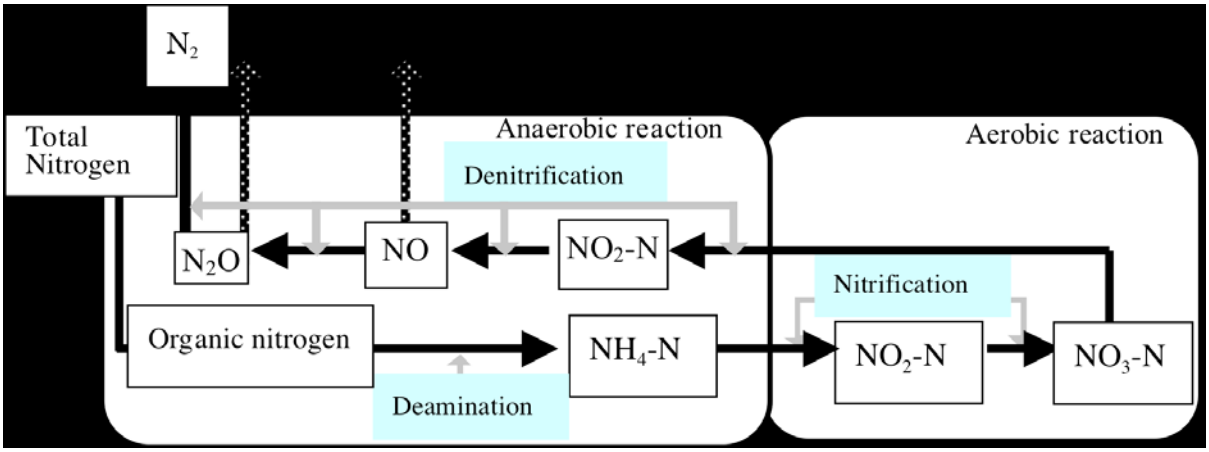


Figure 2.3: Conventional activated sludge process

Biological nitrification, which is a two-step process of ammonium oxidation to nitrite and nitrite to nitrate, is principally carried out by the chemolithoautotrophic AOB and NOB. Although the nitrification process has been extensively studied, it is still difficult to maintain stable nitrification performance in wastewater treatment systems. This is principally due to the slow growth rates of nitrifying bacteria and their sensitivity to toxic shocks, pH and temperature fluctuations. Therefore, quantification and monitoring the physiological activity of nitrifying bacteria is essential for the overall improvement of nitrogen removal from wastewater (Ibrahim *et al*., 2012).

On the other hand, biological denitrification, which is the reduction process of nitrate to nitrite; nitrite to nitric oxide; nitric oxide to nitrous oxide and nitrous oxide to nitrogen gas, is carried out by a host of bacteria and archaea. Most denitrifying bacteria are facultatively anaerobic. Denitrification requires hydrogen donors such as organic materials and sulphides for the reduction of nitrogenous compounds. BNR involves the combination of anaerobic and aerobic treatment to allow for complete N and biologically enhanced P-removal (Tcobanoglous *et al*., 2003). The inclusion of additional zones provides different environmental conditions, especially in terms of oxygen availability, which encourages the growth of particular physiological groups and has expended the performance capabilities of conventional activated sludge to satisfy increased demands. The incorporation of an anoxic zone with low dissolved oxygen but ample nitrite permits denitrification and nitrogen removal and an anaerobic zone lacking nitrate and oxygen are thought to provide the P-accumulating organisms with the selective advantage needed for P- removal (Seviour *et al*., 2003; Oehmen *et al*., 2007).

2.8 Distribution of Nitrifiers in Biological Nutrient Removal (BNR) Plants

The nitrifying bacteria encompass two groups of microorganisms, the AOB and the NOB which catalyse the oxidation of ammonia to nitrite and of nitrite to nitrate, respectively (Huang *et al*., 2010). Consequently, all nitrifiers are slow growing and the sludge age in nitrifying tanks must be higher than in systems for carbon oxidation. This long retention time in turn causes the proliferation of slow growing filamentous bacteria. Additionally, nitrifying bacteria recover very slowly if there are any breakdowns in their populations. This occasionally occurs when there are changes in temperature, pH or wastewater composition. These problems are more common at industrial BNR plants (Ma *et al*., 2009).

2.8.1 Ammonia-oxidising Bacteria (AOB)

Ammonia-oxidising bacteria (AOB) are responsible for the first and rate-limiting step of nitrification, the conversion of ammonia to nitrite. All known AOB are Proteobacteria with the majority being Beta-proteobacteria and *Nitrosococcus halophilus* and *Nitrosococcus oceani* belonging to Gamma-proteobacteria. Beta-proteobacterial AOB has been further sub-divided into four genera, *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*. Head *et al*. (1993) suggested combining the latter three under a single genus, *Nitrosospira*, which is closely related to the genus *Nitrosomonas*. The 16S rRNA-based phylogeny of AOB has been balanced by comparative sequence analysis of the *amoA* gene, which encodes the alpha subunit of ammonia monooxygenase (*amoA*), an enzyme specific for AOB. This gene is suitable as a phylogenetic and a functional marker and allows specific detection and identification of AOB (Purkhold *et al*., 2000).

Beta-proteobacterial AOB is widely distributed in natural and man-made ecosystems. *Nitrosomonas* can survive in aquatic and terrestrial habitats. All known members of the *Nitrosomonas europaea*/*Nitrosococcus mobilis* lineage are halotolerant and survive mainly in eutrophic environments. Their halotolerance and affinity for higher substrate concentrations may explain why these organisms constitute a large fraction of the AOB community in many BNR plants (Wagner and Loy, 2002). They are often found in activated sludge which has high ammonia concentrations. Most of the *Nitrosospira* lineage is found in soil and *Nitrosospira*-like bacteria have been known to dominate terrestrial habitats (Laverman *et al*., 2010). They have however been occasionally detected in nitrifying reactors.

Gamma-proteobacterial AOB are halophilic and are found in brackish water and marine ecosystems (O'Mullan and Ward, 2005; Junier *et al*., 2012). Surveys of AOB from nitrifying reactors result in mainly organisms closely related to *Nitrosomonas europaea* and *Nitrosomonas eutropha*. The genus *Nitrosomonas* is also frequently detected in treatment plants by 16S rRNA and *amoA* sequence analyses and FISH with rRNA-targeted oligonucleotide probes. The distribution patterns of different *Nitrosomonas*-like AOB depend on the type of treatment plant and its operational conditions. Thus the phylogenetic diversity of AOB in sewage treatment plants reflects their adaptations to varying environmental conditions, and this niche differentiation may be relevant to reactor operation, performance and stability. Marine bacterial populations with highly similar or even identical 16S rRNA sequences can differ substantially in their gene content and genomic organisation (Coleman *et al*., 2006). This type of variability may profoundly impact on the nitrification process.

The phylogeny of AOB based on 16S rDNA sequences has been studied over the past few years, resulting in their re-classification. Based on 16S rDNA sequence similarities, the three previously recognized genera *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* were combined into one genus, *Nitrosospira* (Junier *et al*., 2012). 16S rRNA gene sequence information has made it possible to design specific oligonucleotides for the direct monitoring of various subgroups of AOB in the environment (Utaker and Nes, 1998). The use of these specific oligonucleotides together with activity monitoring help us conclude that there is a high diversity of AOB in the environment and that species other than *Nitrosomonas* significantly contribute to ammonia oxidation (Wilén *et al*., 2008). Members of *Nitrosomonas* are also frequently detected using FISH-micro autoradiography (FISH-MAR) analyses, which are considered to be extremely relevant (Purkhold *et al*., 2003).

The distribution of AOB depends on the type of treatment plant and its operational conditions. Some systems are dominated by one species (Juretschko *et al*., 1998), whilst other systems can contain as many as five different populations (Daims *et al*., 2001; Gieseke *et al*., 2003; Kwiatkowska *et al*., 2012). Quantitative FISH revealed that in an industrial plant, both *Nitrosomonas europea* and *Nitrosococcus mobilis* was present, but *Nitrosococcus mobilis* was more abundant (Kindaichi *et al*., 2007). Thus the phylogenetic diversity of AOB in sewage treatment plants reflects adaptations to different environmental conditions and this niche differentiation may be relevant to reactor operation, performance and stability.

2.8.2 Nitrite-oxidising Bacteria (NOB)

Nitrite-oxidising bacteria (NOB) catalyse the second step of nitrification where nitrite is oxidised to nitrate. This functional group is phylogenetically more heterogeneous than AOB. All known nitrite oxidisers belong to one of the following genera: *Nitrobacter, Nitrospira, Nitrosococcus* and *Nitrospina*. The best physiologically characterised NOB, are members of the genus *Nitrobacter* from the class Alpha-proteobacteria (Lebedeva *et al*., 2008), which comprises the four species, *Nitrobacter winogradskyi, Nitrobacter hamburgensis, Nitrobacter vulgaris,* and *Nitrobacter alkalicus*. *Nitrobacter* are found in a range of terrestrial and aquatic habitats. *Nitrococcus mobilis* is a member of Gamma-proteobacteria. The genus *Nitrospira* constitutes the most diverse group of known NOB and contains only two species, *Nitrospira marina* and *Nitrospira moscoviensis*. These species were always considered to be the key nitrite-oxidisers at BNR plants (Zhang *et al*., 2011). Subsequently, the application of the full scale rRNA approach (Amann *et al*., 1995) to full-scale WWTP revealed that novel NOB closely related to the genus *Nitrospira* were the predominant NOB (Daims *et al*., 2001; Gieseke *et al*., 2003).

Although *Nitrobacter* and *Nitrospira* share a common physiological feature, where they belong to two completely different lineages, *Nitrobacter* spp. are considered to be *r*-strategists and *Nitrospira* are the slower growing *K*-strategists (Dytczak *et al*., 2008). This "*K* /r" hypothesis may explain the wide distribution of *Nitrospira* in many natural habitats and their predominance in WWTP. *K*/r selection is a widely used ecological concept to classify the competitive abilities of nitrifiers to survive harsh niche areas. An *r*-strategist grows quickly on easily available substrates whilst a *K*-strategist grows slowly but using limited resources more efficiently. Therefore, the *K*-strategists are more capable of surviving long periods of starvation (Dytczak *et al*., 2008). *Nitrobacter* and *Nitrospira* may co-exist at nitrifying BNR plants (Siripong and Rittmann, 2007). Differences in their competitive abilitities based on their affinities for oxygen and ammonia, have also been noted among *Nitrosomonas* lineages (Geets *et al*., 2007; Park and Noguera, 2008). This may determine which phylotype will succeed at any particular plant. FISH results revealed that communities barely change with time or operational conditions, except possibly during seasonal changes (Siripong and Rittman, 2007). It has also been observed that their cells are not randomly dispersed throughout the floc, but instead organised into large micro-colonies for their survival strategy (Daims *et al*., 2006).

Like other bacteria, nitrifiers are involved in biological interactions with other microbes. AOB and NOB are mutualistic symbionts since AOB produce the nitrite required by NOB, which subsequently prevent the accumulation of toxic nitrite (Stein and Arp, 2008). On the other hand both groups compete for oxygen requirements. Furthermore, the autotrophic nitrifiers are primary producers that release organic compounds used by heterotrophic bacteria (Okabe *et al*., 1999; Kindaichi *et al*., 2006). Current knowledge available on mutualistic or antagonistic interactions between nitrifiers and heterotrophs is limited. Such studies may be important to study the activity and environmental distribution of AOB, NOB, AOA and ANAMMOX at different BNR plants.

2.8.3 Denitrifiers

The second stage of BNR is denitrification, which is an anaerobic process catalysed mainly by facultative anaerobic heterotrophic microbes. These bacteria use organic compounds as electron donors for reducing nitrate and nitrite to gaseous nitrogen compounds, nitric oxide, nitrous oxide and eventually nitrogen gas (Baytshtok *et al*., 2009). Their demand for organic carbon is often unsatisfied during periods with high nitrogen loads and relatively low carbon content. It has become common to increase the denitrifying rate by the addition of readily biodegradable organic compounds like methanol and acetate (Peng *et al*., 2007). Denitrification is a four-step process in which four metallo-enzymes are sequentially involved. This process does not always proceed to result in the complete reduction to nitrogen gas. Depending on their composition and prevailing environmental conditions, denitrifiers may release considerable amounts of the gaseous intermediates such as nitric oxide and nitrous oxide.

Many bacterial species have been isolated from wastewater which was thought to play a role in denitrification. These include members of the genera, *Alcaligenes*, *Bacillus*, *Hyphomicrobium*, *Methylobacterium*, *Parococcus*, *Pseudomonas* and others (Seviour and Nielsen, 2010). Feeding activated sludge with acetate or methanol seems to select for different groups of denitrifiers. Acetate was shown to support members of the *Comamonadaceae* and *Rhodocyclaceae* such as *Comomonas*, *Acidovorax* and *Thaurea* (Beta-proteaobacteria). Methanol favours methylotrophic denitrifiers like *Methylophilus*, *Methylobacillus*, and *Hyphimicrobium* (Ginige *et al*., 2005; Osaka *et al*., 2006).

2.9 Factors affecting Nitrification

Different factors influence the nitrification process. Major factors include MLSS, alkalinity, pH, dissolved oxygen, sludge age (SRT), Temperature (Table 2.3).

Table 2.3: List of factors that influence the nitrification process

FACTOR	ROLE
Mixed liquor suspended solids (MLSS)	Provides a crude estimate of biomass concentrations
Alkalinity and pH	Optimal range is between 6.5 and 8.5. Influent pH is important. The nitrification process is pH dependent, with a pH of 7-8 being optimum for metabolism and growth of autotrophic nitrifiers (Zhang *et al*., 2012).
Dissolved oxygen	Provides valuable information regarding the biological activity of the biomass; often used to control aeration rates, which makes nitrifying bacteria more sensitive to low oxygen concentrations than heterotrophic bacteria. A dissolved oxygen concentration in the range 1.5-2.0 mg/L is therefore recommended in wastewater treatment (Jaroszynski *et al*., 2011).
Sludge age	Determined by sludge loading and is considered to be the fundamental parameter in any design exercise controlled by regulating the rate at which sludge is wasted from the system before clarification (Tchobanoglaous *et al*., 2003). A low sludge age (<5-10 days) may not allow nitrification to occur due to the slow growth rate of nitrifiers. However, a high sludge age (>20-30 days) is important to obtain full N and P removal in temperate climates.
Temperature	All known autotrophic nitrifiers are mesophilic, i.e. their growth rate increases exponentially in the range of 10-25ºC, reaching a constant and optimal growth rate between 25-35ºC, while at 40ºC the growth rate diminishes drastically (Xu *et al*., 2012).

2.10 Detection and Quantification of Nitrifiers from WWTP

2.10.1Cultivation-dependant Techniques

Traditional methods of analysing microbial communities in wastewater treatment systems were carried out either by standard light microscopy (to determine the abundance of floc-forming and filamentous bacteria), cultivation dependent techniques such as plate count or most-probable number counting. Due to the importance of filamentous bacteria for sludge bulking and foaming, keys were developed for provisional identification using their reaction to Gram and Neisser-staining as well as morphological characteristics (Jenkins *et al*., 2004). However, these methods suffer from severe limitations since only a minor fraction of microbes in environmental samples is cultivable on media. Furthermore, bacterial cells present in wastewater treatment systems retain a certain amount of detectable activity despite showing an extremely low level of cultivability when conventional cultivation methods are used. After isolation, bacteria are identified using either physiological parameters or chemotaxonomic markers such as cellular fatty acids or respiratory quinone profiles. The latter two biomarkers can also be used directly for profiling activated sludge microbial communities but provide only relatively low resolution information. Therefore this approach is misleading.

After the introduction of molecular techniques for community analyses it became obvious that only less than 16% of microorganisms can be isolated from activated sludge using standard cultivation techniques and those bacteria that form colonies on the respective media are generally of minor numerical importance *in situ* (Larsen *et al*., 2008). Therefore, cultivation offers a very biased and incomplete view on the bacterial diversity at WWTP. On the other hand, directed cultivation of *in situ* important microorganisms is still a pre-requisite for encompassing physiological and genetic analysis.

2.10.2 Cultivation-independent Techniques

Monitoring abundance and population dynamics of nitrifying bacteria in activated sludge provides important clues regarding correlations between plant operating parameters and microbial community composition. Molecular biological techniques, such as FISH, which are cultivation-independent have been developed and applied to determine the genetic

diversity of microbial communities and to identify individual members based on 16S rRNA genes. FISH with rRNA-targeted oligonucleotide probes is a very powerful research tool and is the method of choice for the cultivation independent detection and identification of microorganisms in activated sludge (Hall *et al*., 2003). Q-PCR is, of recent, becoming more commonly applied to environmental samples.

2.10.2.1 The 16S rDNA Approach

During the past decade, various molecular approaches have been developed and used to study microbial communities in wastewater treatment systems (Sanz and Köchling, 2007). Furthermore, progress regarding gene databases has supported the development of these molecular approaches. These approaches which are not cultivation-dependant are believed to bring new insight to microbial ecology of wastewater treatment systems. Comparative sequence analysis of environmentally retrieved 16S rDNA sequences has become the standard for cultivation-independent assessment of bacterial diversity (Barriuso *et al.*, 2011).

Current 16S rDNA databases contain more than 20,000 entries and thus provide a high-resolution framework for the assignment of sequences obtained (Hugenholtz *et al*., 2001; Giongo *et al*., 2010). The approach consists of DNA extraction, subsequent PCR amplification of (a fragment of) the 16S rDNA gene using primers targeting regions conserved in the bacterial domain, followed by cloning and sequencing. The obtained sequences are analysed together with adequate reference sequences to infer their phylogenetic affiliation. Thus for this approach, efficient DNA extraction techniques should be implemented, with adequate coverage of the selected PCR primers to avoid kinetic and stoichiastic biases introduced during PCR amplification (Suzuki and Giovonnoni, 1996) and cloning.

2.10.2.2 16S rDNA-based Fingerprinting Techniques

The common principle behind these methods is based on the separation of PCR products of the same length but different sequences to visualise the diversity within the amplificate using banding patterns. The most frequently applied fingerprinting technique in wastewater microbiology is denaturing gradient gel electrophoresis (DGGE) (Portillo *et al*., 2011), but terminal restriction fragment length polymorphism (T-RFLP) (Portillo *et al*., 2011), gel

retardation (Schmid *et al*., 2006) and single strand conformation polymorphism (SSCP) (Dabert *et al*., 2010) have also successfully been used.

The main advantage of the use of fingerprinting techniques is that high sample numbers can be processed in a relatively short space of time to obtain an overview of the dynamics of complex microbial communities. DGGE and gel retardation bands of interest can be excised, cloned and sequenced for subsequent identification. DGGE offers higher resolution when compared to gel retardation, but is limited to fragments smaller than 500 nucleotides which are unsuitable for phylogenetic analyses. The organism represented by a T-RFLP or SSCP band can only be identified if a 16S rDNA clone library is established and if the clones obtained are sequenced for identification and used as a reference in the respective fingerprinting protocol. Fingerprinting techniques are affected by the same DNA-extraction and PCR-amplification biases as the 16S rDNA approach and thus cannot provide quantitative data (Engelbrektson, 2010).

2.10.2.3 16S rDNA-based Quantification Method: Fluorescent *in situ* Hybridisation (FISH)

FISH relies on DNA/RNA hybridisations taking place within whole microbial cells. Recently, FISH has been considered the diagnostic method of choice with widespread environmental and medical applications (Amann and Fuchs, 2008). Many FISH probes have been described in literature, most of which are listed in probeBase (Loy *et al*., 2007) that target specific sites on the rRNA of organisms of interest. These probes then hybridise to their target sites forming DNA/RNA duplexes under favourable conditions and permit the detection of target cells by fluorescence after excess probe has been removed.

Detection is carried out by microscopy or flow cytometry. Ideal probes for FISH should offer both high sensitivity and specificity. Probe design and the optimisation of FISH protocols should ensure the elimination of any false positive hybridisations. FISH is extremely effective for specific bacterial detection and for analysing the spatial organisation of a complex microbial community. This is due to the possibility of specific bacterial cell detection at the single-cell level using *in situ* hybridisation with phylogenetic markers labelled with a fluorescent compound (Amann and Fuchs, 2008).

FISH can be used to target populations ranging from the whole taxonomic hierarchy. Many probes are now available to detect individual phenotypes such as nitrifying bacteria (Daims *et al*., 2001; Gieseke *et al*., 2001), denitrifying bacteria (Thomsen *et al*., 2004) as well as other important physiological groups of bacteria in activated sludge (Thomsen *et al*., 2004; Loy *et al*., 2007). FISH does not suffer from the biases inherent in conventional PCR-based methods and is relatively simple. This method has been exploited not only for identification purposes but for quantification of individual populations together with with image analysis software (Daims *et al*., 2006). The application of rRNA targeted probes for activated sludge relies on high ribosome levels in physiologically active cells to generate bright signals which suggest that most cells (about 80%) are metabolically active (Loy *et al*., 2005).

2.10.2.4 -Confocal Scanning Laser Microscopy (CSLM)

Confocal microscopy now provides a powerful tool with which to study microbial community structure, when used in combination with FISH. Samples can be viewed using three dimensions (Sanz and Köchling, 2007). CSLM is widely applied to studies concerning activated sludge, especially for visualisation and quantification during FISH analysis (Schmid *et al*., 2003; Daims *et al*., 2006). Additionally, CSLM with image analysis can also be used with the LIVE/DEAD® method of staining to quantitatively map the distribution of living and dead cells in flocs formed under different operating conditions (Lopez *et al*., 2005). However interpretation of this LIVE/DEAD® staining method may prove difficult with complex samples like activated sludge (Biggerstaff *et al*., 2006).

2.10.2.5 Fluorescent *in situ* Hybridisation (FISH)-Microautoradiography (MAR)

FISH-MAR is another powerful tool that has been applied to a range of activated sludge studies including the detection of cell viability and enumeration of bacteria capable of utilising specific organic substrates, studies of autotrophic uptake of orthophosphate and potential use of various electron acceptors (Kong *et al*., 2005; Eales *et al*., 2006). This method is based on the observation that radio-labelled substrate uptake by individual cells can be visualised with a radiation-sensitive silver halide emulsion. This is placed over the radio-labelled bacteria and subsequently processed by bright field or phase contrast microscopy. FISH-MAR is able to identify *in situ* which populations or phylotypes are

responsible for performing key biochemical changes in activated sludge at a single cell level (Angenent *et al*., 2006).

The quantification of functional groups capable of assimilating particular substrates *in situ* under specified operating conditions is achieved with MAR (Rogers *et al*., 2007). MAR-FISH provides the means to link the identity of individual bacterial populations to their *in situ* physiology. The limitations with MAR-FISH lie in having extensive experience with both techniques, and preferably access to and experience with confocal microscopy. This is however time-consuming and expensive. Also, certain countries require safety regulations with dedicated laboratories and equipment for isotope work. Additionally, the commercial availability of suitable radio-labelled substrates may be a limitation. MAR can be limiting in that with suitable controls it clearly demonstrates substrate assimilation but not necessarily the subsequent substrate metabolic fate (Angenent *et al*., 2006).

2.10.2.6 Polymerase Chain reaction (PCR)-based techniques

PCR is a fundamental technique and is extremely successful in generating amplified regions of DNA. This technique is routinely used for the culture-independent analysis of activated sludge communities and their functions. The 16S rRNA gene has been targeted to determine overall population biodiversity. The average bacterial 16S rRNA molecule (Figure 2.4) is approximately 1,500 nucleotides in length. When partially or fully sequenced it contains sufficient conserved and variable nucleotide regions for reliable phylogenetic analyses (Amann *et al*., 1995; Clarridge, 2004). Therefore 16S rRNA targeted primers have been widely used to obtain PCR amplicons directly from DNA which has been extracted from activated sludge. Universal and domain targeted primers for 16S rRNA genes are still widely used. However as databases increase in size these primers require further modification.

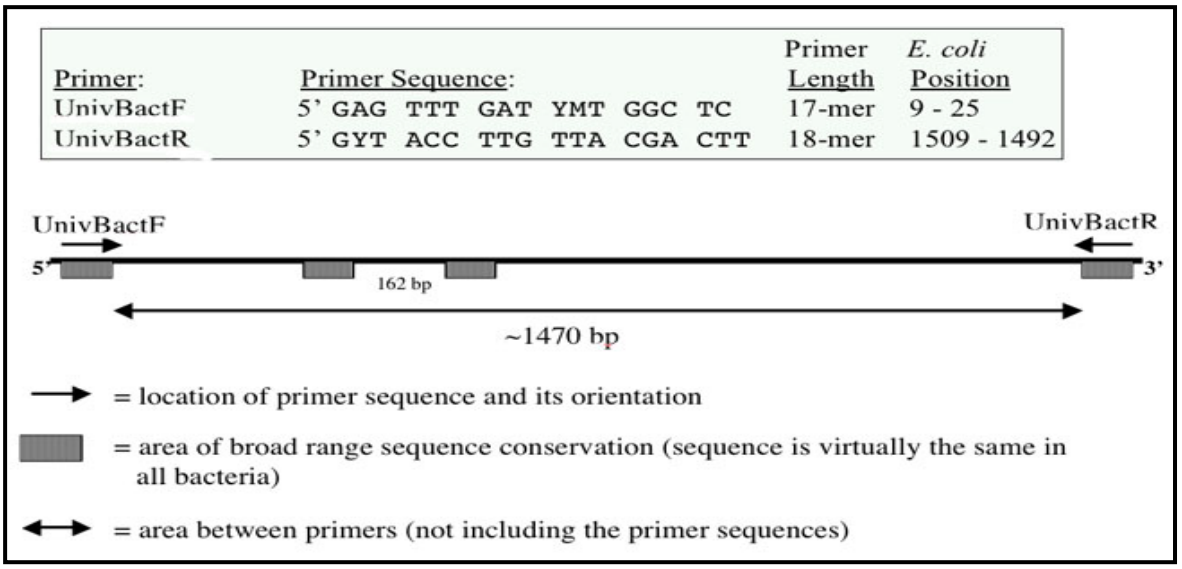


Figure 2.4: A simplified map of the 16S rRNA molecule (Rice, http://serc.carleton.edu/microbelife/research_methods/genomics/modgen.html)

Primers that target a specific phylogenetic group are required. The challenge lies in finding sequence regions that capture 100% of the group required and simultaneously excludes all other organisms. As new sequences are continually added to the database, group specific primers may become obsolete, as they have mismatched sequences for intended species. The repetitive discovery of new higher taxa after 16S rRNA gene sequence analyses indicates that 'prokaryotic' biodiversity is still poorly represented in these databases (Teeling and Glockner, 2012). Thus the design of domain and group specific primers requires further development (Forney *et al*., 2004). A list of universal or domain-targeted primers targeting the 16S rRNA gene is listed in Table 2.4.

Table 2.4: Universal or domain-targeted primers targeting the 16S rRNA gene (Lane, 1991)

PRIMER	POSITION*	TARGET	SEQUENCE (5'-3')
27f	8-27	Most bacteria	AGAGTTTGATCMTGGCTCAG
357f	339-357	Most bacteria	CTCCTACGGGAGGCAGCAG
530f	515-530	Most bacteria, Archaea	GTGCCAGCMGCCGCGG
926f	907-926	Most bacteria, Archaea	AAACTYAAAKGAATTGACGG
1114f	1099-1104	Most bacteria	GCAACGAGCGCAACCC
1406f	1391-1406	Most bacteria, Archaea	TGYACACACCTCCCGT
342r	357-342	Most bacteria	CTGCTGCSYCCCGTAG
519r	536-519	Most bacteria, Archaea	GWATTACCGCGGCKGCTG
907r	926-907	Most bacteria, Archaea	CCGTCAATTCMTTTRAGTTT
1100r	1114-1100	Most bacteria	GGGTTGCGCTCGTTG
1392r	1406-1392	Most bacteria, Archaea	ACGGGCGGTGTGTRC
1492r	1513-1492	Most bacteria, Archaea	TACGGYTACCTTGTTACGACTT
1525r	1542-1525	Most bacteria, Archaea	AAGGAGGTGWTCCARCC

*Based on *E. coli* numbering

Functional genes coding for important proteins in activated sludge communities can be the target for PCR amplification. They may provide additional phylogenetic information on population biodiversity of the functional populations present. Several primers for functional genes have been designed for activated sludge community analyses, including those targeting genes thought to be important for substrate or nutrient removal. A list of PCR primers for the detection of nitrifying functional genes in activated sludge are are displayed in Table 2.5.

Table 2.5: PCR primers for the detection of nitrifying functional genes in activated sludge

PRIMER	TARGET GENE	SEQUENCE (5'-3')	REFERENCE
nirK1F	nitrite reductase (*nir*K)	GGMATGGTKCCSTGGCA	Braker *et al*., 2000
nirK4R	nitrite reductase (*nir*K)	GGRATRARCCAGGTTTCC	Braker *et al*., 2000
nirS1F	nitrite reductase (*nir*S)	CCTAYTGGCCGCCRCART	Braker *et al*., 2000
nirS5R	nitrite reductase (*nir*S)	CTTGTTGWACTCGSSCTGCAC	Braker *et al*., 2000
***amoA*-1F**	ammonia monooxygenase (*amoA*)	GGGGTTTCTACTGGTGGT	Rotthauwe *et al*., 1997
***amoA*-2R**	ammonia monooxygenase (*amoA*)	CCCCTCKGSAAAGCCTTCTTC	Rotthauwe *et al*., 1997

The specificity of a primer is crucial for successful PCR amplification. To amplify only the desired fragment, primers need to be 100% complementary to the target sequence only. Challenges exist when trying to obtain sufficient PCR product and substances interfere with

the properties of DNA, therefore PCR optimisation is essential. PCR-based methods do not provide quantitatively valid information. Therefore, PCR techniques have been developed to overcome some of these problems by increasing amplification specificity and amplicon yield. Q-PCR methods were developed to allow targeted gene copy numbers to be quantified. This has been applied to activated sludge communities by targeting rRNA genes and appropriate functional genes.

2.10.2.7 Real-time Quantitative PCR (Q-PCR)

This method enables quantification of the number of targeted gene copies. A range of different platforms have been developed, including SYBR® Green I (Smith and Osborn, 2009), TaqMan probe (Hoffmann *et al*., 2009), Molecular beacons (Saidac *et al*., 2009) and quenching primer/probe (Smith and Osborn, 2009). The principle of Q-PCR using SYBR® Green I is that fluorescence intensity increases in proportion to the amount of amplified products formed at a given time, which is proportional to the concentration of the specific template DNA in the original sample. Q-PCR has been applied to activated sludge systems to quantify targeted functional genes including *amoA* (Harms *et al*., 2003) and the 16S rRNA gene. Q-PCR is suitable to quantify target organisms in activated sludge even at abundances of less than 1% of the total bacterial population. Q-PCR targeting the rRNA gene can be used to determine relative abundances of targeted bacteria with high sensitivity, reproducibility, and throughput. Thus this technique is a fast and efficient tool to quantify targeted population abundances in activated sludge and is more sensitive than FISH.

Conventional PCR cannot be used quantitatively, Q-PCR (Smith *et al*., 2006) and competitive PCR (cPCR) systems (Dionisi *et al*., 2002) allow for the quantification of individual bacterial populations and copy numbers of functional genes of interest. Q-PCR has been used to quantify many activated sludge populations including nitrifying and denitrifying populations (Geets *et al*., 2007; Limpiyakorn *et al*., 2007) and the levels of their functional genes (Jin *et al*., 2010). Several nitrifying and denitrifying bacterial populations (Geets *et al*., 2007; Limpiyakorn *et al*., 2007) can be quantified using this approach. Q-PCR based methods (Smith, 2005) also help to monitor functional groups in wastewater systems using functional genes.

2.10.3 Microarrays

Microarrays are best known for the genome-wide analysis of gene expression (Martinez-Godoy *et al*., 2006). However, their potential as tools for community composition studies was recognised which led to an increase in the number of studies using arrays (He *et al*., 2011). Current interest in microarrays reflects their advantage over other molecular techniques, especially their potential for high-throughput sample analysis once full automation is achieved. This feature alone is of considerable benefit in monitoring engineered systems like activated sludge plants.Their beauty lies in the highly parallel nature of organism detection i.e. hundreds to tens of thousands of probes can be applied simultaneously to a single sample, compared with only a few as with techniques such as FISH (Gresham *et al*., 2008). This advantage is especially important when dealing with phylogenetically diverse groups of organisms, where no single specific probe or primer might target all members.

Most microchips developed thus far for environmental applications contain either long oligonucleotide probes (Tiquia *et al*., 2004) or probes based on PCR products (Dennis *et al*., 2003). Since many AOB species differ by more than 20% in their *amoA* gene sequences (Koops *et al*., 2003), it may be feasible to discriminate among some using the relevant functional gene and stringent hybridisation conditions. To date, only a few studies have used microarrays with activated sludge samples. However, several diagnostic arrays are suitable (Yu and Zhang, 2012). These include arrays for organisms involved in nitrification (Adamczyk *et al*., 2003), denitrification (Loy *et al*., 2005) nitrogen fixation (Moisander *et al*., 2007) and phosphorus removal (Loy *et al*., 2005).

This study carried out the analyses of autotrophic nitrifying bacterial communities using advanced molecular techniques, which are more rapid and reliable as compared to microbiological and biochemical methods. A study of this nature has not been conducted in South Africa to our knowledge, regarding autotrophic nitrifying bacteria and their levels of activity and dominance across full-scale wastewater treatment systems, treating different influent waste streams. Thus there is a need to correlate plant operating conditions with population dynamics as this will assist to pre-empt problems at full-scale level and allow operators and engineers to source a problem before it reaches an escalated and uncontrolled level.

CHAPTER THREE: Impact of Different Pre-Treatments on Nitrifying Bacterial Community Analyses in Wastewater Treatment Plants

N. Ramdhani, S. Kumari and F. Bux. 2010. Impact of pre-treatments on nitrifying bacterial community analysis from wastewater using FISH and CSLM. *Journal of General and Applied Microbiology*, **56** (2): 101-106.

3.1 INTRODUCTION

Wastewater is treated by a variable and diverse community of microorganisms in an aquatic environment. The activated sludge process is one of the most extensive biological processes used for the treatment of wastewater (Snidaro *et al*., 1997; Wei *et al*., 2003). Bacteria constitute the majority of microorganisms present in activated sludge and are accountable for the degradation process (Thomsen *et al*., 2004). The activated sludge process is based on the formation and arrangement of strong microbial flocs to which bacteria are attached. This bacterial aggregation and flocculation has been primarily attributed to an exopolymeric matrix (Falcioni *et al*., 2006). Nitrifying bacteria mainly appear as dense aggregates within activated sludge flocs (Manser *et al*., 2005). The complex arrangement of activated sludge is complicated to assess with conventional techniques alone (Bourrain *et al*., 1999).

Emerging molecular techniques used for *in situ* analyses may not replace conventional microbiological techniques entirely, but will assist to identify, monitor, and isolate particular organisms of interest from wastewater (Snaidr *et al*., 1997; Falcioni *et al*., 2006). Fluorescent *in situ* hybridisation (FISH) is used to characterise nitrifiers (Sears *et al*., 2005) in conjunction with oligonucleotide probes, thus enabling *in situ*, culture-independent identification (Carr *et al*., 2005). This technique also allows for the assessment of the total bacterial population, including the viable but uncultivable portion (Falcioni *et al*., 2006). However, a limitation of the FISH technique rests primarily in the difficulty of probe penetration to individual cells, since the major fraction of bacterial cells in activated sludge is attached to aggregates. For accuracy during quantification, it is therefore essential to initially disrupt the flocs to release the bacterial cells from within the floc (Biggs and Lant, 2000).

The sludge type and compaction also play a major role during floc dispersion. Therefore, in order to acquire representative results where individual cells are quantified, the detachment of bacteria from within these flocs is essential (Falcioni *et al*., 2006). Confocal scanning laser microscopy (CSLM) in conjunction with FISH (FISH-CSLM) and digital image analysis has been effectively used for the direct analysis of single cells within microbial populations in activated sludge systems. FISH-CSLM makes it feasible to analyse the spatial distribution of nitrifying bacteria in activated sludge flocs and removes disturbing auto-fluorescent signals. The FISH technique is usually associated with a conventional fluorescent microscope. However, FISH-CSLM significantly minimises the effects of background and out of focus fluorescence (Wagner *et al*., 1994). The optical sectioning properties of CSLM also significantly improved the *in situ* detectability of nitrifying bacteria in activated sludge (Delatolla *et al*., 2009).

A fundamental difficulty in the efficient detachment of bacteria from flocculated clumps lies in striking a balance between the use of procedures harsh enough to achieve near-complete detachment and the associative risk of cell disruption. Taking these conflicting factors into consideration, determination of the most efficient pre-treatment method is based on the degree of disruption and the effects on cell integrity (Buesing and Gessner, 2002; Falcioni *et al*., 2006). Physical methods, like sonication, do not contaminate the sample, and is an efficient method for dispersing bacterial aggregates (Comte *et al*., 2006; Li *et al*., 2008), provided excessive energy, which could lyse the cells and contaminate the sample with intracellular polymers, is not used (Jorand *et al*., 1995; Sears *et al*., 2005). The hydro-chemical shear forces produced by sonication are predominantly responsible for sludge disintegration. High temperatures and pressures developed inside the collapsing bubbles can induce many physico-chemical effects which can alter the state of the sample. Therefore sample containers are usually immersed in ice baths.

Chemical methods can also be applied by the use of surfactants. Surfactants are classified in different groups: anionics, non-ionics, cationics and zwitterionics. The mechanism of action of surfactants includes the facilitation of penetration by the control agent into the cells of the target organism (Andreu *et al*., 2007). Chemical surfactants such as Igepal are non-ionic and non-denaturing in nature and enhance cell dispersion by the alleviation of cell-clumping without any obvious damage to the cells when used at the appropriate concentration (Stahl

and Amann, 1991; Singh *et al*., 2006). Enzymatic pre-treatment with lysozyme allows for cell permeability (Furukawa *et al*., 2006), since lysozyme opens up the peptidoglycan layer and allows for increased cell permeability which might otherwise result in insufficient penetration of high molecular weight reagents into nitrifying bacterial cells (Moter and Göbel, 2000).

Although there are reports on floc dispersion studies, the effect of these pre-treatments on activated sludge microbial communities has not well been analysed. Many of these procedures have been employed for years, however little work has been done to compare the efficiency of these pre-treatments on different types of wastewater. Thus, the aim of this aspect of the study was to investigate the impact of pre-treatment methods on bacterial floc dispersion to expose single cells for accurate enumeration of the nitrifying bacterial community, using FISH-CSLM.

3.2 MATERIALS AND METHODS

3.2.1 Sampling and Cell Fixation

The activated sludge samples were obtained from the aerobic zones of activated sludge BNR plants in KwaZulu-Natal (South Africa). These were selected to encompass the treatment of both domestic (Kingsburgh and Northern) and industrial (Hammarsdale, Southern and Darville) wastewater. Sludge samples were collected from the aerobic zones of all five plants (Table 3.1). The mixed liquor was half-filled in the sample bottles so as to maintain aerobic conditions during sample transit, and was stored at -4°C until further use. Samples (1ml) were washed twice and re-suspended in phosphate buffered saline (PBS: 130mM sodium chloride, 10mM sodium phosphate buffer [pH 7.2]). Samples were then immediately fixed in 4% paraformaldehyde to render the cells permeable to oligonucleotide probes during FISH analyses (Amann, 1995). Fixed samples were stored in a 1:1 mixture of PBS and absolute ethanol at -20°C until further hybridisation.

Table 3.1: List of BNR plants and influent waste streams

WWTP	HAMMARSDALE	KINGSBURGH	NORTHERN	DARVILLE	SOUTHERN
WASTEWATER TYPE	Industrial	Domestic	Domestic	Industrial	Industrial
COMPOSITION	Abattoir waste, textiles, dyes (95%) and domestic (5%)	Domestic (80%) and industrial (20%)	Domestic (80%) and industrial (20%)	Mix of domestic (10%) and industrial (90%)	Mix of domestic (20%) and petrochemical (80%)
NUMBER OF SAMPLES	12	12	12	12	12

3.2.2 Pre-treatment of Activated Sludge using Physical, Chemical and Enzymatic Methods

3.2.2.1 Physical Pre-treatment using Sonication

Samples (1ml) were placed in 2ml microtubes and sonicated using a probe sonicator (Virsonic 100; Virtis, USA). Sonication was optimised by comparing the effects of four sonication power levels of 5, 6, 7 and 8 watts. The sonication duration varied from 5 to 8 minutes at a time interval of 1 minute. This process was carried out whilst the sample containers were held in an ice bath during sonication and between each sonication cycle only a few seconds passed. Following sonication samples were evaluated microscopically (1000 × oil immersion) so as to determine the effects of the various sonication times on the integrity of the nitrifying bacterial cells.

3.2.2.2 Chemical Pre-treatment using Igepal

The samples (1ml) were treated with 250µl Igepal (0.1% (v/v); (Igepal CA-630-Sigma, Germany) surfactant following sonication. The samples were then homogenised for 1 minute using a vortex mixer so as to assure complete homogenisation and optimal floc break-up, spotted and stained with DAPI (4, 6-diamidino-2-phenylindole dihydrochloride hydrate – Sigma-Aldrich, Germany), at a final concentration of 1.25µg/ml for image analysis. DAPI is a DNA-intercalating dye that binds to the sample. Following pre-treatment, samples were evaluated microscopically (1000 × oil immersion oil).

3.2.2.3 Combination Pre-treatment using Igepal and Sonication

A combination pre-treatment of sonication together with Igepal was also tested. Samples (1ml) were combined with Igepal (0.1% v/v) in microtubes and homogenised. This mixture was then sonicated. This was thereafter spotted and stained with DAPI for image analysis, and evaluated microscopically (1000 × oil immersion oil).

3.2.2.4 Enzyme Pre-treatment using Lysozyme

Cells were treated with an enzyme to facilitate cell dispersion. Samples (1ml) were placed in 2ml microtubes together with lysozyme (1mg/ml); (Sigma-Aldrich, Germany). Permeabilization buffer was applied for 30 minutes at 37°C (Wallner *et al.*, 1993). The enzyme mixture was thereafter homogenised for 1 minute using a vortex mixer so as to ensure complete homogenisation and optimal floc break-up, spotted and stained with DAPI. Following pre-treatment, samples were evaluated microscopically (1000 × oil immersion oil).

3.2.2.5 Combination Pre-treatment using Lysozyme and Sonication

The samples were then exposed to a combination pre-treatment. Samples (1ml) were combined with lysozyme in microtubes, sonicated and then homogenised. This mixture was thereafter spotted and stained with DAPI for image analysis, and evaluated microscopically (1000 × oil immersion).

3.2.3 Slide Preparation

Teflon coated microscope slides were pre-treated with 1:10 poly-L-lysine solution (Sigma-Aldrich, Germany) according to the manufacturer's instructions. A volume of 10µl of treated sample was applied to each well. Spots were allowed to air dry before dehydrating through an ethanol series of 60, 80 and 100 % (absolute) ethanol for 3 minutes each (Amann, 1995).

3.2.4 Oligonucleotide Probes

Group-specific oligonucleotide probes were selected to target all bacteria and the dominant AOB and NOB populations (Roche Diagnostics, South Africa) in wastewater. All probes were modified on the 5' end with a 5(6)-carboxyfluorescein-N-hydroxysuccinimide (FLOUS)

ester and HPLC purified. The 16S rRNA-targeted oligonucleotide probes used in this study for FISH and their stringencies are listed in Table 3.2.

Table 3.2: List of 16S rRNA-targeted oligonucleotide probes and stringencies

PROBE	SEQUENCE (5'-3')	SPECIFICITY	%FA*	REFERENCE
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	20	Daims *et al*., (1999)
EUB338II	GCAGCCACCCGTAGGTGT	Planctomycetes	20	Daims *et al*., (1999)
EUB338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	20	Daims *et al*., (1999)
NONEUB	ACTCCTACGGGAGGCAGC	Control probe	20	Wallner *et al*., (1993)
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp	40	Wagner *et al*., (1996)
NEU	CCCCTCTGCTGCACTCTA	*Nitrosomonas* spp	40	Juretschko *et al*., (1998)
Nsv443	CCGTGACCGTTTCGTTCCG	*Nitrospira* spp	30	Mobarry *et al*., (1996)

*Percentage of formamide (%v/v) in the hybridization buffer

3.2.5 Whole Cell Hybridisation

Teflon coated microscope slides were pre-treated with 1:10 (v/v) Poly-L-Lysine solution (Sigma, Germany). Poly-L-Lysine solution (100ml) was brought to room temperature (18–26°C) and slides were immersed in this solution for 5 minutes. Slides were subsequently dried overnight at room temperature before use. The samples for all five pre-treatment methods were mixed thoroughly and hybridized with 16S rRNA-targeted oligonucleotide nitrifying bacterial probes (Table 3.2). Samples were hybridised with appropriate hybridisation buffers and probed *in situ* with oligonucleotide probes (Amann, 1995). Hybridised, pre-treated samples (10µl) were applied to each well. Spots were air dried prior to dehydrating through an ethanol series (60, 80 and 100%, v/v) for 3 minutes each.

Filter paper was soaked in the appropriate hybridisation solution and placed in a polypropylene tube to allow the chamber to equilibrate for 15 minutes at 46ºC. The spotted slides were placed in the pre-warmed chamber and incubated to allow for hybridisation at 46ºC for 2 hours. Wash buffer was allowed to pre-heat in a water bath at 48°C. Thereafter, hybridised slides were placed into the wash buffer and into a water bath for 20 minutes at 48ºC. Slides were thereafter rinsed twice with 1 x PBS and air dried.

3.2.6 DAPI Staining

The bacterial cells were stained with 10µl of 0.25µg/ml DAPI for 10 minutes in the dark for image analysis. The slides were thereafter rinsed with PBS solution (pH 7.2), air dried and cells were mounted in VECTASHIELD® anti-fading agent (Vector Laboratories, USA).

3.2.7 Image Analysis and Quantification using FISH and CSLM

Following pre-treatment and whole cell hybridisation, samples were visualised using epiflourescent microscopy (1000 x oil immersion) and enumerated accordingly. Images were captured using a Zeiss AxioCam MRc (Carl Zeiss, Germany) camera and image analysis was carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging software. A total of three random fields per sample were quantified. For CSLM, the slides were viewed using a Zeiss confocal scanning laser microscope (LSM 710 and Axio Observor Z1) and images were captured to ascertain floc structure and its dispersion after exposure to the various pre-treatment methods.

3.2.8 Statistical Analysis

Statistical analysis was carried out using analysis of variance (ANOVA) from the data analysis Add-In Toolpak in Excel (Millar, 2001).

3.3 RESULTS

3.3.1 Effect of Pre-treatment on Floc Dispersion of Different Wastewater Types using CSLM

3.1.1 Physical Pre-treatment: Sonication

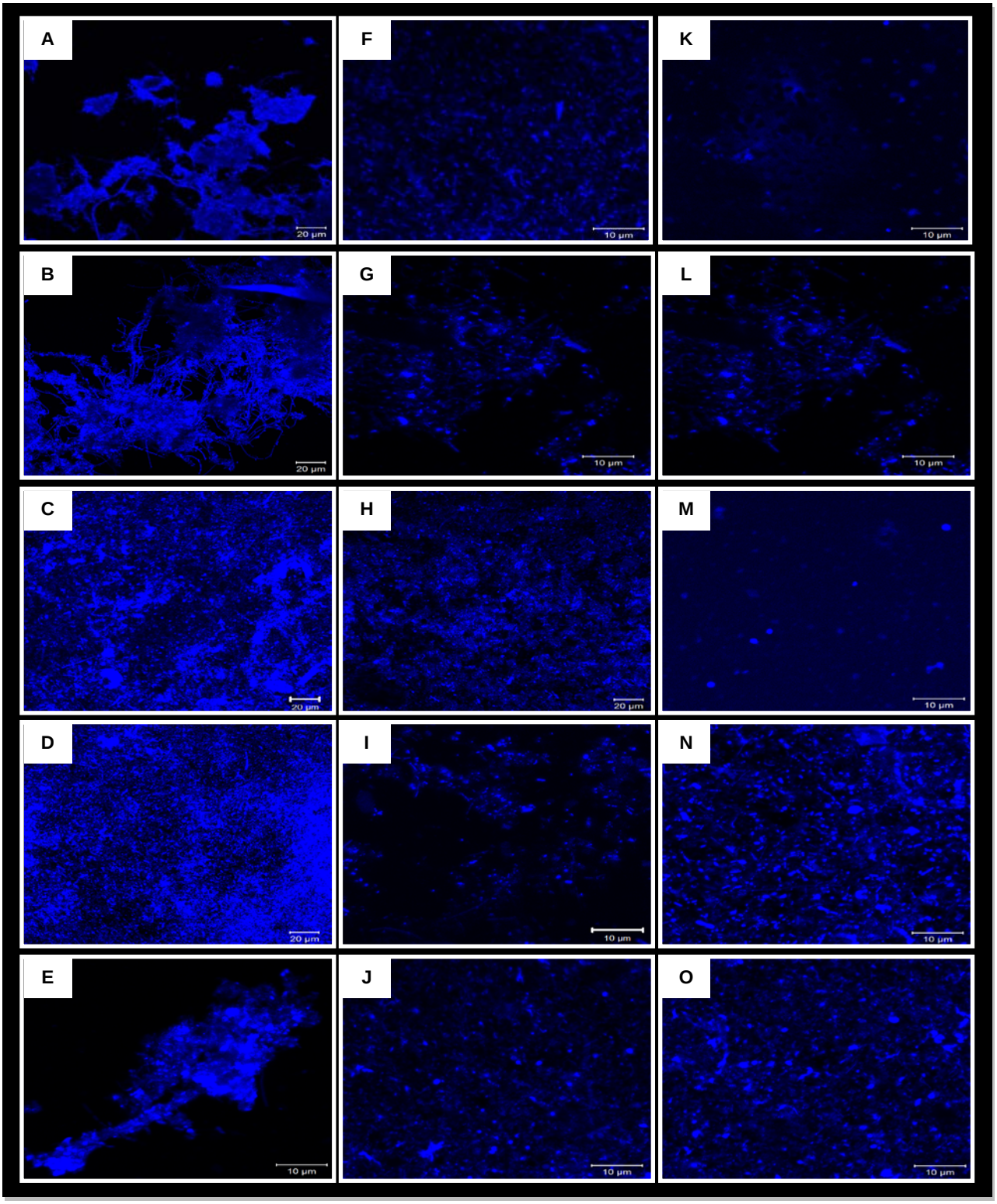


Figure 3.1: CSLM micrographs depicting floc dispersion in the controls (A-E); sonication pre-treatment at 8W (F-J); sonication at 9W (K-O), where: A) Hammarsdale; B) Northern; C) Kingsburgh; D) Southern and E) Darville BNR plant.

The CSLM micrographs above clearly represent the variation in floc size and dispersion for each BNR plant. Figures 3.1A-E (controls) depicts cells clumped together, which made quantification difficult during FISH analysis. Thus it was imperative to disperse the flocs using pre-treatment methods such as sonication to release individual cells without any loss of cell integrity. Activated sludge from these plants underwent a series of experiments using sonication at varying time intervals and power levels. Sonication was optimised at 8 watts thereby allowing accurate and efficient enumeration of individual cells for all BNR plants. Figures 3.1F-J show quantification of individual cells using sonication which resulted in cell counts of 127 individual cells (Hammarsdale); 187 cells (Northern); 167 cells (Kingsburgh); 117 cells (Southern); 126 cells (Darville). However, it was noted that beyond this optimised sonication level, cell integrity (9 watts) was lost since cell lysis occurred. This compromised the efficiency of quantification.

Figures 3.1K-O depict drastic decreases in individual cell counts for all BNR plants, where 28 individual cells (Hammarsdale); 40 cells (Northern); 48 cells (Kingsburgh); 35 cells (Southern); 42 cells (Darville) were quantified. Sonication was therefore optimised at 8 watts, 8 minutes for industrial wastewater (Hammarsdale; Southern; Darville BNR plants) and 8 watts, 5 minutes for domestic wastewater (Northern and Kingsburgh BNR plants). Statistical analysis resulted in a significant difference between the number of cells quantified after 8 and 9 watts at 8 minutes ($P<5\%$; $P=0.02$) Cell quantification after 9 watts is significantly lower than after 8 watts.

3.3.1.2 Chemical Pre-treatment: Igepal

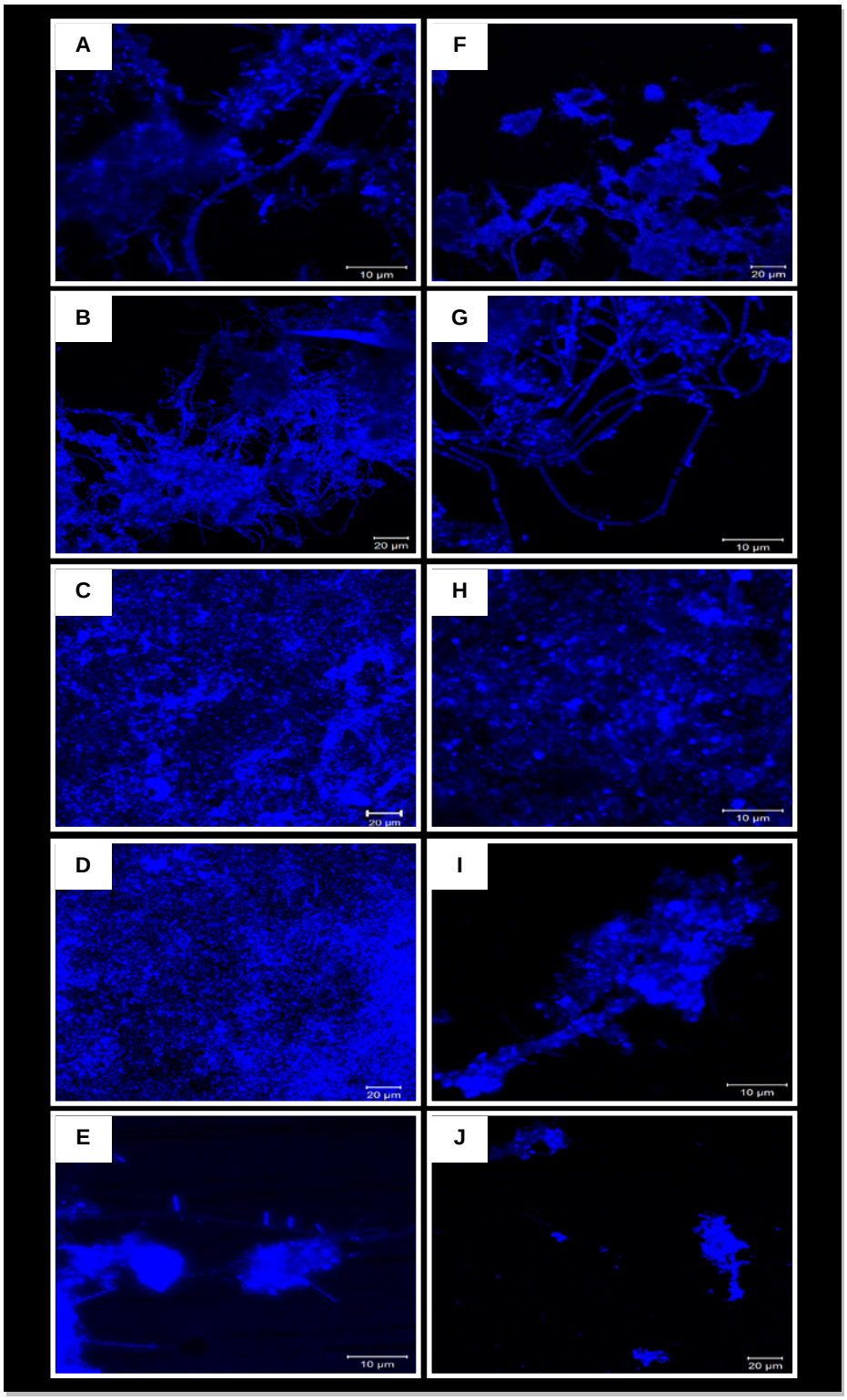


Figure 3.2: CSLM micrographs depicting floc dispersion in the controls (A-E) to Igepal pre-treatment (F-J), where: A) Hammarsdale; B) Northern; C) Kingsburgh; D) Southern; E) Darville BNR plant.

The CSLM micrographs above represent untreated samples (Figures 3.2A-E) which are controls, and samples pre-treated using Igepal. Figures 3.2F-J depict individual cell counts of 38 cells (Hammarsdale); 31 cells (Northern); 26 cells (Kingsburgh); 17 cells (Southern); 14 cells (Darville).

3.3.1.3 Enzymatic Pre-treatment: Lysozyme

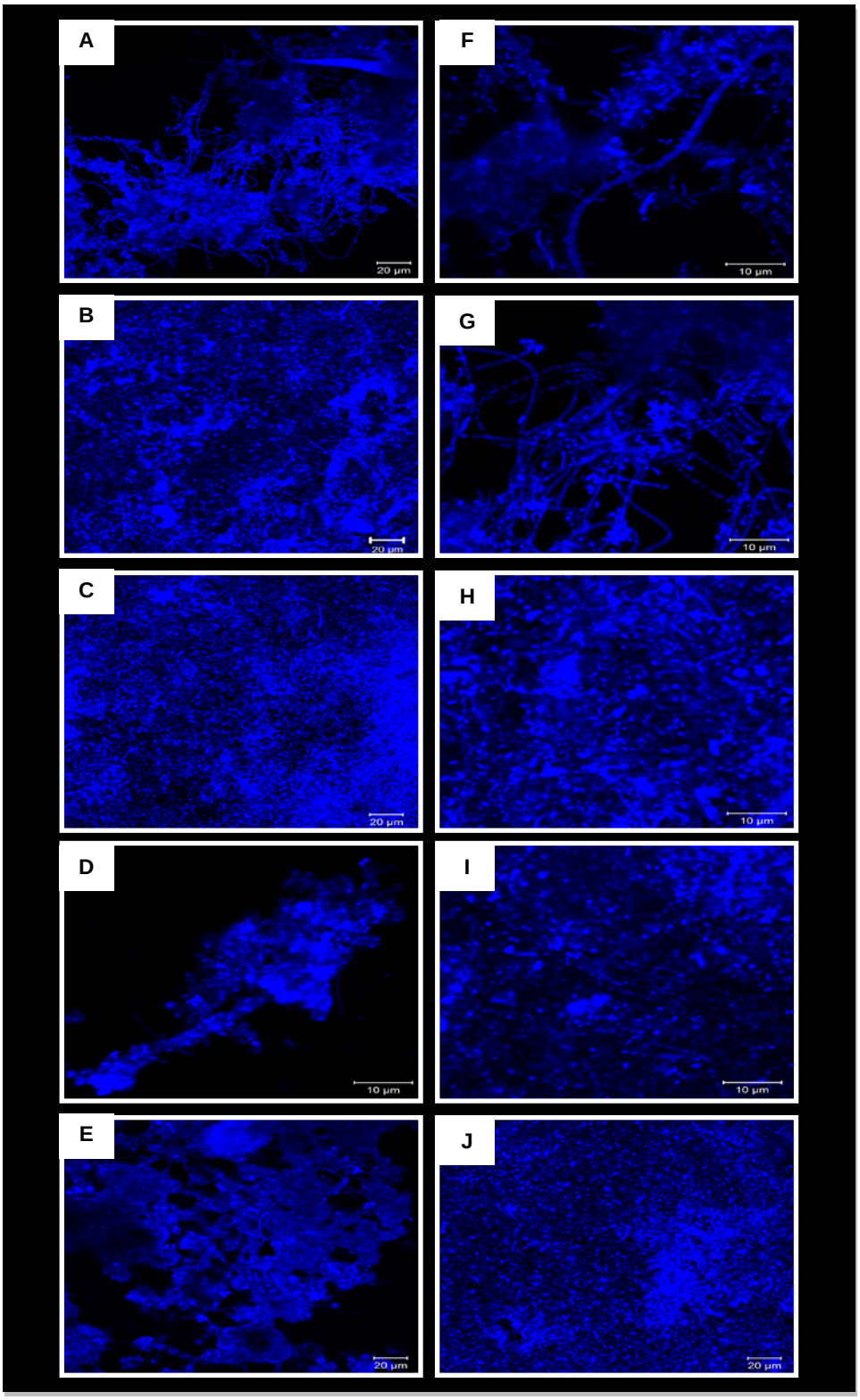


Figure 3.3: CSLM micrographs depicting floc dispersion in the controls (A-E) to lysozyme pre-treatment (F-J), where: A) Hammarsdale; B) Northern; C) Kingsburgh; D) Southern; E) Darville BNR plant.

The CSLM micrographs above represent untreated samples (Figures 3.3A-E) which are controls, and samples pre-treated using lysozyme (Figures 3.3F-J). The images clearly indicate the variation in floc size and dispersion for each BNR plant. Figures 3.3F-J show the dispersion using pre-treatment with lysozyme, which resulted in cell counts of 6 individual cells (Hammarsdale); 46 cells (Northern); 51 cells (Kingsburgh); 12 cells (Southern); 15 cells (Darville).

3.3.1.4 Physical and Chemical Combination Pre-treatment: Sonication and Igepal

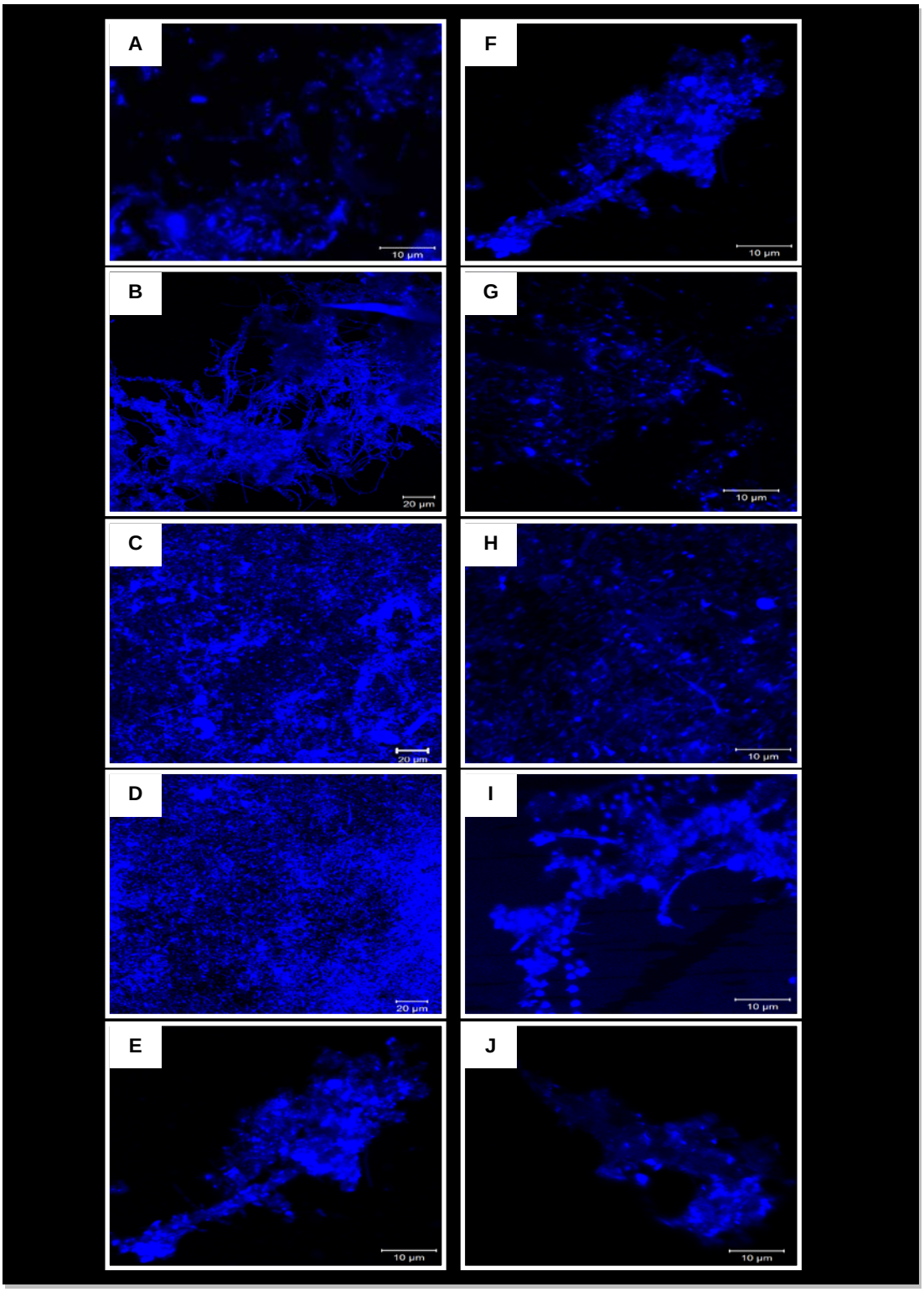


Figure 3.4: CSLM micrographs depicting floc dispersion in the controls (A-E) to Sonication and Igepal pre-treatment (F-J), where: A) Hammarsdale; B) Northern; C) Kingsburgh; D) Southern; E) Darville BNR plant.

The CSLM micrographs above represent untreated samples (Figures 3.4A-E) which are controls, and samples pre-treated using a combination pre-treatment of sonication and Igepal (Figures 3.4F-J). The images indicate the variation in floc size and dispersion for each WWTP. Figures 3.4F-J show the dispersion which resulted in cell counts of 31 individual cells (Hammarsdale); 48 cells (Northern); 52 cells (Kingsburgh); 35 cells (Southern); 38 cells (Darville).

3.3.1.5 Physical and Enzymatic Combination Pre-treatment: Sonication and Lysozyme

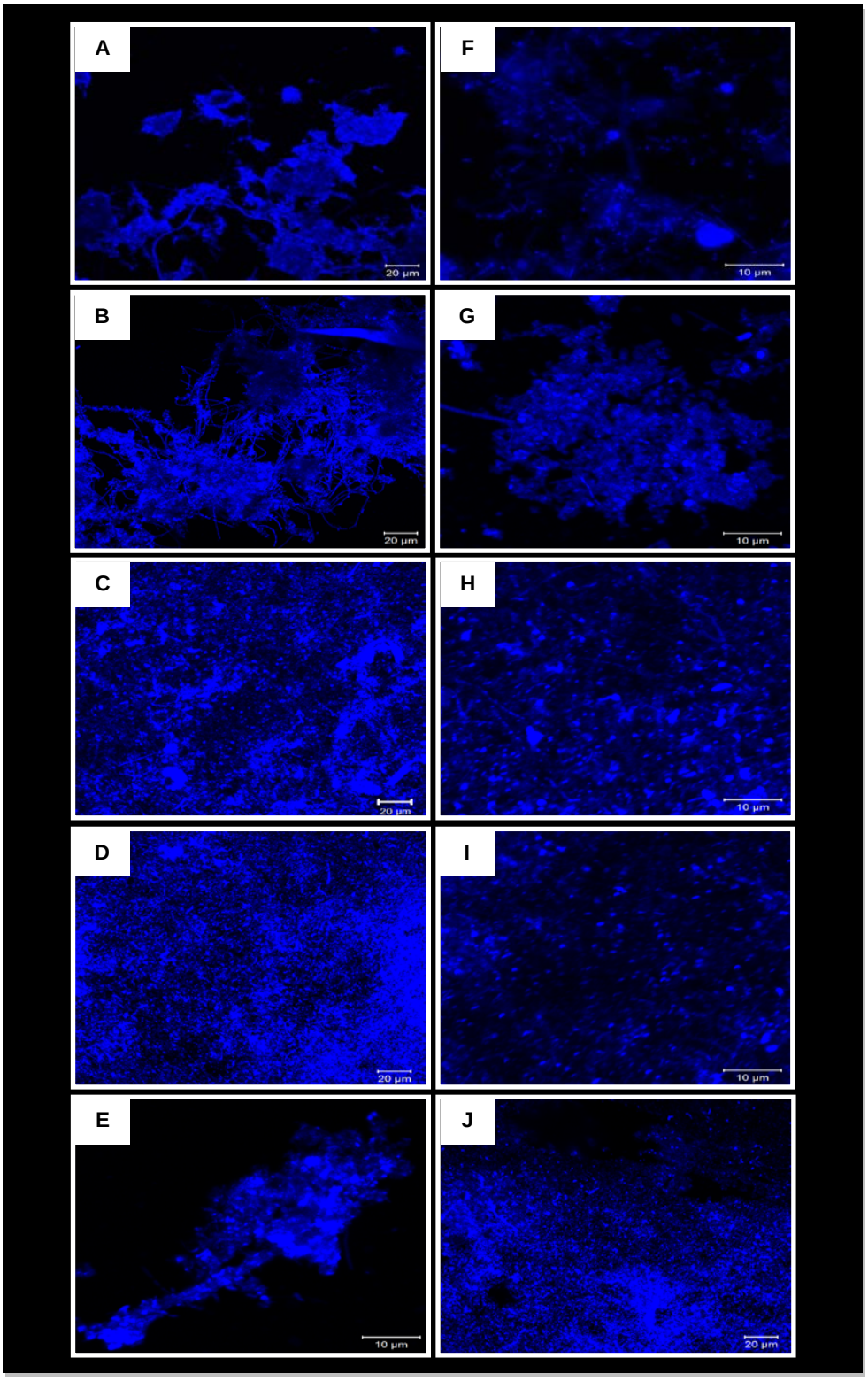


Figure 3.5: CSLM micrographs depicting floc dispersion in the controls (A-E) to sonication and lysozyme pre-treatment (F-J), where: A) Hammarsdale; B) Northern; C) Kingsburgh; D) Southern; E) Darville BNR plant.

The CSLM micrographs above represent untreated samples (Figures 3.5A-E) which are controls, and samples pre-treated using a combination of sonication and lysozyme. Figures 3.5F-J clearly indicate the variation in floc size and dispersion for each BNR plant, where Figures 3.5F-J depict resulting cell counts of 43 individual cells (Hammarsdale); 32 cells (Northern); 48 cells (Kingsburgh); 36 cells (Southern); 41 cells (Darville).

3.3.2 Effect of Pre-treatment on Enumeration of Bacterial Populations from Different Wastewater Types

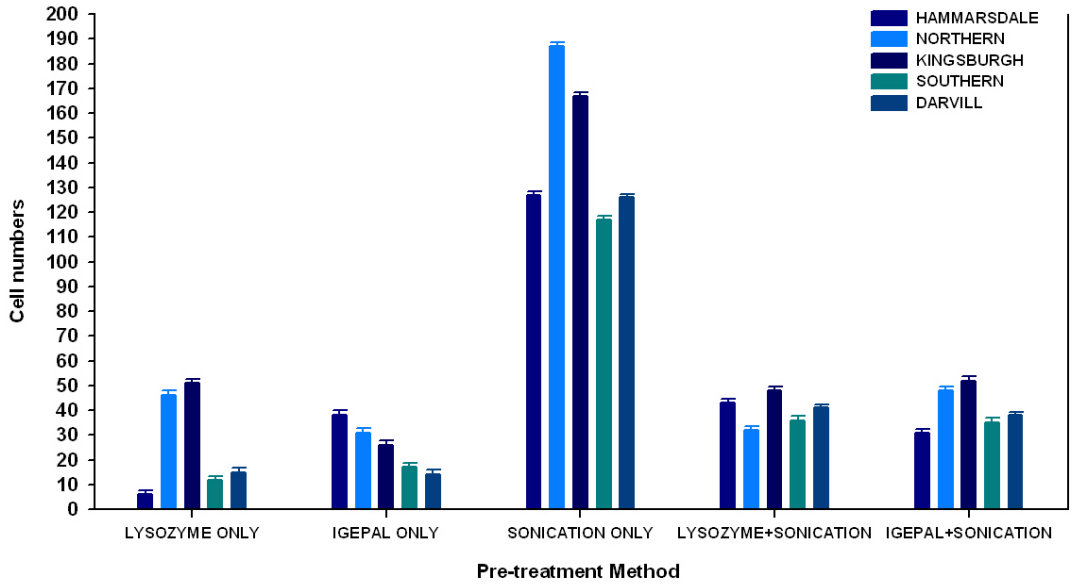


Figure 3.6: Comparison of pre-treatment methods based on cell quantification. Results are represented as medians and standard deviations of the three replicates. Error bars represent the standard deviations of three replicate measurements of the median.

The results of experiments on the effect of pre-treatment on the enumeration of bacterial populations for all five BNR plants are presented in Figure 3.6. All cell enumerations were conducted in triplicate. Error bars represent the standard deviations of the median measurements. There was a significant difference ((P<5%; P=0.96) in the number of individual cells dispersed out of the floc observed for the same sample that was subjected to

different pre-treatment methods. Northern and Kingsburgh BNR plants showed significantly higher cell counts for all pre-treatment methods. There were significant differences with regard to the number of cells that were observed. Sonication was the superior pre-treatment method for individual bacterial cell enumeration from all plants. Pre-treatment with Igepal and lysozyme were ineffective since they resulted in low individual cell counts (Figure 3.6) for the same sample. Therefore, further optimization of sonication was necessary to evaluate the optimum time and power level for bacterial cell dispersion for all five plants (Figures 3.7-3.11), at a power range of 5-9W across a time frame of 3 to 9 minutes. Findings showed that 8 watts was optimum for sonication.

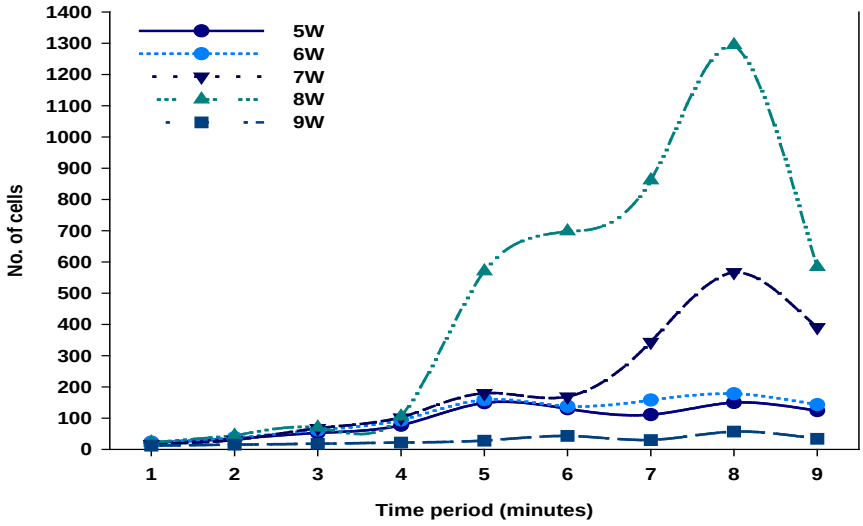


Figure 3.7: Sonication at a power range of 5 - 9 watts across a time frame of 5 – 9 minutes for Hammarsdale BNR plant.

For Hammarsdale BNR plant, it can be noted that an optimised sonication level of 8 watts for 8 minutes resulted in high individual cell counts (Figure 3.7), which reduced the floc size to 10µm (Figure 3.1F). Beyond 8 minutes, there was a drastic decline in the number of individual cells enumerated due to cell lysis. Statistical analysis resulted in a significant difference between the number of cells quantified at 5-9 watts across 5-9 minutes (P<5%; P=0.80). There were a significantly higher number of cells quantified at 7-8 watts compared to the other power levels, with 8 watts, 8 minutes being optimum.

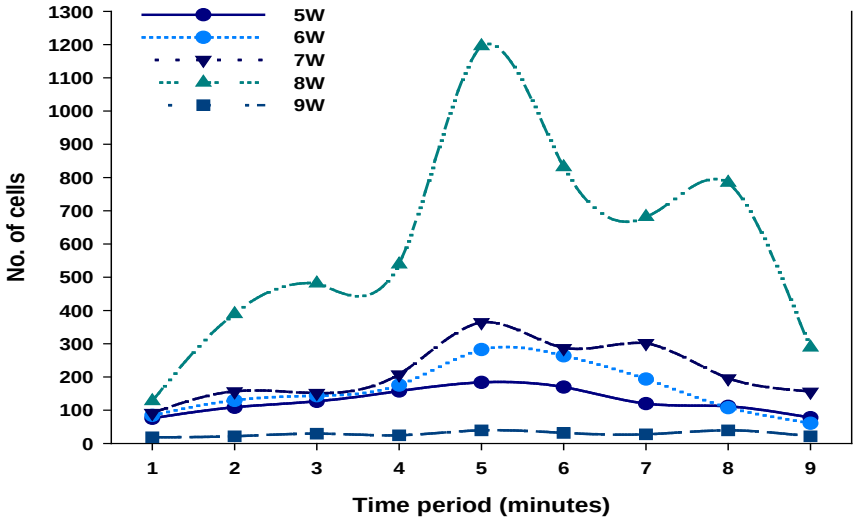


Figure 3.8: Sonication at a power range of 5 - 9 watts across a time frame of 5 – 9 minutes for Northern BNR plant.

For Northern BNR plant, there was a decrease in the number of individual cells enumerated after 5 minutes (Figure 3.8), which reduced the floc size to 10µm (Figure 3.1G). Statistical analysis resulted in a significant difference between the number of cells quantified at 5-9 watts across 5-9 minutes (P<5%; P=0.67). There were a significantly lower number of cells quantified after 5 minutes, with the optimum being at 8 watts, 5 minutes.

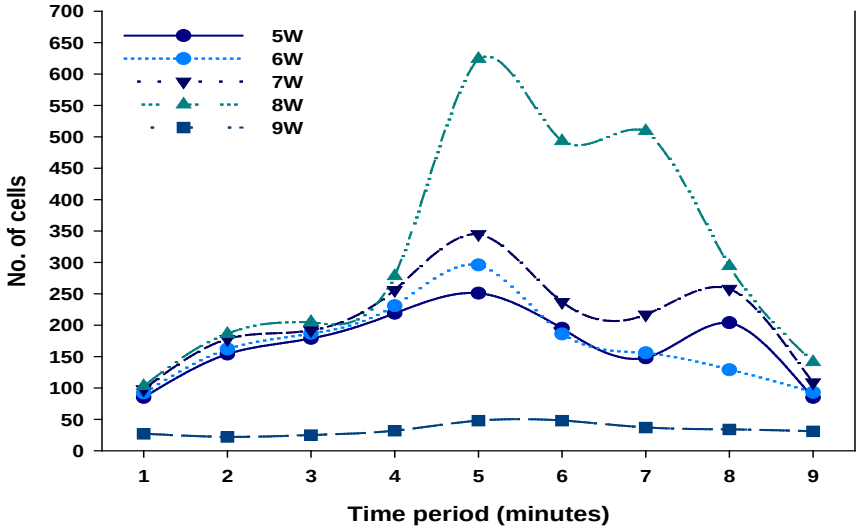


Figure 3.9: Sonication at a power range of 5 - 9 watts across a time frame of 5 – 9 minutes for Kingsburgh BNR plant.

Once again, in keeping with Northern BNR plant, for domestic wastewater (Kingsburgh BNR plant), there was a decrease in the number of individual cells enumerated after 5 minutes (Figure 3.9), which reduced the floc size to 10µm (Figure 3.1H). Statistical analysis resulted in a significant difference between the number of cells quantified at 5-9 watts across 5-9 minutes (P<5%; P=0.27). There was a significantly lower number of cells quantified after 5 minutes, with optimum being at 8 watts; 5 minutes.

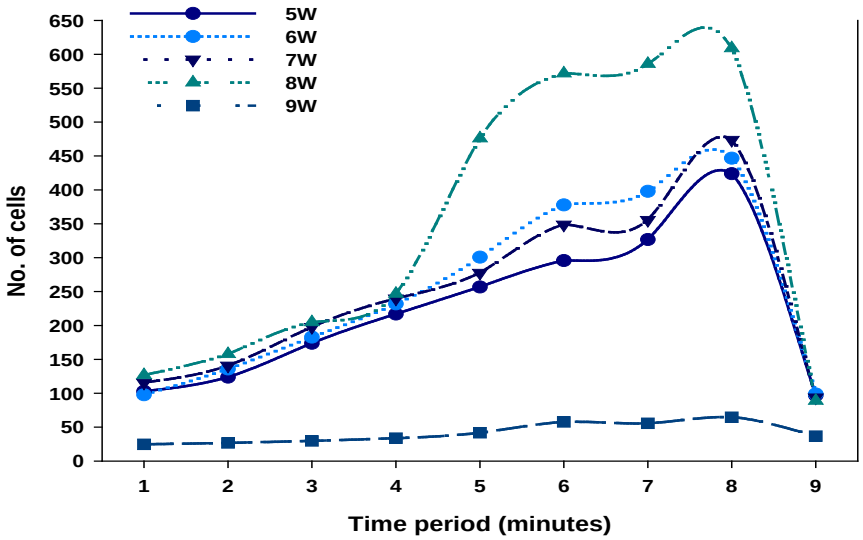


Figure 3.10: Sonication at a power range of 5 - 9 watts across a time frame of 5 – 9 minutes for Southern BNR plant.

For Southern BNR plant, there was a decrease in the number of individual cells enumerated after 8 minutes (Figure 3.10), which reduced the floc size to 10 µm at 8 watts for 8 minutes (Figure 3.1I). Statistical analysis resulted in a significant difference between the number of cells quantified at 5-9 watts across 5-9 minutes (P<5%; P=0.20). There was a significantly lower number of cells quantified after 8 minutes, with optimum being at 8 watts; 8 minutes.

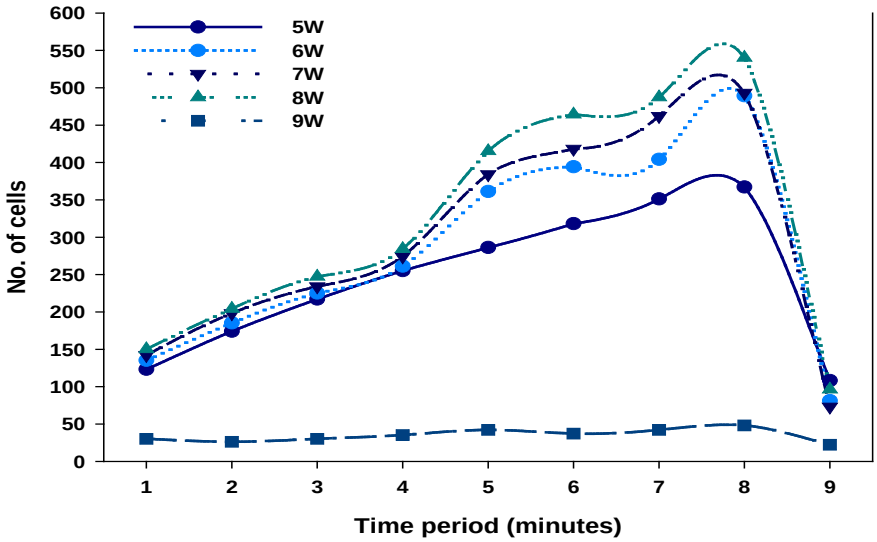


Figure 3.11: Sonication at a power range of 5 - 9 watts across a time frame of 5 – 9 minutes for Darville BNR plant.

For Darville BNR plant, there was a decrease in the number of individual cells enumerated after 6 minutes (Figure 3.11), which reduced the floc size to 10 µm at 8 watts for 6 minutes (Figure 3.1J**).** This too could be attributed to cell lysis, as can be observed from the CSLM images (Figures 9F-J). Statistical analysis resulted in a significant difference between the number of cells quantified at 5-9 watts across 5-9 minutes (P<5%; P=0.15). There was a significantly lower number of cells quantified after 8 minutes, with optimum being at 8 watts; 8 minutes.

3.4 DISCUSSION

The generally low abundance of nitrifying bacteria in activated sludge and their appearance as densely packed aggregates facilitate the need for effective pre-treatment methods for their successful enumeration in microbial population dynamics studies in wastewater. A quantification method using FISH in combination with CSLM was successfully applied in this study to reproducibly determine the representative abundance of nitrifying bacteria in activated sludge. Five major BNR plants were chosen for this aspect of the study to encompass both industrial and domestic wastewater, to provide sufficient data for comparative analysis. The comparative analysis revealed the efficiency of each pre-treatment in enumerating nitrifying bacteria from different wastewater samples (Figure 3.6).

Digital CSLM micrographs (Figures 3.1-3.5) depict the comparative analysis of all five pre-treatment methods tested for all five BNR plants. All five methods showed varying degrees of floc dispersion and cell lysis. Sonication alone proved to be the consistently superior method of dispersion, since there was a clear correlation between sonication and the highest number of individual cells enumerated, that were dispersed out of the floc (Figures 3.1F-J). Due to dispersion of cells from the floc there was a reduction in the floc diameter across the different pre-treatment methods. Although power levels and time frames differed for sonication, Snidaro *et al*. (1997) also reported a reduction in floc size to 10µm.

For the present study, the maximum number of individual cells dispersed out of the floc was observed and enumerated at 8 watts, 8 minutes for industrial wastewater (Hammarsdale, Southern, Darville BNR plants) and 8 watts, 5 minutes for domestic wastewater (Northern and Kingburgh BNR plants) samples. At this stage the size of the floc was reduced from 20µm to 10µm. Beyond these time intervals, cell integrity was lost (Figures 3.1K-O), since very few individual cells could be enumerated. Thus cell lysis had occurred at this point. This phenomenon was common for all plants investigated. These results indicated that the sonication period also varied according to the nature of sludge. The industrial wastewater analysed in this study comprised primarily of not-readily biodegradable substances such as textile dyes, plastics and animal waste from an animal waste processing facility, all of which are not easily disposed. The domestic wastewater was composed primarily of domestic waste (80%); (Table 3.1).

Comparatively, when sonication alone was used as a pre-treatment, it resulted in the highest number of cells that could be dispersed from the flocs and quantified for all BNR plants. This helped in the dissociation of activated sludge bacteria from organic and inorganic materials and flocs (Biggs and Lant, 2000; Daims *et al*., 2006). Sonication is reported to be very effective in breaking up even compact sludges (Pérez-Elvira *et al*., 2010). This could be the underlying reason why sonication was the superior pre-treatment during this study. The most inefficient method was with lysozyme alone, where the least number of cells were quantified. This was expected as the lysozyme alone is ineffective in breaking up the floc structure and the exo-polymeric substance (EPS) surrounding the cells. Lysozyme is mainly used for cell permeability where its action on the peptidoglycan layer mainly for detecting gram positive bacteria. The bacterial cell is surrounded by the EPS and all the cells found to be embedded

in the biofilm. The lysozyme will be effective only when the bacterial cells come into contact with the enzyme. However, most nitrifying bacteria are reported to be Gram negative, where there is no need for enzymatic degradation of the peptidglycan layer.

Optimisation of sonication parameters (sonication duration and power level) resulted in comparatively better cell dispersion. Previous studies by Snidaro *et al*. (1997) showed that it was highly unlikely to totally disrupt the micro-colonies by sonication without causing significant cell lysis. In addition to evaluating different pre-treatment methods, there was a need for evaluating the power level of sonication in terms of watts. The current study was conducted to determine the minimum time and the minimum watts required to disperse the flocs into microcolonies which would prevent cell lysis. For this, the sludge samples were sonicated at different time intervals, ranging from 3 to 9 minutes across power levels of 5 to 9 watts (Figures 3.7-3.11). Preliminary experiments showed that sonication time varies according to the type of sludge being tested.

Findings also showed decreases in the floc size (150µm to 10µm) after 5-8 minutes at 8 watts (depending on the nature of the sludge) and beyond this there was no reduction in floc size. This result, however, is in contrast to the findings of Biggs and Lant (2000) where the optimum sonication time for the production of micro-colonies was 3 minutes. This could possibly be influenced by the differences in sludge type. The industrial sludge was very compact and dense and thus more time was required to break up the cell matrix before cell lysis occurred. Previous findings show that short sonication time intervals destroy sludge floc agglomerates, but do not affect the integrity of the cells. Long sonication time intervals and high power levels, however, breaks cell walls as well as the sludge solids which release dissolved organic compounds, which in turn, lead to the loss of cell integrity (Guangming *et al*., 2006). This is in keeping with our findings (Figure 3.1).

3.5 CONCLUSIONS

- Among the different methods tested in this study, sonication was found to be the superior method for cell dispersion and also for individual cell enumeration
- The combination of 8 watts for 8 minutes of sonication was the most effective method for cell dispersion without the loss of cell integrity for industrial wastewater pre-treatment
- The combination of 8 watts for 6 minutes was the most effective pre-treatment for domestic wastewater pre-treatment
- The results presented here highlight the importance of optimising pre-treatment methods for different types of wastewater for accurate bacterial community analysis using FISH-CSLM and helps to provide a basis for further analysis

CHAPTER FOUR: Quantification and Evaluation of Dominant Nitrifying Bacterial Communities and their Nitrification Performance in Three Full-Scale Biological Nutrient Removal (BNR) Plants

N. Ramdhani, S.K Sheena Kumari and F. Bux. 2011. Distribution of *Nitrosomonas*-related AOB and *Nitrobacter*-related NOB in full-scale Biological Nutrient Removal (BNR) plants. *Water Environment Research* (in press).

4.1 INTRODUCTION

Nitrogen is a major pollutant in wastewater systems and exists in both organic and inorganic forms. In domestic wastewater, nitrogen is present usually in the form of urea or protein, while in industrial wastewater, it generally occurs in the effluent from chemical industries. Discharging wastewater effluent rich in nutrients like nitrogen (ammonia and nitrites) and phosphorus into receiving water bodies causes adverse effects on human health and marine ecology (Bernhard, 2010). An untreated domestic wastewater usually contains 20 to 85 mg/L of total nitrogen, (60% NH_3-N and 40% organic nitrogen). The treated effluent normally contains about 10 to 30% of the influent total nitrogen (Lie, 1996; Mayo and Bigambo, 2005; Makaya *et al*., 2007).

Even though activated sludge is a common process for wastewater treatment, nitrification is a frequent problem of concern, since nitrifying bacteria are very sensitive to environmental changes. Moreover, the inability of nitrifying bacteria to outcompete heterotrophs in stressed conditions makes nitrification the rate-limiting step of nitrogen removal (Daims *et al*., 2006; Wittebolle *et al*., 2008; Huang *et al*., 2010). Traditionally, *Nitrosomonas* sp. and *Nitrobacter* sp. were considered to be the most important nitrifying bacteria in wastewater treatment (Dytczak *et al*., 2008). However, recent studies have shown the predominance of other species including *Nitrosospira and Nitrospira* in most BNR plants (Whang *et al*., 2009).

Previous studies have suggested that different influent waste streams significantly impact on dominant microbial populations in BNR plants (Mulkerrins *et al*., 2004). Several process modifications have been made in recent years for the BNR plants to increase the efficiency of

their nutrient removal (Seviour and Nielsen, 2010). However, not much work has been done to evaluate the nitrification efficacy of BNR plants with varying process configurations. Monitoring functional microbial activities in full-scale systems is extremely complex and requires highly specific and rapid detection techniques such as FISH (Gieseke *et al*., 2001; Seviour and Nielsen, 2010). This approach has been approved to permit the *in situ* detection and quantification of nitrifying bacteria using fluorescently labelled rRNA-targeted oligonucleotide probes (Wagner *et al*., 1993; Seviour and Nielsen, 2010).

In the present study, the FISH technique was employed to detect and quantify AOB and NOB communities and corresponding nitrification efficiencies of three BNR plants in KwaZulu-Natal. It should also be noted that most studies have been carried out at laboratory scale, whereas comparative data and observations from full-scale studies are still limited. Since there is a gap between the conclusions drawn from laboratory and full-scale research, the objective of this study was to evaluate the effect of environmental conditions, wastewater characteristics and process configurations on the occurrence and function of AOB and NOB communities at full-scale BNR plants in KwaZulu-Natal.

4.2 MATERIALS AND METHODS

4.2.1 Selection of BNR Plants

This study focused on three full-scale BNR plants from the KwaZulu-Natal province treating industrial and domestic wastewater (Table 4.1) to encompass the most common types of wastewater treatment systems found in South Africa. All three plants varied in their influent composition, process design and capacity. The Hammarsdale plant is operated using the basic Bardenpho process, which is a complete BNR process (Figure 4.1). The loading capacity of the plant was 27.5 ML/day average dry weather flow (ADWF). This plant was receiving mainly industrial wastewater which comprised of a combination of abattoir and petrochemical waste as well as textiles and dyes. The second BNR plant, Kingsburgh, is of a UCT process configuration and was receiving its influent mainly from domestic wastes (Figure 4.2). The organic loading rate of the plant was determined to be 2980 kg COD/day and the plant was designed to treat 3.1 ML/day (ADWF) of screened and degritted raw sewage. The third BNR plant, Darville, is a modified Johannesburg process without internal recycling and consisted of primary settling, pre-anoxic, anaerobic, anoxic and aerobic zones

(Figure 4.3). There was a return activated sludge (RAS) recycle from the clarifiers to the pre-anoxic zone to allow for phosphate removal and denitrification. The plant had a calculated treatment capacity of 77 ML/day (ADWF) and was receiving 90% of its waste from domestic wastewater and 10% from industry. The industrial waste consisted mainly of oil and waxes.

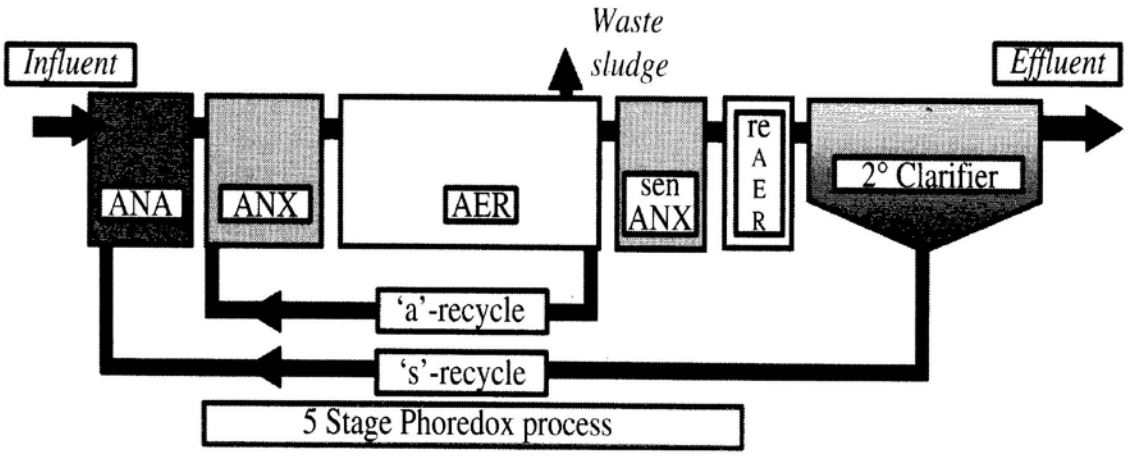


Figure 4.1: Bardenpho process design (Seviour and Nielsen, 2010)

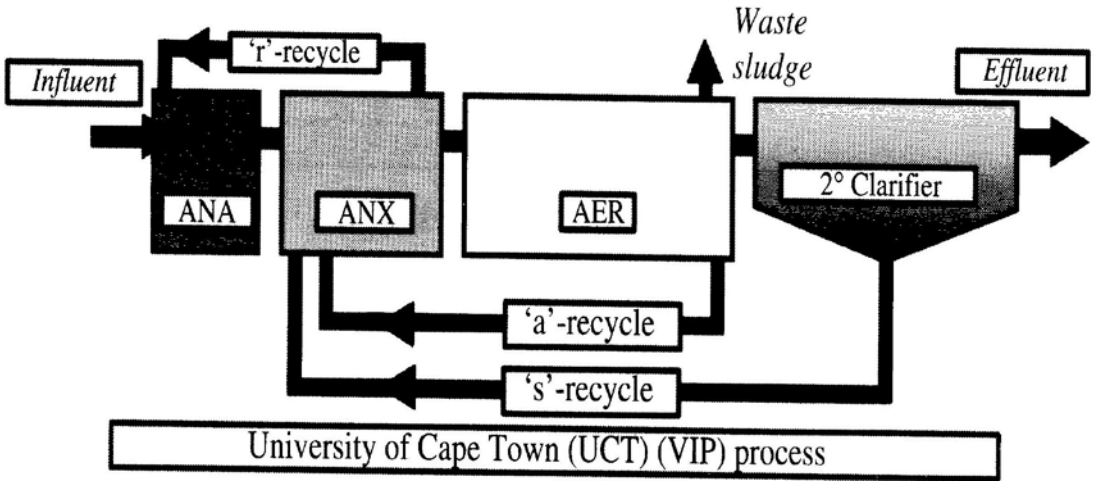


Figure 4.2: UCT process design (Seviour and Nielsen, 2010)

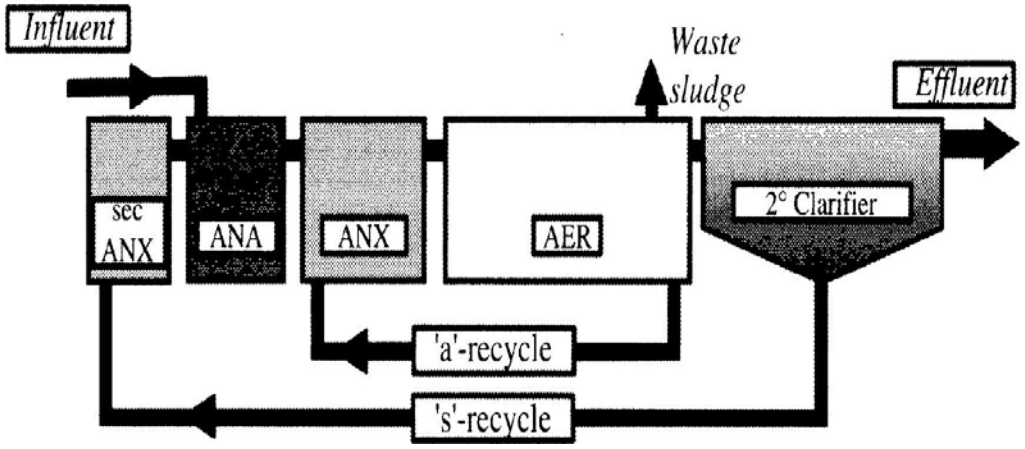


Figure 4.3: Johannesburg process configuration (Seviour and Nielsen, 2010)

4.2.2 Sampling and Operational Parameters

Table 4.1: List of BNR plants

WWTP	HAMMARSDALE	KINGSBURGH	DARVILLE
PLANT CONFIGURATION	Bardenpho process	UCT process	Modified Johannesburg process
INFLUENT COMPOSITION	Abattoir waste, textiles, dyes (95%) and domestic (5%)	Domestic (80%) and industrial (20%)	Industrial (90%) and Domestic (10%)
SAMPLE NUMBER	12	12	12

The information related to operational parameters and wastewater characteristics were calculated based on data provided by the respective plant operators from all BNR plants. Activated sludge grab samples were collected fortnightly from the aerobic zones of the BNR plants over a period of 12 months (January – December), as shown in Table 4.1. The samples were stored at 4°C during transit. Three replicates were used for each set of samples and the average was calculated. All samples were washed twice and re-suspended in sterile phosphate buffered saline (PBS, pH 7.2) for further analysis. For *in situ* hybridization, all samples were fixed immediately in 4% paraformaldehyde (Amann *et al*., 1995), to render the bacterial cells

permeable to the oligonucleotide probes (Table 4.2). Fixed samples were stored in a 1:1 mixture of PBS and absolute ethanol at −20°C until further use.

Table 4.2: List of oligonucleotide probes applied in this study

PROBE	SEQUENCE (5'-3')	TARGET	REFERENCE
NEU	CCCCTCTGCTGCACTCTA	*Nitrosomonas* spp	Mobarry *et al*., (1996)
NmV	TCCTCAGAGACTACGCGG	*Nitrosococcus mobilis*	Pommerening-Röser *et al*., (1996)
Nso1225	CGCGATTGTATTACGTGTGA	ß-*Proteobacterial AOB*	Mobarry *et al*., (1996)
NSV443	CCGTGACCGTTTCGTTCCG	*Nitrosospira* spp	Mobarry *et al*., (1996)
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp	Wagner *et al*., (1996)
Ntspa662	GGAATTCCGCGCTCCTCT	Genus *Nitrospira*	Daims *et al*., (2001)
Ntspa712	CGCCTTCGCCACCGGCCTTCC	Phylum *Nitrospirae*	Daims *et al*., (2001)

4.2.3 Calculation of Nitrification Rate

Nitrification rates were calculated based on the information supplied by the plant operators and was defined as the ammonium removal rate (Carrera *et al*., 2004) in this study. This was done to evaluate the nitrification capacity of the selected BNR plants.

$$R_{nitrification} = \frac{Q\text{in}([NH4^{+} - N]_{in} - [NH4^{+} - N]_{\text{out}})}{V_{reactors}\,[VSS]_{reactors}}$$

Where: $R_{nitrification}$ – rate of nitrification (g N–NH_4^+ g VSS^{-1} d^{-1})

Qin – Influent flow rate (L^3 T^{-1})

$[VSS]_{\text{reactors}}$ — VSS concentration in reactor ($M\ L^{-3}$)

V_{reactors} — Reactor working volume (L^3)

4.2.4 Quantification of AOB and NOB using FISH

4.2.4.1 Whole Cell Hybridization and Probe Selection

FISH analyses were performed for the quantification of the AOB and NOB communities (Amann *et al*., 1995). The probes used in this study are listed in Table 4.2. All samples were pre-treated by sonication at 8 watts for 8 minutes (industrial wastewater); 8 watts for 5

minutes (domestic wastewater) prior to FISH analyses (Ramdhani *et al*., 2010). The samples were then hybridized with 16S rRNA-targeted oligonucleotide probes. Probes specific for dominant nitrifying bacteria in wastewater were selected for this study and were labelled with fluoresceine isothiocyanate (FITC) at the 5' end (Roche, South Africa).

4.2.4.2 Staining and Image Analysis

All nitrifying bacterial cells were stained with 0.25 µg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate – Sigma, Germany) intercalating dye in a dark room for image analysis. The slides were thereafter rinsed with PBS solution, air dried and mounted in VECTASHIELD® anti-fading agent (Vector Laboratories, USA). All samples were visualized using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany). Images were captured using a Zeiss AxioCam MRc camera. Quantification of the population distributions were carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging software. Ten images were randomly chosen for evaluation. Final results reflected the average number of AOB and NOB cells present in ten fields of view in the corresponding wastewater samples per month from January to December. The standard error of the mean (SEM) was calculated as the standard deviation divided by the square root of the number of images analysed.

4.2.5 Statistical Analysis

Statistical analysis was performed using Microsoft Excel software, for Pearson correlation coefficient, unpaired *t*-test and analysis of variance (ANOVA); (Millar, 2001).

4.3 RESULTS

Nitrification efficiency and distribution of AOB and NOB at Hammarsdale BNR plant with a Bardenpho process configuration

FISH analysis revealed the existence of more than one species of AOB (average of 8.5%: 4.3% *Nitrosomonas* sp; 2.2% *Nitrosospira* sp*;* 2% *Nitrosococcus* sp) and NOB (average of 10.1%: 3.3% *Nitrospira* sp; 6.8% *Nitrobacter* sp). Among the different probes used for AOB, *Nitrosomonas* sp dominated throughout the study period and for NOB, *Nitrobacter* sp were found to dominate over *Nitrospira* spp. An overall higher NOB population was also noted

compared to the AOB population (Figure 4.4) and the AOB/NOB ratio of the plant was in the range of 0.8 to 1.0. There were slight variations in the nitrification rate across the months ranging from 0.136 to 0.281 g N–NH_4^+ g VSS^{-1} d^{-1} with an average nitrification efficiency of 0.188 g N–NH_4^+ g VSS^{-1} d^{-1} (Figure 4.4; Table 4.3).

In this study, the nitrification rate of the plant was calculated based on the ammonia removal rate. The DO concentration of the plant was in the range of 0.9 to 2.0mg/L within the reactor. The NH_3-N and NO_2^- concentration in the final effluent was in the range of 2.2 to 3.3mg/L and 0.8 to 1.8mg/L respectively (Figure 4.5; Table 4.3). A higher COD loading rate (407 to 869mg/L) was also recorded during the period of investigation and in the the final effluent it was in the range of 21 to 28mg/L. The plant had an average Sludge Retention Time (SRT) of 25 days (Figure 4.4; Table 4.3) and not much variation was recorded with regard to the environmental temperature and pH (21 to 24°C and 6.9 to 7.6 respectively). It was always within the optimum range for good nitrification (Table 4.3).

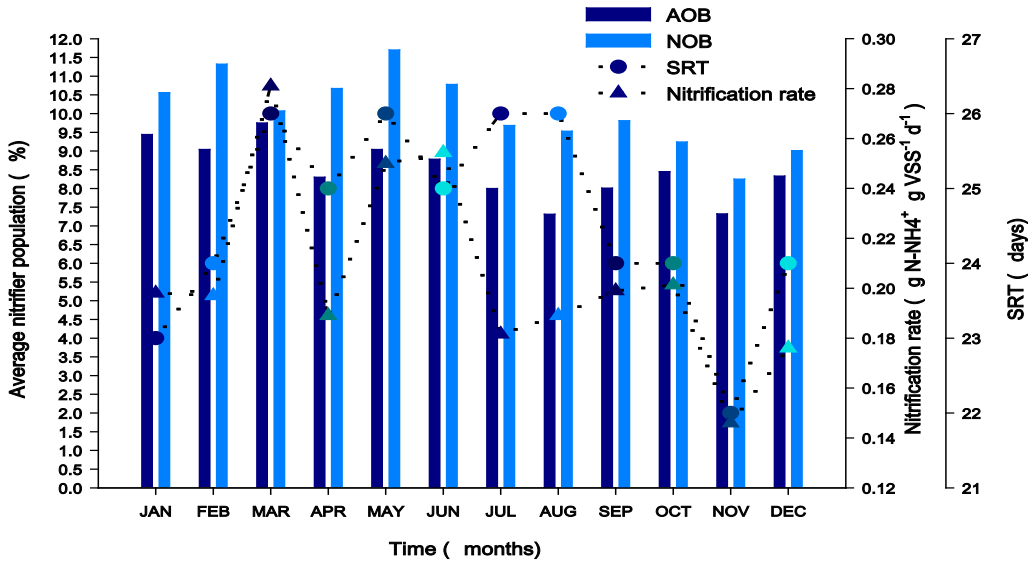


Figure 4.4: Depicts AOB and NOB populations vs. nitrification rate and SRT of Hammarsdale BNR plant. The bar graphs represents the percentage of AOB and NOB against EUB (average) and line graph (Δ) represents average nitrification rate (g N–NH_4^+ g VSS^{-1} d^{-1}) and (○) represents SRT (days).

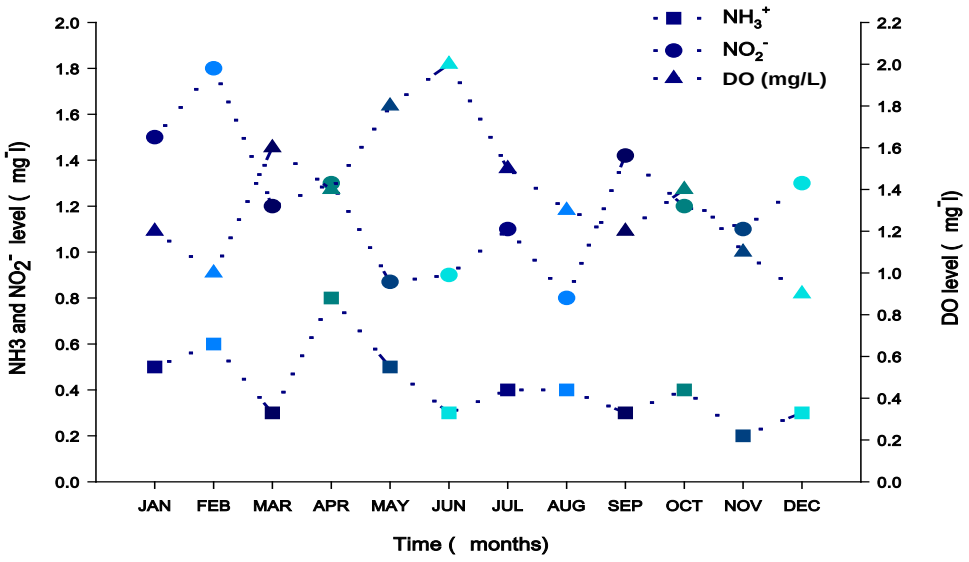


Figure 4.5: The line graph represents the DO level (mg/L) (▼) within the aerobic reactor, NH_3^+ (□) and NO_2^- (○) concentration (mg/L) in the final effluent of Hammarsdale BNR plant. Statistical analysis resulted in a strong positive correlation between the AOB (r =0.67) and NOB (r =0.65) population and their respective nitrification rate. There were weak positive correlations between SRT (r =0.18; r =0.41), DO (r =0.32; r =0.46) and the effluent ammonia (r =0.56; r =0.59) concentrations and the AOB population and NOB populations respectively. There was a significant difference between the AOB and NOB populations (P<5%; P =0.00), with the NOB population being significantly higher (Appendix 4).

Table 4.3: Operational parameters recorded for Hammarsdale BNR plant during 12 months of investigation. The parameters are represented as averages for each month.

	AOB (%)	NOB (%)	AOB/ NOB ratio	Nitrification Rate (g $N–NH_4^+$ g VSS^{-1} d^{-1})	Influent COD (mg/L)	SRT (days)	DO (mg/L)	Influent Ammonia (mg/L)	Effluent Ammonia (mg/L)	Nitrites (mg/L)	pH	T°
JAN	9.4	10.6	0.9	0.184	669	23	1.2	33.41	3.3	1.5	7.0	23
FEB	9.0	11.3	0.8	0.197	447	24	1.0	32.01	3.2	1.8	6.9	24
MAR	9.8	10.1	1.0	0.281	650	26	1.6	31.11	3.1	0.9	7.0	24
APR	8.3	10.7	0.8	0.189	539	25	1.4	31.23	3.1	1.3	7.6	23
MAY	9.0	11.7	0.8	0.250	418	26	1.8	25.09	2.5	0.8	7.0	22
JUN	8.8	10.8	0.8	0.204	508	25	2.0	28.03	2.8	0.9	7.0	21
JUL	8.0	9.7	0.8	0.186	407	26	1.5	26.13	2.6	1.4	7.3	22
AUG	7.3	9.5	0.8	0.179	564	26	1.3	27.21	2.7	0.8	7.0	23
SEP	8.0	9.8	0.8	0.159	623	24	1.2	26.05	2.6	1.4	7.0	24
OCT	8.5	9.2	0.9	0.143	869	24	1.4	22.24	2.2	1.2	7.1	24
NOV	7.3	8.3	0.8	0.146	505	22	1.1	24.37	2.4	1.1	6.9	24
DEC	8.3	9.0	0.9	0.136	480	24	0.9	24.15	2.4	1.3	7.1	23
AVG	**8.5**	**10.1**	**0.8**	**0.188**	**557**	**25**	**1.4**	**27.59**	**2.7**	**1.2**	**7.1**	**23**

Nitrification efficiency and distribution of AOB and NOB at Kingsburgh BNR plant with a UCT process configuration

FISH results displayed a considerable amount of AOB (average of 9.4%: 5.3% *Nitrosomonas* sp; 3.1% *Nitrosococcus* sp; 1% *Nitrosospira* sp) and NOB (average of 8.0%: 4.7% *Nitrobacter* sp*;* 3.3% *Nitrospira* sp). However, the AOB community was found to dominate over the NOB community throughout the investigation period and the AOB/NOB ratio varied from 1.0 to 1.4. Among AOB, *Nitrosomonas* sp which is reported to be environmentally sensitive, was found in significant numbers as compared to the industrial plant (Hammarsdale), and for NOB both *Nitrobacter* sp and *Nitrospira* sp were detected in significant numbers. The rate of nitrification in terms of ammonia removal rate was comparatively higher than that of the Hammarsdale BNR plant (0.303 to 0.520 g $N–NH_4^+$ g VSS^{-1} d^{-1}) giving an average nitrification efficiency of the plant as 0.363 g $N–NH_4^+$ g VSS^{-1} d^{-1} (Figure 4.6; Table 4.4).

A higher DO concentration was recorded in the aerobic reactor which was in the range of 1.8 to 2.5 mg/L (Figure 4.7; Table 4.4) which is optimum for the nitrification process. The final effluent had an average DO concentration of 0.10mg/L. The DO concentration was found to be directly proportional to the AOB population, however, no clear correlation was observed between the NOB population and DO concentration (r =-0.19) with a positive correlation between the NOB population and nitrification rate (r =0.43). DO concentration significantly affects the oxidation of ammonia, with DO concentrations greater than 1 to 2 mg/L being recommended for enhanced nitrification. The NH_3-N and NO_2^- concentration in the final effluent was comparatively lower compared to the industrial plant (Hammarsdale) which was in the range of 1.2 to 2.4mg/L and 0.3 to 0.6mg/L respectively (Figure 4.7; Table 4.4). The influent COD level was at the optimum range (305 to 464mg/L) and an average of 18mg/L COD was recorded in the final effluent. A higher SRT varying from 28 to 36 days was also recorded at this plant during the period of investigation (Figure 4.6; Table 4.4).

Statistical analysis resulted in positive correlations between the AOB and NOB populations and their nitrification rates and effluent ammonia concentrations. There was a weak positive correlation between the AOB population and SRT (r =0.14), DO (r =0.38) and nitrite concentrations (r =0.21) and no clear correlation between the NOB population and SRT (r =-0.09), DO (-0.19) and nitrite concentrations (r =-0.45). There was a significant difference

between the AOB and NOB populations (P<5%; P=0.00), with AOB being significantly higher (Appendix 4).

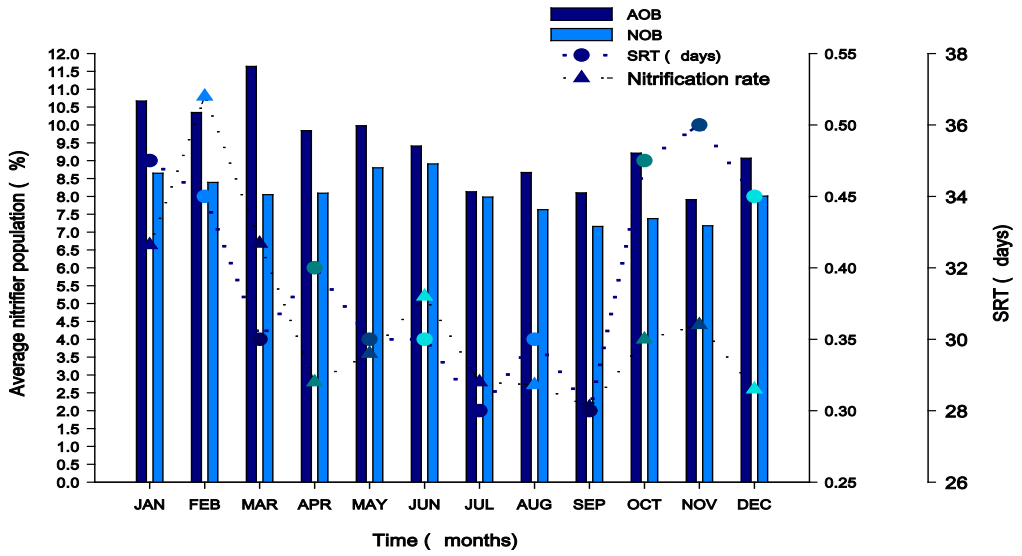


Figure 4.6: Depicts AOB and NOB populations vs. nitrification rate and SRT for Kingsburgh BNR plant. The bar graphs represents the percentage of AOB and NOB against EUB (average) and line graphs (Δ) represents nitrification rate (g $N–NH_4^+$ g VSS^{-1} d^{-1}) and (○) represents SRT (days).

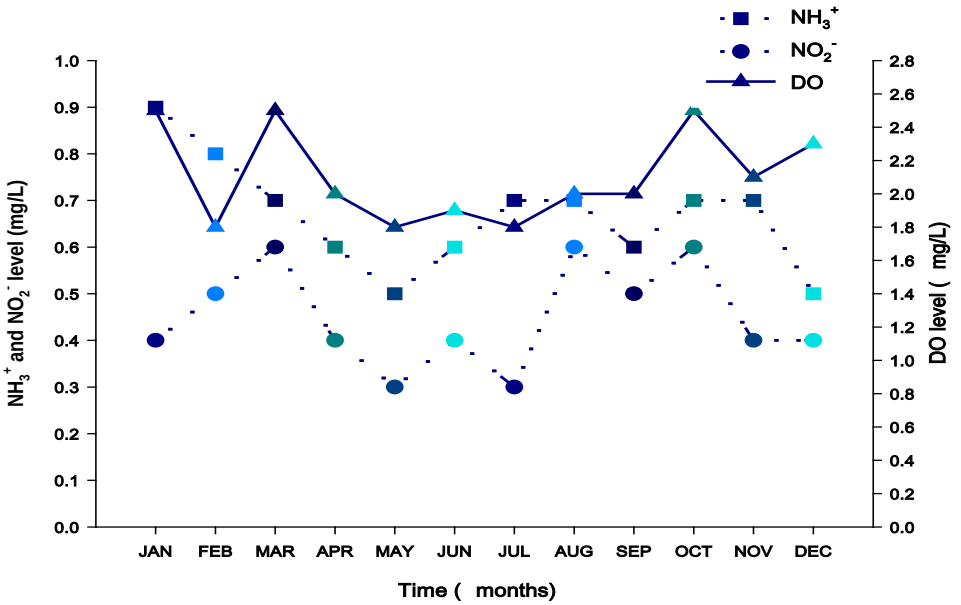


Figure 4.7: The line graph represents the DO level (mg/L) (▼) within the aerobic reactor, NH_3^+ (○) and NO_2^- (□) concentration (mg/L) in the final effluent of Kingsburgh BNR plant.

Table 4.4: Operational parameters recorded for Kingsburgh BNR plant during 12 months of investigation. The parameters are represented as averages for each month

	AOB (%)	NOB (%)	AOB/ NOB ratio	Nitrification Rate ($gNH4^{+}$-$NgVSS^{-1}$/d)	Influent COD (mg/L)	SRT (days)	DO (mg/L)	Influent Ammonia (mg/L)	Effluent Ammonia (mg/L)	Nitrites (mg/L)	pH	T°
JAN	10.7	8.7	1.2	0.416	369	35	2.5	22.86	2.1	0.4	7.2	25
FEB	10.4	8.4	1.2	0.520	447	34	1.8	22.01	1.8	0.5	7.0	23
MAR	11.6	8.1	1.4	0.417	450	30	2.5	21.11	2.4	0.6	7.1	22
APR	9.8	8.1	1.2	0.320	439	32	2.0	27.23	1.6	0.4	7.3	22
MAY	10.0	8.8	1.1	0.340	418	30	1.8	24.91	2.3	0.3	6.9	23
JUN	9.4	8.9	1.1	0.380	308	30	1.9	26.45	1.6	0.4	6.8	21
JUL	8.1	8.0	1.0	0.320	407	28	1.8	25.87	1.7	0.3	7.1	23
AUG	8.7	7.6	1.1	0.318	464	30	2.0	24.69	1.2	0.6	7.2	24
SEP	8.1	7.2	1.1	0.303	423	28	2.0	25.31	1.6	0.5	6.9	25
OCT	9.2	7.4	1.2	0.350	369	35	2.5	23.32	1.2	0.6	7.3	24
NOV	7.9	7.2	1.1	0.360	305	36	2.1	23.69	1.3	0.4	7.0	23
DEC	9.1	8.0	1.1	0.315	380	34	2.3	25.17	1.9	0.4	7.1	22
AVG	**9.4**	**8.0**	**1.2**	**0.363**	**398**	**32**	**2.1**	**24.39**	**1.7**	**0.5**	**7.1**	**23**

Nitrification efficiency and distribution of AOB and NOB at Darville BNR plant with a Modified Johannesburg process configuration

FISH results displayed a significant amount of AOB (average of 7.3%: 1.7% *Nitrosospira* sp*;* 3.9% *Nitrosomonas* sp*;* 1.7% *Nitrosococcus* sp) and NOB (average of 7.5%: 2.5% *Nitrospira* sp and 5% *Nitrobacter* sp). Among AOB, *Nitrosomonas* was found to dominate and among NOB, *Nitrobacter* was found to dominate over *Nitrospira* throughout the study period. The nitrification rate of the plant was comparatively lower (0.076 to 0.107 g $N–NH_4^+$ g VSS^{-1} d^{-1}) as compared to the two other plants investigated. The plant showed an average nitrification efficiency of 0.089 g $N–NH_4^+$ g VSS^{-1} d^{-1} in terms of the ammonia removal rate (Figure 4.8; Table 4.5).

A lower DO concentration was recorded which seems to inhibit the rate of nitrification (0.8 to 1.4mg/L in the reactor and 0.10mg/L in the final effluent). It was also noted that the DO concentration was directly proportional to the NOB population and no correlation was observed for the AOB population. The NH_3-N and NO_2^- concentration in the final effluent was in the range of 2.2 to 4.1mg/L and 1.2 to 2.3mg/L respectively (Figure 4.9; Table 4.5). A higher COD loading at an average of 691mg/L COD in the influent and 22mg/L COD in the final effluent was recorded during the investigation period. The SRT values were in the range of 19 to 28 days (Figure 4.8).

Statistical analysis showed a negative correlation between the AOB and NOB population and their respective nitrification rates. There was a weak positive correlation between the AOB and NOB populations and SRT (r =0.09; 0.03), and nitrite (r =0.25; r =0.32) concentrations respectively. There was a strong positive correlation between the AOB and NOB populations and the DO concentration (r=0.41; r =0.61) respectively, whereas there were no clear correlations between the AOB and NOB populations and effluent ammonia concentrations (r =-0.43; r =-0.26). There was a significant difference between the AOB and NOB population (P<5%; P=0.43); (Appendix 4).

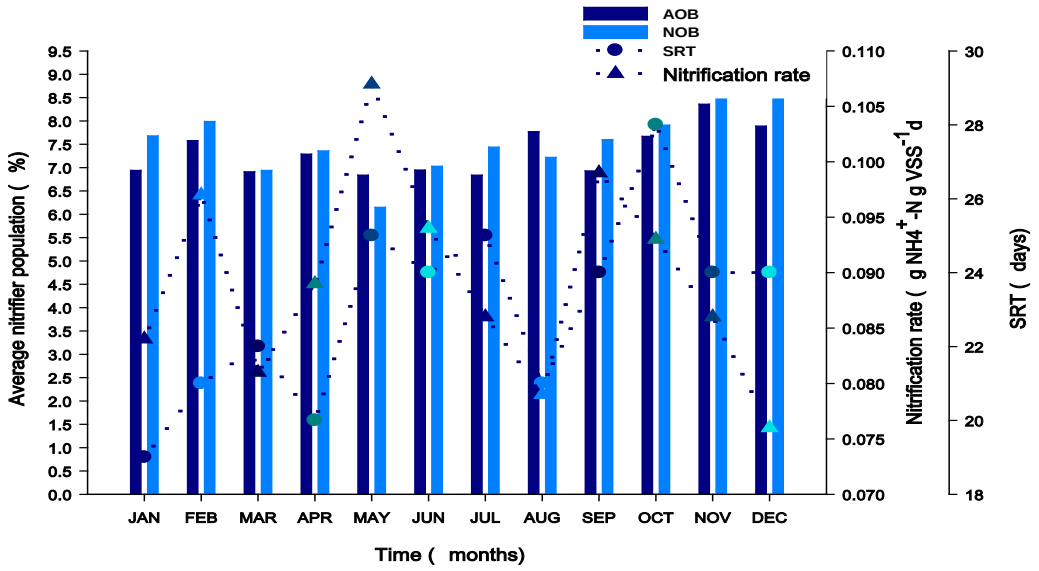


Figure 4.8: Depicts AOB and NOB population vs nitrification rate and SRT for Darville BNR plant. Bar graphs represent the percentage of AOB and NOB against EUB and line graphs (○) represent nitrification rate (g $NH4^{+}$ -$NgVSS^{-1}$/d) and (Δ) represent SRT (days).

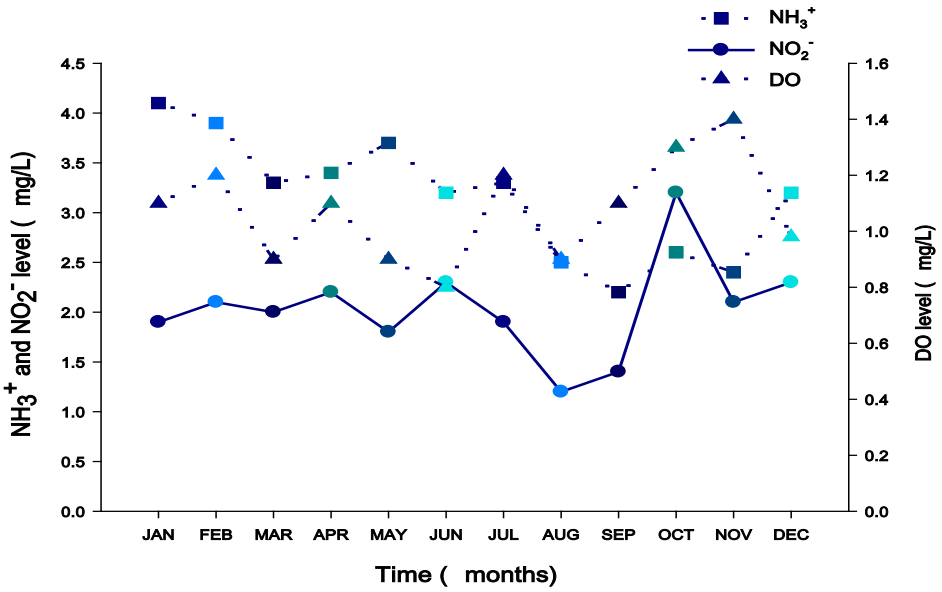


Figure 4.9: The line graph represents the DO level (mg/L) (▼) within the aerobic reactor, NH_3^{+} (□) and NO_2^{-} (○) concentration (mg/L) in the final effluent respectively of Darville BNR plant

Table 4.5: Operational parameters recorded for Darville BNR plant during 12 months of investigation. The parameters are represented as averages for each month.

	AOB (%)	NOB (%)	AOB/ NOB ratio	Nitrification Rate (g $N–NH_4^+$ g VSS^{-1} d^{-1})	Influent COD (mg/L)	SRT (days)	DO (mg/L)	Influent Ammonia (mg/L)	Effluent Ammonia (mg/L)	Nitrites (mg/L)	pH	T°
JAN	6.9	7.7	0.9	0.084	820	19	1.1	30.23	4.1	1.9	6.8	22
FEB	7.6	8.0	1.0	0.097	809	21	1.2	31.09	3.9	2.1	7.0	23
MAR	6.9	6.9	1.0	0.081	794	22	0.9	29.56	3.3	2.0	7.1	23
APR	7.3	7.4	1.0	0.089	395	20	1.1	28.33	3.4	2.2	7.4	24
MAY	6.8	6.2	1.0	0.107	608	25	0.9	27.21	3.7	1.8	6.9	21
JUN	7.0	7.0	1.0	0.094	433	24	0.8	31.98	3.2	2.3	6.8	20
JUL	6.8	7.4	0.9	0.086	802	25	1.2	32.87	3.3	1.9	7.1	20
AUG	7.8	7.2	1.0	0.079	732	21	0.9	28.36	2.5	1.2	7.1	22
SEP	6.9	7.6	0.9	0.099	655	24	1.1	29.07	2.2	1.4	6.8	23
OCT	7.7	7.9	1.0	0.093	596	28	1.3	30.21	2.6	3.2	7.0	22
NOV	8.4	8.6	1.0	0.086	852	24	1.4	31.97	2.4	2.1	7.1	24
DEC	7.9	8.5	0.9	0.076	800	24	0.9	32.74	3.2	2.3	7.2	21
AVG	**7.3**	**7.5**	**1.0**	**0.089**	**691**	**23**	**1.1**	**30.30**	**3.2**	**2.0**	**7.0**	**22**

4.4 DISCUSSION

Nitrification is one of the major functions at a BNR plant which is carried out by two physiologically and phylogenetically unrelated groups of organisms, the AOB and NOB. Several studies have shown that operational conditions and wastewater characteristics have a significant impact on the selection of specific nitrifying bacterial populations (Li *et al*., 2007; Park and Noguera, 2008; Sudarno *et al*., 2011). Thus a better understanding of its diversity at various treatment plants is imperative for maintaining or improving the process parameters and thereby controlling functional failures like nitrification. The FISH technique was employed in this study to target the dominant nitrifying bacterial populations and the oligonucleotide probes selected targeted the dominant AOB (*Nitrosomonas*; *Nitrosospira*; *Nitrosococcus*) and NOB (*Nitrobacter*; *Nitrospira*) populations reported from wastewater treatment plants (Table 4.2), (Haseborg *et al*., 2010).

The present study has focused on the process configurations as well as wastewater characteristics and its impact on the nitrification performance at full scale BNR plants. Developments in molecular techniques such as FISH, Q-PCR and microarrays would make it possible to monitor the *in situ* changes in nitrifying communities (Brodie *et al*., 2006; Kim and Kim, 2006; Seviour and Nielsen, 2010. The FISH technique was employed in this study to target the dominant nitrifying bacterial communities using rRNA-targeted specific probes.

All three BNR plants investigated, varied in terms of nitrification efficiency and their nitrifying community size. However not much difference is noted with regard to the dominance of this species.The AOB/NOB ratio of plants fluctuated throughout the study period; however, it is not yet clearly understood whether the AOB/NOB ratio is consistent for good nitrification. In a few earlier studies, good nitrification was reported when the AOB/NOB ratio was 2.0 to 3.5 (You *et al*., 2003; Li *et al*., 2007) and an increase in ratio from 3.5 to 6.3 was reported in adverse conditions such as low DO concentrations and short hydraulic retention times (HRT); (Li *et al*., 2007). In this study, the Kingsburgh plant with a basic UCT configuration showed a moderately higher AOB/NOB ratio and a higher nitrification rate (Table 4.4) compared to the other two BNR plants. The Hammarsdale BNR plant showed on average a higher NOB population (Table 4.3) compared to Kingsburgh and Darville BNR plant and a lower AOB/NOB ratio (Tables 4.4 and 4.5). The dominance of

NOB over AOB has been reported in few studies earlier, however, a higher AOB community was also seen in adverse conditions such as low DO concentrations and short HRT (Dionisi *et al*., 2002).

Both the Hammarsdale and Darville plant configurations were theoretically designed for maximum nutrient removal, however, it was found to be influenced by many other factors other than the process configuration itself. Both these plants showed a lower nitrification rate and a lower AOB population (Figures 4.4 and 4.8). This could also be due to the adverse effect of the influents which includes textiles dyes, chemicals from petrochemical industries, oils and waxes. The AOB communities are considered to be much more environmentally sensitive than the NOB (Li *et al*., 2007). Among the three plants, Darville showed the lowest nitrification rate in terms of ammonia removal efficiency (Figure 4.8). A lower DO concentration ($\geq 0.8 \leq 1.4$ mg/L) was recorded from this plant for most of the investigation period which might have affected the overall nitrifying community and the nitrification efficiency of the plant (Figure 4.9).

Among the different probes used for AOB, *Nitrosomonas* and *Nitrosospira* sp were found to dominate and this is in accordance with previous reports (Dionisi *et al*., 2002). The dominance and co-existance of more than one species of AOB in various wastewater systems have previously been reported (Tarre and Green, 2004). The FISH results displayed a significant NOB population at the Hammarsdale BNR plant (Figures 4.4) compared to the Kingsburgh and Darville BNR plants (Figures 4.6 and 4.8); whilst the Kingsburgh BNR plant showed a significant AOB population (Figure 4.6) as compared to the Hammarsdale and Darville BNR plants (Figures 4.4 and 4.8). For NOB, *Nitrospira* was found to dominate over *Nitrobacter* in the case of the Darville and Hammarsdale BNR plants, whereas at the Kingsburgh BNR plant, *Nitrobacter* dominated over *Nitrospira*.

It is reported that nitrite concentration is one of the limiting factors that control the selection of these two species. It was previously reported that *Nitrosomonas* and *Nitrobacter* are *r*-strategists (low affinity for substrates, high growth rate, competes successfully at high substrate concentrations) and are highly susceptible to environmental changes (Sudarno *et al*., 2011). However, the Darville BNR plant had higher COD levels (with an average of ≥500mg/L COD) and nitrogen loading rates (average of 30.30mg/L) as compared to the other

two plants (Table 4.5). Both Hammarsdale and Darville had shown a higher variation in their COD levels during the investigation period (Tables 4.3 and 4.5). We suspect that the co-existence of these species at the plants could be due to the variations in the substrate concentrations. Even though higher COD loading rates are usually a characteristic of industrial wastewater plants, it also has been reported in domestic plants (Makaya *et al*., 2007).

These deviations in COD loading rates at these plants could be due to the variation in the type of influents due to commercial and industrial activities occurring around the wastewater treatment plants across the investigation period. Earlier studies have also reported the ability of *Nitrosomonas* and *Nitrobacter* to survive at higher COD levels as a result of their heterotrophic nature, whilst most others viz., *Nitrospina, Nitrococcus, Nitrospira* are unable to grow heterotrophically (Li *et al*., 2007). However, there was not much variation recorded for both temperature and pH during the investigation period at any of these plants. The optimum pH required for nitrifiers is reported to be 7.5 and most BNR plants were found to effectively nitrify when the pH was between 6.5 and 8.0 and when the temperature was between 20 and 30°C (Tarre and Green, 2004).

The presence of all these species in considerable numbers at these BNR plants could be attributed to the variation in DO and substrate concentrations. DO concentrations of 1 to 2 mg/L is reported to be sufficient to ensure complete nitrification of ammonia to nitrate and the establishment of stable communities of AOB and NOB (Vesilind, 2003). *Nitrosomonas* and *Nitrobacter* dominated at mid/high DO concentrations (Li *et al*., 2007) which was evident in this study. The Kingsburgh BNR plant showed a mid/high DO concentration (≥1.8 to 2.5 mg/L) which was dominated by *Nitrobacter* and *Nitrosomonas* sp. It was also noted that these species remained fairly constant at varying DO concentrations within a system indicating that once there was sufficient oxygen in the system; a further increase or decrease in aeration would not affect the nitrifying bacterial population (Li *et al*., 2007). However, a very low DO concentration (≤0.5mg/L) was found to inhibit the nitrification rate of the plant, indicating that, a significant amount of DO is required for these nitrifiers to perform specific functions such as nitrification.

4.5 CONCLUSIONS

- This study has shown that specific AOB or NOB predominant in wastewater rather suggests that the wastewater type and characteristics may establish significantly different microbial environments
- Among the AOB, *Nitrosomonas* dominated in all BNR plants throughtout the study period and for NOB both *Nitrobacter* and *Nitrospira* were found in significant numbers and their dominance varied across the plants
- Dissimilar, distinct distribution patterns recorded of AOB and NOB in BNR plants throughout this study could be attributed to their environment which in turn impacted on the nitrification performance of the system
- The co-existence of more than one group of AOB or NOB at the same plant could help the plant escape functional failures such as nitrification due to sudden changes in temperature and substrate concentrations, as this function can be performed by different groups
- Future work using specific primers targeting functional genes of AOB, NOB, Anammox and AOA with advanced techniques such as high through-put sequencing would shed more light on unraveling the complexity of the nitrifying microbial community and its interaction in wastewater treatment systems
- Although it would have been merotorious to conduct a nitrogen balance in this study, this was not possible since the research focused on full-scale systems

CHAPTER FIVE: 16S rRNA Phylogeny of Dominant Ammonia-oxidising Bacteria (AOB) and Nitrite-oxidising Bacteria (NOB) from Activated Sludge Plants in KwaZulu-Natal

N. Ramdhani, S.K Sheena Kumari and F. Bux. 2011. Distribution of *Nitrosomonas*-related AOB and *Nitrobacter*-related NOB in full-scale Biological Nutrient Removal (BNR) plants. *Water Environment Research* (in press).

5.1 INTRODUCTION

Identification and quantification of nitrifying bacteria have traditionally been executed using culture-dependant methods. However, while these approaches have been successful at gathering data reflecting bacterial presence, they have been criticised for their inability to accurately characterise the microbial diversity in natural environments (Amann *et al.*, 1995; Revetta *et al*., 2010). As a result, culture-dependant methods present both qualitative and quantitative biases (Beneduce *et al*., 2007). To circumvent some of the problems associated with culturing techniques, molecular tools have been used to provide information regarding the composition of complex microbial networks. The quest for less time-consuming, culture-independent methods in the microbiological analyses of AOB and NOB has led to the establishment of various molecular techniques.

The current available molecular techniques can be used to overcome these limitations, to an extent, by identifying and analysing sequences of the 16S rRNA genes to reveal AOB and NOB community diversity in various environments. The 16S rRNA based approach for the identification of nitrifying bacteria from activated sludge, has been routinely used to determine population diversity. However, PCR amplification does not provide quantitatively valid information (Kurata *et al*., 2004). Thus new techniques were developed to overcome these issues by increasing amplification specificity and amplicon yield, such as Q-PCR. This allows targeted gene copy numbers to be quantified (Fukushima and Bond, 2010).

The average bacterial 16S rRNA molecule is approximately 1500 nucleotides in length and once sequenced contains sufficient conserved and variable nucleotide regions for reliable

phylogenetic analyses (Amann *et al*., 1995). Therefore 16S rRNA primers have been widely used for extracting DNA from activated sludge. Universal and domain-targeted primers for 16S rRNA genes are widely used, however, these primers often need to be modified and updated due to the vast increase in database size (Forney *et al*., 2004).

In recent years, 16S rRNA amplification, cloning and sequencing has been used for estimation of AOB and NOB abundance, allowing quantitation specificity up to the genus level (Ward and Bouskill, 2011). The application of specific PCR amplification in combination with clone libraries can provide clarification of the AOB and NOB communities in detail (Zhang *et al*., 2010). Consequently, early detection of a decline in the nitrifying population by rapid and reliable molecular methods may improve process control and prevent washout of these organisms from wastewater treatment systems.

The goal of this study was to determine the diversity of dominant AOB and NOB from activated sludge plants in KwaZulu-Natal based on their 16S rRNA phylogeny.

5.2 MATERIALS AND METHODS

5.2.1 Sample Collection

Activated sludge samples were taken from the aerobic zones of three activated sludge BNR plants treating a variety of influents, which included industrial (Hammarsdale, Darville plants) and domestic wastewater (Kingsburgh); (Table 3.1, Chapter 3). The samples were half-filled in the sample bottles so as to maintain aerobic conditions during sample transit, and they were stored at -4°C until further use.

5.2.2 DNA Extraction from Activated Sludge Samples

Genomic DNA was extracted from the activated sludge samples in duplicate using three different methods of extraction including phenol-chloroform, CTAB (Cetyl Trimethyl Ammonium Bromide Cationic Surfactant), and a modification of the boiling method.

The phenol-chloroform method (Lemarchand *et al*., 2005) involved centrifugation of the activated sludge samples at 1400rpm for 5 minutes at 4°C. Supernatants were discarded and

pellets were re-suspended in 1 x PBS and centrifuged once again at 5000rpm for 3 minutes. Supernatants were discarded and pellets were re-suspended in 75µl of Tris/Ethylenediaminetetraacetic acid (EDTA) buffer (10mM Tris/Hydrochloric acid (HCl); 1mM EDTA; pH 8.0) with 25µl of 10% Sodium disulphide (SDS). Tubes were inverted to ensure thorough mixing and incubated for 2 hours at 65°C. Lysis buffer (500µl) (10% SDS in 0.1M sodium chloride (NaCl) and 0.5M Tris/HCl) was then added to the tubes and vortexed. Cells were then lysed using the freeze-thaw method (Immersion in an ice and ethanol slurry for 2 minutes followed by heating in a water bath at 65°C for 2 minutes). This cycle was repeated five times.

Thereafter, 500µl Tris-saturated phenol was added to the tubes, inverted to mix and centrifuged at 5700rpm for 5 minutes. Samples were then treated using phenol-chloroform (25:24), chloroform and 100% isopropyl alcohol, where each upper layer was immediately transferred to a sterile tube with an equal amount of the next reagent and centrifuged at 5700rpm for 5 minutes. Following the addition of isopropyl alcohol the tubes were incubated for 1 hour at -20°C. The DNA was then centrifuged at 5000rpm for 5 minutes. Supernatants were discarded and the pellets were washed with 70% ethanol, centrifuged and re-suspended in 100µl 10mM Tris-HCl (pH 8.0) and 0.1mM EDTA (Tris-EDTA buffer).

The CTAB method (Worden, 2009) involved the addition of activated sludge samples into 2ml eppendorf tubes. These were centrifuged at 5000rpm for 3 minutes and supernatants were discarded. This process was repeated until an activated sludge pellet of approximately 2g remained. These pellets were thereafter re-suspended in 1ml of 1 x PBS and centrifuged at 8000 rpm for 10 minutes. Supernatants were discarded and pellets were re-suspended in 500µl of pre-warmed CTAB buffer (pH 8.0) and 40µl of 10% SDS. This mixture was incubated at 65°C for 20 minutes. Thereafter, this mixture was cooled to room temperature, 20µl of lysozyme (100mg/ml) was added and this was mixed well. This mixture was incubated for a further 5 minutes at room temperature. Proteinase K (10mg/ml) was added (8µl) and this was mixed well. Once again, this was incubated for 5 minutes at room temperature.

Heat shock was carried out for 5 minutes at 65°C followed by 2 minutes in an ice and ethanol slurry. This combination was repeated 5 times. Chloroform and isoamyl alcohol (0.5ml) was added in a (24:1) ratio respectively and this was mixed well. This mixture was centrifuged at 10

000rpm for 10 minutes at room temperature. The aqueous phase was transferred to sterile eppendorf tubes. The chloroform-isoamyl alcohol step was repeated. This new aqueous phase was once again transferred to sterile eppendorf tubes and 0.6 volumes of isopropanol were added. This was incubated for 1 hour at -20°C. Tubes were then centrifuged at 10 000rpm for 15 minutes. Supernatants were discarded and pellets were re-suspended in 70% ethanol. This was centrifuged at 10 000rpm for 5 minutes, supernatants were discarded and pellets were dried at room temperature for 5-10 minutes. Pellets were finally re-suspended in 100µl nuclease-free water and stored at -20°C.

A modification of the boiling method used by Sepp *et al*. (1994) involved centrifuging the sludge samples at 10000rpm for 5 minutes first. The supernatants were decanted and the pellets were re-suspended in sterile distilled water. This suspension was thereafter boiled at 80°C for 20 minutes and stored at -20°C until further use.

5.2.3 DNA Quantification

The purity and concentration of the extracted DNA from all three BNR plants resulting from all three methods of extraction were determined (Table 5.2) using the Nanodrop ND – 1000 spectrophotometer (Nanodrop Technologies, USA). All DNA samples were quantified by analysing a 1µl aliquot of the extracted DNA sample. Final quantification readings were provided in ng/µl. The extracted DNA was thereafter further diluted to 100 ng/µl prior to PCR.

5.2.4 Polymerase Chain Reaction

PCR amplification was carried out using three group-specific primer sets for targeting the 16S rRNA genes of previously reported nitrifying bacteria in wastewater. Details of these primers are listed in Table 5.1. All PCR reaction mixtures had a total working volume of 25µl and consisted of 6 x Taq buffer (MBI, Fermentas, USA), 200µm dNTP’s, 50-100pm forward and reverse primers, 100µg genomic DNA, nucleas-free water and Taq polymerase (1U).

Table 5.1: List of primers and their specificity

PRIMER	SEQUENCE (5´ - 3´)	SPECIFICITY	REFERENCE
amoA-1F	GGGGTTTCTACTGGTGGT	AOB *amo*A gene	Rotthauwe *et al*., 1997
amoA-2R*	CCCCTCKGSAAAGCCTTCTTC	AOB *amo*A gene	Rotthauwe *et al*., 1997
FGPS872f	CTAAAACTCAAAGGAATTGA	*Nitrobacter* sp.	Degrange and Bardin, 1995
FGPS1269r	TTTTTTGAGATTTGCTAG	*Nitrobacter* sp.	Degrange and Bardin, 1995
NSR1113F	CCTGCTTTCAGTTGCTACCG	*Nitrospira* sp.	Dionisi *et al*., 2002
NSR1264R	GTTTGCAGCGCTTTGTA	*Nitrospira* sp.	Dionisi *et al*., 2002

*Where K=G or T; S=G or C

The PCR conditions for the first primer set of amoA-1F and amoA-2R, achieved amplification with an initial denaturation cycle at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds; annealing at 55.6°C for 30 seconds; extension at 72°C for 30 seconds, and a final extension at 72°C for 8 minutes. Amplification was carried out using a Veriti thermal cycler (Applied Biosystems, USA). All PCR products were electrophoresed on a 1% (w/v) agarose gel and visualised.

Similarly, the PCR conditions for the second primer set of FGPS872f and FGPS1269r, achieved amplification with an initial denaturation cycle at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds; annealing at 43.6°C for 30 seconds; extension at 72°C for 30 seconds, and a final extension at 72°C for 8 minutes. Amplification was carried out using a Veriti thermal cycler (Applied Biosystems, USA). All PCR products were electrophoresed on a 1% (w/v) agarose gel and visualised.

5.2.4 Agarose Gel Electrophoresis

All DNA extracts and PCR products were verified by electrophoresis on 1% (w/v) agarose gels. All gels were made by combining 0.8g high purity agarose (Sigma, Germany) with 50ml (Tris-boric acid-ethylene (TBE) buffer. This mixture was boiled for 1 minute, cooled to approximately 60°C and 1µl ethidium bromide was added and mixed thoroughly. This was then poured into gel casting trays and allowed to set for approximately 20 minutes. After solidifying, these gels were immersed in 250ml TBE buffer in an electrophoresis tank and wells in the gel were loaded with the respective samples. These gels were run at 80 volts (V)

for 60 minutes. All gels were visualised using a gel-documentation system (Vacutec, South Africa).

5.2.5 Design of New Primers

Two new sets of primers were synthesised using the National Centre for Biotechnology Information (NCBI) primer design tools, to target 16S rRNA genes of dominant *Nitrosomonas* and *Nitrobacter* spp. (Nitroso2-F: 5'GGCGCAAGCCTGATCCAGCA3' / Nitroso2-R: 5' ACCGTACTCCCCAGGCGGTC 3') and Nitroba1-F: 5' CGGTACCGGAAGAATAAGCA 3' / Nitroba1-R: 5' GTTTAGGGCGTGGACTACCA 3'). Targeted 16S rRNA gene sequences from GenBank were used to design the new specific primers. Sequence comparison was done using the CLUSTAL X program. New primers were synthesised to target the 16S rRNA region of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) based on previous FISH results where it was noted that these two species of AOB and NOB predominated in all three BNR plants.

5.2.6 Cloning of PCR Products

Cloning of eluted fragments from agarose gels were carried out using the InsT/Aclone™ PCR product cloning kit (M/s MBI, Fermentas Inc., USA), which is a convenient system for one step cloning of PCR amplified DNA fragments. The PCR amplified fragments were electrophoresed on a 1% agarose gel and the desired bands were eluted. After elution, the fragments were run on a 1% agarose gel to conform the product. The eluted products were then ligated with pTeZ57R/T and the ligated mixture was used for transformation. *E. coli* (DH5α) was transferred with the ligated recombinant pTZ57R/T vectors carrying inserts. Colonies started to emerge from 16th hour. The cells that took up DNA would have taken it up at the restriction site made in the *lacZ* gene. The *lacZ* gene codes for β-galactosidase, which hydrolyzes X-gal to yield blue colonies. The cells that contain plasmids with interrupted *lacZ* genes (plasmids with inserted DNA) cannot code for β-galactosidase, and result in white colonies.

5.2.5.1 Ligation

Ligation of the desired PCR amplified purified DNA fragments were performed using a pTZ57R/T vector as described by the manufacturer. The vector (pTZ57R/T) and inserts were taken in 1:3 ratios. Ligation was conducted in volumes of 30µl containing 3µl of plasmid vector pTZ57R/T DNA (0.165µg, 0.18pmol ends), 4µl of eluted PCR product (approximately 0.54pmol ends), 3µl of 10 x ligation buffer, 3µl of PEG 4000 solution and 1µl of T4 DNA ligase (5U). The ligation mixture was incubated at 22°C overnight.

5.2.5.2 Transformation of *E. coli* Strains

Transformation was carried out using the TransformAid^{TM} Bacterial Transformation system. TrasnsformAid^{TM} C-medium (2ml) was inoculated with a loop full of *E. coli* DH 5α frozen culture and incubated overnight at 37°C on a shaker at 180rpm. A 1/10 volume of the overnight culture was added to 1.5ml of pre-warmed C-medium and incubated for 20 minutes at 37°C on a shaker at 180rpm. From this, 1.5ml of fresh culture was dispensed into a micro-centrifuge tube and centrifuged at 10 000rpm for 1 minute at 4°C. The supernatant was discarded; the pellet was re-suspended in 300µl of TransformAid^{TM} T-solution and incubated on ice for 5 minutes. The cells were centrifuged again at 10000rpm for 1 minute at 4°C, re-suspended in 120µl of TransformAid^{TM} T-solution and incubated on ice for 5 minutes. DNA for transformation was prepared by dispensing 2.5µl of ligation mixture into sterile micro-centrifuge tubes and placing them on ice for 2 minutes. To this, 50µl of re-suspended cells were added and incubated on ice for 5 minutes. The cells were finally plated onto pre-warmed LB-agar plates containing ampicillin (100ppm), isopropyl thiogalactoside (IPTG); (100ppm), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal); (160ppm) and incubated overnight at 37°C.

5.2.5.3 Selection of Recombinant Clones

The recombinant clones were identified using a blue/white colony selection procedure (Banerjee *et al*., 2010), where the white colonies generally contain the target inserts. The recombinant plasmids were taken into the bacterial cells via transformation. The cells that

did not take up a vector were unable to survive the presence of ampicillin that was present in the plates. The cells that took up a vector, which contained the *Amp*^{r} gene for ampicillin resistance, survived. The cells that took up DNA would have taken it up at the restriction site made in the *lacZ* gene. The *lacZ* gene codes for β-galactosidase, which hydrolyzes X-gal to yield blue colonies. The cells that contain plasmids with interrupted *lacZ* genes (plasmids with inserted DNA) cannot code for β-galactosidase, and thus result in white colonies.

5.2.5.4 Clone Analysis by PCR (Colony PCR)

Colony PCR was carried out for the direct analysis of the positive transformants. Single white colonies were picked up using sterile toothpicks and suspended in 20µl of nuclease-free water, which represented genomic DNA. This reaction mixture was incubated for 5 minutes at 94°C to lyse the cells and inactivate the nucleases. Thereafter, PCR amplification was carried out and all reaction mixtures had a total working volume of 25µl and consisted of Taq buffer (MBI, Fermentas, USA), dNTP's, forward and reverse primers, genomic DNA, nuclease-free water and Taq polymerase. Amplification was achieved using the optimised PCR protocols mentioned earlier for the Nitroba1, Nitroso2 and Nitroso3 primer sets. All PCR products were visualised using agarose gel electrophoresis.

5.2.5.5 Sequencing and Phylogenetic Analysis

PCR products obtained after the use of the new primer sets were submitted for sequencing. The partial 16S rRNA sequences were thereafter compared to the NCBI database using the Basic Local Alignment Search Tool (BLAST) to determine phylogenetic affiliations (Altschul *et al*., 1997). Clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU), and their representative sequences were used for further analysis. Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap re-sampling analysis for 100 replicates was performed to estimate the confidence of the tree topologies. All the analysis was carried out using CLC DNA Workbench, Version 6.0.

5.2.6 Accession Numbers

The sequences determined in this study have been deposited in the GenBank database under accession numbers HM243223 to HM243230.

5.3 RESULTS

5.3.1 Optimisation of DNA Extraction

Three different protocols were used for isolating DNA from three different wastewater samples. For each procedure, four replicates were analysed and compared to evaluate the performance of the different methods. The quantity and quality (purity) of extracted DNA were assessed by absorbance at 260nm and the ratio of absorbance at 260 and 280nm, respectively based on Nanodrop readings, whereby the DNA quality is basically marked by the ratio of A260 to A280 being as close to 1.8 as possible.

However, for environmental samples, the DNA quality was considered reasonable when the ratio was >1.5 (Lemarchand *et al*., 2005). The occurrence of fragmentation of the extracted DNA was determined by electrophoresis of each DNA through a 1% (w/v) agarose gel. All of the protocols successfully extracted DNA from the samples but with varying degrees of efficiency (Table 5.2). Great variability in extraction efficiencies was observed among these protocols, as well as between replicates. The boiling method yielded the maximum DNA concentration as compared to the other two methods tested. The phenol-chloroform and CTAB methods resulted in very low DNA yields as well as phenol and protein contamination (Table 5.2). The extracted DNA was then pooled together for further evaluation.

Table 5.2: Comparison of (Nanodrop readings) DNA concentrations from three different extraction methods (values are represented as the average of four replicates)

WWTP	METHOD					
	PHENOL-CHLOROFORM		CTAB		BOILING	
	Purity (A260/280)	Concentration (ng/µl)	Purity (A260/280)	Concentration (ng/µl)	Purity (A260/280)	Concentration (ng/µl)
HAMMARSDALE 1	1.58	450.5	0.47	5.51	1.84	1200.52
HAMMARSDALE 2	1.52	528.2	1.86	3.28	1.82	1140.31
KINGSBURGH 1	1.83	161.6	2.25	46.60	1.80	1290.54
KINGSBURGH 2	1.63	193.2	7.97	31.47	1.85	1316.72
DARVILLE 1	1.54	309.2	1.59	100.32	1.91	1368.62
DARVILLE 2	1.54	326.7	1.36	100.75	1.87	1194.3

5.3.2.1 Detection of AOB using amoA-1F and amoA-2R

The primer set *amoA*-1F/*amoA*-2R targeted the ammonia-monooxygenase genes and the expected product length was 491bp. All three wastewater samples analysed produced successful PCR amplicons. These products were then cloned and sequenced for further analysis. Agarose gel electrophoresis was used to determine DNA presence and intensity. Figure 5.1 depicts an agarose gel showing the PCR products that resulted from the use of the *amoA*-1F and *amoA*-2R primers, specifically for the AOB.

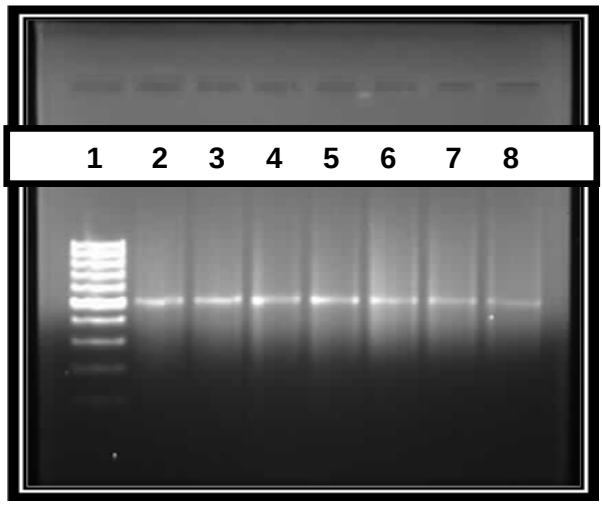


Figure 5.1: Primer specificity: Agarose gel depicting PCR products for AOB. Lane 1 depicts the banding pattern for a 100bp DNA ruler, whilst lanes 2-8 depict the banding patterns of the amplicons that resulted from using this primer set.

5.3.2.2 Detection of NOB using FGPS872F and FGPS1269R

The primer set FGPS872F and FGPS1269R targeted the nitrite-reductase genes of *Nitrobacter* spp and had expected product lengths of 397bp. The optimised PCR conditions as mentioned earlier resulted in samples being amplified at the required bp length (Figure 5.2). The PCR products were then successfully cloned and sequenced for further analysis. Figure 5.2 depicts an agarose gel showing the PCR products that resulted from the use of the FGPS872F and FGPS1269R primers, specifically for Nitrobacter (NOB).

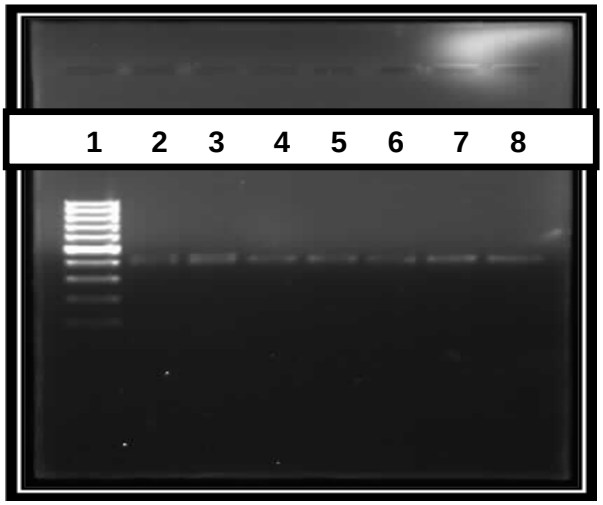


Figure 5.2: Primer specificity: Agarose gel depicting PCR products for *Nitrobacter* spp. Lane 1 depicts the banding pattern for a 100bp DNA ruler, whilst lanes 2-8 depict the banding pattern of the amplicons that resulted from the use of this primer set.

5.3.3 Detection of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB)

Two new set of primers were synthesised during the course. New primers were synthesised to target the 16S rRNA region of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) based on previous FISH results where it was noted that these two species of AOB and NOB predominated in all BNR plants. The expected length of the Nitroba1 set of primers was 420bp whilst the expected length of the Nitroso2 was 450bp. Samples (60 in total) were tested against each of these primer sets. All samples resulted in successful amplification. The successful amplicons were further cloned and sequenced for confirmation. The resultant nucleotide sequence showed 98% similarity to *Nitrosomonas* and *Nitrobacter* sp.

5.3.3.1 Detection using Nitroso2f and Nitroso2r; Nitroso3f and Nitroso3r primer sets

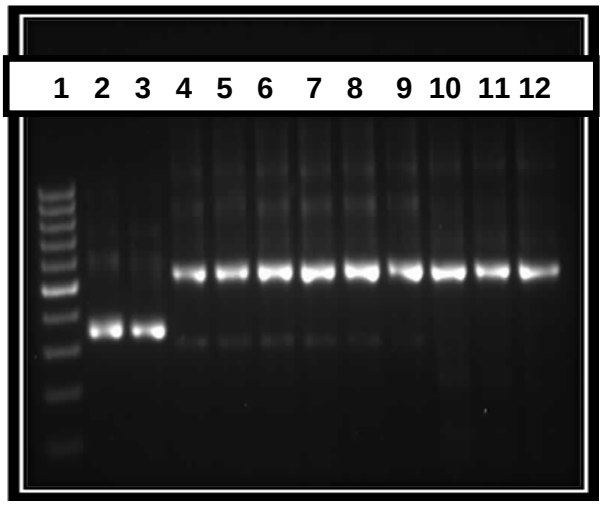


Figure 5.3: Agarose gel depicting PCR products for *Nitrosomonas* sp.

Figure 5.3 depicts an agarose gel showing the PCR products that resulted from the use of the Nitroso2f and Nitroso2r; Nitroso3f and Nitroso3r primers, specifically for *Nitrosomonas* sp (AOB). Lane 1 depicts the banding pattern of a 100bp DNA ruler, whilst lanes 2-3 depict the banding pattern for a control that was run during this experiment for a diffrent primer set which resulted in positive amplification. Lanes 4-12 depict the improved, brighter banding patterns of the amplicons that resulted from the use of these new Nitroso2f and Nitroso2r; Nitroso3f and Nitroso3r primer sets.

5.3.3.2 Detection using Nitroba1f and Nitroba1r Primer Set

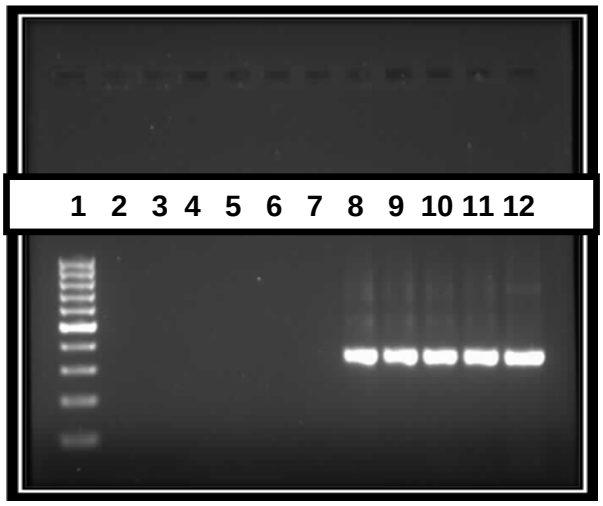


Figure 5.4: Agarose gel depicting PCR products for *Nitrobacter* sp.

Figure 5.4 depicts an agarose gel showing the PCR products that resulted from the use of the Nitroba1f and Nitroba1r primers, specifically for *Nitrobacter* sp (NOB). Lane 1 depicts the banding pattern of a 100bp DNA ruler, whilst lanes 2-7 depict no banding patterns for these samples using this primer set. Lanes 8-12 depict bright and improved banding patterns for the amplicons that resulted from the use of this primer set.

5.3.4 Cloning of the PCR Products

Occurrence of white colonies among the blue ones confirmed the successful transformants (Figure 5.6). The cells that did not take up a vector were unable to survive the ampicillin present in the media. The cells that took up a vector, which contained the *Amp* gene for ampicillin resistance, survived. The agar plates containing the transformed colonies were screened for recombinant-positive clones. White colonies were selected and grown in LB broth medium containing ampicillin. The recombinant colonies were screened for the presence of inserts by colony PCR. For comparison, genomic DNA samples were also used separately for PCR. After PCR, the amplicons were electrophoresed on an agarose gel. There was amplification of the expected size in all the recombinant clones**.** The recombinant plasmids, for which the confirmation tests were performed, were sent for automated nucleotide sequencing at Africa Centre (University of KwaZulu-Natal, Durban South Africa).

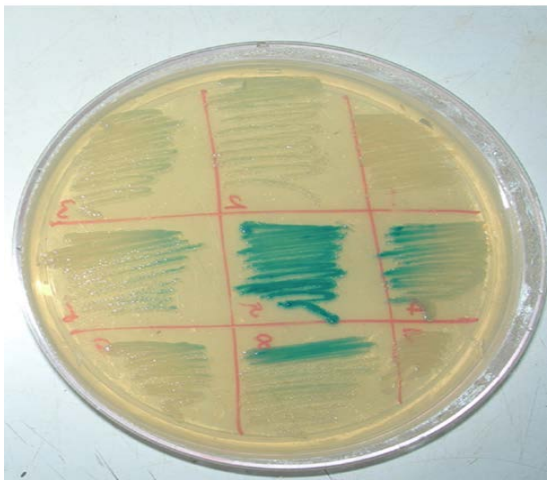


Figure 5.5: Blue/white colony screening, distinguishing recombinant (white) from non-recombinant (blue) colonies on LB media

5.3.5 Phylogenetic Analysis

Cloning and sequencing of gene fragments were performed to investigate the nitrifying population diversity. Sequences were compared to the NCBI database using the Basic Local Alignment Search Tool (BLAST) to determine phylogenetic affiliations (Altschul *et al*., 1997). Clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU), and their representative sequences were used for further analysis. All sequences exhibited more than 98% similarity to either *Nitrosomonas* or *Nitrobacter* sp. These partial sequences were thereafter submitted to the GenBank database to obtain accession numbers and were used for the phylogenetic tree construction. Phylogenetic trees were constructed using the neighbor-joining method and bootstrap re-sampling analysis for 100 replicates was performed to estimate the confidence of the tree topologies. Neighbor-joining phylogenetic trees, which graphically depict the homologies of aligned sequences, are presented in Figures 5.7 and 5.8.

Among the various clones analysed, the majority of them showed the co-existance of more than one species of *Nitrobacter* and *Nitrosomonas* sp at these three BNR plants. Comparative sequence analysis of PCR fragments for AOB confirmed the presence of *Nitrosomonas halophila*, *Nitrosomonas eutropha* and *Nitrosomonas europaea*, an unidentified *Nitrosomonas* spp and an unidentified betaproteobacteria at these BNR plants (Figure 5.7). Comparative sequence analysis for *Nitrobacter* clones showed a higher percentage on *Nitrobacter vulgaris*, *Nitrobacter alkalicus* and an unidentified *Nitrobacter* sp (Figure 5.8) prevalant at these BNR plants.

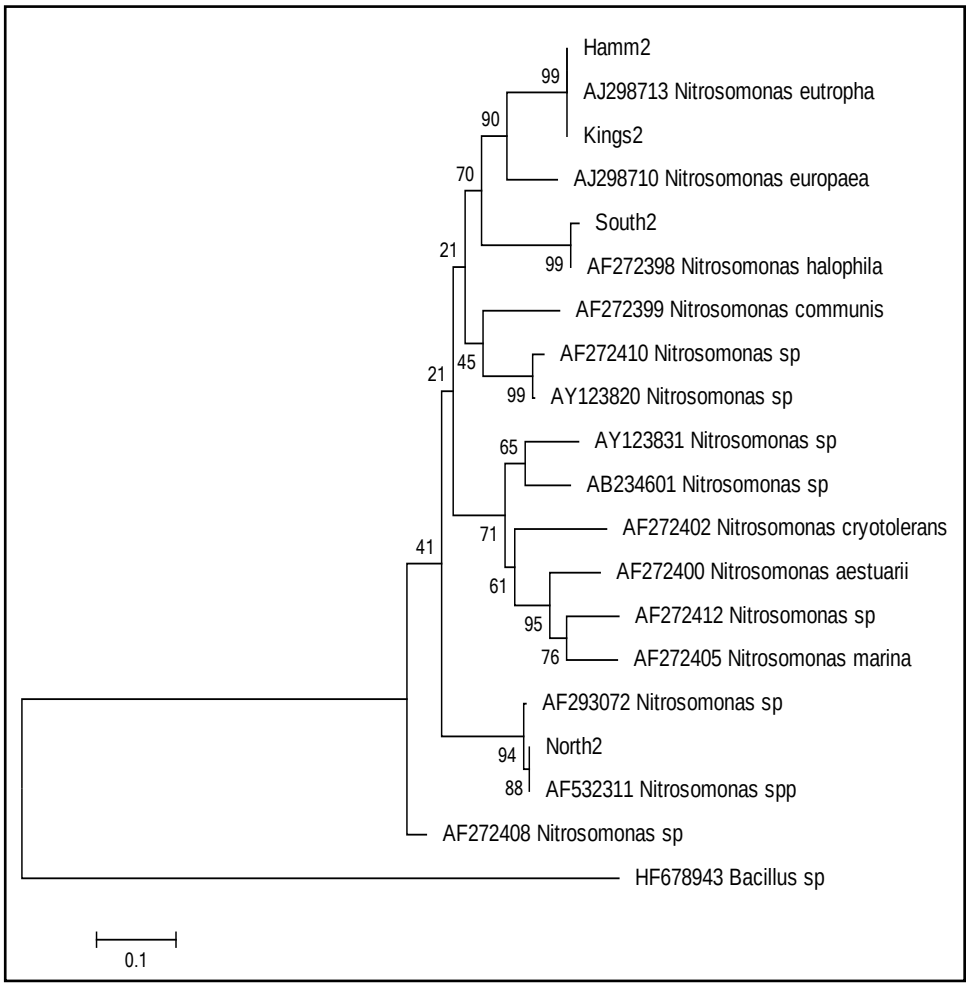


Figure 5.6 Dendrogram showing the phylogenetic position of the *Nitrosomonas* clones obtained after sequencing. The scale bar represents a 0.4 substitution per nucleotide position. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap test was calculated for 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). *Bacillus* sp is used as an out-group.

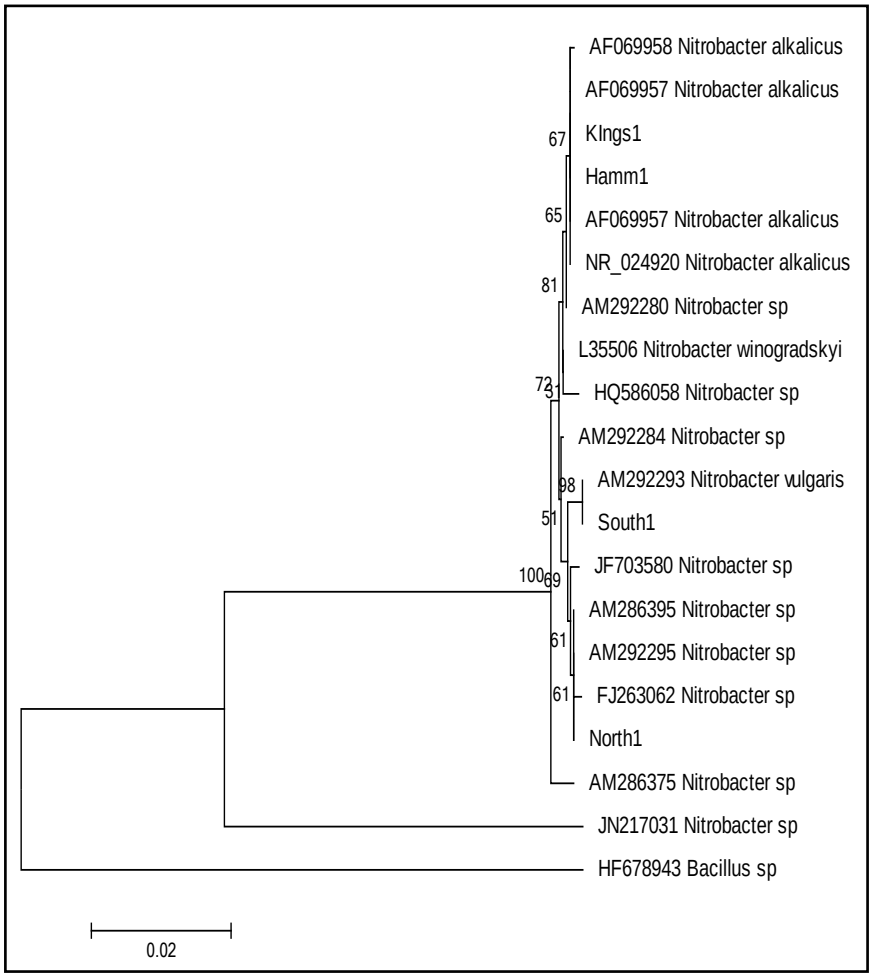


Figure 5.7: Dendrogram showing the phylogenetic position of the *Nitrobacter* clones obtained after sequencing. The scale bar represents a 0.4 substitution per nucleotide position

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap test was calculated for 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). *Bacillus* sp is used as an out-group.

5.4 DISCUSSION

There is great difficulty in obtaining a full picture of the bacterial diversity of an ecosystem by relying solely on conventional methodologies. To overcome the limitations of this, powerful new technical approaches in molecular microbial ecology, which permit a new level of insight into the population structure and function of complex microbial communities in WWTP, can be used to analyse sequences of the 16S rRNA. This helps reveal AOB and NOB communities from various environments. In combination with clone libraries or finger printing methods, the application of specific PCR amplification can provide clarification of the AOB and NOB communities in detail (Sunday and Seun, 2011). Recently, Q-PCR techniques have enabled the quantitation of AOB and NOB communities in the environment since it entails the combination of high throughput with high analytical sensitivity and precision (Harms *et al*., 2003; Winkler *et al*., 2012). A number of studies have been conducted to assess the ecology and microbiology of AOB and NOB in wastewater treatment systems (Okabe *et al*., 1999; Pynaert *et al*., 2003; Haseborg *et al*., 2010).

To supplement the results obtained from FISH analysis in this study, further studies based on PCR and 16S rRNA analysis using specific primer pairs were conducted to study the phylogenetic variation among the dominant AOB and NOB populations. Several studies using molecular techniques including 16S rRNA-targeted methods have shown a great diversity of nitrifiers in activated sludge (Cydzik-Kwiatkowska *et al*., 2012). Therefore it is difficult to obtain a full picture of the bacterial diversity of an ecosystem by relying solely on conventional methods. To overcome these limitations powerful new technical approaches in molecular microbial ecology permit a new level of insight into the population structure and function of complex microbial communities present and active in BNR plants by analysing sequences of the 16S rRNA to reveal AOB and NOB communities. In combination with clone libraries or finger printing methods, the application of specific PCR amplification can provide clarification of the nitrifying bacterial community in detail (Sunday and Seun, 2011). Recently Q-PCR techniques have enabled quantitation of AOB and NOB populations in the environment since they combine high throughput with high analytical sensitivity and precision (Harms *et al*., 2003; Winkler *et al*., 2012).

Previous studies (Okabe *et al*., 1999; You *et al*., 2003) have debated whether *Nitrosomonas* or *Nitrosospira* is the predominant species in various wastewater treatment systems. Some researchers found that the community was dominated by AOB from the genus *Nitrosomonas* (Cao *et al*., 2012). Other studies indicated that *Nitrosospira* sp (Gieseke *et al*., 2001; Coskuner and Curtis, 2002) remained the predominant population in biofilter compost, while *Nitrosomonas* sp is the predominant population in biofilter sludge (Gieseke *et al*., 2001; Daims *et al*., 2001; Dionisi *et al*., 2002; Coskuner and Curtis 2002; Tsuneda *et al*., 2003; Hallin *et al*., 2005; Yin and Xu, 2009).

For this study PCR and sequencing of 16S rRNA was used to investigate the diversity, population structure and physiology of dominant nitrifying bacteria in three full-scale WWTP in KwaZulu-Natal.The importance of the diversity within nitrifying bacterial groups catalyzing critical steps in nutrient removal for process stability, has been previously inadequately addressed by microbiologists and engineers (Kwiatkowska *et al*., 2012). Bacterial diversity allows for changes in the process regime and the design of more robust and reliable nutrient removal plants will be possible. Understanding the diversity of key WWTP microbes such as nitrifiers should also lead to more efficient bioaugmentation strategies. Consequently microbes are added which will not find an adequate niche in the system and thus rapidly disappear either by wash-out or grazing. Addition of the 'desired' microbes should enhance the success of future BNR processes. As detailed microbiological knowledge is required to select suitable nitrifying bacteria to enhance the microbial diversity in treatment plants, thetools offered by molecular biology are indispensable. They enable us to understand the biology of important bacterial 'major players' and to define the range of environmental conditions that allow certain speciesor communities to grow and perform substrate conversions at optimal rates (Bernhard, 2010).

DNA extraction techniques play a pivotal role as this stage can either enhance or inhibit the phylogenetic analysis stage. In this study three different techniques of DNA extraction were implemented and compared to obtain maximum representation of the microbial community present. The specificity of primers from previously published works was evaluated in this study (Figures 5.1-5.3); (Degrange and Bardin, 1995; Rotthauwe *et al*., 1997; Dionisi *et al*., 2002). Primers specific for AOB and NOB predominant in wastewater treatment systems were applied (Loy *et al*., 2008). The new primers were designed from PCR products to

specifically to target *Nitrosomonas* AOB and *Nitrobacter* NOB spp from South African wastewater treatment plants (Figures 5.4-5.5). Neighbour-joining phylogenetic trees, which graphically depict the homologies of aligned sequences, are presented in Figures 5.6 and 5.7. Bootstrap re-sampling analysis for 100 replicates was performed to estimate the confidence of the tree topologies.

The sequence analysis of 16S rRNA genes of AOB confirmed the presence of more than one species of *Nitrosomonas* sp at these plants viz., *Nitrosomonas halophila*, *Nitrosomonas eutropha* and *Nitrosomonas europaea,* an unidentified *Nitrosomonas* sp and an unidentified betaproteobacteria (Figure 5.6). Among *Nitrosomonas* sp, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas halophila and Nitrosococcus mobilis* are frequently reported at many BNR plants (Purkhold *et al*., 2000; Daims *et al*., 2001; Gieseke *et al*., 2003; Li *et al*., 2012). It was reported that the distribution pattern of different *Nitrosomonas* spp. seem to depend on the type of treatment works and its operational conditions (Daims and Wagner, 2010). The 16S rRNA analysis of PCR products using genus-specific primers, revealed the presence of more than one species of the same group at these plants.

Previous studies have also shown that community structures of AOB and NOB were different among different water treatment processes. The influent quality and the treatment processes both influenced the AOB and NOB diversity (Limpiyakorn *et al*., 2005; Bo and Xin, 2012). *Nitrosomonas* spp. including *Nitrosomonas halophila*, *Nitrosomonas eutropha*, *Nitrosomonas europaea*, *Nitrosomonas aestuarii* and an unidentified *Nitrosomonas* sp were found to dominate among the AOB and *Nitrobacter vulgaris, Nitrobacter alkalicus, Nitrobacter hamburgensis* and an unidentified *Nitrobacter* sp were the dominant species for NOB. Among these species, *Nitrosomonas aestuarii*, *Nitrosomonas europaea*, *Nitrobacter hamburgensis* were detected only from the industrial wastewater samples.

In wastewater treatment systems (full- and lab-scale) there appears to be selection for either predominance of a single bacterial population or several different bacterial populations occur together. It was suggested that different plants might support different populations and different levels of species richness (Hallin *et al*., 2005; Dytczak *et al*., 2008). In addition, different environmental conditions during long-term operation should result in the selection of different microbial populations. It might be assumed that the system under different

operation situations may harbor different bacterial community compositions (Ding *et al*., 2011).

The *Nitrobacter* clones showed more affiliations to *Nitrobacter vulgaris, Nitrobacter alkalicus* and an unidentified *Nitrobacter* sp (Figure 5.7). The co-existence of multiple nitrifying species (or strains) and shifts in the dominant nitrifying population have been previously reported (Gieseke *et al.,* 2001). Among the different species *Nitrosomonas halophila* and *Nitrobacter vulgaris* were found to be prevalent at all three WWTP irrespective of the influents and the operational conditions. After sequencing, majority of the clones showed affiliation to these two species indicating their dominance at these BNR plants. These species have been reported to grow in wastewater where there is high concentration of ammonia and salts (Daims *et al*., 2001). According to earlier studies, SRT plays a major role in controlling the total number of nitrifying bacteria and has been associated with their diversity (Purkhold *et al*., 2000). Thus, varying ammonium concentrations, dissolved oxygen levels and longer SRT's (Tables 4.3-4.5-Chapter 4) are all factors that could have supported the growth of these diverse group of nitrifying bacteria at these plants.

The presence of more than one group of organisms can overcome limitations such as low/high dissolved oxygen and ammonium concentrations and could help the system to function normally as these groups differ in their growth requirements. However, substantial numbers of nitrifying bacteria have also been previously reported under conditions such as nitrification failure. This has been attributed to the adaptive ability of these groups to endure periods of energy-source deprivation, when they cannot perform their usual functions (Li *et al*., 2007).

5.5 CONCLUSIONS

- It can be concluded from this study that community dominance and diversity depend on the type of treatment works and its operational conditions
- Phylogenetic variation of AOB and NOB populations across these three BNR plants could be attributed to the variation in their influent characteristics and operational parameters.
- Phylogenetic analysis of the dominant groups showed the presence of more than one species of the same group at these BNR plants
- Future work using specific primers targeting functional genes of AOB, NOB, Anammox and Ammonia-Oxidising Archaea (AOA) with advanced techniques such as high through-put sequencing would shed more light on unraveling the complexity of the nitrifying microbial community and their interaction at these BNR plants

CHAPTER SIX: Comparison of FISH and Q-PCR to Study the Distribution of Nitrobacter spp in Two Full-Scale Wastewater Treatment Plants

6.1 INTRODUCTION

The focus of this aspect was to compare the efficiency of two novel techniques i.e. FISH and Q-PCR as an effective tool for the quantification of nitrifiers from BNR plants. For this purpose *Nitrobacter* was selected to represent the nitrifying population. Bacteria belonging to the genus *Nitrobacter* constitute a key part of the nitrification process since they are responsible for the conversion of toxic nitrites to nitrate in biological wastewater treatment. Failure to promptly detect and correct nitrification leads to the build-up of toxic nitrites in the system. This negatively affects wastewater discharge standards and thus impacts on the ecosystems of receiving water bodies. Thus *Nitrobacter* spp plays a highly significant role in the nitrification process, and a greater understanding of *Nitrobacter* spp is essential for process control and efficiency (Jechalke *et al*., 2011).

A greater understanding of the microbial ecology of nitrifying bacteria in wastewater is essential for improving overall process performance and control. These qualitative and quantitative biases can be avoided by the use of *in situ* as well as molecular biological techniques to study bacterial diversity in wastewater in a cultivation-independent manner. Therefore, it is difficult to obtain a full picture of the bacterial diversity of an ecosystem by relying only on conventional methodology (Bin *et al*., 2010).

To overcome these limitations, molecular biological techniques can be used to reveal nitrifying communities in various environments. Techniques such as FISH and Q-PCR are two widely applied techniques for *in situ* identification and quantification of microbial communities from complex environments (Schmid *et al*., 2006; Isaka *et al*., 2006, Mutlu and Güven, 2011). FISH is a common method (Amann *et al*., 1995; Almeida *et al*., 2010) applied for the quantification of microbial populations from environmental samples. FISH using 16S rRNA-targeted oligonucleotide probes has become a well established technique for the direct,

cultivation-independent identification of complex microbial community structures in environmental and engineered systems (Amann *et al*., 2001; Gilbride *et al*., 2006) *in situ*.

In recent years, Q-PCR is reported to be an effective and rapid technique and has been used for the estimation of nitrifier abundance, allowing quantitation specificity up to the genus level (Banerjee *et al*., 2012) for quantifying microbial proliferation *in situ*. This has eliminated the variability traditionally associated with PCR, allowing for the routine and reliable quantification of PCR amplicons (Converse *et al*., 2012), especially for the detection and quantification of nitrifying bacteria in wastewater. For Q-PCR, amplicons are detected based on the measurement of a fluorescence signal produced.

Q-PCR techniques have enabled quantification of nitrifying populations in the environment because they combine high throughput with high analytical sensitivity and precision (Harms *et al*., 2003; Brankatschk *et al*., 2012). Although a number of studies have been conducted to assess the ecology and microbiology of nitrifying bacteria in wastewater treatment systems (Okabe *et al*., 1999; Haseborg *et al*., 2010), the effect of different methods for quantification of *Nitrospira* communities are poorly understood. The melt-curve analysis of Q-PCR experiments makes distinctions between different amplified sequences possible and has already been successfully applied to nitrifying populations (Loescher *et al*., 2012).

However, both techniques were reported to have their limitations when applied to a complex environment such as activated sludge. The FISH technique has been widely applied for identification and quantification of nitrifying biofilms from wastewater treatment systems (Daims *et al*., 2001; Gieseke *et al*., 2003). The FISH technique is cheap and rapid to detect microorganisms from environmental samples and has already been successfully implemented in the field of wastewater research. It has a low risk of contamination, since it lacks a nucleic acid amplification step. The main advantages of the FISH technique includes rapid detection, feasibility and the need for not much technical equipment as compared to PCR, which requires costly reagents and instrumentation. However, FISH is time-consuming, at times difficult to use in complex microbial samples due to the formation of dense microbial clusters and might be affected by low cell rRNA content (Third *et al*., 2001).

More recently, a Q-PCR assay was developed and applied to assess microbial diversity in wastewater plants (Harms *et al*., 2003). This is based on the continuous monitoring of fluorescence intensity throughout the PCR reaction which is a fast, reliable, sensitive method to enumerate the relative and absolute abundance of a genus or species from a complex environment. However, it should be noted that Q-PCR measures the gene copy number and not the acual cell number which might affect accurate enumeration.

Therefore the goal of this study was to compare the efficiency of these two widely applied techniques viz., FISH and Q-PCR for *in situ* detection and quantification of *Nitrobacter* spp from two different full-scale BNR plants.

6.2 MATERIALS AND METHODS

6.2.1 FISH Analysis

6.2.1.1 Sampling and Cell Fixation

Activated sludge samples were collected from the aerobic zones of two BNR plants (KwaZulu- Natal, South Africa). In addition to comparing the two techniques, research also focused on comparison of two different types of wastewater viz. domestic and industrial. The plants selected treated domestic (Kingsburgh BNR plant) and industrial (Hammarsdale BNR plant) wastewater. Samples were collected weekly from both plants across a period of 9 weeks. Plant operating parameters were obtained from the plant operators at the time of sampling. For *in situ* hybridization, all activated sludge samples were fixed at the time of sampling in 4% paraformaldehyde (Haseborg and Frimmel, 2007), to render the *Nitrobacter* cells permeable to probes. Fixed samples were stored in a 1:1 mixture of phosphate buffered saline (PBS; pH 7.2) and absolute ethanol at -20°C until further hybridisation. All samples were pre-treated with sonication to allow for floc dispersion and thus accurate quantification (Ramdhani *et al*., 2010).

6.2.1.2 Oligonucleotide Probe Selection

Probes were selected to target *Nitrobacter* spp and eubacteria that are commonly found in activated sludge (Table 1). The 16S rRNA-targeted oligonucleotide probes chosen were

labeled with either tetramethylrhodamine-5-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) at the 5' end. The following probes were used for population analysis (Inqaba Biotech, South Africa): EUB338 (Eubacteria), NONEUB (control probe complementary to EUB338); NIT3 (NOB-*Nitrobacter* spp.). Probes EUB338, EUB338II and EUB III were used in a mixture (Yeates *et al*., 2003).

Table 6.1: List of 16S rRNA-targeted oligonucleotide probes

PROBE	SEQUENCE	SPECIFICITY	REFERENCE
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp.	Wagner *et al*. (1996)
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	Daims *et al*. (1999)
EUB338II	GCAGCCACCCGTAGGTGT	Planctomycetes	Daims *et al*. (1999)
EUB338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	Daims *et al*. (1999)
NonEUB	CGACGGAGGGCATCCTCA	Non binding control	Wallner *et al*. (1993)

6.2.1.3 *In situ* Hybridisation

In situ hybridisations were carried out according to methods previously described by Manz *et al*. (1992). Samples were thereafter stained with 10µl of 0.25 µg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate – Sigma, Germany) for 10 min in the dark and mounted in VECTASHIELD® anti-fading agent (Vector Laboratories, USA).

6.2.1.4 Image Analysis

All samples were visualised using an epiflourescent microscope (Zeiss AxioCam MRc - Carl Zeiss, Germany). Image analysis was carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging software. Ten random images were evaluated with final results reflecting the average counts of *Nitrobacter* sp present in the corresponding activated sludge samples (Haseborg *et al*., 2010). *Nitrobacter* sp population fractions were expressed as ratios of EUB and the average cell percentage were reported in this study.

6.2.2 DNA Extraction

Genomic DNA was extracted from 18 samples (9 from each plant) of activated sludge using a modification of the boiling method by Sepp *et al*. (1994). Activated sludge samples were centrifuged at 10 000 rpm for 5 minutes. The supernatants were discarded and pellets were re-suspended in 500µl nuclease-free water. This suspension was mixed thoroughly and boiled in a water bath at 80°C for 20 mins. The concentration and purity of all extracted DNA was determined using the Nanodrop ND–1000 spectrophotometer (Nanodrop Technologies, USA).

6.2.2.1 Standard Curve Preparation

A standard curve was constructed using PCR-amplified 16S rRNA gene fragments of universal primer pairs (27f and 1492r) as well as *Nitrobacter* sp (FGPS872f and FGPS1269r) specific primers (Table 6.2). Conventional PCR was carried out and after agarose gel electrophoresis, the quantity of the PCR products were determined densitometrically by comparison with a HaeIII DNA marker (MBI, Fermentas, USA) and converted to μl^{-1} copies under consideration of the molecular weight of the PCR product and Avogadro's number. The standards were prepared by mixing the PCR products of the universal primer pair and specific primer pair in different ratios (0%, 0.01%, 0.1%, 1%, 10%, and 100%). Conventional PCR was carried out and the quantity of the PCR products were determined densitometrically by comparison with a HaeIII DNA marker (MBI, Fermentas, USA) and converted to μl^{-1} copies. The standards were prepared by mixing the PCR products of the universal primer pair and specific primer pair in different ratios (0%, 0.01%, 0.1%, 1%, 10%, and 100%)

6.2.2.2 Optimisation of PCR Conditions for Q-PCR

Q-PCR was based on the principle of Heid *et al*. (1996), using a Mini-Opticon Real-Time PCR system (Bio-Rad, South Africa). Reaction mixtures were prepared by combining SYBR Green supermix (2×), primers (50 pmol), template DNA (100 ng), ampli-Taq DNA polymerase (0.5 U), and nuclease-free water to make up a total volume of 25µl for each reaction. The reaction mixture was transferred to a thin 96-well PCR plate (Bio-Rad, South Africa), with two negative controls (one excluding primers and the other excluding template DNA) in triplicate. The plate was covered with optical-quality PCR caps (Bio-Rad, South Africa) and centrifuged briefly to allow all the reagents to migrate to the bottom of the wells.

For all experiments, appropriate negative controls containing no genomic DNA were subjected to the same procedure to exclude any possible contamination or carry-over.

The *Nitrobacter* spp specific primer pair and the universal bacterial primer pair was used in the assay to determine the 16S rRNA gene copies of *Nitrobacter* spp in comparison to the total bacterial 16S rRNA gene copies. To evaluate the variation of the Q-PCR technique, all measurements were carried out in triplicate for each run; three runs were performed per quantification, and means and standard deviations were calculated from the means of each run. After completing the PCR amplification cycles, a melting curve was generated for the resulting amplicons by measuring loss of fluorescence over a temperature range of 55°C to 99°C.

Table 6.2: List of primers and their specificity

PRIMER	SEQUENCE (5'-3')	SPECIFICITY	REFERENCE
FGPS872f	CTAAAACTCAAAGGAATTGA	nitrite reductase gene	Degrange and Bardin (1995)
FGPS1269r	TTTTTTGAGATTTGCTAG	nitrite reductase gene	Degrange and Bardin (1995)
27f	AGAGTTTGATCMTGGCTCAG	Bacterial 16S rRNA gene	Dojka *et al*., (2000)
1492r	TACCTTGTTACGACTT	Bacterial 16S rRNA gene	Lin and Stahl (1995)

6.2.3 Statistical analysis

Statistical analysis was performed using Excel software. To detect the statistical significance of differences between the two methods of quantification, the unpaired *t*-test was performed.

6.3 RESULTS

6.3.1 Detection and Quantification of *Nitrobacter* spp using FISH

Samples were equally distributed for both FISH and Q-PCR, where 1 ml was used for FISH and 1ml used for DNA extraction for Q-PCR studies to maintain sample consistency for both the analyses. The probe NIT3 (Loy *et al*., 2007) was selected to target *Nitrobacter* sp and EUB mix was selected to encompass all Eubacteria commonly found in activated sludge. To remove autofluorescence and background interference the pixelated areas obtained were subtracted by the areas of non-bound probe (Non338). Image analysis was carried out using

ten random images with final results reflecting the average percent of *Nitrobacter* sp present in the corresponding activated sludge samples (Haseborg *et al*., 2010). FISH results showed the presence of *Nitrobacter* sp (against EUB) in the range of 2.5 to 4.2% at the domestic WWTP and 2.9 to 4.1% at the industrial WWTP. The domestic WWTP showed a slightly higher *Nitrobacter* sp community compared to the industrial plant (Figure 6.2).

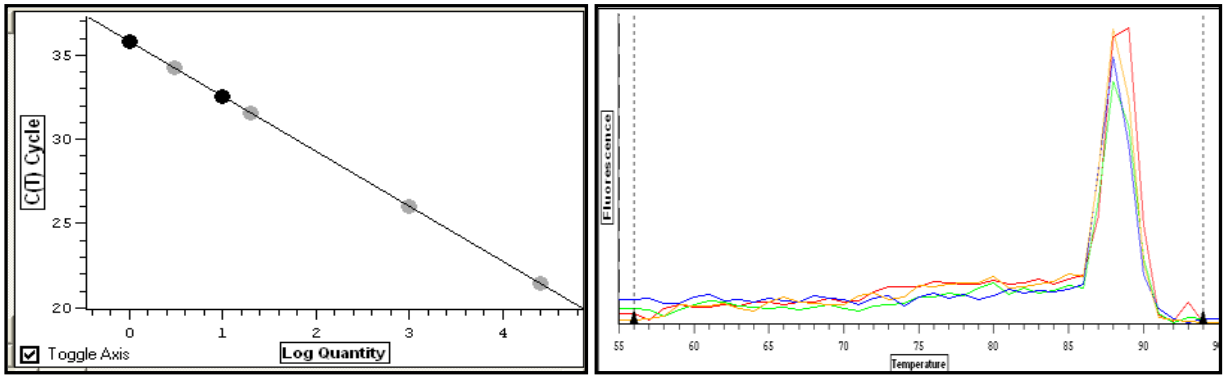
6.3.2 Detection and Quantification of *Nitrobacter* spp using Q-PCR

For Q-PCR, genomic DNA was extracted from 1ml activated sludge samples. A standard curve was constructed using PCR-amplified 16S rRNA gene fragments of the universal primer pair 27f (spanning positions 8 to 27 in *Escherichia coli* rRNA coordinates) and 1492r (commonly spanning positions 1492 to 1507) as well as *Nitrobacter* sp specific primers (FGPS872f [16S rRNA position, 872–891] and FGPS1269r [1269–1286]); (DeLong *et al*., 2006). QPCR was based on the principle of Heid *et al*. (1996). Reaction mixtures were run with two negative controls to exclude any possible contamination or carry-over. The optimised PCR program contained a 10 minute step at 50°C, 5 minute enzyme activation step at 95°C, followed by 39 cycles of 15 seconds at 95°C, 30 seconds at 62°C, and 30 seconds at 60°C. To evaluate the variation of the Q-PCR technique, all measurements were carried out in triplicate for each run; three runs were performed per quantification, and means and standard deviations were calculated from the means of each run.

After completing the PCR amplification cycles, a melting curve was generated for the resulting amplicons by measuring loss of fluorescence over a temperature range of 55°C to 99°C. The average 16S rRNA gene copies of *Nitrobacter* in the samples were calculated against the total bacterial 16S rRNA gene copies. The Q-PCR assays were validated using known concentrations of standard DNA (Figure 6.1A). The regression coefficient ($r2$) values for standard curves for all Q-PCR assays in each run were always above 0.90. A melting curve analysis was done at the end of the PCR run to confirm that no non-specific binding had occurred during the reaction (Figure 6.1B).

The Q-PCR results showed a higher variation percentage of *Nitrobacter* sp compared to the FISH results at both BNR plants investigated. At the domestic plant (Kingsburgh), Q-PCR

yielded *Nitrobacter* sp that ranged from 3.0 to 4.8% whilst the industrial plant (Hammarsdale) yielded counts that ranged from 3.3 to 4.5% (Figure 6.3). A higher percentage of *Nitrobacter* sp was detected from the domestic plant compared to the industrial plant. Significantly higher percentages of *Nitrobacter* sp was detected using Q-PCR compared to FISH. For Q-PCR studies, SYBR Green was used as a fluorescent marker and the resultant PCR amplicons were later confirmed by sequencing.



Figures 6.1A and B: (A) Standard curve obtained for *Nitrobacter* specific primers (r^2 =0.92) and (B) melting curve analysis observed after the Q-PCR run.

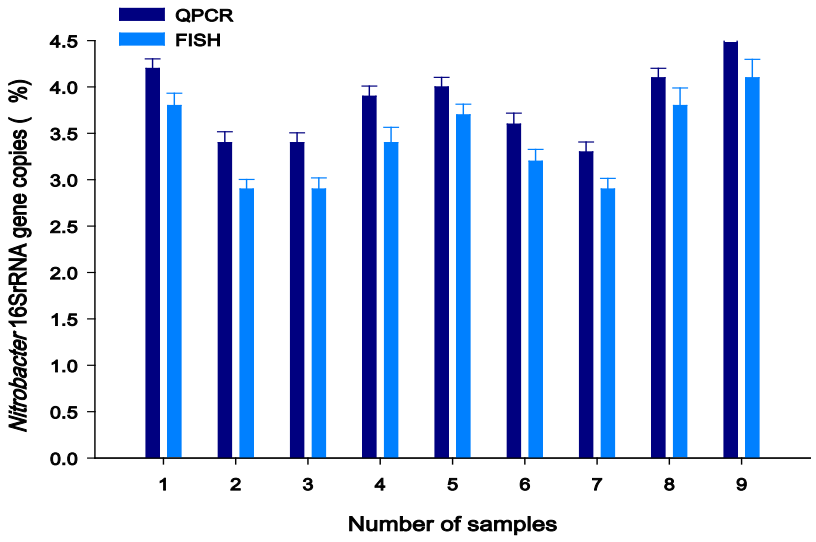


Figure 6.2: Comparison of industrial and domestic BNR plants showing average *Nitrobacter* spp (%) using FISH.

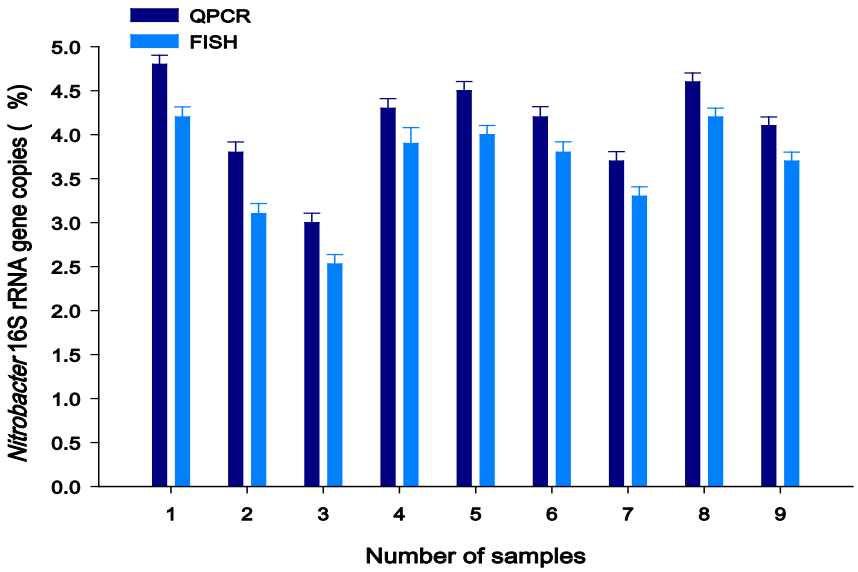


Figure 6.3: Comparison of industrial and domestic BNR plants showing average *Nitrobacter* 16SrRNA gene copies (%) using Q-PCR.

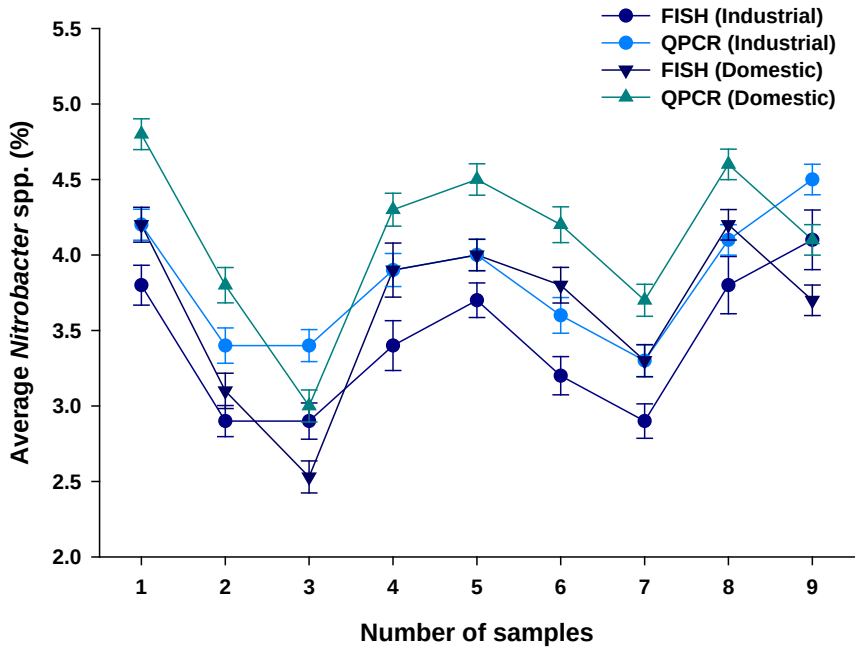


Figure 6.4: Comparison of FISH vs Q-PCR quantification of *Nitrobacter* spp (%) at both BNR plants

6.4 DISCUSSION

FISH and Q-PCR quantification was applied in this study to determine the abundance of *Nitrobacter* sp at two different BNR plants. Both these techniques provided a good indication of the presence of *Nitrobacter* sp at these two plants. However, since FISH has the potential to produce false positives and Q-PCR has the potential to produce false negatives, the use of these two methods provides complimentary data for the detection of *Nitrobacter* sp (Harms *et al*., 2003). Despite being a methodologically robust technique, FISH suffers from several limitations (Wagner *et al*., 2003) which may prevent successful detection of target microorganisms. The most frequently encountered problems are low signal intensity of the detected microbes and strong background auto fluorescence. Dim signals can be caused by a low cellular concentration of the target molecules (Pernthaler *et al*., 2002).

In order to increase the sensitivity of FISH and make it suitable for the detection of microbes with low ribosome content, several strategies have been developed, of which catalysed reporter deposition (CARD)-FISH has found the most widespread application (Hoshino *et al*., 2008). However, CARD-FISH is rather expensive, requires enzymatic pretreatment to allow the large horseradish peroxidase-labeled probes to penetrate the target cells (Pernthaler and Pernthaler, 2007), and causes a dramatically altered melting behavior of the probes (Hoshino *et al*., 2008). Another frequently encountered FISH problem is the low *in situ* accessibility of many regions of the 16S rRNA for single-labeled probes (Fuchs *et al*., 2001). Probes targeting such regions, which comprise about one-third of the *Escherichia coli* 16S rRNA, confer signals which are very dim or even below the detection limit. In order to avoid the selection of poorly accessible target sites for FISH probe design, a consensus 16S rRNA accessibility map for prokaryotes has been established based on detailed accessibility maps of two bacterial model organisms and one archaeal model organism (Behrens *et al*., 2003). Considering this consensus map during probe design is recommended, but it excludes many probes with useful specificities from FISH applications.

The application of PCR in combination with the extraction of nucleic acids from environmental samples has been central to the development of culture-independent approaches in microbial ecology. More recently, Q-PCR assays were developed and applied to quantify microorganisms from complex environments such as BNR plants (Harms *et al*., 2003). These techniques help to overcome the limitations of FISH as this assay is a fast,

reliable, sensitive and convenient method especially suitable for enumeration of a low abundance of dense microcolony-forming biofilms. These methods enable the analysis of total microbial communities present within environmental systems and have revolutionized our understanding of microbial community structure and diversity within an environment. Coupling environmental nucleic acid isolation to subsequent PCR amplification of both taxonomic and functional gene markers and in combination with DNA fingerprinting and sequence-based analyses has enabled the description of the uncharacterised majority of environmental microorganisms. This drives the discovery of new microbial lineages and enables the description of genetic diversity in a wealth of functional gene markers.

In the current study, the Q-PCR results showed a slightly higher (P < 5%) *Nitrospira* sp at both the plants compared to the FISH results (Figure 6.4). The abundance of *Nitrospira* sp varied from 2.5 to 4.2% (domestic plant; Figure 6.2) and 2.9 to 4.1% (industrial plant; Figure 6.2) using FISH; whilst the population varied from 3.0 to 4.8% (domestic plant; Figure 6.3) and 3.3 to 4.5% (industrial plant; Figure 6.3) using Q-PCR. Statistical analysis revealed that at the industrial BNR plant, there was a significant difference between the results obtained for FISH and that obtained for Q-PCR (*t*-test *P:* 0.06; P < 5%). At the domestic BNR plant, there was also a significant difference between the results obtained for FISH and that obtained for Q-PCR (*t*-test *P:* 0.09; P < 5%). The low FISH counts could also be as a result of pre-treatment methods adopted for the analysis which could have a large impact on the final enumeration step.

However, it should be noted that Q-PCR measures the gene copy number and not the actual cell number which might affect accuracy during enumeration. In Q-PCR analyses, quantification is based on the threshold cycle Ct, which is inversely proportional to the logarithm of the initial gene copy number. The threshold cycle values obtained for each sample should be compared with a standard curve to determine the initial copy number of the target gene. For Q-PCR studies, the inhibitory effects of complex genomic DNA at concentrations of 10 to 50ng/ul and other inhibitors could also affect the results. Biodiversity surveys based on comparative sequence analyses are affected by biases of DNA extraction, PCR, and cloning. A critical step for successful PCR-based techniques is the extraction and purification of DNA to increase the yield and reduce the co-extraction of inhibitors that can

weaken the efficiency of nucleic acid amplification. An average quantity and good quality of DNA extracted is essential to perform reproducible, sensitive and specific PCR reactions.

The melt-curve analysis was performed mainly to detect any non-specific PCR amplification and primer-dimer formation. Furthermore, it also allowed clustering of the samples into different groups, with specific melting temperatures. The melting temperature (Tm) reflects the temperature at which 50% of a specific DNA fragment is dissociated and is besides the amplicon's length, dependent on the GC and AT concentration. Thus, it also enables discrimination between different DNA sequences. This can be minimised by dilution of the samples to an extent. However, an over-dilution may also result in the false enumeration of target organisms which are usually present in very minute concentrations, resulting in a high variability in under or sometimes over-estimation of the desired microorganism. Although two quantitative techniques gave comparable and valid results, the Q-PCR assay was easier and faster than the FISH technique for quantification of *Nitrobacter* spp from BNR plants.

6.5 CONCLUSIONS

- Q-PCR proved to be a more efficient method of quantification in this study
- However, considering that all experiments needed to be conducted in triplicate, Q-PCR proved to be a very expensive method of quantification. Thus this may not be the best option in terms of feasibility, since all BNR plants monitor their wastewater at different points in the system on a regular basis
- Therefore, a large number of samples may not be feasibly analysed using Q-PCR However, when combined, Q-PCR and FISH have the potential to considerably accelerate and accurately detect microorganisms from wastewater. To benefit from the various advantages of Q-PCR and FISH, both methods should be combined in a complimentary manner

GENERAL CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

This study employed novel molecular techniques to identify and quantify autotrophic nitrifying populations across full-scale BNR plants in KwaZulu-Natal, South Africa. Techniques included FISH combined with group-specific rRNA-targeted oligonucleotide probes; PCR together with primers specific for the dominant nitrifying populations from these five WWTP; cloning; nucleotide sequencing and phylogenetic analysis; and Q-PCR to compare against the FISH results.

Initially, prior to FISH hybridisation, the effects of 5 different pre-treatment methods on the integrity of individual cells and their dissociation from activated sludge flocs from five different BNR plants were experimentally elucidated. Physical, chemical, enzymatic and combinational pre-treatment methods were compared. Additionally, many different sonication durations and power levels were also compared. The results revealed that Igepal was a far more effective method of pre-treatment than lysozyme. However Igepal still could not achieve the desired results. Sonication was found to be the superior method for cell dispersion. The desired sonication combination was chosen after screening of the sludge samples at various different time intervals and power levels.

This led to the combination of 8W for 8 minutes of sonication being chosen for industrial wastewater pre-treatment since it was the most effective method for cell dispersion without the loss of cell integrity. The combination of 8W for 5 minutes was chosen for pre-treatment of domestic wastewater. It was also concluded that the importance of the optimization of pre-treatment methods for different types of sludge should be emphasised for all bacterial quantification analyses, especially FISH. This objective contributed significantly to the pre-treatment of different types of wastewater in South Africa.

FISH analyses were thereafter carried out for three BNR plants to quantify the nitrifying populations present. Based on FISH analyses evident differences with respect to the

dominant nitrifying populations were found between these four plants. This study further confirmed the highly complex activities of wastewater processes, which depends on a number of factors. Although some of the differences in detected AOB and NOB can be attributed to probe specificity, the lack of consensus on a specific AOB or NOB predominant in wastewater rather suggests that the wastewater type and characteristics may establish significantly different microbial environments. Among the AOB, *Nitrosomonas* dominated in all three BNR plants throughtout the study period and for NOB both *Nitrobacter* and *Nitrospira* were found in significant numbers and its dominance varied across the plants.

These dissimilar, distinct distribution patterns recorded of AOB and NOB in this study could be attributed to their environment which in turn impacted on the nitrification performance of the system. However, the correlation of nitrifying community to the nitrification efficiency of the system is highly complex as there are reports of various phylogenetically diverse groups of organisms involved in this process other than AOB and NOB which includes Anammox and ammonia oxidizing Archaea (AOA). It should also be noted that the co-existence of more than one group of these communities at the same plant could help the plant escape from functional failures such as nitrification due to sudden changes in temperature and substrate concentrations, as this function can be performed by different groups.

The application of *in situ* identification techniques for the monitoring and understanding of nitrifying bacteria will result in more efficient nutrient removal. BNR plants are designed and modeled, and accurate quantitation of the responsible microorganisms would improve these models. Q-PCR has recently been applied and optimized to quantify nitrifying bacteria that usually form dense clusters in biofilms. Q-PCR assays are quick, reliable, sensitive and convenient to enumerate uncultured, slow-growing and cluster-forming bacteria. Reliable quantification methods are essential to determine kinetic parameters of slow-growing bacteria, such as nitrifiers.

This study focused on the distribution pattern of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) from three full-scale BNR plants in South Africa. It can be concluded that community dominance and diversity depend on the type of treatment works and its operational conditions. The phylogenetic variation of AOB and NOB populations across these three BNR plants could be attributed to the variation in their influent characteristics and operational

parameters. Among *Nitrosomonas* spp, NRS2 (industrial), NRS4 and NRS8 (domestic) showed more affiliation to *Nitrosomonas europaea* and NRS6 (industrial) towards *Nitrosomonas halophila*. Among the *Nitrobacter* spp, clone NRS 1 (industrial) showed more affiliations to *Nitrobacter vulgaris*; NRS 3 (domestic) and NRS 5 (industrial) showed affiliations to *Nitrobacter alkalicus* and NRS7 (domestic) to an unidentified *Nitrobacter* sp thus indicating their dominance at these BNR plants. This study has also resulted in the novel design of three new primers for specific PCR amplification of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) from wastewater.

SIGNIFICANCE AND NOVELTY OF THE RESEARCH FINDINGS

The research findings in this study are significant in that AOB and NOB populations were quantified at full-scale plant level and correlated with plant operating parameters.

The novelty of this work lies in the fact that it represents the first comparative study of different pre-treatment methods for application to activated sludge from South African wastewater treatment plants. Additionally, the primers designed are novel and have resulted in phylogenetic affiliations previously unknown in South African wastewaters. This has helped us identify new species and now allows us to compare with international databases.

RECOMMENDATIONS

- While our current understanding of molecular microbial ecology has recently improved, DNA-based studies may not provide accurate information about active members of natural microbial assemblages since DNA may persist in the environment after cell death. Therefore, FISH should be used in conjunction with Q-PCR in a complementary manner for confirmation of quantification results.

- Intracellular RNA is rapidly degraded in stressed cells and is unstable outside of the cell due to the abundance of RNAses in the environment. Consequently, recent studies have focused on developing sequence databases based on extracted rRNA to describe metabolically active members of drinking water biofilm microbial communities. This should be applied to activated sludge.

- Future work using more specific primers targeting functional genes of AOB, NOB, Anammox and AOA with advanced techniques such as high through-put sequencing would shed more light on unraveling the complexity of the nitrifying microbial community and its interaction in wastewater treatment systems.

- The limitation of FISH lies with the databases available for probe design. Although there are now >400 000 16S rRNA sequences, this still represents a small percentage of all the 'prokaryotes' thought to exist on Earth. Thus probes designed using these data sets may target sequences of organisms not yet included, against which it was originally designed, resulting in false positives and negatives, e.g. EUB338 was re-designed to include *Verrucomicrobia* or *Planctomycetales* together with all Bacteria. An additional limitation of FISH is that 16S rRNA genes are highly conserved and it is not always possible to design a probe to distinguish between closely related organisms. This can be overcome with the use of competitor probes or targeting the 23S rRNA instead, for closely related Beta-proteobacteria and Gamma-proteobacteria.

- Although some bacteria like the nitrifiers retain high ribosome numbers no matter what their physiological state is, metabolically inactive cells would generally contain fewer ribosomes and would be difficult to detect via FISH. Inadequate accessibility to

target sites can also lower probe signal strength. Several methods can increase probe signal strength, such as non-fluoresecent helper probes, which alter the conformation of the 16S rRNA, enhancing probe accessibility and may increase fluorescence signal. CARD-FISH and peptide nucleic acid probes achieve similar results. Processes like nitrification and denitrification involve functionally important genes which are not present in such large copy numbers, thus making the identification of their cells problematic even though their gene sequences are known. Thus FISH detecting specific transcribed mRNA have been used for such purposes but not yet for activated sludge communities.

- Future studies on population dynamics in wastewater should place emphasis on correlating this data to plant operating conditions as this would assist plant operators and engineers to pre-empt any problems at the full-scale plant level.

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APPENDICES

APPENDIX ONE: Comparison of Pre-treatment Methods (Chapter 3)

	PRE-TREATMENT METHOD				
	HAMMARSDALE	**NORTHERN**	**KINGSBURGH**	**SOUTHERN**	**DARVILLE**
LYSOZYME	6	46	51	12	15
IGEPAL	38	31	26	17	14
SONICATION	127	187	167	117	126
LYSOZYME + SONICATION	43	32	48	36	41
IGEPAL + SONICATION	31	48	52	35	38
VARIANCE (ANOVA)	2034.25	5685.67	4036.92	1997.58	2402.25
***P*-VALUE**	**0.9645**				

APPENDIX TWO: Comparison of Sonication Optimisation (Chapter 3)

WWTP		TIME (mins)				
	POWER LEVEL (watts)	**5**	**6**	**7**	**8**	**9**
HAMMARSDALE	**5**	149	130	111	150	124
	6	159	138	158	178	143
	7	180	169	344	567	391
	8	570	698	861	1294	584
	9	28	43	30	57	34
NORTHERN	**5**	184	170	120	112	78
	6	283	264	194	108	61
	7	365	287	302	196	156
	8	1196	832	682	785	289
	9	40	32	28	40	22
KINGSBURGH	**5**	251	195	148	204	85
	6	296	186	156	129	93
	7	345	237	217	258	109
	8	624	493	509	294	141
	9	48	48	37	34	31
SOUTHERN	**5**	257	296	327	424	97
	6	301	378	398	447	99
	7	278	349	356	474	94
	8	476	572	586	609	89
	9	42	58	56	65	37
DARVILLE	**5**	286	318	351	367	108
	6	361	394	404	489	81
	7	384	418	462	493	73
	8	415	464	487	540	96
	9	42	37	42	48	22

APPENDIX THREE: Statistical Analysis of Variance (ANOVA); (Chapter 3)

WWTP	TIME (mins)	POWER (watts)	VARIANCE	*P*-VALUE
	5	5	42421.7	
	6	6	69005.3	
HAMMARSDALE	7	7	111359.7	**0.80087**
	8	8	261113.7	
	9	9	51320.7	
	5	5	205965.3	
	6	6	92942	
NORTHERN	7	7	64373.2	**0.662085**
	8	8	93108.2	
	9	9	11174.7	
	5	5	43025.7	
	6	6	26371.7	
KINGSBURGH	7	7	31520.3	**0.26555.1**
	8	8	10875.2	
	9	9	1615.20	
	5	5	31790.92	
	6	6	44956.92	
SOUTHERN	7	7	48156	**0.196945**
	8	8	54528.25	
	9	9	828.9167	
	5	5	30188.33	
	6	6	38544.25	
DARVILLE	7	7	43028.92	**0.154203**
	8	8	53283	
	9	9	1031.333	

APPENDIX FOUR: Pearson's correlation coefficient; T-TEST; ANOVA (Chapter 4)

STATISTICAL ANALYSIS	Plant Operating Parameters	KINGSBURGH		HAMMARSDALE		DARVILLE	
		AOB	NOB	AOB	NOB	AOB	NOB
Pearson's Correlation Coefficient	Nitrification rate	0.638432	0.424552	0.671333	0.653676	-0.37614	-0.422533
	Influent COD	0.321966	-0.031895	0.177864	-0.285747	0.26977	0.441065
	SRT	0.137787	-0.087268	0.177530	0.409379	0.09178	0.029895
	DO	0.379302	-0.190948	0.321286	0.460963	0.412295	0.609662
	Influent ammonia	-0.54282	0.046008	0.549406	0.569479	0.284481	0.622279
	Effluent ammonia	0.72027	0.636236	0.557411	0.585752	-0.4317	-0.264255
	Nitrites	0.21272	-0.449030	0.094933	0.073383	0.248873	0.319265
	pH	0.14679	-0.276049	-0.118791	0.063929	0.422686	0.243500
	T°C	-0.20684	-0.397731	-0.398644	-0.398644	0.391575	0.336748
Unpaired T-TEST	AOB vs NOB	0.0012		0.0003		0.4307	
ANOVA *(P-value)*	AOB			0.997875			
	NOB			0.999968			
	AOB/NOB Ratio			0.978486			
	Nitrification rate			0.999423			

APPENDIX FIVE: Solutions for DNA Extraction (Chapter 5)

Tris-EDTA (Ethylenediaminetetraacetic acid) Buffer

0.12g Tris/HCl (10mM)

20.3mg EDTA(1mM)

Tris-HCl and EDTA were both dissolved in distilled water and made up to 100ml each. These were thereafter adjusted to pH 8.0, autoclaved and stored at 4°C.

10% SDS

100g SDS

SDS was dissolved in distilled water and made up to 1000ml. This was heated to 68°C for solubility and stored at room temperature.

Lysis Buffer

10% SDS

0.1M NaCl

0.5M Tris/HCl

*Make up to 100mL using sterile distilled water.

0.5M Tris/HCl

6.05g Tris/HCl

Tris/HCl was dissolved in distilled water, made up to a final volume of 100ml and autoclaved.

10mM Tris/HCl

0.12g Tris/HCl

Tris/HCl was dissolved in distilled water, made up to a final volume of 100ml, adjusted to pH 8.0 and autoclaved.

0.5M EDTA (pH 8.0)

148g EDTA

EDTA was dissolved in distilled water, made up to a final volume of 1000ml, adjusted to pH 8.0 and autoclaved.

1M NaCl

5.8g NaCl

NaCl was dissolved in distilled water, made up to a final volume of 100ml and autoclaved.

5M NaCl

29g NaCl

NaCl was dissolved in distilled water, made up to a final volume of 100ml and autoclaved.

APPENDIX SIX: DNA Extraction: Phenol-chloroform Method (Chapter 5)

- Samples (1ml) were centrifuged at 1400rpm for 5 minutes at 4°C
- The supernatant was discarded, and the pellet re-suspended in 1 X PBS and centrifuged further at 5000rpm for 3 minutes
- Pellets were re-suspended in 75µl Tris-EDTA buffer (10mM Tris/HCL, 1mM EDTA, pH 8.0) with 25µl 10% SDS
- Tubes were inverted to mix. Samples were incubated for 2 hours at 65°C
- 500µl Lysis buffer was added (10% SDS in 0.1M NaCl and 0.5 Tris/HCl). This was vortexed to mix
- Cells were lysed by the freeze-thaw method (Immersion in a dry ice and ethanol slurry for 2 minutes followed by heating in a water bath at 65°C). This cycle was repeated 5 times
- 500 µl Tris-saturated phenol was added to the tubes, inverted to mix and centrifuged at 5700rpm for 5 minutes
- Samples were treated using phenol-chloroform (25:24), chloroform and 100% isopropyl alcohol, where each upper layer was immediately transferred to a sterile tube with an equal amount of the next reagent and centrifuged at 5700rpm for 5 minutes
- Following the addition of isopropyl alcohol the tubes were incubated for 1 hour at -20°C
- The DNA was pelleted by centrifugation at 5000rpm for 5 minutes
- Pellets were washed with 70% ethanol, centrifuged and re-suspended in 100µl TE buffer (pH 8.0)
- Incubate at 37°C for 1 hr. Freeze at -20°C for 30 min. Add 100µl phenol chloroform, centrifuge, discard the supernatant
- The Nanodrop was used to determine DNA concentration and purity

APPENDIX SEVEN: DNA Extraction: Hexadecyl trimethyl-ammonium bromide Method (Chapter 5)

1M Tris (pH 8.0)

121.1g Tris base

TrisBase was dissolved in 800ml distilled water and pH was adjusted to 8.0. The final volume was adjusted to 1000ml and autoclaved.

CTAB Buffer

2g CTAB (Hexadecyl trimethyl-ammonium bromide)

10ml 1M Tris (pH 8.0)

4ml 0.5M EDTA (pH 8.0)

28ml 5M NaCl

40ml water

1g PVP 40 (Polyvinyl pyrrolidone)

PVP and CTAB were dissolved in 20ml distilled water. All remaining components were added and made up to 50ml with distilled water. This was autoclaved.

APPENDIX EIGHT: Polymerase Chain Reaction (Chapter 5)

Reaction volume	: 25µl
10 x DreamTaq PCR buffer	: 2.5µl
10ng/ml genomic DNA	: 2µl
2mM dNTP	: 2.5µl
50pM stock solution of each primer	: 2µl
*DreamTaq polymersae	: 1µl
Nuclease-free water	: 10µl

*denotes products from Fermentas, Inqaba Biotec, South Africa.

All components are thoroughly mixed together in eppendorf tubes and placed in a Veriti Thermal cycler (Applied Biosystems, South Africa) for PCR.

APPENDIX NINE: Agarose Gel Electrophoresis (Chapter 5)

10 x Tris-Boric acid-EDTA (TBE) Buffer

540g Tris base
275g Boric acid
200ml of 0.5M EDTA (pH 8.0)

Tris base and boric acid were dissolved in 800ml distilled water. EDTA was added and this was made up to a final working volume of 1L using distilled water. This was autoclaved and stored at 4°C.

1 x TBE Buffer

20ml 50 x TBE buffer
TBE buffer was added to sterile distilled water and made up to a final working volume of 1L.

Agarose Gel

50ml 1 x TBE buffer
0.8g Agarose (molecular grade)
1µl ethidium bromide

Agarose was added to 1 x TAE buffer. This was melted in a microwave for approximately 2 minutes and allowed to cool to 60°C. Ethidium bromide (1µl) was added, mixed well, poured into the gel casting tray with comb and allowed to set for 20 minutes at room temperature.

Electrophoresis

DNA ladder (3µl) was loaded into the first well in the gel. Thereafter samples (2µl) were loaded together with (1µl) loading dye (one sample per well). The gel was run at 80 volts (V) for 60 minutes and therafter exposed to UV light in a gel-documentaion sytem and photographed. Good DNA quality was confirmed by the presence of a good resolution high molecular weight band, whilst the presence of smeared bands was indicative of DNA degradation.

APPENDIX TEN: Cloning (Chapter 5)

IPTG (1M)

1g IPTG

IPTG was dissolved in 4ml distilled water and filtered through 0.22µm filters. Aliquots were dispensed into 1ml eppendorfs and stored at -20°C until further use.

X-gal (20mg/ml)

100mg X-gal was added to 5ml DMSO, dissolved and stored at -20°C.

Luria Bertani (LB Agar)

12g LB Agar

LB agar powder was dissolved in distilled water over a heating plate, whilst being stirred continuously until the liquid turned clear. Thereafter the agar was autoclaved, cooled to 60°C and poured into sterile petri dish plates. This was set aside to solify and then stored at 4°C.

APPENDIX ELEVEN: Fluorescent in situ Hybridisation (Chapter 3)

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.

3 x PBS

24g NaCl
0.6g KCl
4.32g Na_2HPO_4
0.72g KH_2PO_4

All components were added together and distilled water was added to make up to 1000ml. pH was adjusted to 7.4 and this was 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 33ml of 3 x PBS was added. The pH was adjusted to 7.2 with HCl, filtered and cooled down to 4°C. This solution was made up fresh for each use.

Cell Fixation

1 x PBS

Paraformaldehyde

Ethanol

The samples were washed twice with 1 x PBS. Three volumes 4% paraformaldehyde 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. Supernatants were discarded. To store, the pellet 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.

Immobilization of Fixed Cells onto Slides

For spotting of samples onto slide, 10µl of sample was spotted onto each well of the slide. This was allowed to air dry. Thereafter cells were dehydrated in an increasing ethanol series of 60, 80 and 100% at 3 minutes each. Slides were stored at room temperature until use.

Hybridization Solutions (pH 7.2; 20%)

5.26g NaCl

0.24g Tris

0.01g SDS

0.186g EDTA

20ml formamide*

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

*All chemicals 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 30ml formamide.

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 50ml. Add 5M NaCl according to the breakdown above, 1 mL of 1M Tris/HCl, 50µl 10% SDS, sterile distilled water to make up to 50mL.

5M NaCl stock soln

2150µl	(20%)
1020	(30%)
700	(35%)
460	(40%)
300	(45%)

Oligonucleotide Probe Preparation

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

Whole cell hybridisation

Strips of filter paper were soaked in the appropriate hybridisation solutions and placed in polypropylene chambers. These chambers were allowed to equilibrate for 15 minutes at a hybridisation temperature of 46ºC. Probe/buffer mixture (10µl) were placed on each well. Slide were then placed in the pre-warmed chamber and incubated to allow hybridisation at 46ºC for 2 hours. Wash buffers were prepared in polypropylene tubes and placed in a water bath to pre-heat at 48°C. Slides were rinsed with wash buffers and thereafter immersed in wash buffer tubes in a water bath for 20 minutes at 48ºC. Slides were washed twice with 1 x PBS and air dried. DAPI solution (10µl) was placed over each well and allowed to stain for 10 minutes in the dark. Slides were then washed with 1 x PBS and air dried. Mounting media (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 TWELVE: Sequences from Genbank (Chapter 5)

>NRS1 (HM243223)

AATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAG
CGTTAWTCGGAATTACTGGGCKTAAAGSGTGCGCAGGCGGTTTTTTAAGTCAGAT
GTGAAATCCCCGGGCTCAACCTGGGAACTGCMTTTGTGACTGCAAGGSTGGAGT
GGGGYAGAGGGGGRTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGG
AGGAACACCGATGGCGAAGGCANTCCCCTGGGCNTGCACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAAATACCCTGGTAGTCCCGCCCCTAAACAA

>NRS2 (HM243224)

ACCCAACAACCAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTGCAGGCCCAGGGGATTGCCT
TCGCCATCGGTGTTCCTCCGCATATCTACGCATTTCACTGCTACACGCGGAATTCC
ATCCCCCTCTGCCGCACTCCAGCTTTGCAGTCACAAATGCAGGTCCCAGGTTGAG
CCCGGGGATTTCACATCTGTCTTACAAAACCGCCTGCGCACGCTTTACGCCCAGT
AATTCCGATTAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTA
GCCGGTGCTTATTCTTACGGTACCGTCATTAGCCCCCTGTATTAGAGAAAGCCGT
TTCGCTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCGTCCTGCACGCGGCA
TTGCTGGATCAGG

>NRS3 (HM243228)

TGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGT
GCATGAGCGTCAGTGCAGGCCCAGGGGATTGCCTTCGCCATCGGTGTTCCTCCGC
ATATCTACGCATTTCACTGCTACACGCGGAATTCCATCCCCCTCTGCCGCACTCC
AGCTATGCAGTCACAAAKGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGT
CTTACAAAACCGCCTGCGCACGCTTTACGCCCAGTAATTCCGATTAACGCTTGCA
CCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTCCGGT
ACCG

>NRS4 (HM243225)

CGCAACCCTGATCCAGCAATCCCGCGTGCAGGACGAAGGCCTTCGGGTTGTAAA
CTGCTTTTGTACGGAGGGAAACGGCTTTCTCTAATACAGGGGGCTAATGACGGT
MCCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGT
AAGACAGATGTGAAATCCCCGGGCTCAACCTGGGACCTGCATTTGTGACTGCAA

AGCTGGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTA
GATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGC
TCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CCTAAACGATGTCAACTGGTTGTTGGGTCTTTACTGACTCAGTAACGAAGCTAAC
GCGTGAAGTTGACCGCCTGGGGAGTACGG

>NRS5 (HM243229)

TCGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGT
TTTGTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCMTTTGTGACT
GCAAGGCTGGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATG
CGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTG
ACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
AGCCCTAAACA

>NRS6 (HM243226)

TGCTTTTGTACGGAGCGAAACCGCTTTCTCTAATACCGGGGGGTAATGACGGTAC
CGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
TGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAA
GACAGATGTGAAATCCCCGGGCTCAACCTGGGAMCTGCATTTGTGACTGCAAAG
CTGGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGA
TATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCKGCACTGACGCTC
ATGCACGAAAGCGTGGGGAGCAA

>NRS7 (HM243230)

TTTCGGTACCGGGAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCG
GTTTTGTAAGACAGAGGTGAAATCCCCGGGCTCAACCTGGGAACTGCCTTTGWG
ACTGCAWAGCTTGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGA
AATGCKTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGT
ACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCC

>NRS8 (HM243227)

ATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGKGCGAGC
GTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGAT
GTGAAATCCCCGGGCTCAACCTGGGACCTGCATTTGTGACTGCAARGCTAGAGTG
CGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGA
GGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGCACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGAT
GTCAACTGGTTG

APPENDIX THIRTEEN: Impacts of pre-treatments on nitrifying bacterial community analysis from wastewater using fluorescent in situ hybridisation and confocal scanning laser microscopy

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Full Paper

Impact of pre-treatments on nitrifying bacterial community analysis from wastewater using fluorescent in situ hybridization and confocal scanning laser microscopy

Nishani Ramdhani, Sheena Kumari,* and Faizal Bux

Centre for Water and Wastewater Technology, Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

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Fluorescent in situ hybridization (FISH) and confocal scanning laser microscopy (CSLM) are the key techniques used to investigate bacterial community structure at wastewater treatment plants. An optimum nitrifying bacterial population is necessary for nitrification, which plays a significant ecological role in regulating the overall quality of water. Nitrifying bacteria mainly appear as dense aggregates within activated sludge flocs. The impacts of five different pre-treatment methods (physical, chemical, enzymatic and combinations) on floc dispersion from two different wastewater treatment plants were determined. The effect of pre-treatment on the enumeration of the nitrifying bacterial population was also investigated. This study on floc dispersion using CSLM images showed sonication was the superior method for all the samples tested, irrespective of the sludge type. For samples from industrial wastewater plants, an optimized sonication level of 8 W for 8 min could reduce the floc size to 10 µm, whereas for domestic wastewater samples, the floc size was reduced to 10 µm at 8 W for 5 min. The maximum number of nitrifying bacterial cells was observed at this optimized level for different samples. A decrease in the number of cells was observed beyond this optimized level for both the plants. The results presented here highlight the importance of optimizing pre-treatment methods for different types of wastewater for accurate bacterial community analysis using FISH-CSLM.

Key Words——CSLM; FISH; nitrifying bacteria; pre-treatment; wastewater

Introduction

The activated sludge process is one of the most extensive biological processes used for the treatment of wastewater, and is dependent on the formation and arrangement of microbial flocs to which many bacteria are attached (Snidaro et al., 1997). This bacterial aggregation and flocculation has been attributed primarily to an exopolymeric matrix. The structural arrangement of activated sludge flocs is too complicated to assess with conventional techniques alone (Bourrain et al., 1999).

CSLM in combination with FISH has been effectively used for the direct analysis of whole cells within microbial populations in activated sludge systems. FISH represents a highly efficient technique that enables the culture-independent identification of bacteria (Carr et al., 2005). Combining FISH with CSLM and digital image analysis makes it feasible to analyze the spatial distribution of nitrifying bacteria in activated sludge flocs and removes disturbing auto-fluorescent signals.

* Address reprint requests to: Dr. Sheena Kumari, Centre for Water and Wastewater Technology, Department of Biotechnology and Food Technology, Durban University of Technology, P. O. Box 1334, Durban, 4000, South Africa.
Tel: +27-31-373-2782 Fax: +27-31-373-2777
E-mail: sheenas@dut.ac.za

The optical sectioning properties of CSLM also significantly improved the in situ detectability of nitrifying bacteria in activated sludge (Delatolla et al., 2009).

There are reports of considerable variation in the nitrifying bacterial population among activated sludge samples from industrial and municipal wastewater treatment plants (Layton et al., 2000). Nitrifying bacteria mainly appear as dense aggregates within activated sludge flocs (Manser et al., 2005). For accurate quantification, it is essential to disrupt the flocs for the release of the bacterial cells from within the sludge sample (Biggs and Lant, 2000). Sonication is one of the commonly used techniques to disperse activated sludge flocs and enumerate bacteria (Daims et al., 2006). This technique does not contaminate the sample and is efficient for dispersing bacterial aggregates, provided excessive energy which could lyse the cells and contaminate the sample with intracellular polymers, is not used (Sears et al., 2005). Other methods tested include enzymatic pre-treatment with lysozyme. Lysozyme opens up the peptidoglycan layer and allows for increased cell permeability which might otherwise result in insufficient penetration of high molecular weight reagents into bacterial cells (Moter and Göbel, 2000). Chemical pre-treatment with a non-ionic surfactant (nonidet) was also tested because it alleviates cell clumping without any obvious damage to the cells and when used at appropriate concentrations, enhances cell dispersion (Stahl and Amann, 1991).

Although there are few reports on floc dispersion, the effect of pre-treatments on the microbial community has not been well analyzed. Many of these procedures have been employed for years; however, little work has been done to compare the efficiency of these pre-treatments on different types of wastewater. Thus, the aim of this study was to investigate the impact of pre-treatments on bacterial floc dispersion and to expose single cells for accurate enumeration of the nitrifying bacterial community, using FISH-CSLM.

Materials and Methods

Sampling and cell fixation. Samples of mixed liquor were collected from the aeration basins of two different activated sludge treatment plants, treating domestic and industrial wastewater. Sample bottles were half-filled with the mixed liquor to maintain aerobic conditions during sample transit, and stored at 4°C until further use. Samples were washed twice with distilled water and re-suspended in phosphate buffered saline solution (PBS: 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2). For in situ hybridization, all activated sludge samples were fixed immediately in 4% paraformaldehyde (Amann, 1995), to render the nitrifying bacterial cells permeable to probes. Fixed samples were stored in a 1 : 1 mixture of PBS and absolute ethanol at −20°C until further hybridization.

Pre-treatment of activated sludge samples using physical, chemical and enzymatic methods. For physical treatment, floc dispersion was achieved by sonication of the fixed sample, placed in an ice bath, using a probe sonicator (Virtis, Virsonic 100; USA). Sonication was optimized by comparing the effects of five power levels of 5, 6, 7, 8 and 9 W. The duration of sonication varied from 3 to 9 min at a time interval of 1 min for each power level. For chemical treatment, all fixed samples were treated with Nonidet P-40 (Sigma, Germany) 0.1% (v/v). A combination of pre-treatments involving sonication together with nonidet was also tested.

For enzymatic treatment, all fixed samples were treated with lysozyme (Sigma). Lysozyme permeabilization buffer (1 mg/ml) was applied to samples for 30 min at 37°C (Wallner et al., 1993). Thereafter samples were exposed to a combination pre-treatment method of sonication together with lysozyme.

Whole cell hybridization and DAPI staining. Teflon-coated microscope slides were pre-treated with 1 : 10 (v/v) Poly-L-Lysine solution (Sigma), which is an effective tissue adhesive. Poly-L-Lysine solution was brought to room temperature (18–26°C) and slides were immersed in this solution for 5 min. Slides were subsequently dried overnight at room temperature before use. The samples for all five pre-treatment methods were mixed thoroughly and hybridized with 16S rRNA-targeted oligonucleotide nitrifying bacterial probes (Table 1). Hybridized, pre-treated samples (10 µl) were applied to each well. Spots were air dried prior to dehydrating through an ethanol series (60, 80 and 100%, v/v) for 3 min. Filter paper was soaked in the appropriate hybridization solution and placed in a polypropylene tube to allow the chamber to equilibrate for 15 min at 46°C. The spotted slides were placed in the pre-warmed chamber and incubated to allow for hybridization at 46°C for 2 h. Wash buffer was allowed to pre-heat in a water bath at 48°C. Thereafter, hybridized slides were placed into the wash buffer and into a water bath for 20 min at 48°C. Slides were thereafter

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