APPLICATION OF BIOAUGMENTATION TO IMPROVE NITRIFICATION AT THE GAMMAMS WASTEWATER TREATMENT PLANT

THESIS

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Abstract

Namibia is among the driest countries in the world, and with the current drought that the country is experiencing, there is a greater need to provide drinking water not only to Windhoek, but the greater Nation at large. The Gammams Wastewater Treatment Plant, which is responsible for treating domestic and some industrial wastewater, regularly experiences toxic pluses that result in the inhibition of nitrification in the activated sludge process, leading to poor removal of ammonia. This is of great concern as the final effluent of the plant is used downstream for the production of potable water. The aims of this study were firstly to isolate and identify the nitrifying bacteria in the activated sludge system. Secondly, it was to test the inhibition capabilities of three test chemicals and thirdly to test the percentage recovery attainable by bioremediation using a fresh sample of activated sludge and by bioaugmentation using pure strains of *Nitrosomonas* and *Nitrobacter*. Sampling was performed once off for the growing on culture media, which was then used for molecular experimentation. Samples were then collected once a week for a duration of 9 weeks for the inhibition experiments, and then once off for the remediation and bioaugmentation experiments. Inhibition was carried out using acetone, toluene and brake fluid at concentrations of 5 ml/L, 10 ml/L and 50 ml/L, respectively. The test chemical that produced the highest inhibition percentage was then used in the remediation and bioaugmentation experiments. Pure cultures of *Nitrosomonas* and *Nitrobacter* were used in the bioaugmentation experiment, whereas the remediation experiment used a fresh sample from the aerated basins. Toluene at 50 ml/L produced the highest inhibition percentage (484%), while acetone at 5 ml/L produced the lowest inhibition percentage (17%). Culturing on solid media resulted in little growth, compared to growth that was obtained on the samples that were cultured on broth, which produced visible growth in suspension. These colonies were then used for the extraction of DNA for molecular identification of the variety of

microorganisms present in the activated sludge basins at the GWWTP. The identity of the isolates was similar to that of *Weissella* species, *Francisella* species as well as *Pseudomonas* species. A 33.7% percentage recovery was attained through remediation, while the bioaugmentation experiment attained a percentage recovery of 35.8%.

Keywords: Nitrosomonas, Nitrobacter, Bioaugmentation, Remediation, Toluene, Acetone, Gammams Wastewater Treatment Plant

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ABBREVIATIONS

GWWTP – Gammams Wastewater Treatment Plant

OUR – Oxygen Uptake Rate

DO – Dissolved Oxygen

BOD – Biological Oxygen Demand

COD – Chemical Oxygen Demand

NGWRP – New Goreangab Water Reclamation Plant

ATU- Allylthiourea

NITROX – Nitrification Toxicity

RODTOX – Rapid Oxygen Demand and Toxicity

TKN – Total Kjeldahl Nitrogen

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DEDICATION

This work is dedicated to my late grandparents, Manuel Kumbe and Henriethe Usuta Kakujaha. You may not be here to witness this achievement and the next milestone in my life, but your hard work and dedication to me when you were here, have led to this success. You may not understand the science but if it were not for the principals you taught me, there would not be a science to talk about. You are missed severely and will forever be loved.

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DECLARATIONS.

I, Ngajozikue Kavikairiua, declare hereby that this study is a true reflection of my own research, and that this work or part thereof has not been submitted for a degree in any other institution of higher education.

Signature ……. Date ……..

CHAPTER 1

1. INTRODUCTION

Namibia is amongst the most arid countries in the world as two deserts flank it, the Namib Desert in the west and Kalahari Desert in the east. More than 80% of the country consists of desert or semi-desert. Windhoek, the capital, is located in the Central Highlands approximately 1,540 m above mean sea level. The annual rainfall in Windhoek is approximately 370 mm, while the potential surface evaporation rate is in the range of 3,200–3,400 mm/annum (Lahnsteiner, Lempert, 2007). The population of Windhoek is approximately 400,000 with a growth rate of 4.4% per year (Councillor Organogram, 2016) in comparison to a natural population growth of 2.4% (Bertelsmann Stiftung's Transformation Index, 2016). Urbanisation has by far been the major contributor to the increase in Windhoek's population, with an increase from 13.7% in 2001 to 16.2% in 2011 (Indongo, 2015). The city's water supply is based on the use of surface and groundwater. All potable water resources within a radius of 500 km have been fully exploited. The rainfall is uncertain and long spells of severe droughts are frequently encountered. Therefore, the supply of water from the central Namibian reservoirs and wells cannot be guaranteed in the near future. The current drought that the country is facing and the increased migration of people from rural areas to Windhoek in search of a better living has increased the strain on the water reservoirs and has such prompted the study to be undertaken so as to provide an improved effluent from the Gammams wastewater treatment plant (GWWTP).

The Gammams Wastewater Care Works was commissioned 56 years ago in 1961 and has since then undergone two upgrades, the first in 1991 and then 10 years later in 2001. These upgrades increased the plant's capacity from 6.1 mega litres per day to the current 26 mega litres per day (Namibia Economist, 2015). Gammams Water Care Works is responsible for the treatment of municipal wastewater in Windhoek as well as industrial wastewater from the

southern industrial area of the city. This is a nutrient removal plant consisting of primary treatment followed by secondary treatment with biological nitrogen and phosphorus removal (Lahnsteiner, Lempert, 2007). Biological treatment entails both an activated sludge process and trickling filters in parallel. Subsequently, the secondary effluent with a Chemical Oxygen Demand (COD) of approximately 60 mg/L is discharged into maturation ponds (Lahnsteiner Lempert, 2007). The final effluent in these ponds serves as raw water for the New Goreangab Water Reclamation Plant (NGWRP) (Lahnsteiner, Lempert, 2007).

Activated sludge consists of a community of microorganism that is recurrently supplied with organic matter and oxygen. The process was developed in the 20^{th} century in England (Verstraete, 1996) and was originally designed for the removal of carbonaceous biodegradable matter (BOD) by heterotrophic bacteria, but was later adopted to include autotrophic bacteria capable of nitrification. Ammonia nitrogen is a more serious threat than nitrate nitrogen, hence nitrification is an operational objective for most if not all wastewater treatment plants. Ammonia is used in this thesis to refer to Ammonia (NH_3) and ammonium (NH_4^+) in the generic sense.

The presence of toxic inhibitory compounds in pre-treated industrial effluents remains an operational problem in the activated sludge process, in particular at the Gammams wastewater treatment plant where periodic problems of incomplete nitrification are experienced. It is plausible that these problems are due to inhibition of the nitrification process caused by organic chemicals that arrive at the plant because of their presence in the influent mainly from the Southern Industrial area of the city. These chemicals may include chemicals such as benzene, turpentine, and a variety of oils. In previous studies, attempts have been made to find remedial additives or pre-treatment methods to decrease the toxicity of industrial wastewater towards autotrophic nitrifying bacteria in order to improve the performance of nitrification in the activated sludge system (Julies, 1997, Nzila Razzak, Zhu,

2016, Yamashita, Yamamoto-Ikemoto, 2014). Therefore, there exists a need to investigate the presence of possible chemical inhibitors and their effect on nitrification. This study was focused on studying the process of nitrification inhibition in the activated sludge system of the GWWTP as well as determining the effectiveness of bioaugmentation to recover nitrification after a toxic pulse. The study also focused on identifying the population of bacteria present in the aerated basins.

1.1 Orientation of the Study

The study looked at identifying the population of nitrifying bacteria in the aerated basins at the GWWTP and it was mainly orientated around two concepts. The first being the inhibition of nitrification caused by organic chemicals present in the wastewater and the second was the use of bioaugmentation to aid the recovery of nitrification after a toxic pulse. Biological nitrification and denitrification processes remove ammonia from wastewater. Nitrification is the most sensitive part in the biological nutrient removal of wastewaters, with the autotrophic nitrifying biomass being approximately 10 times more sensitive than its aerobic heterotrophic counterpart. The growth rate of the autotrophic nitrifiers is much lower and strongly dependent on temperature, substrate concentration, oxygen content, pH, and the presence of inhibiting components (Juliastuti, Baeyens, Creemers, 2003). Since the requirement for nutrient removal is becoming increasingly stringent, a high efficiency of ammonium removal is necessary to achieve a low total nitrogen concentration in the effluent (Campos et al., 2007).

Bioaugmentation is a proposed technique to improve nutrient removal in municipal wastewater treatment and involves the introduction of specific cultures to enhance ammonium removal or other “target pollutants” of special interest. By adding acclimated biomass to the main reactor, activated sludge processes can increase removals of the target

compounds, or operate under conditions that would otherwise be unfavourable to target compound removal (Shao-Yuan Stenstrom, 2010).

1.2 Statement of the Problem

The identity of the population of nitrifying bacteria has mainly been assumed to be *Nitrosomonas* or *Nitrobacter* but, there could be more than just these two species of nitrifying bacteria and getting to know these populations might aid in the improvement of the nitrification process and aid in finding the appropriate remedial additives or pre-treatment methods to better the nitrification process. Nitrification is a problematic process to manage in wastewater treatment plants; since it requires long solids retention time (Yu, Peng, Pan, 2012) and chemical inhibitors present in influents may inhibit nitrifying bacteria. Furthermore, release of effluent rich in nitrogenous compounds has adverse knock-on effects such as eutrophication in natural water bodies and "blue baby syndrome" if water with elevated level of nitrite is consumed by infants. Therefore, removal of nitrogen from wastewater by biological nitrification and denitrification has received increasing attention owing to rigorous discharge regulations (Yu, Peng, Pan, 2012).

The effluent of the GWWTP is the influent into the NGWRP as part of reclamation of domestic water for drinking water production for the City of Windhoek and levels of ammonia in the effluent should not exceed 1 mg/L (Ohio EPA, 2014), because it may lead to high levels of nitrite in drinking water due catalytic action or colonization of filters by ammonium-oxidizing bacteria. This study will aid in identifying active nitrifying bacteria in the activated sludge system of the GWWTP and seek for plausible solutions to the problem of incomplete ammonia removal.

1.3 Objectives of the Study

The specific objectives of the study are to:

- Identify nitrifying bacteria present in the aeration basin using molecular techniques such as DNA extraction using a specialised kit, Polymerase Chain Reaction (PCR) for the amplification of selected regions of the isolated DNA and subsequent sequencing of the amplicons.
- Measure the oxygen uptake rate as an indication of the activity of the nitrifying bacteria present in the aeration basin.
- Determine the effectiveness of bioaugmentation/bioremediation with firstly pure cultures and secondly mixed cultures of active nitrifying bacteria to recover nitrification after a toxic pulse.

1.4 Research Hypotheses

- The nitrifying bacteria population in the activated sludge system of GWWTP is a mixed culture of different species of autotrophic organisms capable of nitrification and includes other genera in addition to *Nitrosomonas* and *Nitrobacter*.
- The oxygen uptake rate OUR of nitrifying bacteria is high in the aerated basin, indicating an active population of nitrifying bacteria and complete ammonia removal and the OUR of nitrifying bacteria is completely inhibited by the presence of acetone, toluene, and brake fluid.
- Bioaugmentation will lead to a minimum recovery of 50% nitrification after a toxic pulse.

1.5 Significance of the Study

Release of partially treated wastewater into receiving water bodies has many repercussions, both for the environment and for human beings. This risk is greater at the GWWTP where its effluent is used for reclamation to provide drinking water to the City of Windhoek. Camargo and Alonso (2006) stated , that below par treated effluent has a high content of inorganic nitrogenous compounds that can stimulate the development, maintenance and proliferation of primary producers in surface water sources, resulting in eutrophication and these compounds can also reach toxic levels that mar the ability of animals to survive, grow and reproduce. Inorganic nitrogenous compounds include NH_4^+ (ammonium), NH_3 (ammonia), NO_2^- (nitrite), NHO_2 (nitrous acid) and NO_3^- (nitrate) with unionized ammonia being the most toxic, while ammonium and nitrate ions are the least toxic (Camargo, Alonso, 2006). The effects of ingesting nitrites and nitrates from contaminated drinking waters are methemoglobinemia in humans, for the most part in young children by hindering the oxygen-carrying of haemoglobin, an ailment known as “blue baby syndrome”, as well as the potential role in developing cancers of the digestive tract through their contribution to the formation of nitrosamines (Camargo, Alonso, 2006). Further effects of ingesting nitrites and nitrates as listed by Camargo and Alonso (2006), might result in mutagenicity, teratogenicity and birth defects, contributing to the risk of non-Hodgkin’s lymphoma and bladder and ovarian cancers, be a factor in the etiology of insulin-dependent diabetes mellitus and in the development of thyroid hypertrophy, or cause spontaneous abortions and respiratory tract infections. The study is significant in that it focuses on improving the removal of inorganic nitrogenous compounds and hence avoiding the knock-on effects associated with the release of an effluent with high nitrogen content.

CHAPTER 2

2. LITERATURE REVIEW

2.1 The wastewater treatment process for domestic wastewater

The domestic wastewater treatment system has several steps. Before looking into the wastewater treatment plant itself, it is important to review the general characteristics of domestic wastewater. These are shown in the following tables (Tables 1, 2 and 3).

Table 1: Constituents present in domestic wastewater (Henze, Comeau, 2008).

Constituents		**Effects**
Microorganisms	Pathogenic bacteria, virus and worm eggs	Risk when bathing, eating shellfish and drinking of water
Biodegradable organic materials	Oxygen depletion in rivers, lakes and fjords	Fish death, odours
Other organic materials	Detergents, pesticides, fat, oil and grease, colouring, solvents, phenols, cyanide	Toxic effect, aesthetic inconveniences, bioaccumulation in the food chain
Nutrients	Nitrogen, phosphorus, ammonium	Eutrophication, oxygen depletion, toxic effect
Metals	Hg, Pb, Cd, Cr, Cu, Ni	Toxic effect, bioaccumulation
Other inorganic materials	Acids, for example hydrogen sulphide, bases	Corrosion, toxic effect
Thermal effects	Hot water	Changing living conditions for flora and fauna
Odour (and taste)	Hydrogen sulphide	Aesthetic inconveniences, toxic effect
Radioactivity		Toxic effect, accumulation

There exist a great variety of constituents present in domestic wastewater ranging from a variety of microorganisms, nutrients, metals and odours (Tables 1 and 2). These can be present as a result of several sources.

Table 2: Physical and chemical characteristics of wastewater and their sources (EPA, 1997).

CHARACTERISTIC	**SOURCES**
Physical Properties	
Colour	Domestic and industrial wastewater, natural decay of organic materials
Odour	Decomposing wastewater, industrial wastewater
Solids	Domestic water supply, domestic and industrial wastewater, soil erosion, inflow/infiltration
Temperature	Domestic and industrial wastewater
Chemical constituents	
Carbohydrates, fats, oils and grease, proteins, surfactants, volatile organics	Domestic, commercial and industrial wastewater
Pesticides	Agricultural wastewater
Phenols	Industrial wastewater
Other	Natural decay of organic material
Alkalinity, chlorides	Domestic wastewater, domestic water supply, groundwater infiltration
Heavy metals	Industrial wastewater
Nitrogen	Domestic and agricultural wastewater
Ph	Domestic, commercial and industrial

	wastewater
Phosphorus	Domestic, commercial and industrial wastewater natural runoff
Hydrogen sulphides, methane	Decomposition of urban wastewater
Oxygen	Domestic water supply, surface water infiltration

Table 3: Typical composition of raw municipal wastewater with minor contributions of industrial wastewater (mg/L) (Henze, Comeau, 2008).

Parameter	**High**	**Medium**	**Low**
COD Total	1200	750	500
COD soluble	480	300	200
COD suspended	720	450	300
BOD	560	350	230
Volatile Fatty Acids (VFA) as acetate	80	30	10
Nitrogen total	100	60	30
Ammonia-N	75	45	20
Phosphorus total	25	15	6
Ortho-Phosphate	15	10	4
Total Suspended Solids (TSS)	600	400	250
Volatile Suspended Solids (VSS)	480	320	200

The different characteristics and constituents of raw wastewater presented in Tables 2 and 3, influence the treatment options for wastewater. These may also influence the efficiency of the selected method of treatment. Most wastewater treatment plants are comprised mainly of the

primary and secondary treatment levels. The primary treatment being the physical mechanisms, whereas the secondary treatment involves biological mechanisms. A few plants, perform tertiary treatment which involves the treatment of wastewater with chemicals.

The foremost phase of the wastewater treatment takes place in the preliminary treatment plant where constituents of the wastewater such as oils, fats, grease, grit, rags and large objects are removed (EPA, 1997). This is achieved using a set of screens, which include the coarse screens to remove larger objects, such as plastic bag, rags amongst others. The finer screens remove smaller wastes such as ear buds, toilet paper, and other small objects. The degritters are responsible for the removal of sand, slit, rocks, seeds and other large objects. The primary settlement stage is used prior to biological treatment. Radial or horizontal flow tanks are customarily used to decrease the rate at which the wastewater flows such that a proportion of suspended solid gravitates out of the wastewater (EPA, 1997). The solids are then further treated anaerobically, while the effluent from the primary treatment stage is treated aerobically.

Biological treatment of wastewater takes place in stationary media or suspended growth reactors by means of activated sludge, biofiltration, rotating biological contactors, constructed wetlands or deviations of these processes. Nitrification, denitrification and biological elimination of phosphorus can be integrated at this stage and diminishes nutrient concentration in the effluent (EPA, 1997). Biological treatment is generally followed by a secondary settling phase that permits for the solids and effluent from the biological treatment to be separated. A report by the United States Environmental Protection Agency (1997), indicates that chemical treatment can be used to expand the settling abilities of suspended solids preceding to a solids removal stage or to fine-tune the properties or constituents of wastewater before biological treatment (e.g. pH modification, lessening of heavy metals or nutrient alteration).

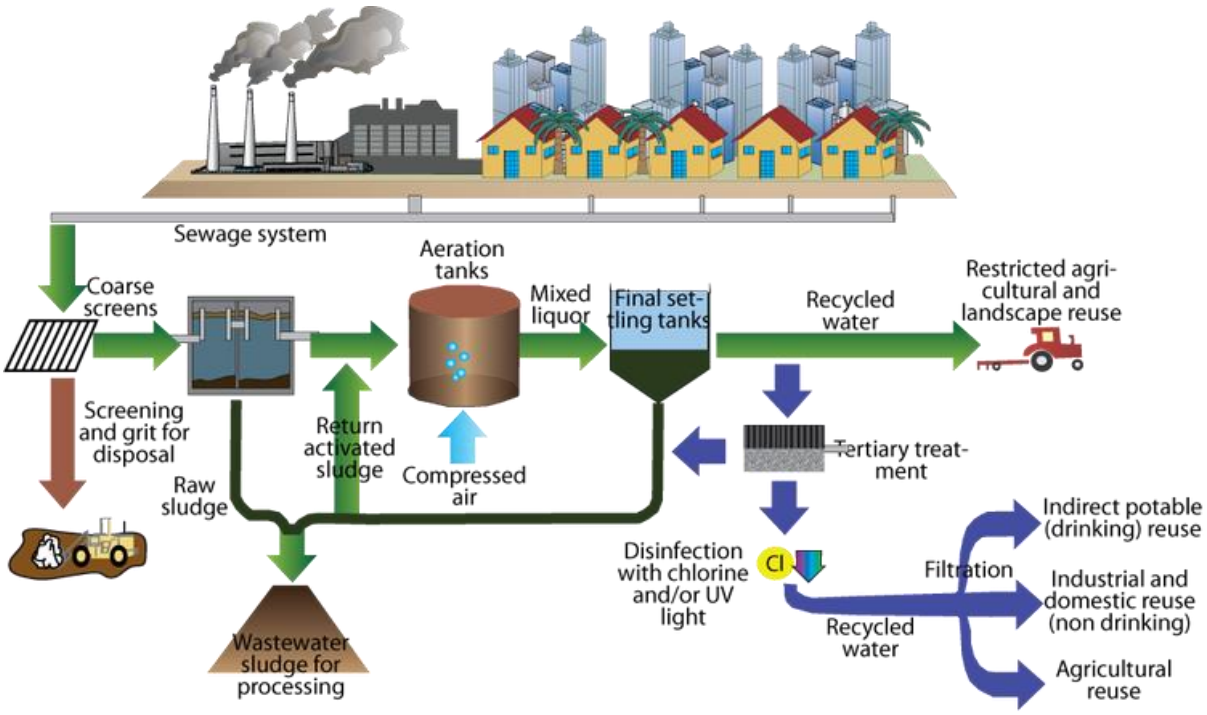

Figure 1: Physical and biological processes in a typical domestic wastewater treatment plant (Krucznski, Fletcher, 2012).

Wastewater treatment is desired so that we can use our rivers and streams for entertainment activities such as fishing and swimming as well as for sanitary purposes and drinking water. For the first half of the 20th century, pollution in the urban waterways resulted in repeated incidences of low dissolved oxygen, fish kills, algal blooms and bacterial contamination (EPA, 2004). The focus of this study was around the activated sludge process, where nitrification takes place. The process is the most commonly used system for the treatment of municipal wastewater, and it is probably the most versatile and effective of all wastewater treatment processes.

2.2 Nitrification

Nitrogen in municipal wastewater is mainly the result from human excreta and gaseous wastes and is primarily in the organic form, including ammonia, nitrate, nitrite and gaseous nitrogen. According to Julies (1997), roughly 40% of nitrogen in wastewater is in the form of

ammonia and the remaining 60% is bound to organic matter, while the amount of nitrate is insignificant. In any nutrient balance for land application of wastewater or treated wastewater (effluent), the attentions of averting nitrogen from contaminating groundwater or any water body are frequently assume priority over the impending impression of other wastewater contaminants such as sodium (Patterson, 2003) and this makes nitrification an important part of the treatment of wastewater. To moderate the adverse impacts of nitrogenous wastes upon the receiving water, an activated sludge process may be mandated by regulatory agencies to reduce the quantity of nitrogenous wastes in its final effluent. The activated sludge process then has the responsibility of nitrifying and denitrifying the nitrogenous wastes (Gerardi, 2002).

Nitrification and denitrification are defined by Gerardi (2002), as chemical reactions that occur inside living cells or bacteria and therefore these chemical reactions are considered ‘‘biochemical’’ reactions. Nitrification is recognized as the initial step of the nitrogen removal process in wastewater treatment plants. The autotrophic process is largely acknowledged to be the slowest step, more sensitive to temperature dissimilarities and inhibitory effects by toxic amalgams than its heterotrophic counterparts (Juliastuti, Baeyens, Creemers, 2003). Nitrification is traditionally thought to be carried out by only specific types of nitrifying bacteria, namely the *Nitrosomonas* and *Nitrobacter* bacteria. However, recent studies (Veuger et al, 2013, Thymann, 2013 and Yan et al, 2005) have demonstrated unequivocally that nitrite/nitrate production is not restricted to autotrophic ammonia oxidizers (e.g. *Nitrosomonas*) or nitrite oxidizers (e.g. *Nitrobacter*), but is a widespread phenomenon among different genera of fungi and heterotrophic bacteria. Nevertheless, these assumptions continue to be speculations, due to the fact that methods were failing to establish that microorganisms with the potential of heterotrophic nitrification are existent in these systems.

Moreover, there is no selective enrichment or isolation method for heterotrophic nitrifying microorganisms (Yan et al, 2005).

Nitrification is a two-step process:

$NH_4^+ + 1.5\ O_2 \longrightarrow 2H^+ + H_2O + NO_2^-$ (1)

This step is performed by NH_4^+-oxidizing bacteria that oxidize NH_4^+ to NO_2^- and include mainly the bacteria from the genus *Nitrosomonas*

$NO_2^- + 0.5\ O_2 \longrightarrow NO_3^-$ (2)

This step is performed by NO_2^- - oxidizing bacteria that oxidize NO_2^- to NO_3^- and include mainly bacteria from the genus *Nitrobacter.*

There are several factors that influence the effectiveness of nitrification such as pH, dissolved oxygen (DO), temperature and the concentration of ammonia and these will be discussed briefly in the next section.

2.2.1 Effect of temperature on nitrification

Temperature is one of the relatively difficult factors to regulate in a large setting like the activated sludge process and thus has a big influence on biological process, as well as the nitrification process. Water temperature vacillates based on exposure to environmental factors or other factors and therefore, temperature change impacts the metabolic process and bacterial kinetic of the nitrification procedure in the activated sludge process (Saidu, 2009). Bacterial reaction rates tend to increase with rising temperatures and this is indicated by Saidu (2009).

The maximum satisfactory operating temperature for typical activated sludge systems is limited to about 35°C to 40°C, which corresponds to the maximum temperature for the growth of mesophilic organisms. Even transitory temperature variations above this range must be circumvented since thermal inactivation of mesophilic bacteria ensues rapidly.

Though there is an optimum temperature range for the efficient growth of bacteria, efficacious operation has similarly been obtained when temperatures are reliably maintained above about 45°C to 50°C (Grady, Daigger, Lim, 1999). Temperature has a strong effect on the growth of nitrifiers, but quantifying the effect has been difficult. Part of the difficulty in enumerating temperature effects is the fact that the ideal temperature and pH are not fixed, but diverge as the total ammonia-N concentration changes (Sabalowsky, 1999). The optimal temperature for nitrifying activity was reported by Sabalowsky (1999), as low as 15^{o}C, but more typically appears to increase with increasing temperature up to approximately 30^{o}C, decelerating as the temperature increases further than that.

2.2.2 Effect of ammonia concentration on nitrification

Ammonia (NH_3) is a nitrogenous compound that is oxidized during the process of nitrification, which is a component of raw domestic water. The nitrification of wastewater is essential to remove or reduce the quantity of nitrogen compounds in wastewater; these compounds can act as environmental contaminants in the receiving water bodies (Pigue, 2013). According to Constantine (2008), low concentration of ammonia would result in the limiting of nitrification owing to a lack of substrate, whereas an extremely high concentration of ammonia can result in free ammonia that can also obstruct nitrification through opposition for dissolved oxygen by aerobic microorganisms. Each mg/L of ammonia nitrogen transformed into nitrate will consume about 7 mg/L of alkalinity (buffering capacity). This is due to the hydrogen ions being liberated from the ammonia as an acid, and this acid then reduces the pH of the entire system. Nevertheless, it is customarily not a great apprehension in the treatment of domestic wastewater (Wright, 1994, Joeng, Park, Kim, 2013).

2.2.3 Effect of pH on nitrification

The pH range for prime nitrification is 7.0 to 8.8 as recorded by Saidu (2009). The optimum range is determined by three effects that pH exerts on nitrifying bacteria; a) Activation – deactivation of nitrifying bacteria; b) Nutritional effect linked with alkalinity and; c) Inhibition through free ammonia and free nitrous acid. The nitrification process uses up alkalinity (HCO_3^-) and as a result, produces carbonic acid (H_2CO_3). Changes in alkalinity due to HCO_3^- consumption depresses water resistance, while the carbonic acid lowers the pH. The discrepancy of pH is to a large degree concomitant with meagre aeration of the system, which infers that aeration is considered a fundamental part of the system dynamics in the entire nitrification process. Appropriate pH management is a prerequisite for obtaining optimum performance (Saidu, 2009) for activated sludge systems.

Secondly, nitrifying bacteria are very sensitive to pH. *Nitrosomonas* has an optimal pH between approximately 7.0 and 8.0, and the ideal pH range for *Nitrobacter* is approximately 7.5 to 8.0. Some utilities have reported that an increase in pH (to greater than 9) can be used to reduce the occurrence of nitrification (Cates, Lavinder, 1999, Skadsen, Sanford, 1996). However, several other features contribute to the feasibility of nitrifying bacteria and as a result, nitrification episodes have been detected at pH levels extending between 6.6 to 9.7 (EPA, 2002).

2.2.4 Effect of dissolved oxygen on nitrification

Dissolved Oxygen (DO) is the level of molecular oxygen in the wastewater solution expressed as milligrams per litre (mg/L). The DO level is controlled by the quantity of air added to the basin. The prime DO level for an aeration basin is referred to as the dissolved oxygen set point (Pigue, 2013). The source of oxygen for bacteria in the nitrification process is known to be the principal limiting factor in the activated sludge process and oxygen

concentrations should not fall underneath the limiting bulk water concentration of 2 mg/L (Saidu, 2009). The nitrification process consumes approximately 2.04 kilograms of molecular oxygen (O_2) for each kilogram of ammonia that is nitrified. The nitrification process can proceed at DO levels as low as 1 mg/L. Raised levels of DO can similarly decrease the levels of Ammonia in the wastewater through the process of desorption/striping in which surface turbulence caused by mixing and aeration releases or strips the ammonia molecules from the wastewater solution into the atmosphere (Pigue, 2013). This can be advantageous in that it rids the wastewater of some ammonia, but can be disadvantageous in that it negatively affects the nitrification process by decreasing the concentration of ammonia that is required for nitrification to proceed efficiently.

2.3 Toxicity and Inhibition

Inhibition is an impermanent, short-term (acute) or long-term (chronic) forfeiture of enzymatic activity. Toxicity is the perpetual loss of enzymatic activity or irretrievable destruction to cellular structure (Gerardi, 2002). As stated by Sabolowsky, 1999, oxidation of ammonia to nitrite by *Nitrosomonas* is pondered to be the more sensitive of the two steps in nitrification, since *Nitrosomonas* is in general more susceptible to reticence from other compounds than *Nitrobacter*. Most inhibitors halt ammonia oxidation, nonetheless they repeatedly inhibit nitrite oxidation similarly in high enough concentrations. Extreme Chemical oxygen demand (COD) or Biological oxygen demand (BOD) loading have a habit of causing inhibition of nitrification (Sabolowsky, 1999). Certain compounds may well directly be inhibitory in elevated enough concentrations albeit they are not essentially explicit inhibitors. For example, methanol toxicity to ammonia oxidation was reported by Sabolowsky (1999). Conversely, nitrification inhibition by the occurrence of organic matter can be accredited to DO diminution by heterotrophic organisms consuming the organics

present. Granting, BOD levels up to 40-50 mg/L can be endured in nitrifying reactors, it has been publicised that BOD levels in excess of 40 mg/L can lead to as diminutive as 50% nitrification (Sabolowsky, 1999).

2.4 Nitrification inhibitors and their modes of action

A variety of inhibitory compounds are present in wastewater including a wide range of organic compounds and heavy metals. Elements that influence the degree of inhibition of nitrification include pH, concentration of the inhibitor, type of microorganism present, suspended solids concentration, sludge age, solubility of the inhibitor, and the concentration of the other cations and molecules present (Juliastuti, Baeyens, Creemers, 2003). The inhibitory effects on nitrification have been well documented (Luca et al., 2004; Ginestet et al., 1998; Tindaon, 2010).

Due to the comparatively trivial quantity of energy accessible for acclimation, nitrifying bacteria are delicate to very low concentrations of inorganic and organic wastes (Gerardi, 2002). Wastes that are greatly toxic to nitrifying bacteria encompass cyanide (0.5 mg/L), halogenated compounds, heavy metals, hydantoins, mercaptans, phenols as well as thiourea. The capability of nitrification inhibitors to diminish nitrification is reliant upon both climatic and substrate circumstances and dissimilar inhibitors retort contrarily (Suter et al., 2010). Some inhibitors employ their effects circuitously, whereas others are understood to act by way of chelating copper and others are proposed to either thwart the uptake or utilization of ammonia, or perform as a copper chelator as well (Lehtovirta-Morley et al., 2013).

Some inhibitors, for instance, dicyandiamide (DCD), nitrapyrin (N-Serve, NS), and thiourea (TU) were used in experiments to hinder the growth and metabolism of *Nitrosomonas europea* by Zacheri, Amberger (1990). This is one of the approaches of inhibition of nitrification that has been well studied and documented. The inhibition functions by

retardation of the growth of the nitrifiers in so doing plummeting ammonia oxidation in addition to lessening or inhibiting the respiration of cells in the culture (Zacheri, Amberger, 1990). Other modes of inhibition effort their effects by inhibiting ammonia oxidation through obstruction of catalytic pathways. Compounds encompassing C=S bonds are heady inhibitors of ammonia oxidation in *Nitrosomonas europea* (Julies, 1996). These compounds are comprised of carbon disulfide, thiourea, allythiourea, thiosemicarbazide, thioacetamide, potassium ethylxantate and diethylcarbamate. Despite the fact that these compounds all have the same functional group (C=S), they do not devise matching inhibitory mechanism (Julies, 1996).

Julies (1996) indicates that, enzyme and heme-protein binding compounds which inhibit ammonia oxidation consist of ethyl xanthate, 3-aminotriazole, phenoxyethyldiethylamine, ethylamine hydrobromide, thiosemicarbazide and carbon monoxide. In *Nitrosomonas*, cytochrome P-460 has an extraordinary attraction for CO and NH_2NH_2 and both these compounds inhibit ammonia oxidation. Allythiourea and diethyldithiocarbamate bears a close resemblance to the structure of 3-aminotriazole and inhibit a catalase or catalase-like enzyme, suggestive of the participation of a catalase-like enzyme in ammonia oxidation (Julies, 1997). The inhibition of nitrite oxidation is carried out through the inhibition of the enzyme nitrate reductase which is inhibited by chlorates which subdues *Nitrobacter* proliferation, but does not disturb its nitrite oxidizing ability (Julies, 1997). A few compounds are uncouplers of oxidative phosphorylation and inhibit nitrite oxidation in *Nitrobacter.*

2.5 Respiration Measuring Techniques

2.5.1 Chemical Methods

Monitoring the rates at which NH_4^+ is consumed or NO_2^- and NO_3^- are produced in a batch experiment using activated sludge is one of the most prevalent means to quantify the

nitrification rate of the activated sludge samples (Gernaey et al., 1998b). Consistent sampling and chemical analysis of the samples is indispensable, which brands this technique affluent and somewhat time consuming. On the other hand, observing NO_2^- production or depletion in the presence of a discerning inhibitor for NO_2^- and NH_4^+-oxidizing bacteria singly, was recommended as a useable process to ration the activity of NH_4^+ and NO_2^- oxidizing bacteria disjointedly (Gernaey et al., 1998b).

2.5.2 Direct Probes

DO, pH and ORP probes have in repetition established their stoutness, dependability and limited upkeep demand (Vanrolleghem, Coen, 1995). For numerous years, there is an inclination to excerpt a considerable amount of information as conceivable from the raw data delivered by means of these sensors when immersed in the mixed liquor. As a coherent outcome of these exertions, particularly DO, but also pH and ORP electrodes are used as the emphatic sensor component in respirometers, nitrification activity meters and denitrification activity meters (Vanrolleghem, Coen, 1995).

Dissolved oxygen probes

DO electrodes are applied in aerobic stages of wastewater treatment processes. The broad quantifying principle of the electrode is founded on the diffusion of O_2 molecules through a gaspermeable membrane, subsequently the electrochemical reaction of the O_2 in an amperometric or polarographic measuring cell (Gernaey et al., 1998a). Solicitation of DO electrodes is pervasive owing to the high asking price of aeration in wastewater treatment plants and the cost lessening that can be achieved by DO-based aeration control. One of the most mutual problems is the choice of an evocative gauging point in the aeration tank. DO electrodes are commonly useful for guarding a fixed DO set point in the aeration tank to

minimalize aeration expenditures. The moderation of the aeration is attuned centered on the DO measurement in the mixed liquor (Gernaey et al., 1998a).

pH

pH measurements are a regular occurrence in wastewater treatment plants. When the buffer aptitude of the mixed liquor is excessively low to recompense for the proton production due to nitrification, a pH measurement method joined to a dosing system can be applied for pH amendment in nitrifying activated sludge components. Electrode ensnarling can contribute to the distress in a system, but stretched periods deprived of maintenance of the electrode can be attained by providing a preset hydraulic (water spray), mechanical (brush), chemical (rinsing with cleaning agent) or ultrasonic cleaning system (Gernaey et al., 1998a). As with DO probes, a problem arises with selecting a location to place the probe.

Oxidation -reduction potential (ORP) electrodes

ORP electrodes afford a broad-spectrum indication of the oxidative eminence of the scrutinized system. ORP electrodes, in comparison to DO electrodes, furthermore make available information about the biological processes arising under anoxic and anaerobic circumstances. From a methodical perspective, the ORP quantity can be considered precise and without a hitch (Gernaey et al., 1998a). The out-and-out ORP values oscillate with the definite load and hinge on the surface treatment and maintenance procedure for the electrode.

2.5.3 Indirect Probes

Even though in use, these probes are not the utmost desirable alternative. On-line NH_4^+ and NO_3^- analyzers have been technologically advanced for nitrogen abstraction monitoring and process control. Fundamentally, the on-line analyzers make use of renowned laboratory

measuring ideologies. Conversely, it is imperative to annotate that the quantifying ideologies have habitually been amended, paralleled to the customary laboratory method when applied in on-line analyzers. Maneuver of on-line NH_4^+ and NO_3^- analyzers necessitates sample pretreatment for most solicitations in biological process tanks. Gernaey et al., (1998a) points out that these probes include, NH_4^+ analyzers that are either centered on ion-selective electrodes or colorimetric reactions and NO_3^- analyzers, which are either founded on ion-selective electrodes, colorimetric reactions or UV absorption.

2.5.4 Respirometry

Respirometry is the measurement and clarification of the proportion of biological activity in activated sludge. Vanrolleghem (2002) defines respiration rate is the amount of oxygen per unit of volume and time that is consumed by organisms. Respirometric methods have been used to illustrate heterotrophic and nitrifying biomass in activated sludge samples since the oxygen uptake is a significant activity in both carbon oxidation and nitrification. Respirometry is desired above substrate particular observation methods, because respirometry is commonly pertinent, easy to automate and sensitive even for reasonably small substrate concentrations (Gernaey et al., 1998b). Based on Gernaey et al., (1998b), respirometers can be differentiated centered on two benchmarks. The first being the phase where the oxygen concentration is measured (liquid or gas), and the second, whether there is input and output of liquid and gas (flowing or static) or not (Gernaey et al., 1998b).

Stationary gas respirometers can be run with a static or a flowing-liquid phase. The static gas-static liquid respirometer is functioned by extracting a sample of activated sludge from a plant, transporting it into a small reactor vessel with nitrification and subsequently observing the decline of DO concentration with time succeeding a short aeration phase. Gernaey et al., (1998b) states that usages of static gas–static liquid respirometers are constrained because of

the hazard for oxygen limitation. This category of respirometer is nonetheless very prevalent, because of its unpretentious manufacture and measurement standard.

Flowing gas–static liquid respirometers function contrarily and are incessantly aerated and have the benefit that higher sludge concentrations can be used, as there is an unceasing input of oxygen and oxygen restriction is implausible. In this instance, the oxygen transference coefficient and the saturation DO concentration has to be recognized to allow for the calculation of the respiration rate. Static-liquid respirometers can be programmed to function in a semi-continuous approach, that is, the respirometer carries out a recurring batch experiment. Static gas–flowing liquid respirometers quantify the DO concentration individually at the inlet and the outlet of a closed respiration chamber. Aerated sludge is impelled unremittingly through the respiration chamber. Gernaey et al., (1998b) indicates that the oxygen uptake rate (OUR) is calculated by making an oxygen mass balance over the respiration chamber using the input and output DO concentration and the residence time in the vessel. In nitrogen elimination systems, respirometric instruments are advantageous for observing purposes. Respirometry can be applied efficaciously to quantify the nitrifying sludge activity, due to the elevated oxygen consumption for nitrification. The disadvantage of this experiment as stated by Gernaey et al., (1998b) however lies in extricating the nitrification oxygen uptake from the oxygen uptake for carbon substrate oxidation and endogenous metabolism. Habitually this problem is overcome, through the use of batch experiments with a sludge sample in the endogenous state (Gernaey et al., 1998b). There are two kinds of biosensor systems that are frequently used in respirometry and these will be discussed next.

NITROX

As per Gernaey et al., (1998a), the NITROX (NITRification tOXicity tester) was developed as a respirometric on-line toxicity uncovering system, conjoining a high sensitivity with a short retort time. Julies (1998), goes on to describe the system in more modest terms, as the system was advanced as an premature cautioning toxicity system and is founded on OUR measurements of nitrifiers and heterotrophs. ATU is used to differentiate between the OUR of heterotrophs and nitrifiers, since ATU is an inhibitor of the nitrifiers. The procedure of NITROX is centered on two phases: A contact phase and a measuring phase. For the duration of the contact phase, a sample of sludge is mixed with an explicit amount of toxic compound. Ammonia is added to the concoction to prevent substrate restriction for nitrifiers and to warrant naught order kinetics of nitrification. The mixture is subsequently ventilated for a few minutes to safeguard the reaction of sludge with the toxic compound, after which aeration is brought to a standstill and the mixture is agitated to retain the sludge in suspension, while the total OUR (OURt) is enumerated. As soon as the OUR becomes stable, ATU is added and the OUR of the heterotrophs (OURh) is measured and the dissimilarity between OURt and OURh provides the OUR of the nitrifiers (OURn). The experiment is then repeated for a control sample deprived of the toxic chemical. The gradation of inhibition triggered by the toxic chemical can be calculated by:

$$\%\ inhibition = \frac{\text{OURn of control} - \text{OURn of intoxicated sample}}{\text{OURn of control}}\ x\ 100$$

The foremost plus of the NITROX biosensor is that the measurement is rapid and henceforth permits for the prompt discovery of toxic compounds (Julies, 1997)

RODTOX

The flowing gas-static liquid respirometer, the RODTOX (Rapid Oxygen Demand and Toxicity tester) was industrialized in the eighties and consents for the approximation of

kinetic nitrification strictures grounded on a solitary substrate diminution experiment (Gernaey et al., 1998a). For static gas–static liquid respirometers, a number of experiments may be desired to acquire analogous information about the nitrification kinetics. Gernaey et al., (1998a) articulates that a technique for the concurrent categorization of carbon oxidation and nitrification was recommended, by means of an applicable mixture of a willingly biodegradable carbon source and NH_4^+, which is added to a nitrifying activated sludge sample in the reactor vessel of the RODTOX sensor (Gernaey et al., 1998a).

Respirometric solicitations for nitrogen removal procedures are merely not restricted to the approximation of nitrification proportions. Approaches based on the interpretation of respirograms documented after addition of a wastewater sample to activated sludge in the endogenous state, was used to elucidate the concentration of nitrifiable nitrogen accessible in the influent of an activated sludge plant (Gernaey et al., 1998a).

2.6 Bioaugmentation and Bioremediation

Joubert (2012), defines bioaugmentation as the addition of bacteria, nutrients, and other growth features to augment the biodiversity and effectiveness of wastewater or other pollution-degrading systems. The nitrification procedure obliges lengthier solids retention time (SRT) as compared to the orthodox method which merely treats the carbonaceous BOD and lengthening the SRT possibly will necessitate for larger aeration tanks or clarifiers, but in various prevailing treatment plants, extension is not conceivable (Leu, Stenstrom, 2010). An alternate solution to this delinquent is the usage of bioaugmentation, where by the conventional process is supplemented with bacteria to diminish the minutest mandatory SRT (Leu, Stenstrom, 2010) or aid in the recovery of nitrification after a toxic pulse. Leu, Stenstrom (2010), add that in addition to upgraded nitrogen removal, bioaugmentation

practices have been proved in numerous other applications, for instance, biomass flocculation, amended elimination of suspended solids and the treatment of perilous wastes.

Remediation can be demarcated as the reversal or discontinuing of impairment, and well in this case environmental damage of some kind. Remediation can either be chemical or biological. Chemical treatments comprise the use of agents to endorse abstraction of the precarious substances, and physical treatments encompass deduction of the menaces through physical means (Hamby, 1996). Biological ways and means make the most of the assorted dilapidation aptitudes of microorganisms to transform the multifaceted chemical constituents of a waste product to innocuous products. As per Odokuma and Akponah (2010), bioremediation makes use of three processes, biodegradation, bioaccumulation and biosorption.

Biodegradation consist of the biological reactions that alter the chemical structure of the compound, so, this infers a decline in toxicity (Valequez-Fernandz et al., 2012). Rani and Dhania (2014) indicate that biodegradation of a compound is frequently an outcome of the activities of various organisms. Most bacterial species which vitiate pesticides have their place in the following genera *Flavobacterium*, *Arthrobacter*, *Azotobacter*, *Burkholderia* and *Pseudomonas*. The style of degradation diverges amongst species and the targeted compound. *Pseudomonas* sp. and *Klebsiella pneumoniae* have been publicized to possess hydrolytic enzymes that are capable of breaking down s-triazine herbicides, such as atrazine. Correspondingly, several enzymes such as oxygenases, hydroxylases, hydrolases and isomerases present in *Pseudomonas* and *Alcaligenes* sp. have been publicized to cut down herbicides. (Rani, Dhania, 2014). Any influence which can amend growth or metabolism, would correspondingly distress biodegradation. Henceforth, physicochemical appearances of the environmental matrix, for example temperature, pH, water potential, oxygen and substrate obtainability, would influence the biodegradation efficiency. Two more factors that are worth

mentioning. These are co-metabolism and consortia condition. According to Valequex-Fernandz et al., (2012), some biodegraders entail additional substrates to destroy pollutants. Biodegradation has had several tenders in debasing petroleum hydrocarbons (Das, Chandran, 2010), pesticides such as DDT ((Valequez-Fernandz et al., 2012) along with oil spillages in oceans (Rani, Dhania, 2014).

Biosorption may well modestly be defined as the subtraction of affluences from solution by biological material. These substances can be both organic and inorganic, and in gaseous, soluble or insoluble forms. Biosorption is a physico-chemical practice and takes into account mechanisms which include absorption, adsorption, ion exchange, surface complexation and precipitation. Biosorption is encompasses both living and dead organisms (and their components) and has been foreshadowed as an encouraging biotechnology for impurity abstraction from solution, and/or contaminant retrieval, for a number of years, because it is efficient, ingenuous, can be manoeuvred parallel to orthodox ion exchange technology, and accessibility of biomass. Most biosorption research work has been carried out on microbial systems, chiefly bacteria, microalgae and fungi, and with toxic metals and radionuclides, comprising actinides like uranium and thorium (Gadd, 2008).

The heavy metal can move into the cell traversing the cell membrane by means of the cell metabolic cycle. This manner of metal uptake is denoted as ‘‘active uptake’’. The metal uptake by both active and passive means can be dubbed as ‘‘bioaccumulation’’ (Malla, et al., 2015). Bioaccumulation is somewhat analogous to biosorption and the two are habitually understood to be identical despite the fact that is not the case.

CHAPTER 3

3. METHODS

3.1 Research Design

The research involved both quantitative and qualitative data. The study was comprised of both a field study and laboratory experiments.

3.2 History and Site Description

The Gammams wastewater treatment plant is domestic wastewater treatment plant, which was commissioned in 1961. The plant is responsible for treatment of domestic wastewater of the city of Windhoek as well as some industrial wastewater that is produced by the city's Southern Industrial Area. Upon commission the plant was designed to manage 6.1 mega litres a day, however the plant underwent two upgrades in 1991 and 2001, respectively to the current capacity of 26 mega litres. With an increase in the population of the City of Windhoek, the plant is currently running at an overload of approximately 63% (41 mega litre per day). Reports from the Municipal laboratory indicates that the influent usual has a BOD that ranges between 400 mg/L to about 600 mg/L and leaves the plant with a BOD that ranges between 60 mg/L-80 mg/L. The secondary treatment plant is comprised of the Activated Sludge Process (ASP) as well as the Biological (Trickling) Filters (BFs). Currently the effluent from the Primary Settling Tanks is divided at a ratio of 7:3 (BFs: ASP). Samples for the study were obtained from the activated sludge reaction basins of the GWWTP (Figures 2 and 3).

3.3 Sampling

Sampling was done once weekly in the morning hours between 07:30 and 08:30 and again between the hours of 11:30 and 12:30 whenever experiments were carried out at the plant. Two 5 litre samples were collected for the performance of inhibition experiments at the plant.

The initial 5 litre sample was used to determine the total OUR of all the organisms present in the sample, as well as to determine the OUR of the nitrifying bacteria. The second 5 litre sample was then used for the inhibition experiment. Sampling for the microbial isolation was carried out once-off from the aerated basins of the GWWTP. During the time of sampling throughout the study, sampling was only performed when nitrification in the activated sludge basin exceeded 80% removal of ammonia, since the use of sludge with incomplete ammonia removal would have caused bias in the experimental results. The extent of ammonia removal was evaluated from weekly measurements performed by the laboratory at Gammams WWTP.

Figure 2: Aerated basins at the Gammams Wastewater Treatment Plant.

Figure 3: Sampling point at the aerated basins.

3.4 Microbial isolation and DNA extraction

Samples obtained from the aerated basins at the Gammams WWTP were diluted using standard serial dilution techniques. Samples were diluted (10^{-6}) and all dilutions were initially inoculated on two different media. Two different media by Alexander and Clark (1965) were used for cultivating and enumerating nitrifying bacteria. The first medium (Modified Alexander and Clark ANO medium) was used for the cultivation and enumeration of all nitrifiers (ammonium and nitrite oxidizers). The chemical composition (g/L) of this medium was: 0.5 $(NH_4)_2SO_4$; 1.0 K_2HPO_4; 0.03 $FeSO_4 \cdot 7\ H_20$; 0.3 NaCl; 0.3 $MgSO_4 \cdot 7\ H_2O$; 7.5 $CaCO_3$. The composition of the second medium (Modified Alexander and Clark NO medium): (0.006 $NaNO_2$; 1.0 K_2HPO_4; 0.3 NaCl; 0.1 $MgSO_4 \cdot 7\ H_2O$; 0.03 $FeSO_4 \cdot 7\ H_2O$; 0.3 $CaCl_2$ and 1.0 $CaCO_3$) and it was used for the cultivation and enumeration of nitrite oxidizers

only (Elbanna, El-Shahawy, Atalla, 2012). Samples were then also grown on broth to allow for DNA extraction. Samples were grown on broth that had a similar composition as that of the solid media on which the samples were originally grown. DNA was extracted using the ZYMO DNA Extraction kit (ZYMO Research). The extraction process was carried out per the manufacturer's instructions. The 16S rRNA genes were amplified by PCR using the universal primers: forward primer 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 1492R 5'- GGT TAC CTT GTT ACG ACT T-3' (Saha, Sarkar, Bandhophadhyay, 2013). Polymerase chain reaction and amplification (PCR) was carried out and the following cycling program was performed using a Thermal Cycler (Thermo Scientific) with an initial denaturation step at 95°C x 3 min, 35 cycles of denaturation at 94°C x 1 min, annealing at 55°C x 1 min, and extension at 72°C x 2 min, and final extension at 72°C x 3 min (Saha, Sarkar, Bandhophadhyay, 2013). The presence of the PCR products was examined and visualized by electrophoresis in 1.5% agarose gel in Tris Borate EDTA (TBE) buffer (Saha, Sarkar, Bandhophadhyay, 2013). PCR amplicons were then sent in for sequencing at Inqaba Biotec and the resulting sequences were cleaned and aligned using the software program BioEdit.

3.5 Oxygen Uptake Rate measurements/ Inhibition experiments

The experiment was performed in one well-mixed reactor, fitted with a dissolved oxygen probe, temperature probe and a pH probe connected to an output device. The reactor was mixed using a magnetic stirrer and was aerated using compressed air. The experiment was a two-part experiment. The first part of the experiment was to determine the OUR of the nitrifying bacteria in the aerated basins and acted as the control for the experiment, while the second part of the experiment was the inhibition experiment. The methods used in this study were a modification of those used by Mbaya in 2011.

In the first part of the experiment (Phase A), 2000 ml of the active sludge sample was added to the reactor and the sample was aerated until DO in the sample reached a level had reached a level of 9.99 mg/L. The DO level was then allowed to drop to 9.5 mg/L, after which the DO and temperature were recorded at 3 minute intervals for 60 minutes. In phase B of the reaction, 30 ml of 15 mg/L allythiourea (ATU) and azide were to the reactor and the reactor was mixed and aerated for 15 minutes to allow the inhibition of the nitrifiers to take place. ATU inhibits *Nitrosomonas* and the first step of the nitrification reaction, while azide inhibits *Nitrobacter* and the second step of the nitrification reaction (Julies, 1997). After the inhibition had taken place, the DO is allowed to reach 9.99 mg/L again and the OUR recorded again at 3 minute intervals for 60 minutes. Allylthiourea and azide were chosen as the organic compound inhibitors, because their use in inhibition experiments has been well documented such as by (Lehtovirta-Morley et al., 2013). In the second part of the experiment, phase A was carried out as in the first part of the experiment. In phase B, the addition of the organic chemical was administered and the reactor was mixed and aerated for 15 minutes to allow for inhibition, after which the DO was allowed to reach 9.99 mg/L, allowed to drop to 9,50 mg/L and recorded again at 3-minute interval for 60 minutes. Each organic solvent was tested at four different concentrations (5 ml/L, 10 ml/L and 50 ml/L). The experiment was carried out at pH 6.5-7.5. Phase C of the second part of the experiment was then similar to phase B of the first part of the experiment.

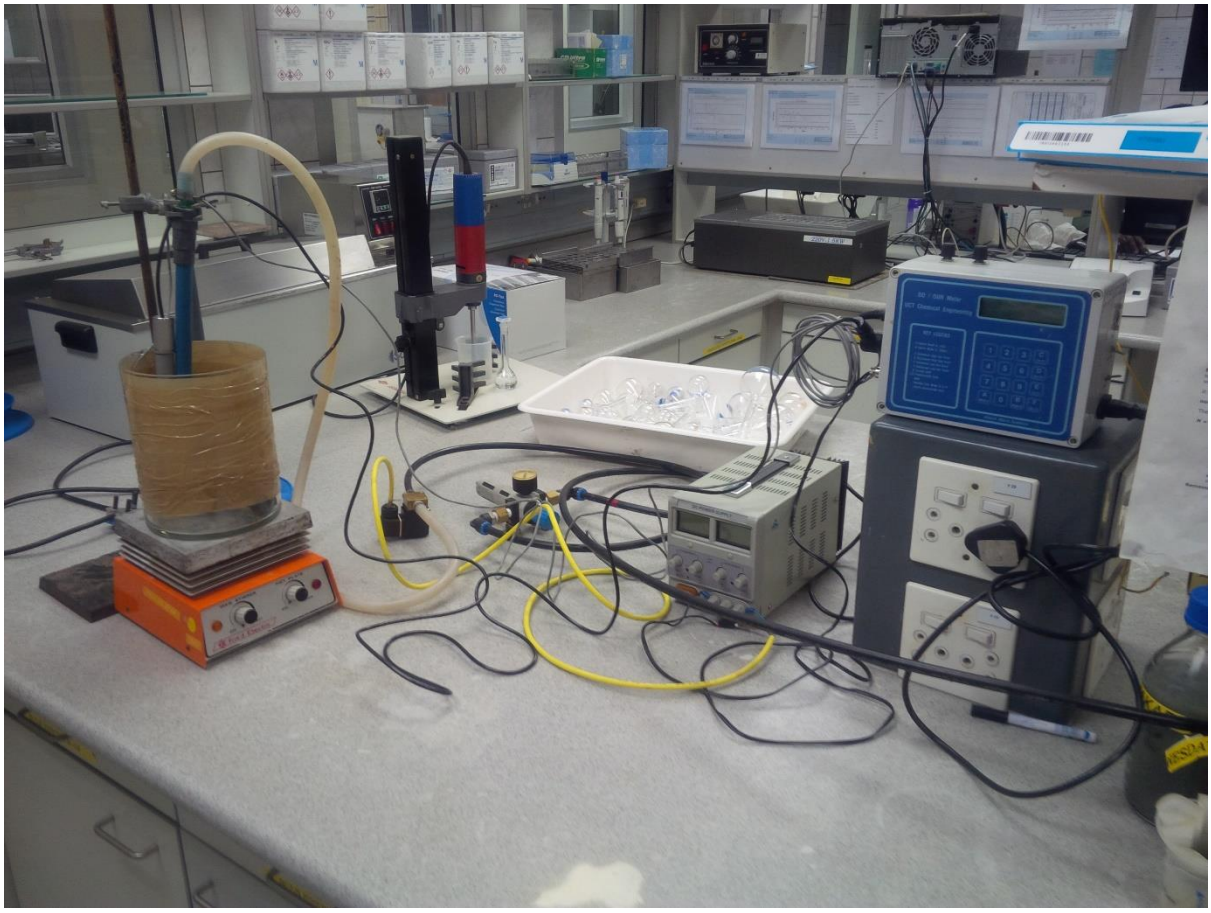

Figure 4: Setup of the OUR measuring apparatus.

Figure 5: Setup of the DO and temperature probes as well as the aeration pipe.

For the first part of the experiment OUR, was measured during two different phases in the reactor. Determining OUR of nitrifiers in the aeration basin.

- Phase A: Wastewater sample – OUR of all bacteria.
- Phase B: Wastewater sample + (ATU and Azide) – OUR of heterotrophs only.

A - B = OUR of nitrifying bacteria before inhibition nitrifiers.

For the second part of the experiment, OUR was measured during three different phases, determining the inhibition of the selected organic chemicals

- Phase A: Wastewater sample – OUR of all bacteria
- Phase B: Wastewater sample + Organic chemical – OUR of all bacteria after inhibition.
- Phase C: Wastewater sample + Organic Chemical + (ATU and azide) – OUR of the heterotrophs only after inhibition.

A-C = OUR of nitrifiers after inhibition.

$$\%\ inhibition = \frac{\text{OUR nitrifiers before inhibition} - \text{OUR of nitrifers after inhibition}}{\text{OUR of nitrifiers before inhibiton}} \times 100$$

OUR was calculated by determining the slope of the dissolved oxygen curve after a brief period of aeration. The rate of change in the dissolved oxygen i.e. the OUR, is computed by the least squares technique (Randall, 2009). OUR was calculated as the absolute value of the slope of the linear portion of the DO versus time curve when an oxygen probe is used. The calculations were done as specified by the manufacturer of the respirometer (Telliard, 2001).

3.6 Bioremediation Experiment

For the remediation experiment, the organic chemicals with the lowest and highest percentage inhibition were selected. A 2000 ml sample of activated sludge the placed in the reactor and aerated until the DO level reached 9.99 mg/L and then allowed to drop to 9.5 mg/L after which the DO and temperature were recorded at 3-minute intervals for a 60 minute period. As with the inhibition experiments, this first part was to determine the total OUR and acted as the control of the experiment. The second part of the experiment was the addition of the 50 ml/L of toluene, which had the highest percentage inhibition from the inhibition experiments. 30 ml of toluene was added to the reaction vessel and allowed to stand for 15 minutes while being mixed and aerated to allow for inhibition to take place.

After the inhibition, the DO was allowed to get to 9.99 mg/L and drop to 9.50 mg/L, after which the DO and temperature were recorded again at 3-minute intervals for 60 minutes to record the total OUR after inhibition. Once this had been carried out, a fresh 2000 ml activated sludge sample was added and allowed to stand while being well-mixed and aerated for 30 minutes. The mixing was done using a magnetic stirrer, while the aeration was carried out using compressed air. The DO was again allowed to increase to 9.99 mg/L before being dropped to 9.50 mg/L. The OUR was then measured again and this was then compared to the OUR of the inhibition step in the experiment to determine whether remediation has occurred. Part of this experiment included the measurement and recording of ammonia, nitrate and TKN concentrations to confirm that remediation had taken place.

$$.Percentage\ Recovery\ (\%) = \frac{OUR\ of\ inhibited\ sample - OUR\ of\ Bioaugmented\ sample}{OUR\ of\ the\ initial\ sample}\ x\ 100$$

3.7 Bioaugmentation Experiment

100 milliliter enrichment stock cultures were setup and maintained using *Nitrosomonas* and *Nitrobacter* pure cultures obtained from ATCC. The culture known as a continuous culture was fed once daily with regular mixing as well as aeration. The culture was fed with a stock solution the following composition in g/L: 1.2 NH_4Cl, 0.1 KH_2PO_4, 0.4 $CaCO_3$ and 0.1 Nutrient supplement (Vandevivere et al., 1998). The culture was maintained at a pH of 7.0 and this was done by the addition of 1-M NaOH. This culture was then added to an activated sludge sample that was exposed to inhibitors (ATU and Azide) which are known to inhibit nitrification over a 24-hour period. Three different concentrations of the respective inhibitors (10, 50 and 100 mg/L in dH_2O) were used (Lehtovirta-Morley et al., 2013). The efficiency of the stock culture was measured by measuring the concentration of ammonium, TKN and nitrate before and after the stock culture was added as well as by measurement and calculation of the oxygen uptake rate of the stock culture nitrifiers. These measurements were

in turn used to determine the percentage recovery of nitrifying bacteria after a toxic pulse (Vandevivere et al., 1998). The same formula that was used to calculate the percentage recovery for the remediation experiment was also used to calculate the percentage recovery for the bioaugmentation experiment.

$$Percentage\ Recovery\ (\%) = \frac{OUR\ of\ inhibited\ sample - OUR\ of\ Bioaugmented\ sample}{OUR\ of\ the\ initial\ sample}\ x\ 100$$

3.8 Data Analysis

Qualitative data included the identification of bacteria and was analyzed by running the sequences through the NBCI database to compare for similarities against known sequences to determine the identity of the isolated strains. Quantitative data included data such as the oxygen uptake rate for the different phases of the experiment as well as the percentage recovery because of bioaugmentation. These data were tested for normality with a Shapiro-Wilk test. If the data was normally distributed, the significance and the recovery effect by bioaugmentation were tested with a paired sample t-test. .

3.9 Research Ethics

Permission was obtained from the City of Windhoek to conduct sampling and OUR measurements at the GWWTP. Protective clothing will be worn at all times when handling sludge samples and in the laboratory. All biological and chemical waste generated was disposed of in the special marked containers that were collected by the City of Windhoek and disposed of at the hazardous waste cell at the Kupferberg waste dumpsite. Since this study did not investigate the efficiency of nitrification at the plant, the City of Windhoek agreed that results from laboratory experiments can be published as an MSc thesis.

CHAPTER 4

4. RESULTS

4.1 Isolation of Strains

There was growth of isolates on selected solid growth media and the morphology of the strains that grew on the selective media varied. The (+) is an indication of growth, whereas (-) indicates no growth. The initial isolation shows a growth on both media. Colonies isolated from both media were small. However, the ammonium oxidizing media produced colonies that were rough on the edges with a smooth surface with a pale-yellow color, whereas the nitrogen oxidizing circular colonies with smooth edges and a light green color. Unfortunately, no pure cultures were obtained (Table 4 and Figure 6).

Table 4: Growth on selected solid media.

Growth Media	Initial Isolation	Pure Culture	Morphology of Colonies
Ammonium Oxidizing	+	-	Small irregular colonies with rough edges and smooth surface, pale yellow in color
Nitrogen Oxidizing	+	-	Small circular/ smooth edges light green colonies

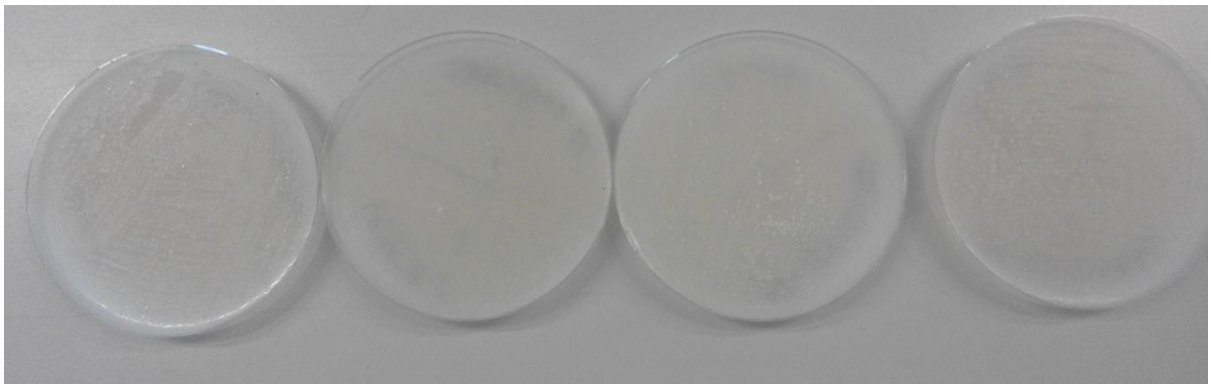

Figure 6: Colonies visible on the solid media. The first two petri dishes from left show colonies grown on the ammonium oxidizing media and the two petri dishes on the right show colonies grown on the nitrogen oxidizing media.

Table 5 shows the growth on selected broth and the morphology of the strains that were isolated from the broth. The (+) being an indication of growth, whereas (-) indicates no growth. As with the solid media, there was also growth on the broth, with the ammonium oxidizing media producing a milky/white solution that small white colonies suspended colonies. In contrast, the nitrogen oxidizing media produced an extremely pale yellow solution.

Table 5: Growth on selected broths.

Broth Type	**Growth**	**Description**
Ammonium oxidizing	+	White/Milky color with small white colonies suspended in the solution
Nitrogen oxidizing	+	Pale yellow color with pale yellow colonies suspended in the solution

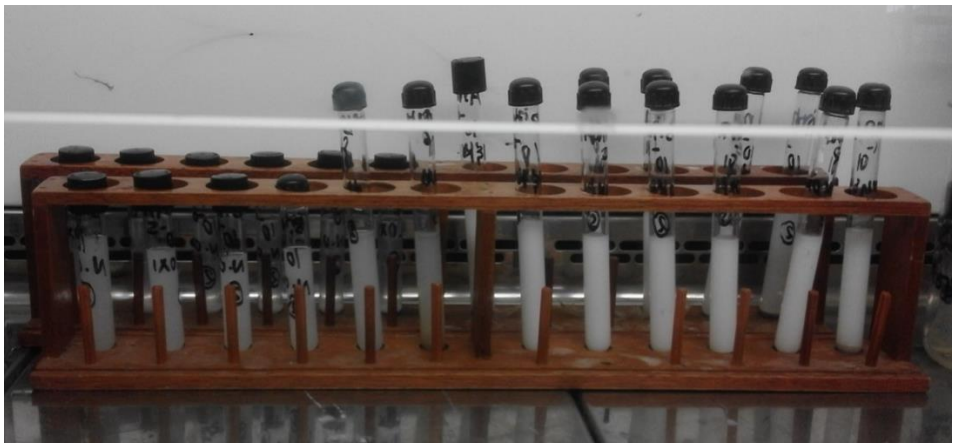

Figure 7: Isolates on respective broths

Figure 7 shows the growth of colonies in the broth at test tube level, before being transferred to larger growth containers, to allow enough growth for the extraction of DNA. The white/milky colour is clear visible in this figure. The broth used had the same composition as the solid media from which the strains were originally grown on.

4.2 Identification of Strains

Both isolates that were tested were gram negative with their cells being rod shape under the microscope (Table 6 and Figure 8).

Table 6: Gram stain result with isolates.

Strain Name	**Gram Positive or Negative**	**Cell Morphology**
Isolate 1NO	-	Rod shaped cells
Isolate 4AO	-	Rod shaped cells

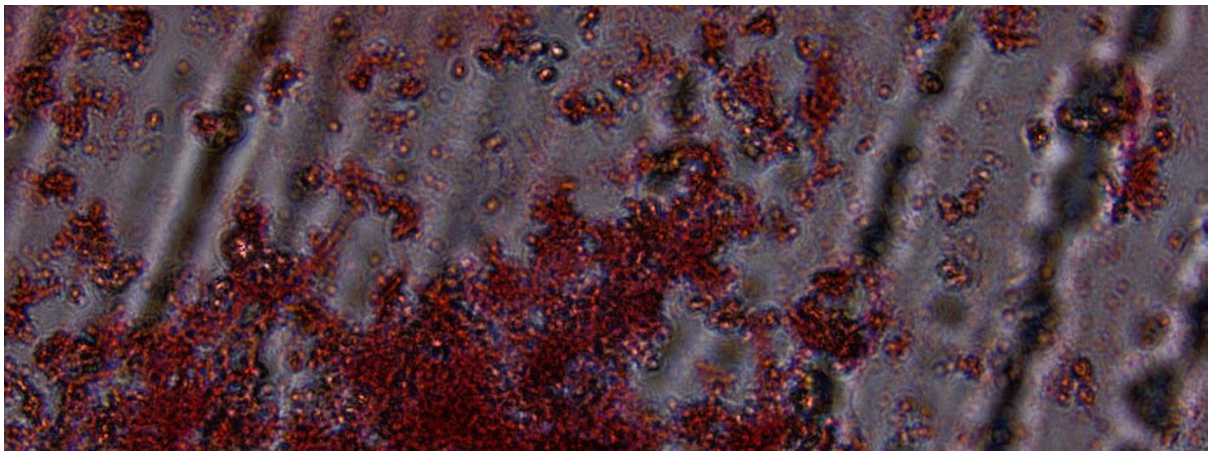

Figure 8: Image of cells viewed under a microscope after the gram stain test.

The broth cultures from which the gram stains and DNA extractions were made were not started from a pure culture, hence the contaminants were detected during the Gram stain (Figure 8).

Two of the samples from which DNA was extracted were identified by comparing the sequences to known sequences found in the BLAST database. Strains isolated have been seen to be similar to *Weissella* species, *Pseudomonas* species and *Francisella* species as obtained from the sequences blasted through the NCBI database (Table 7). The highest percentage similarity was obtained from *Weissella* species with a query cover of 88 % which is substantially low.

Table 7: Description of strains as obtained from the NCBI database.

Isolate Name	**Strain name**
Isolate 1NO	*Weissella* sp.
Isolate 4AO	*Pseudomonas* sp.
	Francisella sp.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

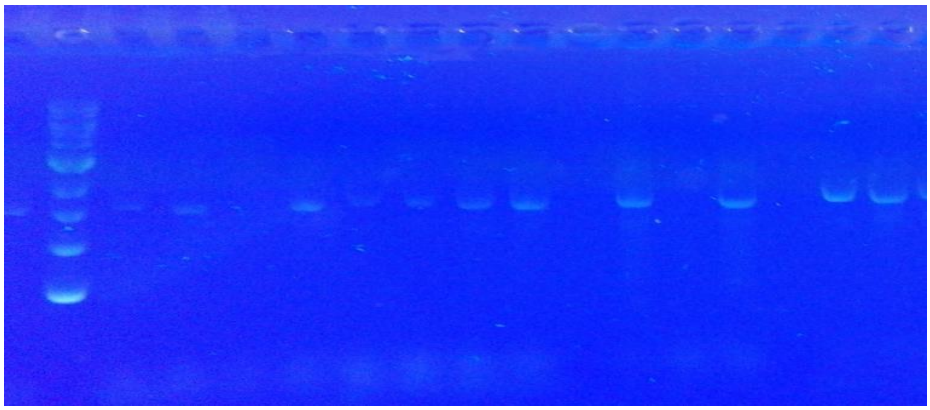

Figure 9: Gel electrophoresis results.

The size of the DNA isolates were 1000-1500 bp as indicated by the gel electrophoresis on a 1% agarose gel using a Kb ladder (Figure 9). The gel had 17 wells and of which well number 1 was the control and number 2 was the ladder. Wells 3-17 represented the PCR amplicons.

4.3 Inhibition Experiments

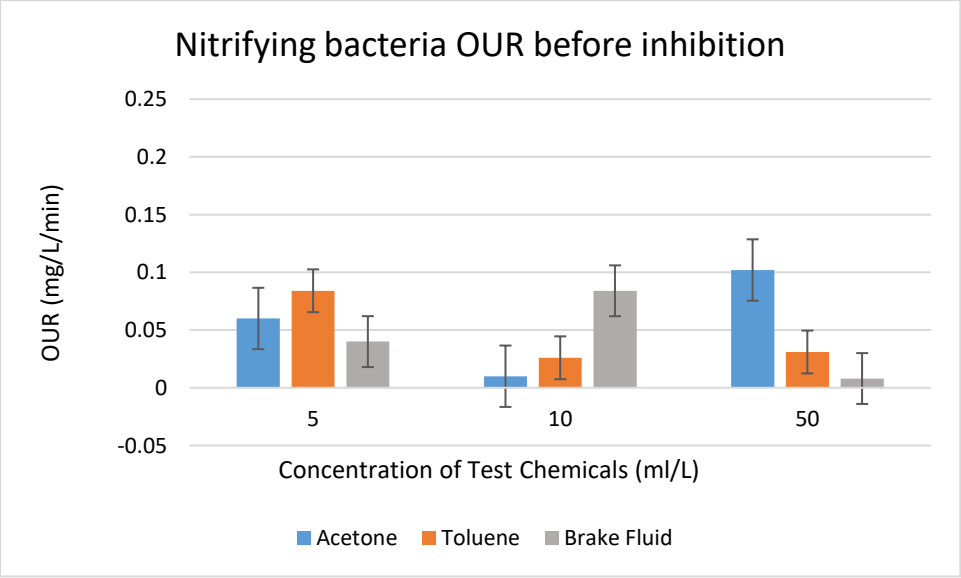

Figure 10: Graph comparing the OUR of nitrifying bacteria before inhibition.

The OUR varied greatly for the different concentrations of the test chemicals and this may be due to the different days on which the experiments were carried out (Figure 10).

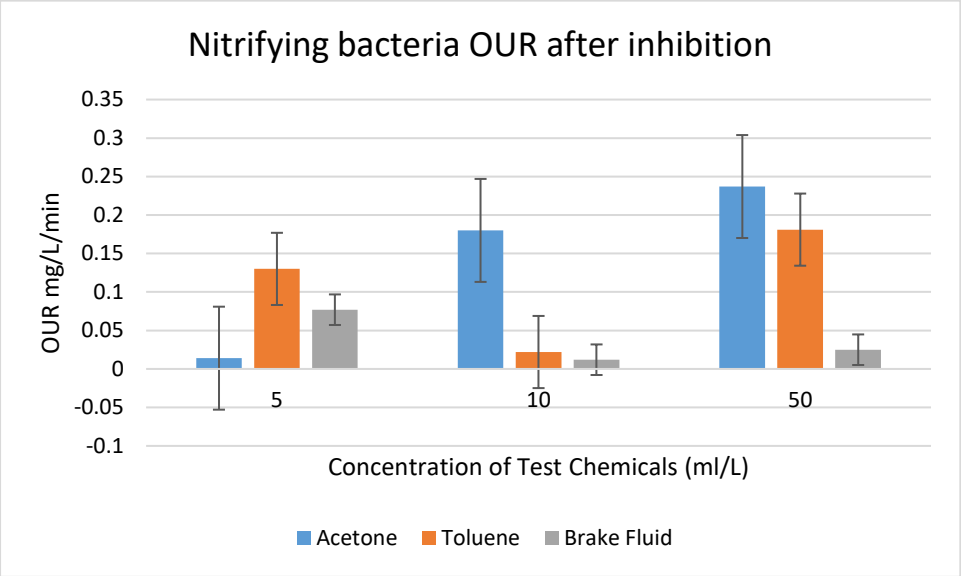

Figure 11 : Graph comparing the OUR of nitrifying bacteria after inhibition.

As before inhibition, the OUR also varied after inhibition at the different concentrations of the test chemicals. Acetone had its lowest OUR at a concentration of 5 ml/L (0,014 mg/L/min), 0,18 mg/L/min at 10 ml/L and highest OUR at 50 ml/L which was also the highest OUR for the experiment. Whereas toluene, had its lowest OUR at a concentration of 10 ml/L (0,022 mg/L/min), 0,13 at 5 ml/L and the highest at 50 ml/L (0,181 mg/L/min) as with acetone. Brake fluid on the other hand had its highest OUR observed at 5 ml/L while the lowest was observed at 10 ml/L (0,012 mg/L/min). 0,025 mg/L/min was recorded for brake fluid at 50 ml/L (Figure 11).

Toluene at a concentration 50 ml/L, showed the highest inhibition concentration, with a percentage inhibition of 483.9 %, while the lowest percentage inhibition (17%) was attained with acetone at a concentration 10 ml/L.

Table 8: Percentage inhibition of selected chemicals on an activated sludge sample.

Test Chemical	Concentration (ml/L)	Percentage Inhibition (%)
Acetone	5	76.7
	10	17
	50	332.4
Toluene	5	54.8
	10	184.6
	50	483.9
Brake Fluid	5	292.5
	10	85.71
	50	412.5

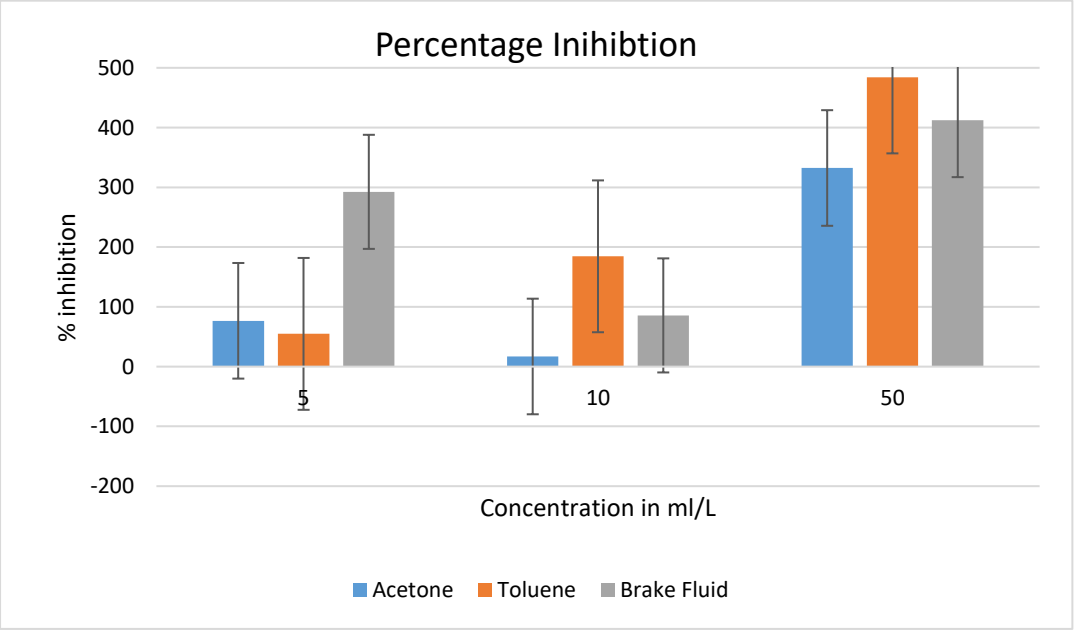

Figure 12: Comparison of percentage inhibition by the three test chemicals at the three respective concentrations.

The three test chemicals at the three different concentrations were compared in terms of their percentage inhibitions. 5 ml/L brake fluid had the highest inhibition (292.5%), whereas at 10 ml/L and 50 ml/L toluene showed the highest inhibitions (184.6% and 483.9%, respectively). The highest inhibitions were attained at the highest concentration (50 ml/L) for all three test chemicals (332.4% for acetone, 483.9% for toluene and 412.5% for brake fluid). The lowest inhibition was attained using acetone at 10 ml/L (17%) (Figure 12).

4.4 Bioremediation

The change in the concentration of Nitrate, Ammonia, and Total Kjeldahl Nitrogen from the initial sample, to the sample after inhibition and finally to the sample after bioremediation had taken place were measured (Table 9). From these results, it is evident that bioremediation using a fresh sample of sludge had occurred. After inhibition with 30 ml of toluene with a concentration of 50 ml/L, the concentration of nitrate decreased from 14.6 mg/L to 0.17 mg/L and then increased to 4.45mg/L after the bioremediation indicating a recovery of approximately 30% (Table 9). The concentration of ammonia increased from 0.58 mg/L to 1.91 mg/L after inhibition and then decreased to 0.90 mg/L after bioremediation. TKN concentrations reacted the way same as those of ammonia, increased after inhibition (from 346.8 mg/L to 402.1 mg/L) and decreased after bioremediation (to 334.4 mg/L).

Table 9: Concentrations (mg/L) of nitrate, ammonia, and TKN before inhibition, after inhibition and after bioremediation.

Samples	NO_3^-	NH_3	TKN
Initial Sample	14.6	0.58	346.8
Sample after inhibition	0.17	1.91	402.1
Sample after Bioremediation	4.45	0.90	334.4

A recovery of 33.68 % was attained using the fresh sample of activated sludge, after the sample was inhibited with 30ml of toluene with a concentration of 50 ml/L. The OURs of the different phase were as expected. With the total OUR being the highest (0,6658 mg/L/min) followed by the lowest OUR observed after inhibition (0,4438 mg/L/min) and increased again after the bioremediation (0,6679 mg/L/min) (Figure 13).

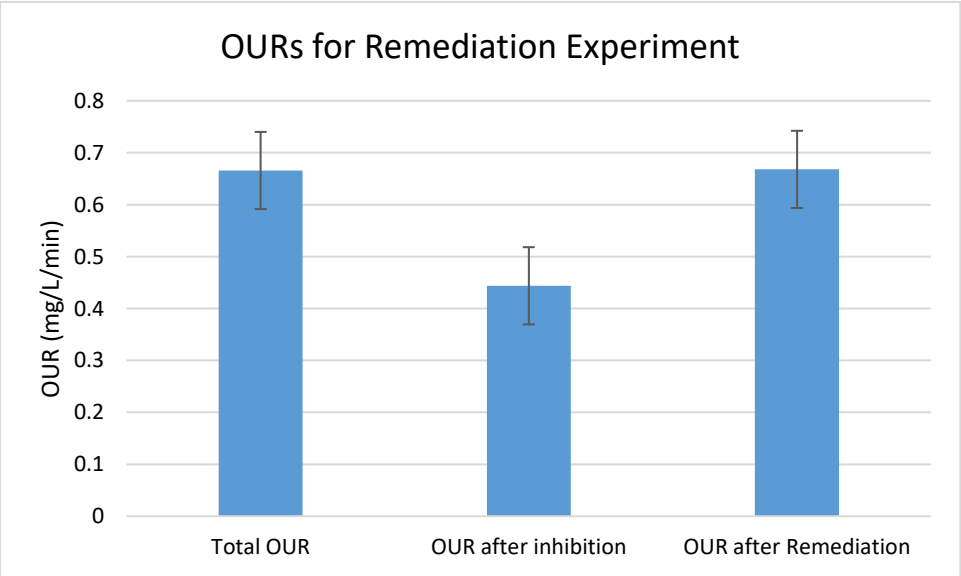

Figure 13: OUR at different stages of the bioremediation experiment

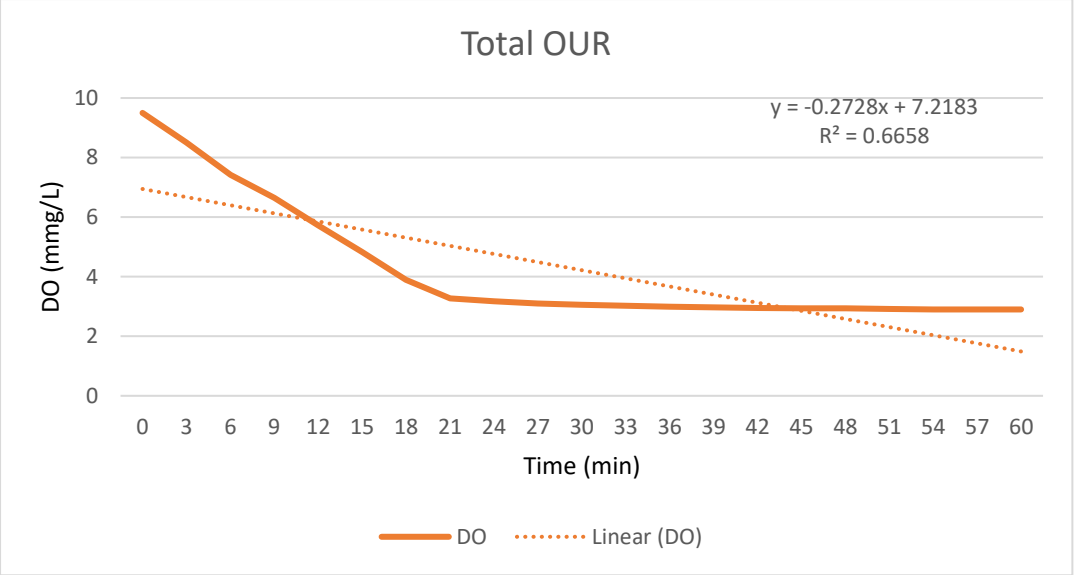

Figure 14: Graph indicating the total OUR before inhibition for the bioremediation experiment.

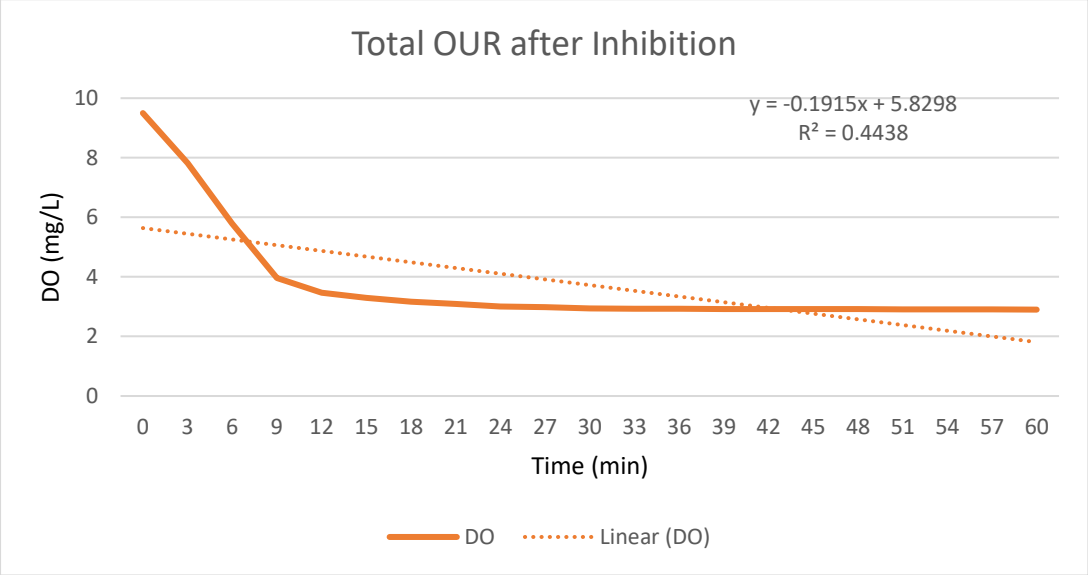

Figure 15: Graph indicating the total OUR after inhibition for the bioremediation experiment.

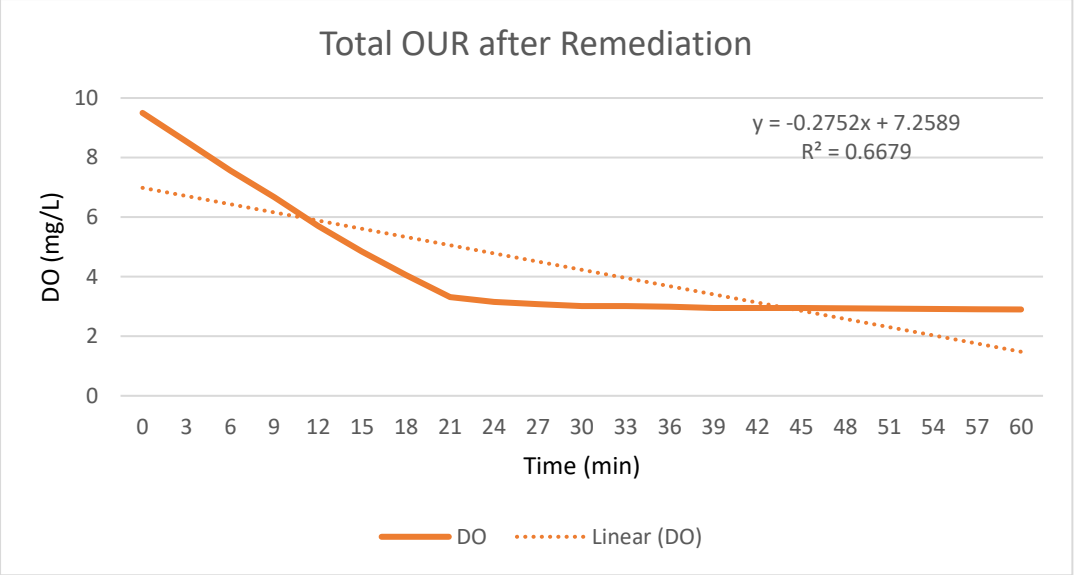

Figure 16: Graph indicating the total OUR after bioremediation for the bioremediation experiment.

Figures 14, 15, and 16 are graphs showing the total our (represented by R^2 on the graphs) before inhibition (0,6658 mg/L/min), after inhibition (0,4438 mg/L/min) and after bioremediation (0,6679 mg/L/min) respectively.

4.5 Bioaugmentation

Results from the bioaugmentation experiment were similar to those of the bioremediation experiment, with regards to the nitrate and ammonia but different with regards to the TKN (Table 10). The concentration of nitrate decreased after inhibition from 5.04 mg/L to 1.36 mg/L and then there was an increase in the concentration after augmentation from 1.36 mg/L to 22.6 mg/L. Regarding the concentration of ammonia, it increased from 6.09 mg/L to 41.8 mg/L as a result of inhibition but then decreased from 41.8 mg/L to 39.6 mg/L as a result of thee augmentation that occurred. However, when looking at the concentrations of the TKN, there was an increase after inhibition as well as after the augmentation step. The concentration increased from 250.1 mg/L to 367.7 mg/L after inhibition and then increased to 406.4 mg/L after the bioaugmentation step of the experiment.

Table 10: Concentrations (mg/L) of nitrate, ammonia and TKN before and after inhibition as well as after bioaugmentation.

Samples	NO_3^-	NH_3	TKN
Initial Sample	5.04	6.09	250.1
Sample After Inhibition	1.36	41.8	367.7
Sample after remediation	22.6	39.6	406.4

A percentage recovery of 35.78% was attained in the OUR after bioaugmentation using pure cultures of *Nitrosomonas* and *Nitrobacter*. As with the remediation experiment the total OUR was the highest (0,91 mg/L/min) but however in this case the OUR after bioaugmentation was the lowest (0,5202 mg/L/min) (Figure 17).

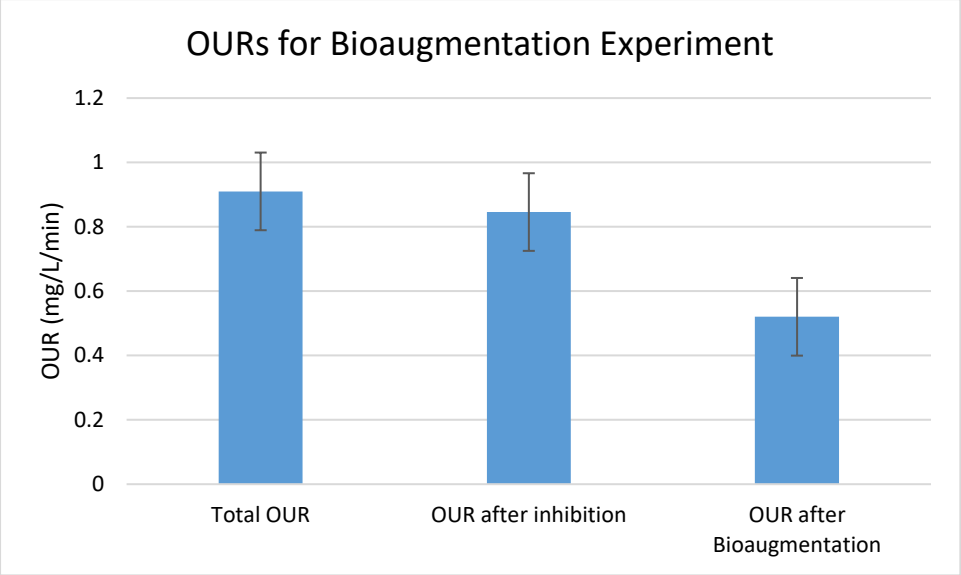

Figure 17: OUR at different stages of the bioaugmentation experiment.

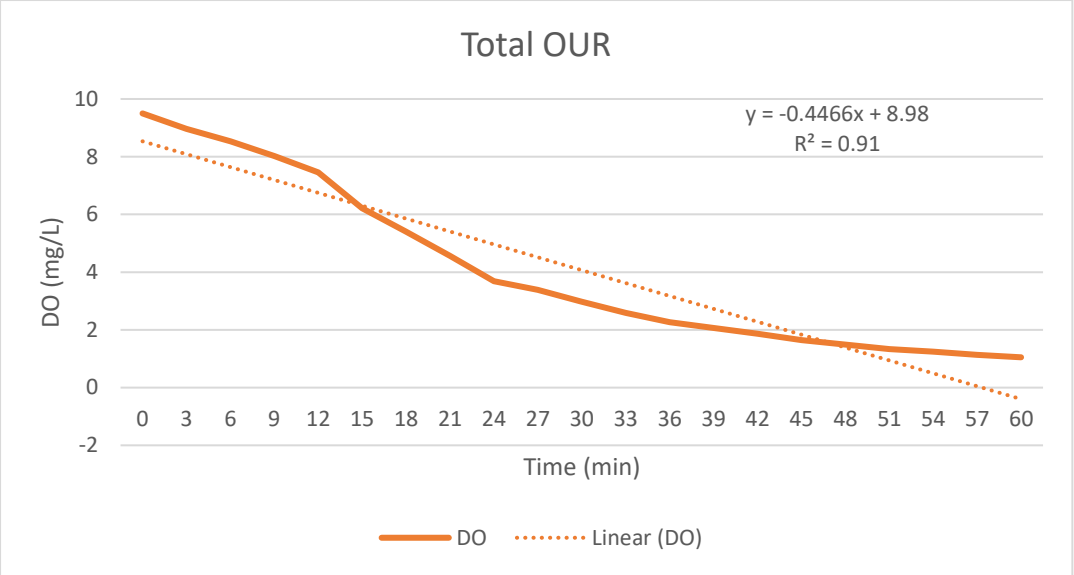

Figure 18: Graph indicating the total OUR before inhibition for the bioaugmentation experiment.

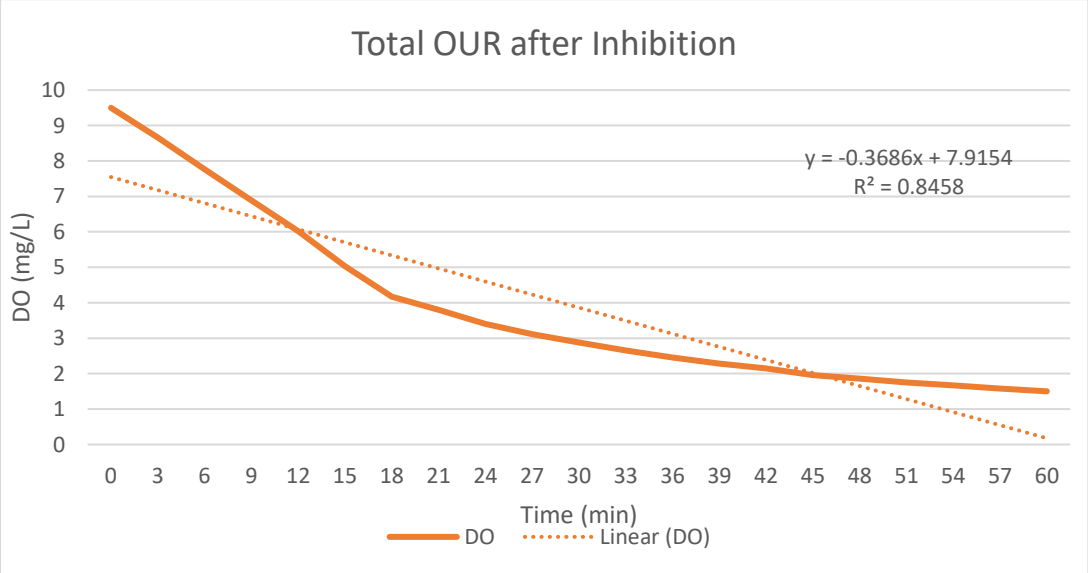

Figure 19: Graph indication the total OUR after inhibition for the bioaugmentation experiment.

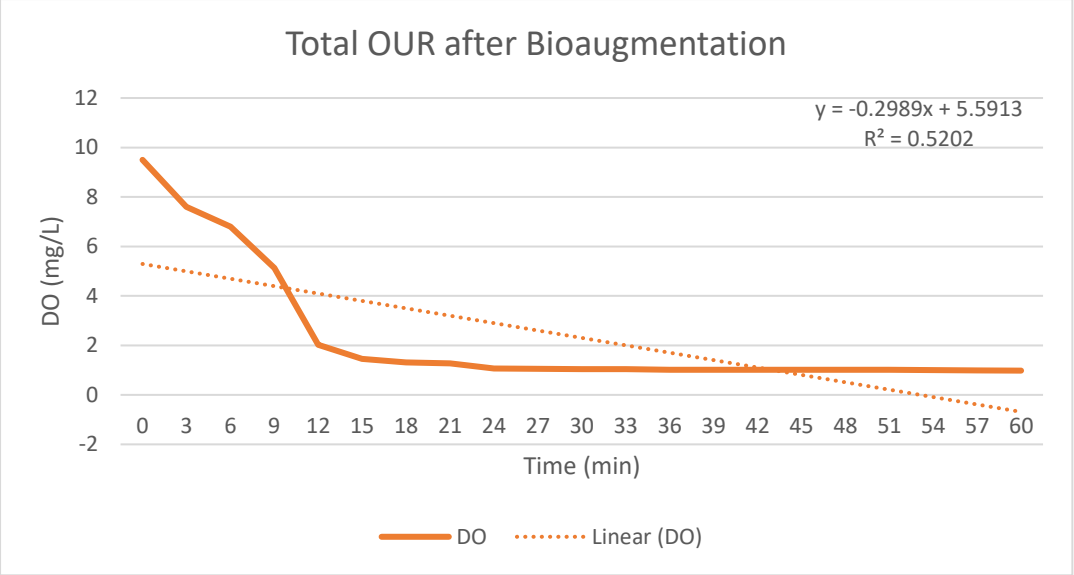

Figure 20: Graph indicating the total OUR after bioaugmentation for the bioaugmentation nexperiment.

Figures 18, 19 and 20 show the respective OUR values (represented by R^2 on the graphs) for the bioaugmentation experiment. The figures each show the total OUR before inhibition (0,91 mg/L/min), after inhibition (0,8458 mg/L/min) and after bioaugmentation (0,5202 mg/L/min) respectively.

CHAPTER 5

5. DISCUSSION

5.1. Isolation of Strains

Nitrifying bacteria are usually slow growing bacteria and proof rather difficult to isolate under laboratory conditions. From the two media (ANO media and NO media) that were used very few colonies were produced and of those that were produced, most turned out to be contaminants or other microorganisms that were present in the wastewater samples. Also contaminants may have been introduced because incubators were shared with other researchers and these may have been a contributing factor. Since nitrifying bacteria are aerobic microorganisms, they require a constant feed of oxygen. Reported values of the limiting DO concentrations range from 0.5 to 4 mg/L (Stenstrom, Song, 1994), however it is rather impossible to effectively aerate samples grown on the solid without risking contamination. In this study, the samples that were cultured on solid media were not aerated and hence this may have been the greatest limiting factor in this regard.

Growing the samples on broth (ANO broth and NO broth) produced better results and this was visible by the turbidity of almost all the broth cultures, indicating growth in all cultures. However, once again purity of the cultures could not be guaranteed since the broth cultures were not prepared from pure cultures. Also, growth could have been improved by provision of an increased amount of oxygen, as the samples were only grown at atmospheric air pressures and hence, isolate growth may have been limited. It is also important to take into consideration that nutrients are more readily available in liquid form as compared to being available in solid form. Another factor that could prove pivotal is that the media used was from a study that researched nitrifying bacteria from a farmyard manure sample and hence may have been more specific for soil bacteria as compared to wastewater bacteria. The media

was used in Egypt where environmental conditions prove to be more extreme as compared to those in parts of Namibia where the experiments were carried out. More favourable results can be attained with media that is more closely related to central Namibia in environmental conditions such as that used by Ramdhani in 2012 in South Africa.

Previous studies had greater success in their attempts to isolate and identify nitrifying bacteria, both on solid media (Rezaee et al., 2010) and broth (Lin et al., 2007). High concentrations of ammonia of up to 1000 mg/L were used in the preparation of the solid media (Rezaee et al., 2010) compared to the 500 mg/L used in this study. This allows for a greater opportunity for the nitrifying bacteria to grow despite competition from contaminants. With broth, most studies achieved better growth due to the fact that the samples were grown aerobically with the added air, whereas the sample in the study were simply grown using atmospheric air. There have been studies that have used molecular techniques only, to isolate and identify nitrifying bacteria (Reagan, Harrington, Noguera, 2002).

5.2. Identification of strains

One of the identification techniques used was the Gram-staining, which allows the cell morphology of isolates to be identified as well as determining whether the strains are Gram-negative or positive. Both isolates were gram negative and both had rod shaped cells. With regards to being Gram-negative, the test was consistent with other studies that indicate nitrifying bacteria as being gram negative (Phirke, 2014). Regarding the cell morphology, nitrifying bacteria are known to have a range of cell morphologies differing from one species to another. These morphologies comprise simple rod shaped and coccus cells, also observed in this study. Some nitrifying bacteria in other studies have been found to have oval or coin shaped cells, some being flagellated at different locations of the cell and some having

multiple appendages (Puzyr' et al., 2001 and Zavarzin, Legunkova, 1959) At this point the possibility of both isolated strains being nitrifying bacteria was still high, due to the fact that both isolates were gram negative and that nitrifying bacteria can have a range of morphologies. However, this was put to bed when the DNA isolated from the isolated bacteria was sequenced.

The sequences that were obtained from the DNA extracted from isolates, were used to find similarities of these sequences, to aid in identifying the isolates. Results indicated that the isolates were similar to species of *Weissella*, *Pseudomonas*, and *Francisella*. From this information it is clear that there is a variety of microorganism present in the sample. This can either be perceived as a variety of nitrifying bacteria present in the aerated basins considering that the media used was specific for nitrifying bacteria, or either contaminants or microorganisms that were part of the samples collected from aerated basins. From literature (Xu et al., 2014, Aquilanti et al., 2004, Hovanec et al., 1996) it is observed that these bacteria are part of the *Proteobacteria*, of which known nitrifying bacteria are part of as well. *Weissellas* are Gram-positive asymmetrical short rods with rounded ends or coccoid rods, occurring individually, in twosomes or in short chains. On de Man, Rogosa and Sharpe (MRS) agar, *Weissellas* produce small translucent colonies, circular in shape of a somewhat eminent profile, older cultures form a concentric structure. These microorganisms are optionally anaerobic, and grow briskly under microaerophilic incubation and are not recognized to retain nitrifying proficiencies (Duskova, Kamenik, Karpiskova, 2012). Bacteria of the genus *Weissella* can be prevalent in extremely varied environments. Duskova, Kamenik and Karpiskova (2012), indicate that these bacteria are recurrently sequestered from plant material, e.g. fresh vegetables, cassava and silage, cacao beans, as well as from meat and meat products, fish, kimchi, soil and, in isolated cases, they can likewise transpire in clinical material of human or animal origin.

Pseudomonas is a bacterium typically saprophytic in nature, and is found in soil, water and other moist environments (The National Institute of Open Schooling, 2014). It has arisen as an imperative source of health care associated and unscrupulous infections. *Pseudomonas* is a stringent aerobe, rod shaped, willowy (dimension: 0.5 - 0.8 µm by 1.5 - 3.0 µm) Gram negative organism, motile by polar flagella, occasionally more than two flagella may be existent. *Pseudomonas* is a strict (obligate) aerobe, but every so often it can grow anaerobically if nitrates (NO_3 act as respiratory electron acceptor) are present in the growth medium. This means it has nitrification capacities and can consequently be an additional nitrifying bacteria – in addition to *Nitrosomonas* and *Nitrobacter*. *Pseudomonas* can propagate at wide ranges of temperature; the peak temperature being 37° C. It can grow on commonplace media, for instance nutrient agar and grows practically on all the culture media used customarily in the bacteriology lab. *Pseudomonas* has been seen to grow in distilled water, as well. *Pseudomonas* yields huge, opaque, even colonies with asymmetrical brims and idiosyncratically fruity aroma colonies. The segregates from water and soil yield small round colonies. The isolates that are sequestered from clinical varieties like respiratory, urine, etc. may possibly yield mucoid colonies. *Pseudomonas* can contaminate any tissue, any organ system in an immune-compromised host. *Pseudomonas* ordinarily are not able to infect regular hosts. Since compromised hosts are found in hospitals, *Pseudomonas* has occurred as a common cause of health care concomitant or nosocomial or hospital affiliated infections. In addition, *Pseudomonas* produces numerous diverse organ system infections in the humans. Most of the clinical isolates of *Pseudomonas* are impervious to various antibiotics. *Pseudomonas aeruginosa* is also a pathogen of plants (The National Institute of Open Schooling, 2014). *Pseudomonas* have been seen to possess strong nitrification characteristics and therefore it is suggest that a vested interest be taken in studying their potential in the nitrification process and advancing their usage.

Francisella is a genus of Gram-negative, coccobacilli, non-motile and aerobic bacteria. Presently, five species have been documented in the genus: *F. tularensis*, *F. philomiragia*, *F. noatunensis*, *F. hispaniensis*and *F. halioticida* (Gu et al., 2015). *F. tularensis* is the most common specie and causes classic tularaemia in humans. Tularaemia is a zoonotic disease that is prevalent all the way through the Northern Hemisphere and is every so often allied with acquaintance to an assortment of disease-ridden wild mammals, most outstandingly lagomorphs, or the bites of blood-feeding arthropods (ticks and deerflies) (type A) or natural water sources (Whitehouse et al., 2012).

Based on the characteristics of the strains that were identified presented above, it can be concluded that the strains might have been part of the initial sample that were collected from the Gammams aerated basins or be part of the population of nitrifying bacteria population present in the aerated basins. This is an indication that further and more specific experiments are required to make the distinction between the above mentioned options. Considering that the Gammams Wastewater Treatment Plant takes in wastewater from industrial areas that process meats and restaurants that deal with fish and several other meat and plant products. More importantly the plant treats human faecal waste and since these are pathogenic bacteria, one can therefore suggest that they are part of the wastewater. However, considering the fact that the segregated organisms grew on media that is meant to be nitrifying bacteria specific, leads to one to not completely ruling out the possibility of the organisms being part of the nitrifying bacteria. On the other hand, these microorganisms may not have similar growth requirements as the usual nitrifying bacteria but can however grow under similar conditions if needed, since they are opportunistic. They also are faster growing organisms as compared to the characteristic slow growing nitrifying bacteria. This explains why the isolated bacteria out growing the traditional nitrifying bacteria and hence being isolated instead of the anticipated bacteria.

5.3. Inhibition Experiments

Three different organic solvents were used as test chemicals during these experiments, at concentrations of 5, 10, and 50 ml/L. Toluene, acetone and brake fluid were the respective chemical test for inhibiting nitrification. The chemicals were chosen since, the GWWTP receives wastewater from the Southern Industrial area of Windhoek which houses several paint production companies as well vehicle garages. This means the waste from this area possibly contains all three test chemicals. Toluene and acetone are components of paint thinners, which is widely used in this area. Vehicles have brake fluid replaced and topped on a regular basis and in that way brake fluid might end up in the wastewater. Toluene at a concentration of 50 ml/L produced the highest percentage inhibition of nitrification at 483,9 %. It is also important to note that, all three chemicals, were most effective in inhibiting nitrifying bacteria at the highest concentrations. However, even the lower concentrations had high percentage inhibitions and this goes to show that even at lower concentrations, inhibition does occur. There is a need to investigate the actual concentration of these chemicals in the influent of the plant.

There are several contributing factors to the experiments performed in the current study, which could affect the results of inhibition. As stated earlier, nitrifying bacteria are very sensitive to change and require optimum conditions to operate efficiently. The GWWTP experiences periodical mechanical problems that lead to low solid contents in the activated sludge process basins. This in turn leads to there being very little food for the nitrifying bacteria and a decrease in the populations of microorganisms in the activated sludge process basins. This could have affected the experiments in that on some days, the solids content may have been lower than others, leading to poor OURs and resulting higher percentages of inhibition and vice versa. If the study is repeated, it would be good to also monitor the suspended solids concentrations. Previous studies are mainly focused on the effects of known

inhibitors such Allylthiourea (ATU), which was used in this study, dicyandiamide (DCD), nitrapyrin (Lehtovirta-Morley et al., 2013), allylsulfide (Neufeld, Knowles, 1999) and 3,4-dimethylpyrazole phosphate (Dong et al., 2013).

5.4. Bioremediation

The bioremediation experiments were carried out to determine by what percentage a fresh sample of activated sludge would help an inhibited sample recover. This was observed in two ways, the first being the measurement of the concentrations of ammonia, nitrate and TKN, before inhibition, after inhibition and after bioremediation. The second way of observation, was the use OURs to determine the percentage recovery. The concentration of the nitrates dropped after inhibition and increased after bioremediation, whereas the concentration of ammonia and TKN both increased after inhibition and decreased after bioremediation. Referring to the formula for nitrification to aid in explanation of the results, it is noticeable that once inhibition had taken place, NO_3^- which is the final product of nitrification decreased as it was not being produced sufficiently.

$$NH_4^+ + 1.5\ O_2 \longrightarrow 2H^+ + H_2O + NO_2^- \qquad (1)$$

$$NO_2^- + 0.5\ O_2 \longrightarrow NO_3^- \qquad (2)$$

This holds similar consequences for the ammonia as the initial substrate for the nitrification reaction. However, there is an increase in the concentration after bioremediation had taken place which was after the introduction of the fresh sample of activated sludge. Hence, the sample had started producing NO_3^-, showing that the sample had become more active. The increase in concentration after inhibition is an indication that the ammonia was not being consumed by the nitrifying bacteria and hence accumulated in the sample. However, the decrease in concentration after the fresh sample of activated sludge was added is indication of

the ammonia being consumed and eventually depleted in the sample. A similar trend is observed as far as the TKN is concerned in the sample.

The results are indication that bioremediation had occurred and are further supported by the OUR results that revealed that there was a recovery of 33,68% in nitrification. This a reasonable result, however, a higher percentage recovery could possibly have been attained by increasing the exposure time that the fresh activated sludge sample had before measurements were made. Alternatively, a larger volume of the fresh sample could have been used to ensure a better percentage recovery. However, time constraints made these solutions less probable. These results show the ability of an activated sludge system to recover after a toxic pulse and hence continue with normal operations. The question raised becomes how effective this self-remediation process is in larger volumes of the aerated basins. These experiments indicated the potential use of mixed cultures from a healthy performing activated sludge system to stimulate recovery of nitrification after a toxic pulse in activated sludge systems. A follow-up experiment should investigate this in larger scale reactors.

Previous studies show bioremediation carried out with different species as compared to using a fresh sample from the same source where inhibition had taken place. The use of algae has seen great success with an efficiency ranging between 80-97% (Sharma, Khan, 2013, Rahman, Ellis, Miller, 2012). Some studies focus on the overall bioremediation of sewage and use a consortium of bacteria to have a greater effect on the entire treatment of wastewater (Kumar et al., 2013, Reddy, Dysdale, Bux, 2003).

5.5 Bioaugmentation

A similar approach to that of the remediation experiments was followed with the regards to the bioaugmentation experiments. The difference being that for the bioremediation, a known

pure culture of *Nitrosomonas* and *Nitrobacter* species were used. As with the bioremediation experiments, the bioaugmentation experiments were performed to produce two sets of results, to determine the percentage by which bioaugmentation recovered the nitrification process. Firstly, monitoring the concentrations of the three respective chemicals as with the bioremediation experiments and secondly the OURs from which the percentage recovery after bioaugmentation was calculated. As with the inhibition reaction, the concentrations of the nitrates decreased after inhibition and then increased drastically after the addition of the pure culture. As nitrate are the final product of the nitrification reaction, it is expected to decrease as in the sample if inhibition has taken place. The increase in the concentration of the nitrate after bioaugmentation is an indication that recovery of the nitrification process had indeed taken place. With ammonia, there was an extreme increase in the concentration after the inhibition step. This was followed by a decrease in the concentration after the bioaugmentation step. As the precursor of the nitrification reaction, it is expected that there will be an increase in its concentration as the reaction is inhibited and then decrease as the reaction rate is increased by the addition of the pure culture of known nitrifying bacteria. This again proves the occurrence of bioaugmentation in the sample. Nevertheless, TKN concentration, increases after inhibition and increases again after bioaugmentation. Even though there was a decrease in the nitrates, the increase in ammonia concentration was greater lending to the increase. After the bioaugmentation step of the experiment, the TKN is increased even though there is a decrease in the ammonia concentration, and this due to the increase in the concentration of the nitrates leading to an overall increase in the concentration of nitrogen in the sample.

These results are further cemented by the percentage recovery calculated from the OUR measurements. A percentage recovery of 35.8% in the OUR of nitrifying bacteria was attained, however a higher percentage was expected, considering that a pure culture of known

nitrifying bacteria was used. Previous studies have produced augmentation percentages ranging between 40-45% when using *Nitrosomonas* and *Nitrobacter* species (Kumar et al., 2013). These results are not far greater than those obtained in this study. With improvement and altering of certain experimental parameters, a percentage recovery that is greater than 50% can sure be achieved.

A greater percentage recovery could possibly have been achieved by increasing the contact time with the inhibited sample as this would allow the nitrifying bacteria in the pure culture to better acclimatise to the conditions in the reaction vessel. Another influencing factor would have been the temperature. The pure cultures, were grown in controlled conditions at 27^{o}C, but were then subjected to the colder temperature of the activated sludge sample, which was at a temperature of 18^{o}C and hence the efficiency of the pure culture was affected. A third possible of a factor that could have affected the efficiency of the pure culture, is the competition from the heterotrophic bacteria already present in the activated sludge sample. This competition for food and DO might have led to the low percentage recovery of nitrification. Nevertheless, the fact that bioaugmentation occurred, renders this a plausible solution to the nitrification problems experienced at the GWWTP. This combined with the natural remediation abilities proven through the remediation experiment should be able to aid in an improved final supernatant quality.

A comparison of cost implications regarding the maintenance of a pure culture vs mixed cultures should be done if application of bioremediation and/or bioaugmentation is considered. This was not part of this study but can be explored in future studies that up-scale the application at the GWWTP. In previous studies, there was a use of different species of nitrifying bacteria such as *Pseudomonas fluorescens*, *Pseudomonas putida*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens* and *Bacillus simplex* as well as a simulation plant to simulate an activated sludge process, also different

parameters were used to gauge the efficiency of bioaugmentation (Abu-Danso, 2015). Also most studies are focused around the bioaugmentation or remediation of polluted soils (Deni, Penninkx, 1999).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The study showed that the population of microorganisms in the activated sludge system at GWWTP is not limited only to those organisms that play a role in the removal of organic matter and nutrients but also accommodates a range of pathogenic bacteria and other bacteria as supplies by the different source of wastewater to the plant. The identification strains of *Pseudomonas*, *Francisella* and *Weissella* species is an indication of this diverse population of microorganisms present in the aerated basins. The study identified *Pseudomonas* species that that are capable to perform nitrification and hence proved that the nitrifying bacteria population in the activated sludge system of GWWTP is a mixed culture of autotrophic organisms capable of nitrification. Furthermore, the study failed to enrich and isolate nitrifying bacteria that includes other genera in addition to *Nitrosomonas* and *Nitrobacter* and this was because no pure cultures were obtained. Moreover, the study showed that nitrifying bacteria are rather susceptible to inhibition by chemicals that may be common in the wastewater system. Chemicals do not have to be present in large concentrations, as even the very low concentrations (5 ml/L) inhibit nitrification, with the higher concentrations (50 ml/L) completely inhibiting nitrification. OURs of the nitrifying bacteria shows an active population of nitrifying bacteria that lead to the complete removal of nitrogen.

The bioremediation experiments revealed the ability of the nitrifying bacteria found in the activated sludge system to recover by itself after a toxic pulse leading to inhibition. This was shown by the 33,68% recovery after inhibition as well as the changes in the concentrations mainly of ammonia and nitrates. The changes in these concentrations after inhibition and bioremediation were an indication that indeed bioremediation had taken place. However, a

higher percentage recovery would be required to be indicative of efficient bioremediation abilities of the of the innate nitrifying bacteria population.

Bioaugmentation experiments resulted in recovery of nitrification but though, the percentage recovery was greater than that of remediation at 35,78%, it was not enough to meet the expected 50% as part of the hypothesis of the study and hence the hypothesis was rejected. However, the change in concentrations of nitrates and ammonia as with the bioremediation experiment were an indication of the recovery of nitrification in response to bioaugmentation.

6.2 Recommendations

Regarding isolation of pure cultures, more work should be done in identifying media and culturing conditions that are more specific for nitrifying bacteria from activated sludge samples, as well as specificity to the environmental conditions. This would aid in limiting growth of contaminating and unwanted bacteria that then prevent the isolation of pure cultures. Even though there was growth on solid media it was very poor as aeration cannot be administered. It is therefore advised that the use of solid media not be used as it is rather difficult to obtain decent growth results on solid media due to poor aeration as well as not being able to replenish nutrients once depleted in the growth medium. Once pure cultures have been obtained, it aids in the better identification of isolated strains.

With regards to the inhibition experiments, more chemicals should be used as test chemicals, considering that nitrifying bacteria are highly sensitive to a variety of chemicals that could be present in the wastewater at any point and time. This would aid in identifying the types of chemicals that inhibit the nitrification and the concentrations at which they do so. An increased volume of the fresh sample as well as an increased time in contact with the inhibited sample would improve the percentage recovery caused by remediation. Finally, with bioaugmentation, the propagation of the pure cultures obtained from the factory requires

extreme caution as these are extremely sensitive microorganisms. Also, one should take into consideration improving the temperature to have the pure strains working at optimum temperatures to be more efficient. Increasing the volume of the pure strains as well as increasing the contact time of the pure cultures with the inhibited would greatly improve the percentage recovery attained by the pure cultures. It is finally recommended that the remediation and bioaugmentation experiments be carried out in activated sludge systems with much higher volumes.

CHAPTER 7

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APPENDICES

Appendix 1: Full results from the inhibition experiments.

Inhibition Chemical	Concentration of Inhibition Chemical (ml/L)	Before Inhibition			After Inhibition		
		Total OUR	Heterotroph OUR	Nitrifying Bacteria OUR	Total OUR	Heterotroph OUR	Nitrifying Bacteria OUR
Acetone	5	0.851	0.911	(-0.06)	0.934	0.948	(-0.014)
	10	0.779	0.789	(-0.01)	0.309	0.489	(-0.18)
	50	0.706	0.604	0.102	0.227	0.464	(-0.237)
Toluene	5	0.613	0.697	(-0.084)	0.567	0.697	(-0.13)
	10	0.873	0.847	0.026	0.562	0.584	(-0.022)
	50	0.858	0.827	0.031	0.907	0.726	0.181
Brake Fluid	5	0.712	0.672	0.04	0.84	0.917	(-0.077)
	10	0.853	0.769	0.084	0.65	0.638	0.012
	50	0.81	0.818	(-0.008)	0.746	0.721	0.025

Appendix 2: Sequences obtained from the isolated DNA

Sequence 1: Isolate 1NO

AGCGGAGTGCTTATGCGTTAGCTGCGACACTAAGGGTGAGGAGCCCGCGACTAC

CAACTTGGTTAAGGGGGGGAAAAACCGGGAAATAAAACCGGGTTGGCCCCCCAC

GTTTTCGCCCCTCAGGGTAAGATAAAGCCCGGAGAGGGCTTTTCCCCAGGGGGG

TTCTTCCATAATTAAAGGTTTTTCCCGGTAACACGGAAATTCCCGCTCCTTTTTCC

TGCACTAAATATACCCTGTTTGCGGATTCCCCAGGTTGGGCCCGGGGTTTTCCCC

CTGAATTTAAAAAACCCCCTTGCGCGCGTTTACCCCCAAAAATTCCCAAAAAAGG

TTGGCCCCTTATAATTACCCGGGTTGGTGGGCAGAAATTAACCGGGGTTTTTTTC

GTTGGGAACGGCTAAGGTGCGGCGATATAAGAACTTTACTTTTCTTCCAACTTAA

AGAGATTTAAAAAATGAAAACATCTTTTTGGCTGGCGGGGTGGGGGTTTCCCGA

ATTGTCCCATATGGCGAAATGTTCCCTACGGGTCCCTCCCAGAGGGGGTGGCCGT

GCCCCTGRGCCCGAGGGGGTTCGCACACCCTCTAAGGATGTATAGATTGCGGCC

Sequence 2: Isolate 4AO

ACCCCCTAGGGAAGCGGCCTCCTTGCGGGAGACTAGCTACTTCTGGAGCAAACC

ACTCCCATGGGGTGACGGGGGGGGTGTACAAAGGCCGGGAACGTATTTTCCGCG

AGATATGGTTACCCGAGTTTACTAGATTCTGACTTTTCGTGGACGAGTTGTAGAC

TGCGATACGCAATATGATAGGGTTTTTGTGATTATCTCCGCCTCGCGAGTTTTCGA

CCCTCTTTACCGCGCATTTGAGAACGGGTGGAAACCCACCCAAAAAGGGCATGA

GAACATTACCTCCTTCCCCCCTTTTTCCGGGTTTTGACCGGGAGTATCTTTTAGAT

TTCCCTGCTTAAGACGACCGATAAAAAGTGGGGTGTGTGCTGGATTTGGGCCAA

AACCTCCCGTCTCCAATGAAACTGCATGAGCCATCTTGCATTGGGTTATAGTCCC

AAAGAAACTAATACATTTTGGAAGGGGCTTTGTATGACAAGAACAGGTAATGTK

GTTTTCGCTGTTTATAATAAAAACACCTGCTCCTCCGCTTGTGGGGGCCCCCCCCT

TTTTTTTTAATATTAACCTTGTGGCCGCCCTCCCCACGAGGGGAAGTGTTTGCTCA
G

Appendix 3: Data analysis

Paired sample t-test. Comparison of two proportions / percentages

Ho: $\mu \geq 50\%$

Ha: $\mu \ngeq 50\%$

$$t* = \frac{(\overline{d} - б)}{\frac{sd}{\sqrt{2}}}$$

$$\overline{d} = \frac{x + \overline{x}}{2}$$

$$sd = \sqrt{(\sum |x - x*|^2)/n}$$

α = 0,05

Degrees of freedom = n – 1

= 2 – 1

= 1

Rejection Region: $\overline{d} + t_{\frac{\alpha}{2}} x \frac{sd}{\sqrt{n}}$

$$\overline{d} = \frac{(50 + 35.7)}{2}$$

$$\overline{d} = 42,89$$

Rejection region: From the table of significance we reject if t^* > 12,71

$$t* = \frac{42,9}{(7,11/\sqrt{2})}$$

$$t* = 8.51$$

$t* \ngeq 12,71$ ***and hence we reject Ho and in turn accepts Ha.***