NITRIFICATION AND AEROBIC DENITRIFICATION IN CYANIDE-CONTAINING WASTEWATER

by

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DECLARATION

I, **Ncumisa Mpongwana**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology or the National Research Foundation of South Africa.

All intellectual concepts, theories, methodologies and material derivations and model developments used in this thesis and published in various scientific journals (except those of which the candidate is not the first author) were derived solely by the candidate and first author of the published manuscripts. Where appropriate, intellectual property of others was acknowledged by using appropriate references. The contribution of co-authors to conference papers and published manuscripts was for training capacity (SA Dyantyi and YP Mpentshu), research assistance (L Mekuto, E Akinpelu) and supervisory capacity (Prof SKO Ntwampe)

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ABSTRACT

Anthropogenic activities that utilise cyanide in various chemical forms have resulted in the disposal of cyanide-contaminated effluents into drainage systems that ultimately reach wastewater treatment plants (WWTP), without prior treatment. Cyanides (CN) and soluble salts could potentially inhibit biological processes in WWTP, which are responsible for the removal of contaminants from incoming wastewaters. The removal of nitrogenous compounds from such waters in processes such as nitrification and denitrification is among the core biological processes used to treat wastewaters in WWTP. Electroplating and mining industries are among the perpetrators of cyanide contamination of WWTP. The presence of these hazardous contaminants results in the alteration of metabolic functions of the microbial populations that are utilised in WWTP, thus rendering the wastewater treatment process ineffective.

In this study, bacterial isolates that were able to carry out nitrification and aerobic denitrification under high salinity cyanogenic conditions were isolated from poultry slaughterhouse effluent. These strains were referred to as I, H and G. The isolated bacterial species were found to be able to oxidise ammonium nitrogen (NH_4-N) in the presence of free cyanide (CN^-) under halophilic conditions. Isolates I, H and G were identified using the 16S rDNA gene and were identified to be *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively. Furthermore, Response Surface Methodology was used to optimise the physicochemical conditions suitable for the proliferation of the isolates for free-cyanide degradation, nitrification and aerobic denitrification.

The optimum conditions for microbial growth, as determined by Response Surface Methodology, were found to be 400 mg CN^-/L, 65.9 mg NH_4-N /L and 4.5% NaCl, respectively, for all the tested strains. Operational parameters, that is, pH and temperature, for total nitrogen oxidation in the presence CN^- and NaCl were studied, and the results showed that *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. growth improved at 34 ºC. Moreover, the maximum total nitrogen and chemical oxygen demand (COD) reduction was observed at pH 7 for all the strains, with a maximum NH_4-N reduction of 70.5, 73.3 and 61.6% for *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively. Therefore, a pH of 7 and temperature of 34 ºC were selected as optimum conditions for total nitrogen removal experiments, for which 81% (*Enterobacter* sp.), 71% (*Serratia* sp.), and 75% (*Yersinia* sp.), of 400 mg NH_4-N/L was oxidised within 72 h, with the rates of biodegradation being suitably

described by a first-order reaction. The rate constants (*k*) were observed to be: 4.2 h^{-1} for *Enterobacter* sp., 3.8 h^{-1} for *Serratia* sp. and 4.2 h^{-1} for *Yersinia* sp., respectively, with the model having correlation coefficients (R^2) ranging from 0.82 to 0.89, when compared with experimental data. Overall, isolates *Enterobacter* sp., *Serratia* sp. and *Yersinia* sp. were found to be capable of simultaneous nitrification and aerobic denitrification under both cyanogenic and halophilic conditions.

The *Serratia* sp. was then selected to study the effect of different carbon sources on nitrogen oxidation and the application of a Membrane Aerated Biofilm Reactor (MABR) in nitrification and aerobic denitrification. This decision was based on the novelty of this strain in this particular study, as there are limited studies associated with this particular strain for simultaneous nitrification and aerobic denitrification under cyanogenic conditions. Furthermore, there are limited reports on the cyanide degrading potential and tolerance to high salinity conditions by *Serratia* sp. demonstrated by cyanide degradation, including high salinity tolerance compared with *Enterobacter* sp. and *Yersinia* sp. Several carbon sources, succinate, glucose and yeast extract, were used as sole carbon sources for nitrogen removal by *Serratia* sp. in batch reactors, in the presence of 100 mg CN^-/L.

The *Serratia* sp. strain was able to nitrify 74%, 68% and 24% of 400 mg NH_4-N/L within 120 h, when succinate, glucose and yeast extracts were utilised as carbon sources, respectively. There was an observed trace accumulation of both NO_2-N and NO_3-N within the media. Additionally, succinate was found to be the suitable carbon source for nitrogen oxidation by *Serratia* sp.; hence, it was selected as the sole carbon source in the continuous oxidation of ammonium in a MABR system, in which 97% of 610 mg NH_4^+-N/L was continuously removed within 96 h, in the presence of 100 mg CN^-/L. The maximum concentrations of nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N) in the effluent of the MABR system between 96 to 120 h were observed to be 13 mg NO_2-N/L and 32 mg NO_3-N/L, respectively. The concentrations of NO_2-N and NO_3-N further decreased between 120 and 192 h, an indication that suggested that the MABR was stabilising. Moreover, ammonia monooxygenase (AMO), nitrate reductase (NR) and nitrite reductase (NiR) enzyme activity were assessed to confirm nitrification and subsequent denitrification. These enzymes demonstrated that they were successfully expressed in the MABR system, thus confirming the nitrification and aerobic denitrification potential of *Serratia* sp. using a MABR system. Therefore, this study demonstrated the potential of the *Serratia* sp. used to nitrify and denitrify aerobically under saline and cyanogenic conditions.

DEDICATION

Challenging work requires self-discipline as well as guidance of elders, especially those who are close to our hearts. I dedicate this thesis to my sweet and loving mother and my late father

Thobeka Florence Mpongwana (maMthembu)
and
Ntozikabawo Mpongwana (uBhukula, Ukhwanase)

Your affection, love, encouragement and prayers gave me strength to be able to accomplish things that I have always wanted to achieve.

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RESEARCH OUTPUTS

The following outputs are the contributions made by the candidate towards scientific knowledge during her master's candidacy:

Posters

Mpongwana, N & Ntwampe, S. (2015). Single-stage nitrification-aerobic denitrification in a continuous membrane aerated biofilm reactor for wastewater containing cyanide. 4th YWP-ZA Biennial, & 1st African IWA YWP Conference, 16–18 November, Pretoria, South Africa.

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Publications

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LAYOUT OF THESIS

The aim of this study was to isolate microorganisms that are able to perform nitrification and aerobic denitrification and further use these microorganisms for the oxidation of total nitrogen under high cyanogenic and halophilic conditions. This thesis is divided into the following chapters:

- **Chapter 1**: Introduction. This chapter provides the background information about total nitrogen removal and the biology behind inhibition of nitrification and denitrification. Furthermore, it provides a problem statement, hypothesis, objectives, the significance of and delineation of the study.
- **Chapter 2**: Literature review. In this chapter, different methods for nitrogen removal and their disadvantage are reviewed, including biological methods such as nitrification and denitrification, which are normally used in wastewater treatment plants as separate processes. Moreover, the chemistry, process parameters and factors that may lead to the inhibition of both nitrification and denitrification are also discussed in this chapter.
- **Chapter 3**: Materials and methods. This chapter lists materials, methods, and equipment used in this study to determine oxidation of total nitrogen, isolation of strains, and the bioreactor designed, including its operation.
- **Chapter 4**: This chapter comprises the results and discussion of the experiments.
- **Chapter 5**: This chapter presents the overall conclusions and also provides answers to research questions in Chapter 1. Recommendations for future research are also listed in this chapter.

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LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
r	Specific substrate uptake rate	mg/L.h
K_{IS}	Substrate inhibition constant	mg/L
r_{max}	Maximum specific substrate uptake rate	mg/L.h
K_I	Non-competitive inhibition constant	mg/L
S	Substrate concentration	mg/L
K_{IA}	Aiba inhibition constant	mg/L
K_S	Half-saturation constant	mg/L
K_{IL}	Loung constant	mg/L
R^2	Correlation coefficient	-

GLOSSARY

Abbreviation	Description
AMO	Ammonia monooxygenase
AnAOB	Anoxic ammonium-oxidising bacteria
ANR	Anaerobic regulation of arginine
AOB	Ammonia-oxidising bacteria
BC	Batch culture
CCD	Central composite design
CDB	Cyanide degrading bacteria
COB	Cyanide oxidising bacteria
COD	Chemical oxygen demand
DO	Dissolved oxygen
DNR	Dissimilative nitrate respiration regulator
ED	Electrodialysis
FA	Free ammonium
F-CN	Free cyanide
HAO	Hydroxylamine oxidoreductase
HCN	Hydrogen cyanide
Hist-gate	Distal histidine gate
His-Fe^{2+}-His	Heme iron atom of the heme protein
IE	Ion exchange
KCN	Potassium cyanide
MAP	Magnesium ammonium phosphate
NaR	Nitrate reductase
NiR	Nitrite reductase
NOB	Nitrite-oxidising bacteria
NoR	Nitric oxide reductase
NoS	Nitrous oxide reductase
NH_4-N	Ammonium nitrogen
NO_3^--N	Nitrate nitrogen
NO_2^--N	Nitrite nitrogen
NXR	Nitrite oxidoreductase
PCR	Polymerase chain reaction
PN	Partial nitrification
RO	Reverse osmosis

RSM	Response surface methodology
SND	Simultaneous nitrification denitrification
N-fixing	Nitrogen fixing
TN	Total nitrogen
WWTP	Wastewater treatment plant

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Background

Reactive nitrogen can be produced in a number of processes such as the Haber process, cultivation of nitrogen-fixing (N-fixing) crops and combustion of fossil fuels. These processes constitute about 45% of global nitrogen production, resulting in surface water containing high concentrations of total nitrogen (TN) (Warneke et al., 2011). The removal of TN from surface and ground water has evoked interest from water professionals in developing remedial interventions, since such waters are a major source of municipal water supply for domestic and industrial use. The use of fertilisers and nitrogen-containing compounds may also increase the concentration of nitrates (NO_3^--N) in drinking water, which is thought to be the reason for methaemoglobinaemia and various kinds of human cancers (Han et al., 2014). High concentrations of TN in discharged wastewater as a result of inadequate treatment can also cause eutrophication into the receiving waters (Zhou et al., 2014). TN is normally removed from wastewater treatment plants (WWTP) through autotrophic nitrification and denitrification (Chen et al., 2014).

Different parameters, including environmental conditions, can affect nitrification, thereby decreasing the quality of treated water. These parameters include dissolved oxygen (DO) and other contaminants present in the wastewater, such as the temperature, pH, etc. Shoda and Ishikawa (2014) showed that aeration rates, agitation, temperature, and high salinity can decrease DO concentration, resulting in the decrease in nitrification efficiency, thus demonstrating the impact that such parameters have on nitrification and denitrification rates. Contaminants that can negatively affect nitrification include acrylonitrile, salt, acrylic acid, acetonitrile and cyanide. However, Han et al. (2014) reported that free cyanide (F-CN) is a pollutant that largely inhibits nitrification. This was demonstrated in a study in which nitrification was inhibited by the presence of F-CN, although nitrification recovered by up to 44% within 10 days. This meant that the presence of CN^- did not deactivate the microorganisms but inhibited the nitrification pathway. Furthermore, Kim et al. (2011) also reported total inhibition of a nitrification process by the presence of cyanide in a coking wastewater treatment facility.

CN^- has a tendency to act as a ligand as it is able to form a variety of complexes with different metals such as cadmium (Cd), nickel (Ni) and zinc (Zn), for which the stability of the complexes is highly dependent on the oxidation state of the metal (Wild et al., 1994).

Similarly, CN^- can inhibit nitrification by acting as an exogenous ligand to the heme iron atom of the heme protein (His-Fe^{2+}-His) with exogenous ligands competing to bind to the heme iron atom. The process of binding by the exogenous ligands occurs in a three-step process: (1) the ionic exchange of the endogenous ligand, followed by (2) the formation of a reactive pentacoordinated species subsequent to (3) the binding of the external ligand (De Sanctis et al., 2006).

His-Fe^{2+}-His has several functions, such as the mediation of redox processes, including respiration. This is important for bacteria, as they require respiration for the reduction of almost all available compounds in wastewater. Another important role of this protein is to generate signals to the heme sensor proteins in order to bind specifically to dioxygen (O_2), nitric acid (NO) and carbon monoxide (CO); this is how the His-Fe^{2+}-His receives and transmits signals to the protein domain (Mayfield et al., 2011). Myoglobin is one of the proteins from the family of heme proteins that can bind reversibly to diatomic ligands such as O_2, NO and CO (Nienhaus & Nienhaus, 2011). The binding of these exogenous ligands directs the functioning of this protein. An example is when a heme sensor protein binds to NO; these NO-sensing domains belong to bacterial transcription factors, i.e., dissimilative nitrate respiration regulator (DNR).

DNR was found to regulate the denitrification pathway of *Pseudomonas aeruginosa*. The binding of these exogenous ligands is believed to occur through the distal histidine gate (His-gate), with axial water molecules having been shown to have a role in the opening of the His-gate. However, other substrates, such as alkyl isocyanides and butyl isocyanides, can also open the His-gate (Lin & Wang, 2013). De Sanctis et al. (2006) reported the binding of CN^- to the heme protein (haemoglobin 1) of *Drosophila melanogaster.* The binding of CN^- to myoglobin restricts the binding of NO, thereby inhibiting nitrification/denitrification.

1.2 Research problem

Cyanides (CN^-) are toxic compounds that bind irreversibly to heme proteins, thus resulting in the inactivation of microbial growth. Despite their toxicity, CN^- are commonly used in a number of industries, resulting in wastewater containing high concentrations of residual CN^- (Dash et al., 2006) which end up in local municipal sewage/domestic wastewater treatment facilities. This results in the inactivation of the activated sludge, which leads to poor wastewater treatment efficiencies, as the activated sludge microorganisms are sensitive to the presence of cyanide. The presence of CN^- will further affect the quality of the discharged effluent into local receiving water sources such as rivers, perpetuating environmental degradation downstream of the wastewater treatment plant. Overall, simple soluble CN^- from

industrial wastewater sources are simply being mixed with domestic wastewater, owing to the lack of monitoring in place in South Africa – a problem which affects municipal domestic wastewater treatment plants. This requires advanced biological wastewater treatment plants in which CN^- tolerant and degrading bacteria are utilised.

1.3 Hypothesis

Nitrification and aerobic denitrification can be achieved in a single stage when microorganisms that can tolerate CN^- and high salinity conditions are used.

1.4 Research aims and objectives

The experiments were divided into three phases: Phase 1 (Aim 1): Isolation, characterisation and identification of suitable strains; Phase 2 (Aim 2): Nitrification subsequent to denitrification under high CN^- and salinity conditions; and Phase 3 (Aim 3): Evaluation of the viability of a continuous membrane aerated biofilm reactor in simultaneous nitrification and denitrification. All the phases had their individual objectives, as shown below:

Phase 1: Aim 1: Isolation, identification and characterisation of strains with an ability to nitrify and denitrify in a single-step process. To achieve this aim, the study focused on the following objectives:

Objective 1: Isolate and identify nitrifying/denitrifying bacteria from a halophilic environment that has wastewater containing high concentrations of nitrogenous compounds.
Objective 2: Assess the ability of the isolates to grow under high CN^- and salinity concentrations, focusing on novel isolates not reported to nitrify and denitrify in a single-step process.

Phase 2: Aim 2: Nitrification subsequent to denitrification under high CN^- and salinity conditions. To achieve this aim, this part of the study focused on the following objectives:

Objective 1: Identify suitable/optimum physicochemical conditions, i.e., pH and temperature, for the maximum treatment of saline CN^- containing wastewater, using response surface methodology.
Objective 2: Study the CN^- and nitrogenous compounds degradation efficiency of the isolates.
Objective 3: Study the effect of CN^-, nitrogenous compound concentrations and salinity on microbial growth, focusing on operational parameters on ammonium-nitrogen degradation in closed systems, i.e., batch cultures.
Objective 4: Assess nitrification and denitrification efficiency under high CN^- and salinity conditions.

Phase 3: Aim 3: Evaluation of the viability of a continuous membrane aerated biofilm reactor in simultaneous nitrification and denitrification. To achieve this aim, this part of the study focused on the following objectives:

Objective 1: Select the best performing and/or novel isolate (which has not been previously reported to be able to nitrify and denitrify in a single step process, and use it to,

Objective 2: Study the effect of several preselected carbon sources on the isolate to nitrification and subsequent denitrification, in order to,

Objective 3: Assess the feasibility of an open system, i.e., continuous membrane aerated biofilm reactors (MABRs), in a single-step nitrification and subsequent denitrification process, and

Objective 4: To analyse enzyme activity for nitrate reductase (NaR), nitrite reductase (NiR) and ammonia monooxygenase (AMO) in immobilised biofilms in the MABRs designed.

1.6 Significance of study

This study investigated nitrification/aerobic denitrification in CN^- containing bioreactors. This study therefore, provided knowledge and alternative strategies to overcome the inhibitory effect of CN^- in nitrification/aerobic denitrification to ensure effective removal of TN in municipal wastewater, even under halophilic conditions.

1.7 Delineation of study

The following were not considered in this study:

- The application of the isolated strains in pilot and/or large-scale bioreactors.
- The effect of the isolated strain on indigenous microorganims normally found in sludge used in wastewater treatment plants.
- The effect of dissolved oxygen (DO) on the performance of the isolates used, both in closed (batch cultures) and open (MABRs) systems, as in these systems DO limitations are normally minimal.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2
LITERATURE REVIEW

Contamination of wastewater by nitrogenous compounds has become a global problem due to the extensive use of nitrogen fertilisers, animal faeces, industrial effluent and general human wastes. The increase of these compounds in the environment may result in eutrophication (Feng et al., 2003). In local river water, nitrates can also be reduced to nitrites, and as indigenous people drink such water, the ingested nitrates will combine with haemoglobin, forming methaemoglobin, which is fatal in human beings. Although this condition mostly affects infants under the age of six months, it can result in brain damage and even death in adult humans (Lee et al., 2006). Owing to these health risks, several methods have been designed in an attempt to reduce the levels of nitrogen-based compounds in wastewater (Li et al., 2009). These methods comprise biological and chemical treatment methods using ozone, chlorides, and coagulants, including electrochemical treatments.

2.1 Chemical precipitation of ammonium nitrogen

Ammonium nitrogen precipitation by magnesium ammonium phosphate was discovered in the early 1990s in Los Angeles at the Hyperion wastewater treatment plant when a white crystalline solid precipitate was discovered during wastewater treatment. This residue was examined and it was found to be an inorganic mineral, magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$), commonly known as struvite (Stratful et al., 2001). Struvite crystallisation has been observed in bovine manure wastewater, coking wastewater, leather tanning wastewater and swine wastewater (Lee et al., 2003). In this process, ammonium nitrogen is removed by the addition of magnesium and/or phosphate salt, to form magnesium ammonium phosphate hexahydrate (MAP). MAP is a white crystaline substance that contains concentrations of magnesium, ammonium and phosphorus. The chemical reaction is expressed in Eq. 2.1 (Zhang et al., 2009).

$$Mg^{2+} + NH_4^+ + PO_4^3 + 6H_2O \leftrightarrow MgNH_4PO_4 6H_2O \qquad (2.1)$$

This method has been studied for the treatment of high-strength ammonium nitrogen wastewater. Iron and aluminium are the most suitable metals that can be used for precipitation and may be added as chloride or sulphate salts. Lime can also be used for the precipitation of nitrogenous contaminants in wastewater. Chemical precipitation is a flexible approach that can be used for the removal of several wastewater contaminants, with its application being done in several stages during wastewater treatment (Morse et al., 1998).

However, struvite crystallisation is restricted, as magnesium tends to be low compared to ammonium nitrogen concentrations in many wastewaters, and the cost of adding magnesium salts increases operational cost (Lee et al., 2003). Moreover, this process involves the addition of a high quantity of inorganic salts, resulting in the disposal of wastewater containing unreacted chemicals (Feng et al., 2003).

2.2 Ammonium-nitrogen stripping

Ammonium-nitrogen striping is a similar process to that used for the removal of gases from water by aeration (Reeves et al., 1972). Ammonium nitrogen can exist as either ammonium or ammonia and hydrogen ions, depending on the pH of the wastewater. When the pH of the wastewater increases above 7.0, it exists in a form of NH_4^+ and if the pH increases above 10.0, it will exist as ammonia and hydrogen ions – see Eq. 2.2 and 2.3. More than 85% of ammonia/ammonium present in wastewater can be liberated into the atmosphere through agitation or sparging of the wastewater; it can therefore be absorbed from the air using a strong acid solution resulting in an ammonium-salt precipitate which can be crystallised (Bonmati & Fotats, 2003; Guštin & Marinšek-Logar, 2011). A stripping tower is used for this process, for which the operational aspects are as follows: wastewater is pumped to the top of the tower, trickles down through packing material with air being blown in the opposite direction of the wastewater resulting in the liberation of ammonia to the air (Liao et al., 1995), subsequent to the precipitation in an acid solution.

$$\mathrm{NH_4^+ + OH + NH_3OH \leftrightarrow NH_3 + H^+} \quad (2.2)$$

A complete equation is as follows:

$$\mathrm{NH_4^+ + OH + NH_3OH \rightarrow NH_3 + H_2O} \quad (2.3)$$

When the pH exceeds 10, the excess hydroxyl ions convert the ammonium ion into ammonium hydroxide, which can be removed by sparging. This can be done in a packed tray that has an air blower. Ammonium stripping is used in wastewater containing low chemical oxygen demand to total nitrogen (COD/TN) ratio (1:3), since low COD/TN ratio is not sufficient to facilitate efficient TN removal by nitrification and denitrification. This process requires wastewater with a COD/TN ratio of up to 4:5 (Lei et al., 2007). Although this process is easy and inexpensive, it requires a stripping tower; therefore, it will add to some initial construction costs with operational and maintenance costs being incurred for the life span of the facility (Liao et al., 1995).

2.3 Electrochemical conversion

This technology involves the reduction of the nitrate ions to nitrite and finally to nitrogen gas on a suitable cathode surface (Li, Feng, Zhang, Lei et al*.,* 2009; Mook et al., 2012). Figure 2-1 illustrates an electrochemical cell that can be used for the conversion of nitrogen-based compounds.

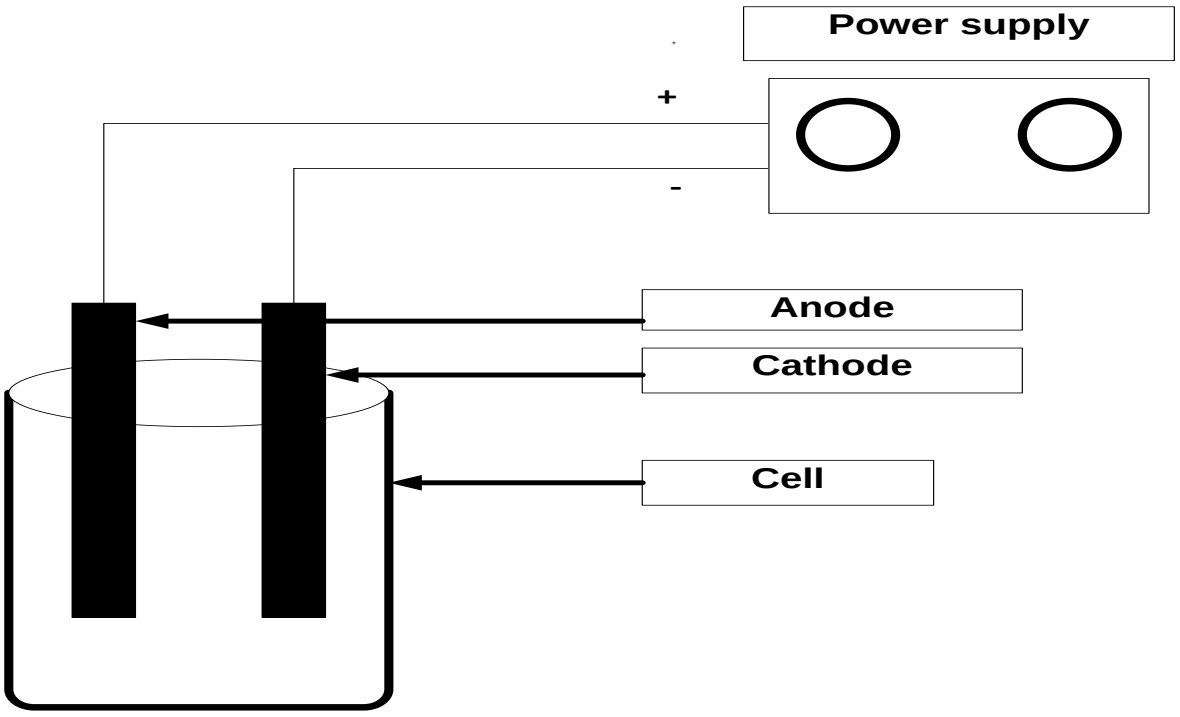


Figure 2-1: Schematic diagram of an electrochemical cell (Li, Feng, Zhang & Sigiura, 2009)

The reduction of nitrogenous contaminants in wastewater using an electrochemical reduction method results in a broad spectrum of by-products. For example, nitrates are reduced at the cathode to nitrite, ammonia and nitrogen gas, which is electrochemically inactive. However, the application of this technology is limited, owing to some unfavourable by-products produced and high-energy requirements (Li, Feng, Zhang & Sugiura, 2009).

2.4 Physicochemical treatment

Physicochemical treatment processes include ion-exchange (IE), reverse osmosis (RO), electro dialysis (ED) and activated carbon adsorption (Paraskeva & Diamadupoulos, 2006). Reverse osmosis is commonly used to treat organic chemicals in brackish water and wastewater because of its high permeability of selective ions and low production cost; it is also environmental friendly. However, RO has high-energy requirements. This results in

increased operational cost. Efforts have been made to overcome this problem by the use of solar systems and wind- driven RO systems. Nevertheless, this technique still has some limitations, as the waste stream for the process will still require further treatment since other contaminants such as nitrates accumulate in the brine stream (Mook et al., 2012).

Alternatively, ion exchange can be used, as it functions by exchanging chloride ions with anions that flow out of the system. If contaminated water is passed through the resin beads, chloride ions bind to functional groups in the ion-exchange resin. Resin beads are normally produced using a sodium chloride solution. However, this is a complicated method since anions have more affinity to the resin than chloride ions. This increases operational costs, as additional treatment steps are needed in order for the anions to be efficiently exchanged before the resultant solution/wastewater is discharged to the environment.

Similarly, electrodialysis (ED) is also used for the desalination of brackish water to produce potable water. The advantage of this technique is that minimal feed pre-treatment is required since membrane fouling and scaling is minimised by using a reverse polarity operation in such systems. Furthermore, a higher brine concentration is also produced in ED. However, ED only removes ions and no other harmful microorganisms or organics. ED also requires high-energy input during processing of solutions with high salt concentrations.

2.5 Biological removal of ammonium nitrogen

As previously discussed, accumulation of nitrogen-based compounds in domestic water, including industrial wastewater, can result in ecological degradation and deterioration of drinking, industrial, agricultural and recreational water quality (Li et al., 2014). Hence, treatment of such wastewater has become a core activity for wastewater treatment plants (WWTP), with a focus on biological treatment, which is a common, efficient and cost-effective way to remove TN in wastewater (Hibiya et al., 2003). This technology uses two sequential processes, nitrification and denitrification (Yao et al., 2013; Pal et al., 2015). These processes use microorganisms that convert nitrogenous compounds into nitrogen gas (Yang et al., 2012). Nitrifying bacteria are autotrophic and they oxidise ammonia or nitrite for their energy, while denitrification bacteria oxidise nitrification by-products.

2.5.1 Nitrification

Nitrification is defined as microbial oxidation of reduced inorganic nitrogen compounds and it plays a crucial role in the reduction of TN in the wastewater, since the disposal of water containing TN to aquatic systems can result in eutrophication and various environmental problems (Chen et al., 2014). This process is performed by certain microorganisms that have the ability to oxidise NH_4-N and NO_2-N. These microorganisms are known as ammonium-

oxidising and nitrite-oxidising bacteria (AOB and NOB). This method has been extensively used for removal of ammonium nitrogen in WWTP (Shoda & Ishikawa, 2014). Nitrification is a two-step process carried out by AOB, and the first step in this process is the oxidation of ammonium to nitrite, a process classified as nitritation.

2.5.2 Nitritation

In most cases wastewater from different industries contains low carbon to nitrogen ratios and high concentrations of ammonium nitrogen. An example is wastewater from the fertiliser, explosives and pharmaceutical industries. A feasible treatment method of this kind of wastewater is a combination of partial nitritation (PN) subsequent to ammonia oxidation (anammox) (Dosta et al., 2008). Anammox is a process whereby anammox bacteria oxidise the ammonium nitrogen to nitrogen gas, in anoxic conditions, while fixing carbon dioxide for growth. Nitritation is required for pre-treatment of wastewater prior to anammox (Yamamoto et al., 2008). In previous years it was thought that ammonium-nitrogen removal through nitrification was the only way to remove nitrogen-based compounds from wastewater until other mechanisms for nitrification were discovered via the nitrite route, instead of nitrification via the nitrate route (Egli et al., 2003). The stoichiometry of nitritation is as follow – Eq. 2.4 (Mousavi et al., 2014):

$$\mathrm{NH_4^+} + \frac{3}{2}\mathrm{O_2} \rightarrow \mathrm{NO_2^-} + \mathrm{H^+} + \mathrm{H_2O} \qquad (2.4)$$

This step is also known as partial nitrification (PN) and is known to be heavily inhibited by free ammonium (FA) and free nitrous acid. However, most studies have reported that high concentration of FA and HNO_2 inhibit nitrification. Blackburne et al. (2007) investigated the inhibition effect of FA and HNO_2 on *Nitrobacter* sp. and *Nitrospira* sp. and found that *Nitrospira* sp. is more sensitive to low concentration of FA than *Nitrobacter* sp.; moreover, NOB are inhibited by ammonium-nitrogen concentration higher than 0.1 to 1 mg NH_3/L and a concentration higher than 0.2 to 2.8 mg HNO_2/L.

2.5.3 Nitratation

This is a second step of nitrification in which anoxic ammonium oxidising bacteria (anAOB) convert the by-product of nitritation, which is nitrite, to nitrate; however, oxidation of nitrite to nitrate decreases the efficiency of TN removal; hence, sufficient dissolved oxygen is required for successful nitritation, particularly in large-scale WWTP. The stoichiometry of nitratation is as follows – Eq. 2.5 (Mousavi et al., 2014):

$$\mathrm{NO_2^-} + \frac{1}{2}\mathrm{O_2} \rightarrow \mathrm{NO_3^-} \qquad (2.5)$$

2.6 Inhibition of nitrification

Nitrification can be carried out by a few genera often known as *Nitrosoma* and *Nitrobacter*. The effectiveness of nitrification relies in the ability of the nitrifying organisms to oxidise the ammonium nitrogen to nitrate. Additionally, factors such as adequate dissolved oxygen and the minimal presence of inhibitory compounds are required for successful nitrification (Anthonisen et al., 1976). Since these genera have slow growth, it makes nitrification prone to inhibition, thus influent in WWTP must not contain toxic compounds, because a shock load of toxicants can be harmful to the nitrification process with its recovery taking several weeks. Similarly, sustained toxicant load can reduce nitrification capacity; additionally, nitrification can also be inhibited by excessively saline wastewater, including substrates. The inhibition of nitrification by the availability of growth-enhancing substrates can be described by the Haldane kinetic model. See Eq. 2.6 (Carrera et al., 2004; Han et al., 2014).

$$r = \frac{r_{max} S}{K_S + S + \frac{S^2}{K_{IS}}} \quad (2.6)$$

If the substrate is not sufficient to induce inhibition, the process can be simply described by the Monod equation (Eq. 2.7):

$$r = \frac{r_{maxS}}{K_s + S} \quad (2.7)$$

Furthermore, other contaminants than the essential substrates can also inhibit nitrification. Examples of these are fluoride and cyanide. This process can be described by the following kinetic equation (Eq. 2.8):

$$r = \frac{r_{max} S}{K_s + S} \frac{K_1}{1 + K_1} \quad (2.8)$$

There are several other models that can be used to describe nitrification inhibition such as the model proposed by Aiba et al. (1986) – Eq. 2.9:

$$r = \frac{r_{max} S}{K_s + S} \, exp(K_{IL} I)^n, \quad (2.9)$$

and a model proposed by Luong (1987) – Eq. 2.10:

$$r = \frac{r_{max} S}{K_s + S} \, (1 - (\frac{1}{K_{IL}})^n) \quad (2.10)$$

Other inhibitors are listed in Table 2.1.

Table 2-1: Example of inhibitors that inactivate ammonia monooxygenase (AMO) responsible for nitrification (Ruser & Schulz, 2015)

Mode of action Inhibitors	Examples	Comments
Competitive vs NH_3	Methane, ethylene, carbon monoxide	Alternative substrate
Non-competitive vs NH_3	Methane, propane, *n*-butane, chloromethane, chloroethane, bromomethane, iodoethane, nitrapyrine	Alternative substrate
Metal chelators	Thiourea, carbon disulfide, potassium-cyanide, Dicyandiamide, nitrapyrin, 3,4-Dimethylpyrazole phosphate	Cu-selective
Mechanism-based inhibitors	Acetylene, Allyl sulfide, *p*-anisidinenitrapyrin (weak effect)	Requires enzyme turnover with O_2

2.7 Inhibition mechanisms of nitrification

The first mechanism of inhibition involves the interaction through the direct binding to ammonia monooxygenase (AMO), which is responsible for nitrification. In this mechanism of inhibition, compounds either bind directly to the active site of an enzyme which catalyses the oxidation of ammonium nitrogen or they can either bind to the second site that is not involved in the oxidation of ammonium nitrogen (Ruser & Schulz 2015). The second inhibition mechanism is through removal of nitrification co-factors by the chelation of such compounds; with numerous studies showing that the availability of a Cu co-factor of AMO affects the oxidation of ammonium nitrogen, i.e., the availability of Cu chelators will affect the activity of AMO, whereas the addition of Cu salts stimulates NH_3 oxidations. The third inhibition mechanism involves substrate oxidation that results in the substrate being highly reactive, which in turn inactivates the AMO and other enzymes. In this inhibition mechanism, enzymes are activated through the covalent modification of the by-products of catalysis, resulting in covalent modification of proteins (Ruser & Schulz, 2015), which in turn affects cellular proteins responsible for the nitrification resulting in inhibition. Figure 2-2 and 2-3 illustrate the profile, i.e., accumulation, formation and consumption of nitrogenous compounds during nitrification when there is minimal inhibition and in the presence of an inhibitor, respectively.

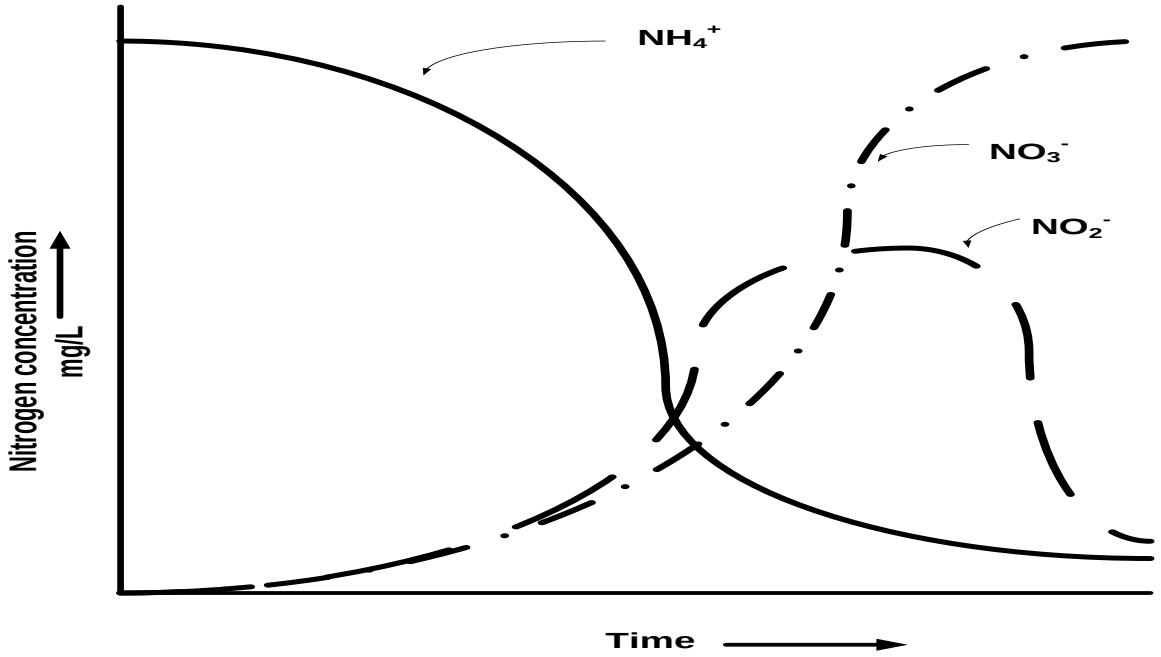


Figure 2-1: Schematic diagram representing formation of nitrogenous compounds during nitrification when there is minimal inhibition (Anthonisen et al., 1976)

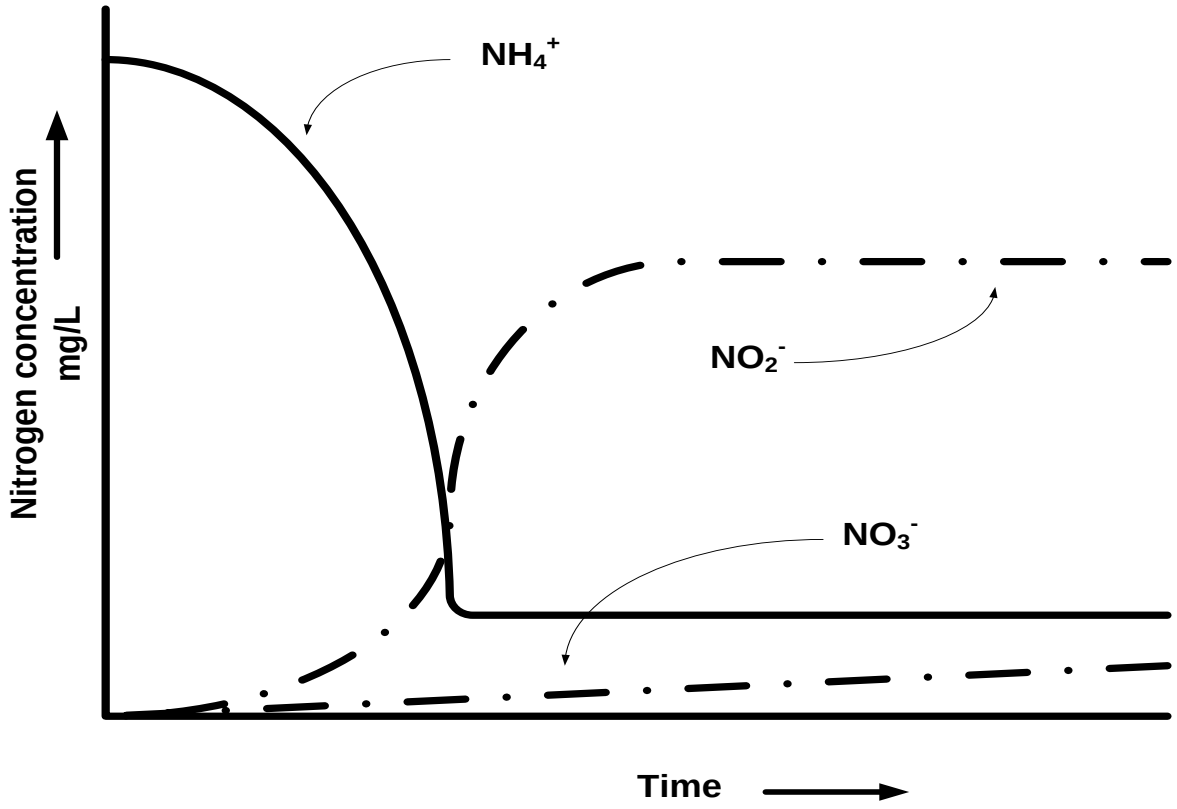


Figure 2-2: Schematic diagram representing nitrogenous compound formation during nitrification in the presence of an inhibitor (Anthonisen et al., 1976)

2.8 Nitrification inhibition by salinity

Nitrification has been practically applied in WWTP; influent may also contain additional ions such as salt ions from various industries. Furthermore, the utilisation of seawater in coastal areas for lavatory flushing may also introduce significant quantities of dissolved salts into a WWTP. Salinity is a major problem in most WWTP, as high concentrations of salt may inhibit the survival, growth and reproduction of organisms, resulting in difficulty in carrying out biological processes performed in WWTP (Cortés-Lorenzo *et al*., 2015; Zhang *et al*., 2015). Reduction of salt in the wastewater prior to biological processes with technologies such as reverse osmosis, ion exchange or electrodialysis is costly. High salt content above 1% (m/m) can result in the disintegration of cells, since the loss of cellular water or recession of the cellular cytoplasm activated by osmotic differences across the cell wall can result in outward flow of intracellular water, lowering microbial activity causing cell dehydration. This may result in low ammonium nitrogen removal (Abou-Elela et al., 2010).

Additionally, phosphorus removal is also inhibited by high salt concentrations in such systems. Nitrification has been found to be more sensitive to influent with high salinity. Some scientists have proposed the use of halophilic organisms for TN removal (Campos et al., 2002), which are adapted to saline conditions. This may reduce the effect of salt stress on bacterial metabolism (Sadurno et al., 2011), resulting in a suitable, and thus appropriate removal response.

2.9 Nitrification inhibition by cyanide

Free cyanide (CN^-) is a carbon-nitrogen radical broadly found in a number of inorganic compounds. Large quantities of CN^- are produced during the extraction of metal ores, in photographic processes and the manufacture of synthetic fibres, and in organic chemical production and steel making (Chen et al., 2008; Özel et al., 2010). CN^- production from these industries is estimated to be 14 million kg/year and because of its toxicity to the environment and to the microbial community used in WWTP, it has to be detoxified in wastewater prior to biological treatment (Ebbs, 2004; Gupta et al., 2012). Commonly used methods for detoxification of simple CN^- include alkaline chlorination; this method is an effective method in treating free CN^-. However, it can result in the production of toxic intermediates (Cui et al., 2014), particularly chlorination by-products which are highly oxidative and thus unsuitable in biological processes.

Other methods such as photo-oxidation, oxidation by ozone and use of inorganic acids, oxidation by SO_2/air, biological degradation, electrochemical processes and adsorption on activated carbon (Yeddou et al., 2011) can also be used to treat CN^- to less toxic compounds (Ebbs, 2004). During the treatment of CN^-, ammonium nitrogen can accumulate as a result of CN^- degradation and other sources in wastewater. The accumulation of this nitrogenous compound in aquatic ecosystems can also result in environmental deterioration as previously discussed, and thus its removal from wastewater has always been the core function of WWTP (Chen et al., 2014).

2.10 Cyanide assimilation in nitrification

Cyanide has been reported as a contaminant that has a higher inhibition effect to nitrification processes (Han et al., 2014). This compound inactivates the respiratory system of a living microorganism by binding to terminal oxidases. However, research findings have shown that some microorganisms are capable of carrying out their metabolic functions even in CN^- concentration higher than 1 mM. These microorganisms have been shown to have the ability to degrade cyanide to a less toxic form to overcome inhibitory stress caused by this compound (Chen et al., 2008). Cyanide is also found in environmental matrices in its natural state, i.e., as simple CN^- and metallic complexes of cyanide. These compounds are released

into domestic wastewater from different industries, i.e., electroplating, mining, photography, jewellery making, coking plants, and steel (Gupta et al., 2012).

2.10.1 Mechanism of biological degradation of cyanide

This process is a process whereby toxic compounds are hydrolysed into simple and non-toxic compounds. Cyanide can be also degraded into simpler and non-toxic compounds through the activity of bacteria, fungi, algae and plants. Although all these organisms are capable of detoxifying cyanide, bacterial detoxification is safer, quicker and cheaper than chemical treatment. Under aerobic conditions, cyanide oxidising bacteria (COB) break down the cyanide into cyanate (Naveen et al., 2011) – see Eq. 2.11:

$$2CN^- + O_2 \xrightarrow{\text{enzyme}} 2CNO^- \quad (2.11)$$

The cyanate is then hydrolysed into ammonium nitrogen and carbon dioxide as shown in Eq. 2.12:

$$CNO^- + H_2O_) \rightarrow NH_4^+ + CO_2^- \quad (2.12)$$

Under anaerobic conditions cyanide degradation only occurs in the presence of HS_2 or H_2S. The presence of sulfur species is dependent on the pH of the wastewater. HS_2 is dominant when the pH is greater than 7 (Eq. 2.13), while H_2S is dominant at a lower pH (Eq. 2.14).

$$CN_2 + H_2S_3 \rightarrow HCNS + H_2 \text{ (High pH)} \quad (2.13)$$

$$HCN + HS_2 \rightarrow HCNS + H_2 \text{ (Low pH)} \quad (2.14)$$

Anaerobic CN^- degradation is slower than aerobic CN^- degradation since anaerobic bacteria have a cyanide toxicity threshold of 2mg/L as opposed to aerobic bacteria that have a toxicity threshold of 200 mg/L; hence, anaerobic biodegradation is less effective than aerobic biodegradation (Dwivedi et al., 2011), and cannot be applied in large-scale operations.

2.10.2 Selection and characterisation of cyanide-oxidising bacteria

The oxidation state of carbon (C), and nitrogen (N), in cyanide makes the C a less desirable carbon source and N a good nitrogen source for bacterial growth. Owing to the high concentration of CN^- in industrial effluent, diluted residues can be used as a sole nitrogen source when a carbon source and other mineral nutrients such as N, P and K, are available. Microorganisms metabolise cyanide into a bioassimilable product (NH_4^+). Additionally,

microorganisms capable of degrading cyanide must be able to assimilate Fe^{2+} from the medium since Fe^{2+} forms a stable complex with cyanide, which is not bioavailable. Biological treatment of cyanide-containing wastewater requires an alkaline pH to avoid formation of volatile HCN (pK_a = 9.2). Hence, the first step in the biodegradation of cyanide is the selection of microorganisms capable of tolerating and degrading cyanide under alkaline conditions (Igeño et al., 2007).

2.11 Aerobic denitrification

Overall, denitrification is a process whereby bacteria use oxidised by-products of nitrification as electron acceptors. Denitrifying organisms require reduced organic compounds for energy since a majority of them are heterotrophic (Sliekers et al., 2002). As previously mentioned, it was believed that denitrification only occurs under anaerobic conditions until recent studies revealed novel aerobic denitrifying bacteria, capable of oxidising nitrate to nitrous gases under aerobic conditions by performing aerobic respiration through specialised enzymes known as periplasmic nitrite reductases (NaR) which are enzymes that facilitate the conversion of nitrate to nitrite under aerobic conditions. Sensitivity of these enzymes to oxygen can result in low enzyme activity. This in turn reduces the ability of a particular microorganism to reduce nitrite under aerobic conditions. Furthermore, nitrite reductases (NiR), nitric oxide reductases (NoR), and nitrous oxide reductases (NoS) also play a role in the reduction of oxynitrides (Sun et al., 2015).

Conventional ways of nitrogen removal include nitrification by autotrophic nitrifying bacteria subsequent to denitrification by heterotrophic denitrifying bacteria. This makes it difficult to achieve completely anoxic conditions as it is difficult to remove DO completely in such systems, which can result in the suppression of activity of most anaerobic denitrifying bacteria due to the insufficient anaerobiosis. This can further result in the emission of NO and N_2O due to the sensitivity of NO and N_2O reductases to dissolved oxygen (Zheng et al., 2014). There are some microorganisms that are able to carry out these reactions simultaneously. This process is known as simultaneous nitrification denitrification (SND). In contrast to conventional autotrophic nitrification and aerobic denitrification, SND (heterotrophic nitrification and aerobic denitrification) occurs in the same reactor. This is done by combining biochemical characteristics of the microorganisms responsible for SND (Rajesh et al., 2015). Additionally, Zhang et al. (2015) reported simultaneous reduction of NH_4^+ and NO_3^- by *Paracoccus versutus* LYM in an SND medium. Nitrifiers and aerobic denitrifiers are affected by a number of factors, including high salinity, heavy metals, extremes of pH and low temperature (Chen et al., 2014).

2.12 Anaerobic denitrification

Anaerobic denitrification is a respiratory process that allows microorganisms to thrive in low or under minimally dissolved oxygen conditions (Cathrine et al., 2009). These microorganisms can use nitrates as electron acceptors in their respiration processes (Su et al., 2015). In these processes, nitrates are reduced to nitrite, nitric oxide, nitrous oxide and nitrogen as a result of the dissimilatory nitrate reductase (NaR), nitric oxide reductase (NoR), nitrite reductase (NiR), and nitrous oxide reductase (NoS) activity, which catalyses the conversion during denitrification. Table 2-2 highlights some of the physiological parameters of aerobic and anaerobic oxidation of ammonium nitrogen.

Table 2-2: Physiological parameters of aerobic and anaerobic oxidation of ammonium (Ahn, 2006)

Parameters	**Anammox** $NH_4^+ + NO_2^- \rightarrow N_2$	**Nitrification** $NH_4^+ + O_2 \rightarrow NO_2$	**Units**
pH range	6.7–8.3	Variable	-
Temperature range	20–43	≤42	ºC
Free energy	-367	-275	kJ/mol
Biomass yield	0.07	0.08–0.1	g protein/g NH_4-N
Aerobic rate	0	200–600	n/mol/min/mg protein
Anaerobic rate	60	2	n/mol/min/mg protein
Growth rate	0.003	0.04	/h
Doubling time	10.6	0.73	days
$K_{s\ (NH4+)}$	5	5-2,600	µM
$K_{s\ (NO2)}$	<5	n.a.	µM

Table 2-2: ***Cont.:***

Parameters	Anammox $NH_4^+ + NO_2^- \rightarrow N_2$	Nitrification $NH_4^+ + O_2 \rightarrow NO_2$	Units
$K_{s\ (O2)}$	n.a	10-50	µM
NO_2 inhibition of NH_4^+ consumption	$K_i = 0.8/\alpha = 0.8$	Usually	g NO_2-N/L
NO_2 inhibition of NO_2-consumption	$K_i = 1/\alpha = 0.7$	n.a	g NO_2-N/L
Protein content of biomass	0.6	Variable	g protein/g SS
Protein density	50	Variable	g protein/L biomass

2.13 The biology behind nitrification and denitrification

2.13.1 Important enzymes in ammonium-oxidising bacteria

The ammonium-oxidising bacteria are facilitated by enzymes called ammonia monooxygenases (AMO). These enzymes are membrane-bound enzymes of the microorganisms containing Cu as primary co-factor and Fe^+ as secondary co-factor. AMO is coded for by three gene subunits, AMO-C (31.4 kDa), AMO-A (31.4 kDa) and AMO-B (38 kDa). However, a portion of the AMO-A is performed as a gene function of the AOB (Ruser & Schulz, 2015). Synthetic (*n* = 64) compounds, which have been suggested to be nitrification inhibitors by Subbarao et al. (2006), were found to inhibit the first step of nitrification. AMO can be activated by different substrates. Subbarao et al. (2006) further suggested 60 compounds that can act as alternative substrates for AMO. Other nitrification enzymes are called hydroxylamine oxidoreductases (HAO), which were isolated and characterised as enzymes responsible for NO_2^- oxidation and are located in the periplasm. Additionally, these enzymes can contain multi-c-heme and homotrimer subunits coded for by a gene cluster of *hao* which showed highly conserved gene encoding proteins, especially in the β-subdivision (Ge et al., 2015).

2.13.2 Important enzymes in nitrate-oxidising bacteria

Nitrite oxidation is catalysed by nitrite oxidoreductases (NxR). The active form of NxR was first isolated from *Nitrobacter hamburgensis*, which showed an efficiency for the oxidation of nitrite to nitrate in the presence of ferricyanide. NxR is generally composed of 2–3 subunits with iron molybdenum, sulfur and copper. NxR α-subunits, NoR-A, β-subunits including NoR-B, and NoR-A consist of the catalytic site of NoR, whereas NoR-B functions as an electron-

channelling protein between NoR-A and the membrane integrated electron-transport chain (Ge et al., 2015).

2.13.3 Expression of denitrification genes

Nitrogen oxide is used as a substitute for terminal electron acceptor in order to conserve energy for the repertory chain under anaerobic conditions. Expression of denitrification enzymes is likely to occur under conditions with low dissolved oxygen concentrations (Nogales et al., 2002). An analogue of fumarate and nitrate reductase regulator (FNR) protein was suggested to be responsible for the induction of denitrification genes in anoxic conditions. FNR is known to regulate the expression of the genes that are responsible for anaerobic metabolism in *Escherichia coli* (Arai et al., 1995). The structure of FNR is comparable to that of CRP, which is known to regulate catabolite expression and control; however, FNR does not contain amino acid residues that are required for the binding of cyclic AMP. FNR and NaR-L are also necessary for the expression of the narGHJI operon (Hartig & Zumft, 1999). FNR homologues retain four cysteine residues involved in sensing anoxia. The conserved binding motif for the promoter that is regulated by FNR is TTGAT-----ATCAA, at 40bp upstream of the transcriptional starting point. A majority of denitrification genes has been found to possess analogous sequences to the FNR binding motif in their promoter regions (Arai et al., 1995; Bothe et al., 2007). ANR is also known to be responsible for the expression of the denitrification pathway, with the arginine deiminase pathway being responsible for the production of cyanide. Transcription of the NiRS and NoRCB, which are structural genes for nitrite reductase (NiR) and nitric oxide reductase (NoR) respectively, was found to be controlled by ANR. A CPR/FNR-related regulator has been found in the denitrification gene cluster of *P. aeruginosa* (Vollack & Zumft, 2001). Cyclic AMP-binding residues and cysteine residues of FNR are not conserved in DNR, which means the effectors of DNR are different from those of CPR or FNR.

2.13.4 Genes that code for denitrification enzymes

The NO_3^- reductase subunits are coded for by genes of a naarGHJI operon. The organisation of this operon is conserved in most species that possess the ability to express NaR. The structural subunits are coded for by narGHI genes where NaR-J codes for a dedicated chaperon, which is required for the maturation and insertion of NaR to the cellular membrane. In most species, the NaR-K gene, which codes for NO_3^- and NO_2^- transportation, leads the narGHJI genes (Philippot, 2002). A set of NaR-XL genes that code for the two-component regulatory system required to transform the expression of NaR in response to concentrations of NO_3^- or NO_2^-, is present in the upstream region of the NaR gene cluster from denitrifying *Pseudomonads* sp. (Philippot, 2002; Mesa et al., 2004). In α-*Proteobacter*,

narXL genes are substituted by FNR-like genes that code for an FNR homologue designated NaR-R. The function of this particular protein is to regulate transcription and expression of the NaR operon in response to intracellular NO_2^- or NO_3^- concentrations. The majority of the operon that codes for periplasmic NO_3^- reductase (NaP) subunits is composed of napABCD genes.

The structural subunit is coded for by napABC and napD, which have a similar role with the chaperone. NaP operons can also consist of one or more napKEFGH genes in some species; however their organisation varies from species to species (Philippot, 2002; Bueno et al., 2010). The napFG is responsible for an alternative electron transfer pathway to NO_3^- reductase. Research has shown that the distinct operon organisation does not have a phylogenetic pattern of the species from which they originate. This is suggestive of the lateral transfer of the NaP operon. Genes that code for structural monomer of cd1-type NO_2^- reductase NiR-S form part of the nir-gene cluster. The organisation and number of nir genes in these clusters differ in different species. Moreover, they share a number of genes that code for multimeric and multifunctional enzyme complexes which are involved in maturation and insertion of specific heme-d, into NO_2^- reductases (Bothe et al., 2007). The NiR-K gene codes for Cu-type NO_2^- reductases; in some species NiR-K gene clusters and NiR-V genes are located downstream of the protein that have an unknown function. NO-reductase is expressed by the norCBQD operon with norCB genes coding for structural subunits I and II; NoR-Q and NoR-D proteins are important for the activation of NoR. Moreover, species have additional norEF genes involved in maturation and stability of nor activity. N_2O reductases (NaS) coded for by nos gene clusters are highly conserved in denitrifiers, in particular the DFYL genes. The function of NoS-RX is still unknown; however it is assumed that they play an important role in the regulation, activation and Cu assemblage of NoS (Philippot, 2002; Mesa et al., 2004; Bueno et al., 2010).

2.13.5 Control of nitrification and denitrification at genetic level

AOB obtain their energy for growth purposes by oxidising ammonium-nitrogen to NO_2^- in a process known as nitrification, whereas NOB obtain their energy through the oxidation of NO_2^- to nitrate NO_3^-. This process is known as denitrification (Cantera & Stein, 2007). These microorganisms are able to reduce NH_4^+ to NO_2^- and further to NO_3^- through the activity of certain genes. There are two classes of dissimulative nitrite reductases, known as cytochrome-cd type, which are coded by nirS, and the copper-containing type encoded for by nirK (Cantera & Stein, 2007). NOBs are capable of anaerobic growth through the reduction of NO_2^- to N_2 (Giardina et al., 2008).

Denitrification enzymes are expressed under environments with low dissolved oxygen concentration. It is suggested that ANR $[4fe\text{-}4s]^2$ regulatory protein is involved in the anoxic activation of denitrification genes. This protein was found in *Pseudomonas aeruginosa* and it was also reported to be responsible for transcription of nirS and norCB, which are structural genes for nitrite reductases (NiRs) and nitric oxide reductases (NoRs), respectively (Arai et al., 1997). These genes are thought to be responsible for nitrification. There are also reports that ANR is responsible for the arginine deiminase pathway, the production of cyanide and the control of the aerobic respiratory chain of *Pseudomonas aeruginosa.* Two mechanisms of the aerobic respiratory chain of *Pseudomonas aeruginosa* can be terminated by cytochrome-c-oxidases. The mechanisms are also sensitive to micromolar concentrations of potassium cyanide. Denitrifiers can produce hydrogen cyanide (HCN), which enables them to facilitate aerobic respiration under cyanogenic conditions, thus being able to proceed with the denitrification of nitrogenous compounds in the presence of cyanide. Mutants that lack ANR are unable to carry out these functions (Ray & Williams, 1997). However, concentrations of NO can also affect ANR by changing its iron-sulphur cluster, inducing the expression of DNR as well.

2.13.6 Proteins that are responsible for denitrification

Enzyme for denitrification receives electron from a respiratory chain system which forms part of the cytoplasmic membrane. This means that denitrification is a form of respiration which shares respiratory components with the electron-transporting system that delivers electrons for dissolving oxygen through a terminal oxidase. Electrons which originate from the NAHD, fatty acid and succinate can be used in the reduction of ubiquinone to ubiquinol. The formed ubiquinol is then directly oxidised by membrane-bound respiratory NO_3^- reductases, also known as NaR. Ubiquinol oxidation takes place in the periplasm surface of the membrane. This results in the release of H^+ to the periplasm. The transfer of electrons across the membrane to the active site located on the globular domain that protrudes into the cytoplasm (Bothe et al., 2007), is such that NO_3^- is required for the location site of NO_3^- reduction on the cytoplasmic side of the membrane. This is provided for by NaR-K proteins. These proteins have been reported to be fused together in *P. denitrificans*. Furthermore, it has been shown that these proteins catalyse NO_3^- symport with one or more H^+ that facilitates NO_3^- entrance to the cell, thus initiating respiration. The steady state availability of NO_3^- will result in the exchange with NO_2^- during the periplasm process; hence it is not affected by the H^+ motive force across the membrane (Wood et al., 2002). Export of NO_2^- to the periplasm is required since it is the location of NO_2^- conversion using reductases responsible for the denitrification system (Korner & Zumft, 1989; Bueno et al., 2010).

2.14 Factors affecting simultaneous nitrification and denitrification

Successful removal of TN depends on exposing wastewater to both aerobic and anaerobic conditions. This makes it difficult to completely remove dissolved oxygen (DO) to create anoxic conditions. This results in a decrease in nitrogen removal efficiency and the significant emission of NO and N_2O due to the sensitivity of enzymes responsible for the reduction of these gases. Hence, a significant quantity of these gases will be produced through nitrifiers and denitrifiers under alternative DO conditions (Zheng et al., 2014). Research has also suggested that DO is related to agitation, energy consumption and aeration (Shoda & Yoichi, 2015; Sun et al., 2015;). There are reports that indicated that high DO concentration negatively affected the nitrite reduction process. Sun et al. (2015) reported a lower nitrite utilisation rate of 62.37% at 160 rpm, and 71.38 % at 100 rpm a, respectively – with cultures under high agitation having a higher DO concentration. Nitrification and aerobic denitrification bacteria generally prefer pH ranges of 6–9; however, Chen et al. (2014) reported *Aeromonas* sp. HN-02 demonstrated strong acid and alkali resistance of pH 2.3 and pH 11. Characteristics of wastewater and their disposal standards are highlighted in Table 2-3.

Table 2-3: Characteristics of wastewater and disposal standards (Cui et al., 2014)

Parameter	Maximum	Minimum	Discharge standard
COD (mg/L)	329	250	80
BODs (mg/L)	20.1	16.5	-
pH	8.05	7.95	6-9
CN^- (mg/L)	49.0	26.5	0.3
TN (mg/L)	289	266	20
Cu^{2+}	19.5	14.0	0.5
Ni^{2+}	9.80	7.82	0.5

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

Experiments were divided into three phases to achieve the aims and objectives as reported in Section 1.4 and 1.5 of Chapter 1.

3.1 Phase 1 experiments

3.1.1 Microbial isolation and identification

Microorganisms used in this study were isolated from poultry slaughterhouse wastewater (Western Cape, South Africa). Initially, the wastewater samples were serially diluted in sterile distilled water, followed by spread plating on nutrient agar containing: 100 mg CN^-/L, 500 mg NH_4-N /L and 2% (w/v) NaCl. The plates were incubated at 34 °C and 37 °C for a period of 72 h, with 34 °C being identified as a suitable growth temperature, since minimal growth was observed at 37 °C. Furthermore, minimal microbial growth was observed at temperatures below 30 °C. Single colonies were transferred into fresh agar plates for colony purification, and thereafter gram staining was performed on the pure isolates. Genomic DNA was extracted from the isolates using a DNA extraction kit (Promega). This was done according to Miller et al. (1988), with minor modifications (see Appendix A.3). The 16S rDNA of the isolates was amplified using a forward primer 27F (5'-AGAGTTTGATCATGGCTCAG-3') and a reverse primer 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The amplification process included an initial denaturing step at 94 °C for 2 min, followed by 30 cycles at 95 °C for 40 s, 55 °C for 30 s, and 72 °C for 1.5 min, with the final extension at 72 °C for 10 min followed by cooling and storage at 4 °C. The PCR products were sequenced at Stellenbosch University (South Africa). The obtained sequences were deposited and compared to those in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.1.2 Toxicant tolerance experiments

Nutrient broth (100 mL in 250 mL Erlenmeyer flasks) was inoculated with 24 h-old cultures followed by incubation in a shaking incubator operated at a speed of 160 rpm, at a temperature of 34 °C for a period of 24 h. Subsequently, serial dilutions were performed using samples (1 mL) obtained from the nutrient broth cultures and grown on nutrient agar containing toxicants: 500 mg NH_4-N/L, 100 mg CN^-/L and salinity (2% to 6% w/v) using NaCl. Colony counts were used to assess the toxicant tolerance of the isolates. Thereafter, response surface methodology was used to determine the maximum toxicant limit that the isolates could tolerate. These are stipulated in Section 3.2.1, i.e., 400 mg NH_4-N/L, 65.91 mg

CN^-/L and 4.5% (w/v) NaCl, based on the cell concentration obtained under different saline conditions.

3.2 Phase 2 experiments

3.2.1 Response surface methodology experiments

A basal medium containing: 1.5 g KH_2PO_4, 7.9 g Na_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 1 mL trace element per litre. The trace element solution contained (per litre): 50 g EDTA, 2.2 g $ZnSO_4.7H_2O$, 5.5 g $CaCl_2$, 5.06 g $MnCl_2.4H_2O$, 5.0 g $FeSO_4.7H_2O$, 1.1 g $(NH_4)_6Mo_7O_2.4H_2O$, 1.57 g $CuSO_4.5H_2O$, 1.61 g $CoCl_2.6H_2O$. Disodium succinate (7.8 g/L) was used as the sole carbon source. The medium was filter-sterilised using a 0.22 µm filter. From a fresh culture, a loopful of each isolate was inoculated into 100 mL of basal medium in a 250 mL Erlenmeyer flask. Toxicants NH_4^+-N, CN^- and NaCl were mixed as stipulated by the Central Composite Design of the Response Surface Methodology (RSM) software as stipulated in Table 4.1. The flasks were incubated in a shaking incubator (160 rpm) at 34 °C for 72 h. Samples (1 mL) were withdrawn after 72 h for spread plating. The plates were incubated in an oven at 34 °C for 72 h subsequent to cell counting in a form of colony- forming units (CFU).

3.2.2 Effect of pH

A basal medium similar to the one used in 3.2.1 was used for these experiments. A loopful of the isolates was inoculated into 250 mL multiport Erlenmeyer flasks with 150 mL sterile basal medium containing disodium succinate as previously described. The medium pH was adjusted to pH 2, 4.5, 7, 10 and 12. After inoculation, the flasks were incubated in a shaking incubator (160 rpm) at 34 °C for 48 h. Toxicants, i.e., 400 mg NH_4^+-N/L, 65.91 mg CN^-/L and 4.5% (w/v) NaCl, were added to the 48 h-old cultures subsequent to incubation under the same conditions for a further 72 h. Samples (10 mL) were collected in duplicate using 20 mL syringes after 72 h to analyse for CN^-, NH_4-N, NO_2-N, NO_3-N and COD. The multiport Erlenmeyer flasks had sampling ports sealed to avoid cyanide (CN^-) volatilisation. A photograph of the multi-port flask used is shown in Appendix B.1.

3.2.3 Total nitrogen removal

A similar nutrient medium to that was used in Section 3.2.1, was also used in these experiments. The experiments were conducted in a similar manner as reported in Section 3.2.1, over a period of 168 h. However, a maximum pH of 7 was chosen for these experiments. Samples (10 mL) were collected in duplicate using 20 mL syringes at 24 h intervals to analyse for NH_4-N, NO_2-N, NO_3-N and CN^-.

3.2.4 Analytical methods

Residual NH_4-N, CN^-, NO_2-N and NO_3-N concentration was measured as per the manufacturers' instruction using Merck ammonium (NH_4^+) (00683), cyanide (CN^-) (09701), nitrite (NO_2^-) (110057) and nitrate (14773) test kits (see Appendix C.1). A Merck Spectroquant® Nova 60 instrument was used to quantify the concentration of the analytes. The cyanide test kit functions on the basis that cyanide reacts with chloramines-T and pyridine-barbituric acid (Lambert et al., 1975). The ammonium test kit is based on the fact that the Berthelot's reagent reacts with ammonium, chlorine and phenolic compounds to form indophenol dyes (Patton & Crouch, 1977). The nitrate test kit uses concentrated sulphuric acid in the presence of a benzoic acid derivative to form a calorimetrically quantifiable by-product, while the nitrite test kit is based on the concept that nitrite ions react with sulfanilic acid in order to form diazonium salt (Hassan et al., 2003). The chemical oxygen demand (COD) method function on the basis that the inorganic substances in the wastewater sample can be oxidised by potassium dichromate in 50% sulphuric acid solution at a suitable temperature. The COD was also quantified using Merck test kits (14555). The microbial growth rate in the cultures was determined using a UV-VIS spectrophotometer at 660 nm.

3.2.5 Modelling of NH_4-N degradation and accumulation

Third polynomial equations were used to model degradation and accumulation of NH_4-N for each of the isolates using an Ordinary Differential Equation Solver, Polymath®. The equations are as follows:

Isolate (H): $\frac{\mathrm{dy}}{\mathrm{dx}} = -12.4\mathrm{x}^2 + 123\mathrm{x} - 245$ (3.1)

Isolate (G): $\frac{\mathrm{dy}}{\mathrm{dx}} = -12.7\mathrm{x}^2 + 125\mathrm{x} - 250$ (3.2)

Isolate (I): $\frac{\mathrm{dy}}{\mathrm{dx}} = -13.7\mathrm{x}^2 + 133\mathrm{x} - 256$ (3.3)

3.3 Phase 3 experiments

Serratia sp. was selected for further studies based on its high tolerance to F-CN and halophilic conditions including its ability to nitrify and denitrify ammonium-nitrogen. The effect of carbon sources on simultaneous nitrification and denitrification under cyanogenic-halophilic conditions by *Serratia* sp. using membrane aerated reactors was also evaluated.

3.3.1 The effect of carbon source in batch experiments

The basal medium comprised: 1.5g KH_2PO_4, 7.9g Na_2HPO_4, 0.5g $MgSO_4.7H_2O$ and 1mL trace elements per litre. The trace element solution contained 50g EDTA, 2.2g $ZnSO_4.7H_2O$, 5.5g $CaCl_2$, 5.06g $MnCl_2.4H_2O$, 5.0g $FeSO_4.7H_2O$, 1.1g $(NH_4)_6Mo_7O_2.4H_2O$, 1.57g $CuSO_4.5H_2O$, 1.61g $CoCl_2.6H_2O$ and made up to 1 L with sterile distilled water. Different carbon sources in the form of succinate, glucose and yeast extract (7.8 g/L) were supplemented in the basal medium, where the pH was adjusted to a pH of 7. Toxicants were in the form of NH_4-N (400 mg NH_4-N/L), and CN^- (100 mg CN^-/L) were also added. The batch cultures were incubated for a period of 168 h under similar conditions used to prepare the inoculum. Samples (8 mL) were collected at 24 h intervals, and analysed for NH_4-N, NO_2-N, NO_3-N and CN^-. Microbial growth was determined using a UV-VIS spectrophotometer at a wavelength of 660 nm.

3.3.2 Membrane aerated biofilm reactor (MABR)

The MABR design used in this study is shown in Figure 3-1, and the pictorial demonstration of the reactor is shown in the appendices (refer to Appendix C.2). The reactors used were cylindrical and made of glass with a volume of 14 mL. A single hollow-fibre membrane was inserted into the middle of the reactor, with wastewater supplied at the bottom of the reactor to the lumen side of the membrane by a Watson–Marlow 504s pump at a flow rate of 15 rpm. The MABR system was incubated at 34 °C. Initially, the reactor was inoculated through the shell side of the reactors using a dead-end filtration mode, by using 24 h old cultures grown on succinate containing nutrient medium. The inoculation of the organisms on the shell side of the reactor was done to immobilise the microorganism onto the membranes. Hollow-fibre α-Al_2O_3 membranes with an inside and outside diameter of 2 mm and 3 mm respectively were used as biofilm supporting matrices. The length of the membrane was 20 cm and the membrane pore size was 40 nm. The nutrient medium was fed through the shell side of the reactors using a dead-end filtration mode for an additional 72 h to allow for microbial growth, adaptation, acclimatisation, and firm attachment of the microorganism to the membrane. Wastewater containing toxicants, i.e., 610 mg NH_4-N/L and 100mg CN^-/L, was then supplied to the MABR systems for a further 192 h. Compressed air was pumped through the lumen of the hollow-fibre membrane at 10 kPa using the dead-end filtration mode so that the air could be directly and evenly distributed to the immobilised microorganisms. Samples (4 mL) were collected at 24 h intervals and analysed for NH_4-N, NO_2-N, NO_3-N and CN^-.

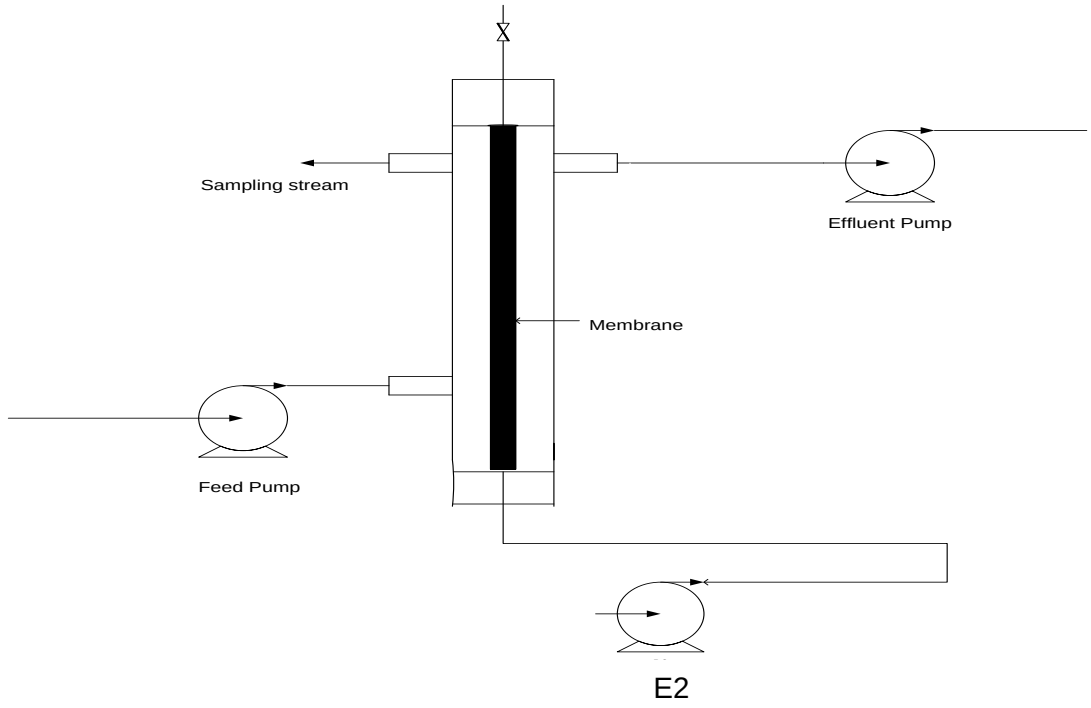


Figure 3-1: Schematic diagram of the membrane aerated biofilm reactor used in this study. *Key: E2-compressed air*

3.3.3 Analytical methods

The analyses were conducted as reported in Section 3.2.4.

3.3.4 Ammonia monooxygenase, nitrite and nitrate reductase enzyme activity experiments

After the MABR experiments, the biofilms on the membranes were harvested by sonication of the membrane fibres in a basal medium. This was done to remove the biofilm from the membrane for further processing. The cells were thereafter harvested by centrifugation at 5000 *g* for 15 min. The pellet was re-suspended in a 20 mmol/L phosphate buffer solution (pH 7.4). The bacterial suspension was lysed using glass beads. The cell-free extract, obtained by centrifugation at 10,000 *g* for 15 min, was then used for enzyme activity experiments for the confirmation of enzymes responsible for nitrification and denitrification. The activity of the ammonia monooxygenase (AMO) was measured by monitoring the change in NH_4-N after the addition of 5 mmol/L NH_4Cl to the free extracts. The formation of nitrate from nitrite and the disappearance of nitrite was analysed to determine enzyme activity of nitrate reductases (NR) and nitrite reductases (NiR) in harvested biofilm, respectively. All experiments were performed in triplicate and the average values obtained were used to generate schematic representation for all parameters evaluated.

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4
RESULTS AND DISCUSSION

This chapter is divided into three phases:

- **Phase 1: Aim 1:** Isolation, identification and characterisation of strains with an ability to nitrify and denitrify in a single-step process.
- **Phase 2: Aim 2:** Nitrification subsequent to denitrification under high CN^- and salinity conditions.
- **Phase 3: Aim 3:** Evaluation of the viability of a continuous membrane aerated biofilm reactor in simultaneous nitrification and denitrification.

4.1 Phase 1: Isolation and characterisation of strains

4.1.1 Introduction

The habitat of microorganisms greatly influence their metabolic functions, since most microorganisms can mutate their existing genes or gain foreign genes either through transformation, transduction or conjugation in order to have competitive advantage in their environment. Thus, the isolates used in this study were isolated from poultry slaughterhouse effluent containing high concentrations of ammonium nitrogen and salt, as well as minute concentrations of CN^- from commercial cleaning and sanitising chemicals used in the facility. It was hypothesised that the isolates found in the wastewater from the poultry slaughterhouse are able to break down CN^- and ammonium nitrogen-based compounds into less harmful products; hence they are able to survive such conditions.

4.1.2 Aims and objectives

The objectives for this part of the study were to:

- isolate and identify nitrifying/denitrifying bacteria from a halophilic environment which has wastewater containing high concentration of nitrogenous compounds; and
- assess the ability of the isolates to grow under high CN^- and salinity concentrations, focusing on novel isolates which have not been reported to nitrify and denitrify in a single-step process.

4.1.3 Microbial isolation

Three microorganisms able to grow on solid media containing CN^-, NH_4-N and high salt content, were isolated using a culture-based approach. The isolates subjected to a Gram-

staining procedure as shown in Appendix A.1 were determined to be gram-negative rods. The biochemical characteristics of the isolates were conducted using a Vitek® 2 Compact system and the results of the biochemical tests are shown in Appendix A.2. The isolates were identified by using molecular methods (16S rDNA gene) whereby the extracted genomic DNA was visualised in agarose gel (Fig. 4.1) and the resultant PCR amplicons were sequenced. The results indicated that strains I, H and G had 95%, 93%, and 97% similarities to *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively.

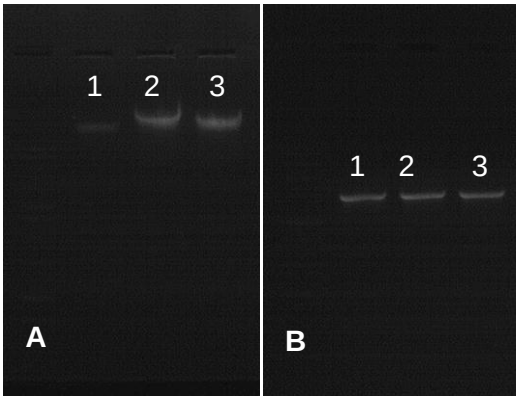
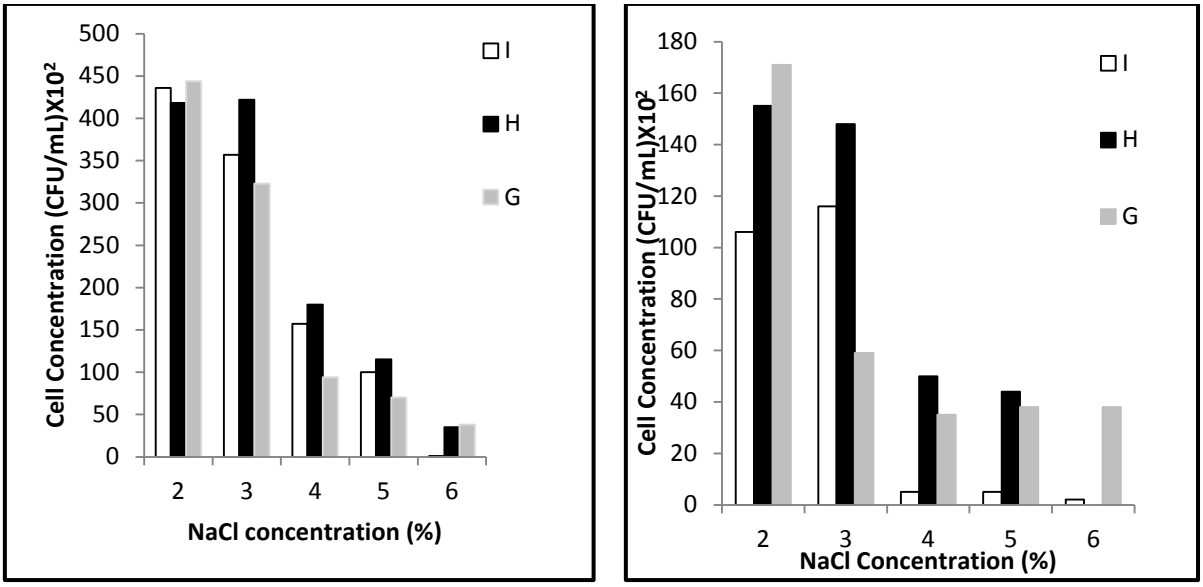


Figure 4-1: (A) Genomic DNA – Lane 1: *Enterobacter* sp., Lane 2: *Yersinia* sp. and Lane 3: *Serratia* sp. (B) PCR products – Lane 1: *Enterobacter* sp., Lane 2: *Yersinia* sp. and Lane 3: *Serratia* sp.

4.1.4 Salinity tolerance test

Nitrification and denitrification are inhibited by high salinity; however, inhibition of nitrification and denitrification by high salt concentration is not well documented in literature (Dinçer & Kargi, 2001). Some authors have proposed the use of halophilic organisms to achieve the highest treatment efficiency of wastewater containing high salinity concentrations (Campos et al., 2002). In this study, bacteria were isolated from poultry slaughterhouse wastewater, where large quantities of quaternary ammonium cleaning reagents (Gantzhorn et al., 2014) and aluminium salts (Ikeda et al., 2002) are used for cleaning and sterilisation. These strains were confirmed for their ability to facilitate nitrification and aerobic denitrification in the presence of NaCl and cyano compounds. Initially, numerous bacteria were isolated; however only three strains were capable of ammonium oxidation at temperatures of 34 °C and 37 °C (Fig. 4-2A and B). High colony counts were observed at 34 °C and in salinity conditions less than 4% for all the three isolates. Although these strains were able to tolerate up to 6% (w/v) NaCl, growth at a salinity concentration of 6% (w/v) NaCl was minimal. Isolate H, i.e., *Enterobacter* sp., had the lowest colony counts at 37 °C and minimal growth was observed

for isolate *Yersinia* sp. at 37 °C and 6% (w/v) NaCl, with 4.5% (w/v) NaCl being chosen as the limit for subsequent experiments.



A **B**

Figure 4-2: Cell concentration at different salinity concentration at (A) 34 °C and (B) 37 °C, in plates containing 500 mg NH_4-N/L, and 100 mg CN^-/L

4.1.5 Summary

In this part of the study, microbial isolates were isolated from poultry slaughterhouse wastewater and identified using molecular methods. The isolation media contained elevated concentrations of ammonium nitrogen, free cyanide and sodium chloride. Identification revealed that these isolates were *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. These isolated organisms were observed to be highly tolerant of high free cyanide and salinity concentrations. In the following phase, ammonium degradation efficiency using these isolates was evaluated.

4.2 Phase 2: Nitrification subsequent denitrification under high CN^- and salinity conditions

4.2.1 Introduction

Nitrification and denitrification are the most crucial processes in WWTP; yet they are the most sensitive processes that can be easily inhibited by toxicants found in WWTP influent. This sensitivity is due to the character of the microorganisms used in these processes, as they grow slowly. However, influent to WWTP may contain different toxicants such as CN^- and salt ions which may result in inhibition of these processes, thus rendering the WWTP ineffective. Some studies have proposed the use of halophilic and CN^- degrading bacteria to minimise the effect of these contaminants in nitrification and denitrification; hence microorganisms used in this study were selected based on their ability to survive high CN^- and salinity concentrations. Furthermore, the isolates used were required to perform nitrification/denitrification under aerobic conditions in a single closed system.

4.2.2 Aim and objectives

The objectives for this part of the study were to:

- identify the maximum physicochemical conditions, i.e., pH and temperature, for the maximum treatment of saline CN^- containing wastewater, using response surface methodology;
- study the CN^- and nitrogenous compounds degradation efficiency of the isolates;
- study the effect of CN^-, nitrogenous compounds concentrations and salinity on microbial growth, focusing on operational parameters on ammonium-nitrogen degradation in closed systems, i.e., batch cultures; and
- assess nitrification and denitrification efficiency under high CN- and salinity conditions.

4.2.3 Maximum biodecomposition efficiency of contaminants

The effect of the combined contaminants used in this study was determined by using Response Surface Methodology (RSM). This was done to establish the maximum bio-decomposition of the contaminants by varying three independent variables, i.e., NH_4-N, CN^- and NaCl. The optimum microbial growth rate was found to be under 400 mg NH_4-N/L, 65.9 mg CN^-/L and 4.5% (w/v) NaCl, with the colony counts under these conditions for *Serratia* sp., *Yersinia* sp., and *Enterobacter* sp. being 260, 323, 323 CFU/mL, respectively (Table 4.1). Moreover, CN^- and NaCl were found to have a greater influence on microbial growth than NH_4-N. This was also demonstrated in the analysis of the variance (ANOVA) for which only CN^- and NaCl had a significant impact on microbial growth for all three isolates.

Previous studies, have shown that CN^- inactivates the respiration system of living microorganism by binding to terminal oxidases (Chen et al., 2008), thus resulting in cell death, while NaCl results in cell plasmolysis, thus inhibiting growth and reproduction of the organisms (Cortés-Lorenzo et al., 2015; Zhang et al., 2015).

Table 4-1: Experimental design matrix of independent variables NH_4-N (A), CN^- (B) and NaCl (C)

Run	Factor 1 A: NH_4^+ mg/L	Factor 2 B: CN^- mg/L	Factor 3 C: NaCl %	Response 1 Log CFU/mL G	Response 1 Log CFU/mL H	Response 1 Log CFU/mL I
1	300	100	3	107	99	118
2	500	200	3	0	29	30
3	500	100	6	0	7	0
4	400	150	4.5	0	5	47
5	300	200	6	0	0	0
6	400	150	4.5	8	5	47
7	500	200	6	0	10	0
8	300	200	3	8	7	9
9	300	100	6	15	0	0
10	400	150	4.5	8	5	47
11	500	100	3	37	0	162
12	400	150	4.5	0	5	47
13	400	234.09	4.5	0	2	25
14	400	65.9104	4.5	260	323	323
15	400	150	7.02	0	4	4
16	231.821	150	4.5	0	5	0
17	400	150	1.98	58	23	23
18	400	150	4.5	0	5	0
19	400	150	4.5	0	5	0
20	568.179	150	4.5	69	6	6

A quadratic model was used to evaluate microbial growth rate (Eq. 3.1) and the significance of the parameters was determined by $p<0.05$. The ANOVA analysis showed that the model was inappropriate to be used to determine the maximum biodecontamination efficiency for the combination of contaminants used for *Enterobacter* sp. and *Serratia* sp. Table 4-2 shows that the microbial growth for the *Serratia* sp. was poorly described by the model, with correlation coefficient (R^2) of 0.4 being observed. Furthermore, variables A and C had an insignificant influence, while parameter B had a significant influence on microbial growth; hence Eq. 3.1 was reduced to Eq. 3.2.

$$\text{Log} = 200.289 + 0.017\text{A} - 0.861\text{B} - 11.449\text{C} \quad (3.1)$$

$$\text{Log} = 200.289 - 0.861\text{B} \quad (3.2)$$

Table 4-2: Analysis of variance (ANOVA) of the quadratic parameters used to estimate colony-forming units of *Serratia* sp.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Comment
Block	5450.96	2	2725.48			-
Model	29406.40	3	9802.13	3.62	0.0400	Significant
A-NH_4^+	38.88	1	38.88	0.014	0.9063	-
B-CN^-	25339.44	1	25339.44	9.37	0.0085	-
C-NaCl	4028.08	1	4028.08	1.49	0.2424	-
Residual	37857.64	14	2704.12			-
Lack of Fit	37793.64	11	3435.79	161.05	0.0007	Significant
Pure Error	64.00	3	21.33	-	-	-

Table 4-3 indicates an ANOVA analysis of isolate *Yersinia* sp. with a determination coefficient (R^2) of 0.7001, an indication that the model was suitable to be used to describe microbial growth for this isolate. The quadratic model used to evaluate microbial growth rate is shown in Eq. 3.3. Table 4-3 shows that the influential parameters, B, AB and B^2, had a significant influence in the description of microbial growth, thus Eq. 3.3 was reduced to Eq. 3.4.

$$\text{Log} = 780.489 - 0.077A - 8.099B - 27.293C + 0.003AB + 0.078AB + 0.110BC - 0.000A2 + 0.018B2 - 3.096C2 \quad (3.3)$$

$$\text{Log} = 780.489 - 8.099B + 0.003AB + 0.018B2 \quad (3.4)$$

Table 4-3: Analysis of variance (ANOVA) of the quadratic parameters used to estimate colony-forming units of isolate *Yersinia* sp.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Comment
Block	6165.54	2	3082.77	-	-	-
Model	66443.94	9	7382.66	2.07	0.1587	Not significant
A-NH_4^+	249.03	1	249.03	0.070	0.7981	-
B-CN^-	26347.69	1	26347.69	7.40	0.0262	-
C-NaCl	1646.52	1	1646.52	0.46	0.5156	-
AB	1922.00	1	1922.00	0.54	0.4834	-
AC	1104.50	1	1104.50	0.31	0.5927	-
BC	544.50	1	544.50	0.15	0.7059	-
A^2	1381.17	1	1381.17	0.39	0.5506	-
B^2	30093.07	1	30093.07	8.46	0.0196	-
C^2	698.59	1	698.59	0.20	0.6694	-
Residual	28468.27	8	3558.53	-	-	-
Lack of Fit	28468.27	5	5693.65	-	-	-
Pure Error	0.000	3	0.000	-	-	-
Corr. Total	1.011E5	19	-	-	-	-

Furthermore, the quadratic model used to model microbial growth rate of *Enterobacter* sp. is presented by Eq. 3.5. Table 4-4 shows that the ANOVA analysis for *Enterobacter* sp., variables A and C, was found to have an insignificant influence on the model used to describe microbial growth, while variable B had significant influence; thus Eq. 3.5 was reduced to Eq. 3.6; however the model's determination coefficient was found to be relatively low, with R^2 of 0.4729.

$$\text{Log} \quad = 314.038 - 0.052A - 1.087B - 17.132C \tag{3.5}$$

$$\text{Log} \quad = 314.038 - 1.087B \tag{3.6}$$

Table 4-4: Analysis of variance (ANOVA) of the quadratic parameters used to estimate colony-forming units of isolate *Enterobacter* sp.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	
Block	4089.66	2	2044.83	-	-	-
Model	49626.22	3	16542.07	2.39	0.1441	Not significant
A-NH4+	274.35	1	274.35	0.040	0.8471	-
B-CN-	40333.05	1	40333.05	5.83	0.0422	-
C-NaCl	9018.82	1	9018.82	1.30	0.2865	-
Residual	55319.33	8	6914.92	-	-	-
Corr. Total	1.090E+005	13	-	-	-	-

Figure 4-3 shows the effect of CN^- and NH_4-N on microbial growth. The optimum microbial growth was obtained when the contaminant concentration used was 400 mg/L, 65.9 mg/L and 4.5% (w/v) for NH_4-N, CN^- and NaCl, respectively, for all the three isolates. The colony counts for isolate *Serratia* sp., *Yersinia* sp*.* and *Enterobacter* sp*.* under such concentrations was found to be 260, 323, and 323 CFU/mL, respectively. Duan et al*.* (2015) reported high NH_4-N removal by *V. diabolicus* SF16 at high salinity concentrations between 1% and 5%. Furthermore, biochemical analysis by the Vitek® system used showed that isolate *Serratia* sp*.* and *Yersinia* sp*.* can grow at 6.5% (w/v) NaCl (see Appendix A.2).

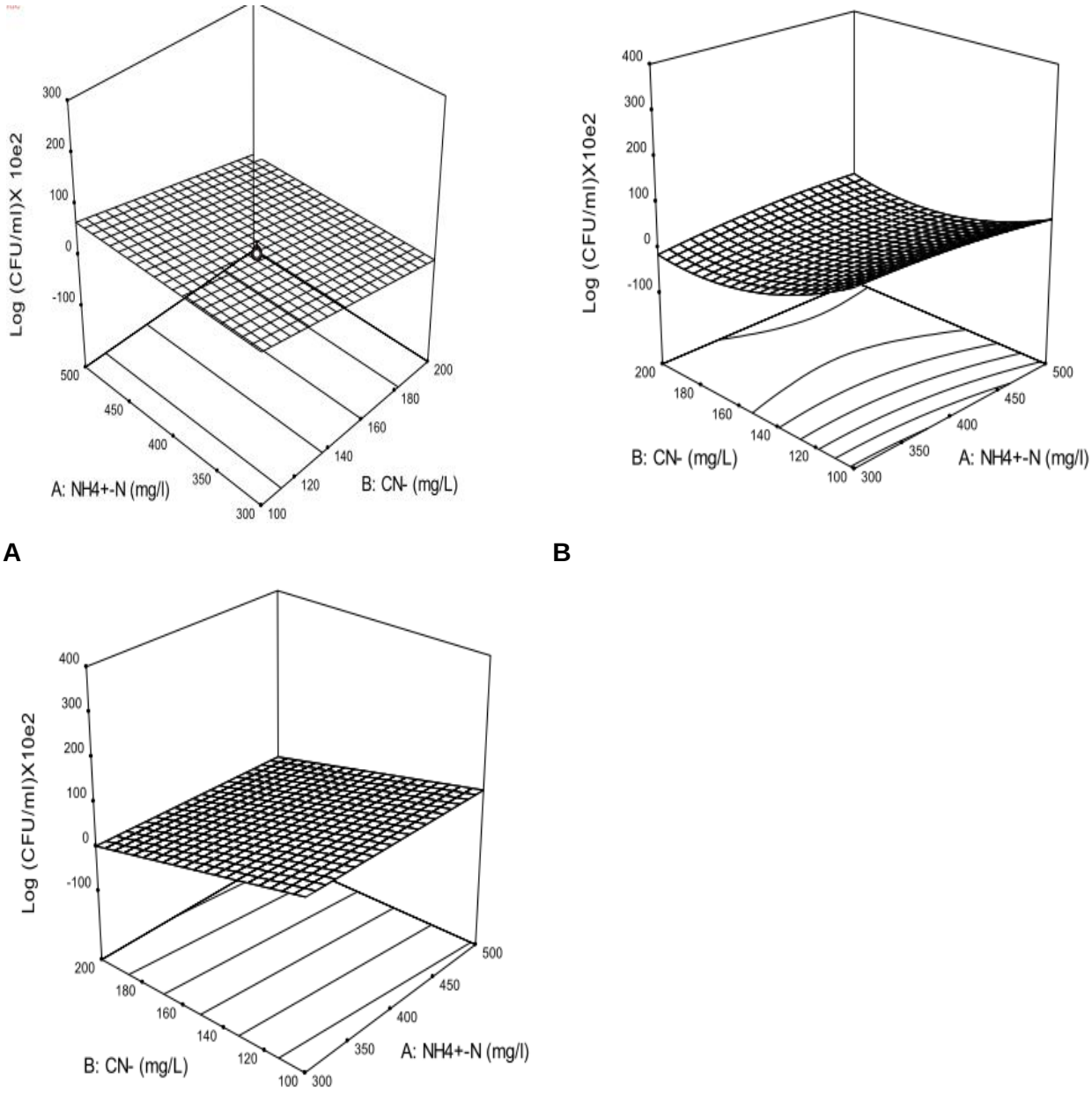


Figure 4-3: Respective graphs showing interaction between CN^- and NH_4-N. Key: A: Strain *Serratia* sp., B: Strain *Yersinia* sp. C: Strain *Enterobacter* sp.

4.2.4 Effect of pH

The effect of pH in conditions containing high salt content and CN^- concentration was evaluated. Many studies have shown that nitrification occurs at neutral pH. The maximum reduction of ammonium nitrogen was observed at slightly alkaline conditions (pH 7), with 73.3%, 70.5% and 60.6% being reduced by *Yersinia* sp., *Enterobacter* sp. and *Serratia* sp. respectively (Fig. 4-4A).

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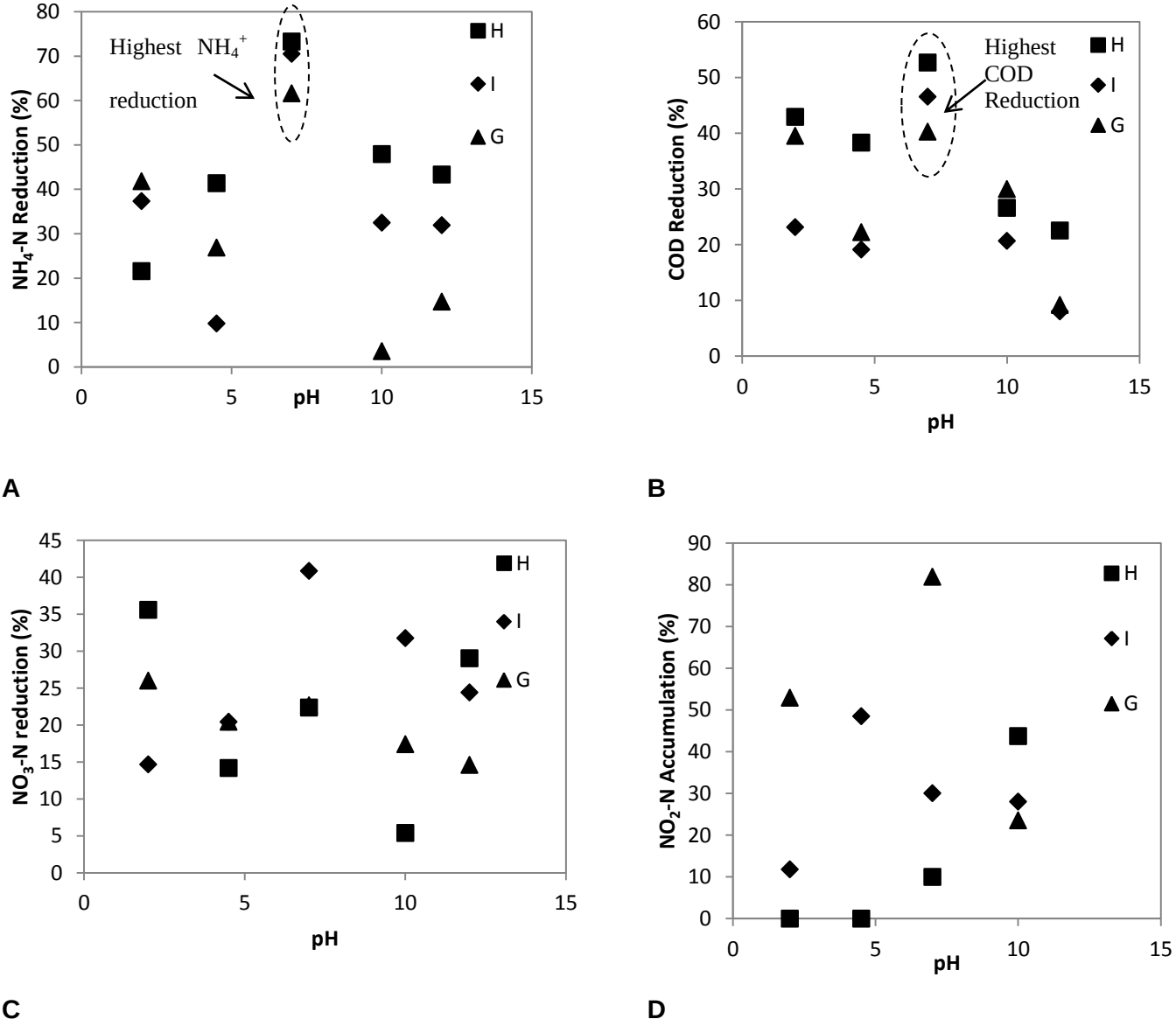


Figure 4-4: Effect of pH in nitrification and denitrification under high saline and cyanogenic conditions. (A) NH_4-N reduction, (B) COD reduction, (C) NO_3-N reduction and (D) NO_2-N accumulation

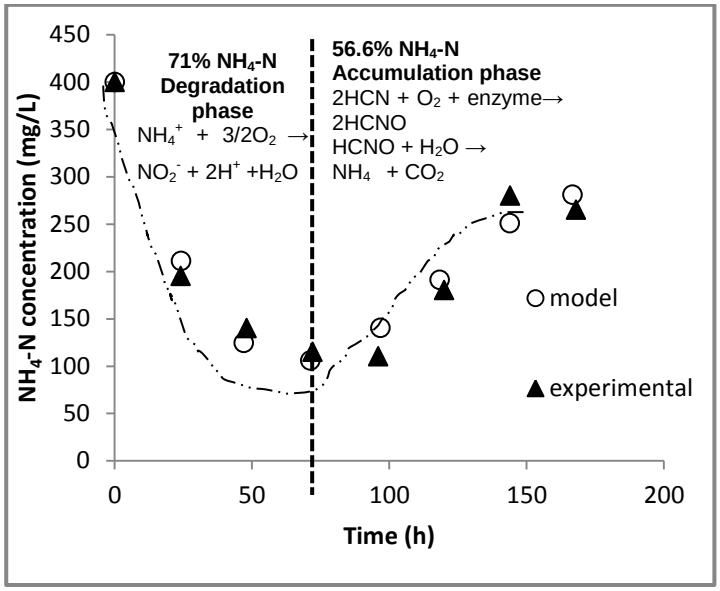
However, nitrification efficiency of *Yersinia* sp*.*, *Enterobacter* sp*.* and *Serratia* sp*.* was low under acidic (pH 2 and 4.5) and high under alkaline conditions (pH 10 and 12). The highest chemical oxygen demand (COD) reduction was also observed at pH 7 for all the isolates (Fig. 4-4B). However, the reduction of NO_3-N was low at pH 7 for *Yersinia* sp*.* and *Serratia* sp*.* Duan et al*.* (2015) showed a 93% NH_4-N reduction at pH 7.5–9.5, while Zhang et al. (2012) also found that strain *B. methylotrophicus* L7 performed nitrification efficiently at pH 7–8, while acidic (pH 5–6) and alkaline (pH 9–10) conditions negatively affected the growth of the strain. NO_2-N accumulation was low at high alkaline conditions, i.e., pH 10–12.

4.2.5 Nitrogenous compound removal efficiency

Ammonium nitrogen degradation by the three isolates from the poultry wastewater effluent was studied in batch cultures in the presence of 65.91 mg CN^-/L and 4.5% NaCl. As previously mentioned, the maximum efficiency for the combination of the studied contaminants was 400 mg /L NH_4-N, 65.91 mg/L CN^- and 4.5% (w/v) NaCl. The maximum ammonium removal was observed until 72h (Fig. 4-5), with first-order reaction rate constants (*k*) being 0.57, 0.53, and 0.52h^{-1} respectively. Ammonium nitrogen utilisation was approximately 81% (*Enterobacter* sp.), 75% (*Yersinia* sp.), 71% (*Serratia* sp.) by 72 h and drastically increased after 96h by 75%, 62.9%, and 56.6%, as the isolates started to degrade the CN^- to NH_4-N which accumulated in the bioreactors when the residual NH_4-N reached a concentration below 100 mg/L from an initial concentration of 400 mg/L. Furthermore, bacterial proliferation was observed at OD_{660nm} from 120 h (Fig. 4-6D).

Dinçer and Kargi (2001) reported a decrease in nitrification from 2.9 mg N/L.h to 2.6 mg N/L.h and further to 2.2 mg N/L.h when the salinity concentration was increased from 0% to 3% and further to 5%, respectively. Additionally, Uygur and Kargi (2004) observed a similar decrease in the nitrification efficiency from 96% to 39% when salinity increased from 0% to 6%. Therefore, it appears that the isolates from this study had higher nitrification rates than those previously studied. The characteristics of the isolates are similar to that of *Rhodococcus* sp. CPZ24 (Chen et al., 2012), with low accumulation of NO_2-N and NO_3-N being observed between 48 and 96h (Fig. 4-6A and B) subsequent to the further reduction of these by-products after 120h. Moreover, there was minimal correlation between ammonium nitrogen oxidation and NO_2-N accumulation within the cultures. Chen et al. (2012) observed a similar phenomenon with *Rhodococcus* sp. CPZ24, with less than 2% and 7% of NO_2-N and NO_3-N accumulating during the study, respectively.

Therefore, NH_4-N removal could be due to rapid bacterial assimilation or simultaneous conversion of total nitrogen (TN) to nitrogen gas – an observation reported in *Acinetobacter* sp. Y16 (Huang et al., 2013) and *P. stutzeri* YZN-001 (Zhang et al., 2015) cultures, whereby ammonium nitrogen was oxidised with minimal nitrite or nitrate accumulation. Wild et al. (1994) showed that despite CN^- toxicity, some microorganisms could adapt to high concentration of CN^- after being exposed for prolonged periods to high CN^- concentrations. This was also confirmed by Do et al. (2008), who observed ammonium nitrogen oxidation after 28 days of non-reactivity during a prolonged lag phase period in cultures containing 10 mg/L of CN^-.



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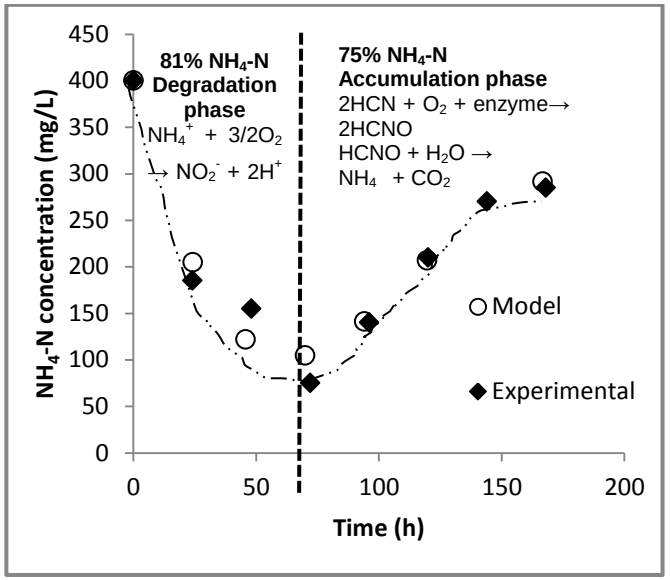

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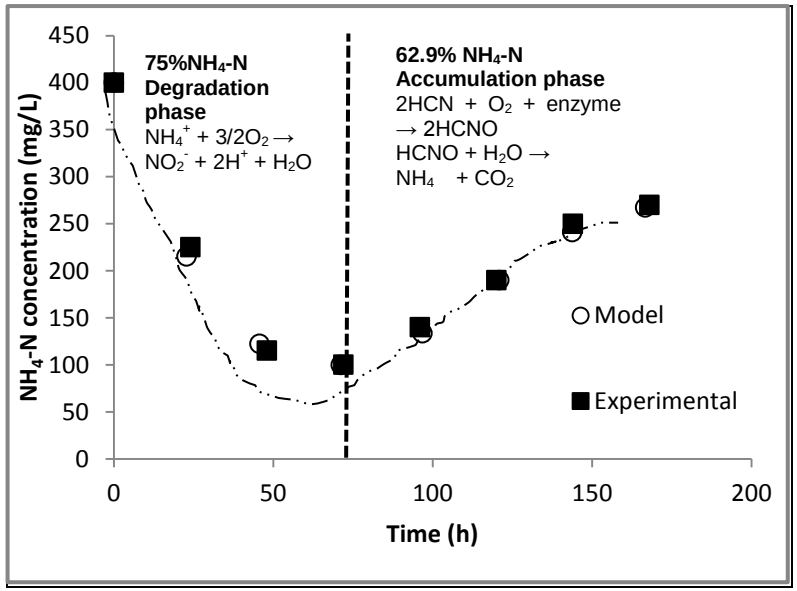


C

Figure 4-5: Comparison between the model and NH_4-N degradation and accumulation for *Serratia* sp. (A), *Enterobacter* sp. (B), and *Yersinia* sp. (C). This includes mechanisms for each part of the reaction

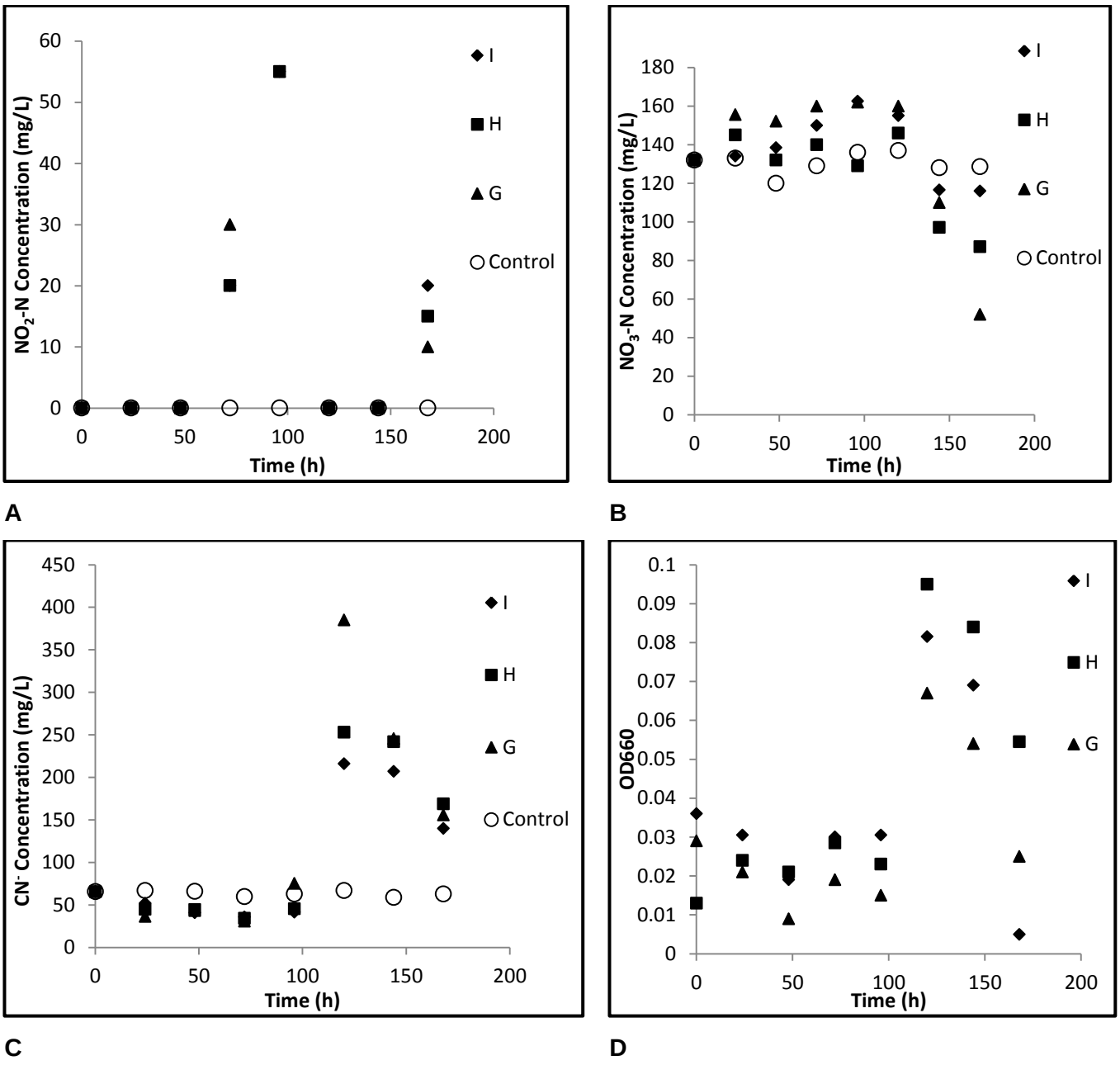


Figure 4-6: (A) NO_2–N accumulation and subsequent degradation, (B) NO_3–N removal, (C) CN^- production and degradation, (D) Cell growth, for isolates I; close circles, H; closed square, G. closed triangle

The ability of isolates *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., to facilitate nitrification in the presence of salts and CN^- could be linked to the environment, which contains significant quantities of nitrogen, phosphorus and other heavy metals from which they were isolated. Furthermore, Magaji and Chup (2012) observed that salt, CN^- and other heavy metals were higher in receiving freshwater bodies of effluent from a slaughterhouse located in Gwagwalada, Abuja (Nigeria). Overall, the presence of CN^- did not affect nitrification as it was unutilised from when the bioreactor operation was initiated to 96 h, with a drastic

increase from 96 h (Fig. 4-6C), an observation which was deemed incomprehensible. However, the increase in CN^- concentration from 96 h may be ascribed to the expression of anaerobic regulation of arginine (ANR).

Arai et al. (1997) reported production of cyanide by *Pseudomonas aeruginosa* when ANR is expressed. This protein is responsible for the dissimilative nitrate respiration regulator (DNR) proteins which produce enzymes necessary for denitrification. This can confirm the expression of the denitrification pathway (Fig. 4-7); moreover, with a decrease in NO_3-N at 120 to 168h, it was plausible that the denitrification pathway was activated in the isolates used. The increase in cyanide did not result in the deactivation of all three isolates as observed in the rapid increase in the OD_{660nm} for the cultures after 120h (Fig. 4-6D). The isolates were also determined to utilise CN^-, with all three isolates modifying their metabolism, starting to degrade CN^- after 120h, subsequent to the accumulation of NH_4-N (Figure 4-6C and 4-5). This observation was modelled using an Ordinary Differential Equation Solver, Polymath®. The initial degradation and subsequent accumulation of NH_4-N was described by a third-order polynomial with R^2 values of 0.96(I), 0.99(H) and 0.95(G), respectively (also see Fig. 4-5).

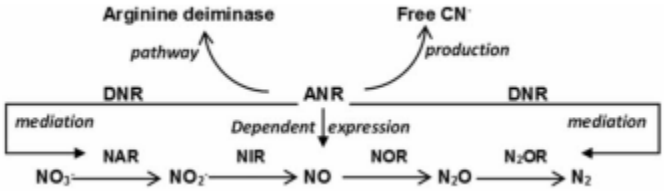


Figure 4-7: Diagram representing regulation cascade of ANR and DNR in *P. aeruginosa*, the production of cyanide, arginine deiminase (ADI) pathway and the expression of denitrification enzymes (Arai et al., 1997)

4.2.6 Summary

Ammonium-nitrogen oxidation by *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. was assessed. In addition, operational conditions were also evaluated. Initially the toxicity of the combination of CN^-, NH_4-N and salinity on microbial growth was evaluated using Response Surface Methodology and the results indicated that the optimum conditions for microbial growth were 400 mg NH_4- /L, 65.9 mg CN^- /L and 4.5% (w/v) of NaCl. Furthermore, *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., were found to reduce high concentrations of ammonium nitrogen when succinate is used as a sole carbon source at pH 7. Additionally, *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. can to degrade up to 81%, 75% and 71% NH_4-N, respectively. The following section evaluates the feasibility of a membrane-aerated biofilm reactor in simultaneous nitrification and denitrification, as well as the activity of

nitrification and denitrification enzymes in the immobilised biofilms. At this stage, *Serratia* sp. was selected as the appropriate isolate to utilise in the next phase of the experiments, since the isolate is scarcely reported as a cyanide degrader nor has it been used in a single-step nitrification/denitrification process.

4.3 Phase 3: Evaluation of viability of membrane-aerated biofilm reactor in simultaneous nitrification and denitrification

4.3.1 Introduction

Heterotrophic microorganisms require an organic carbon source for their cell growth so that they will be able to remove total nitrogen (Isaacs & Henze, 1995; Yang et al., 2012). However, different types of carbon sources affect denitrification differently, hence it is important to identify an appropriate carbon source for a successful denitrification process. Moreover, microorganisms remove toxicants better when in a biofilm state than when they are in a planktonic state. Microorganisms in biofilm state produce a protective structure known as an extracellular polymeric substance that protects them against toxic substances. Thus the practicality of MABR to simultaneous nitrification and denitrification was studied. Initially, the carbon source evaluation experiments were performed in batch cultures, prior to the selection of a suitable carbon source for use in MABRs.

4.3.2 Aims and objectives

The aims and objectives of this part of the study were to:

- select the best performing and/or novel isolate (which has not been previously reported to be able to nitrify and denitrify in a single-step process), and use it to,
- study the effect of several preselected carbon sources on the isolate to nitrification and subsequent denitrification, in order to,
- assess the feasibility of an open system, i.e., continuous membrane aerated biofilm reactors (MABRs), in a single-step nitrification and subsequent denitrification process, and
- to analyse enzyme activity for NaR (nitrate reductase), nitrite reductase (NiR) and ammonia monooxygenase (AMO) in immobilised biofilms in the MABRs designed.

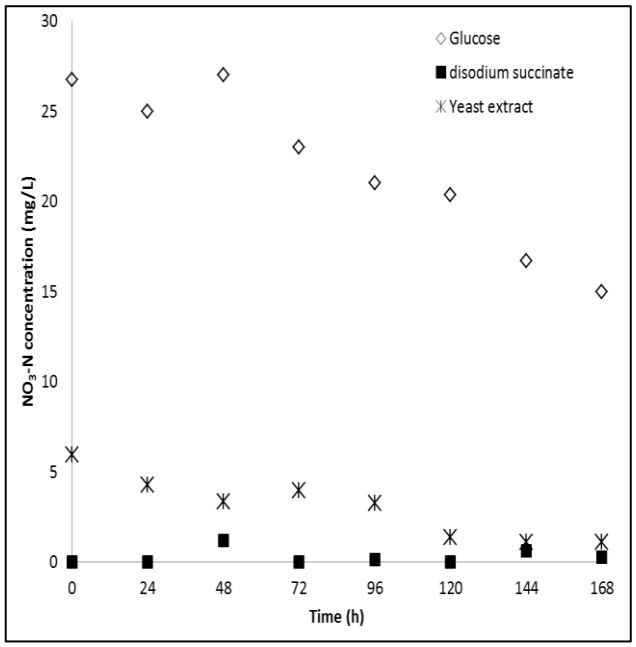
4.3.3 Selection of a carbon source

In this study, the effect of three different carbon source in nitrification by *Serratia* sp. was evaluated. Synthetic wastewater containing 100mg CN^-/L and 400mg NH_4-N/L was used. *Serratia* sp. was cultured into a basal medium containing glucose, succinate and yeast extract in batch cultures. Succinate and glucose were found to be the most efficient carbon

source, followed by yeast extract, achieving nitrification rates of 74%, 68% and 24% respectively, from an initial concentration of 400mg NH_4-N/L, respectively (Fig. 4-8). Qiu et al. (2012) observed rapid bioconversion of ammonia/nitrate to nitrogen gas when succinate and citrate were used as carbon sources, achieving removal efficiency of 87.23%/46.01% and 75.66%/44.90% respectively when compared with cultures in which glucose, ethanol and acetate were used. Contrary to this, Kundu et al. (2014) found that glucose and succinate were good substrate for *Chryseobacterium* sp. obtained from an abattoir's wastewater, while acetate and citrate were determined to be unsuitable substrates for nitrogen removal by this strain.

For nitrification and denitrification studies, it is considered that the disappearance of one constituent will result in the molar equivalent formation of the end/by-product of the biotransformation. This was not the case in this study, as there were trace accumulations of NO_2-N and NO_3-N throughout the experiment (Fig. 4-8A and 8-B) particularly when a succinate carbon source was used, suggesting simultaneous metabolism of NH_4-N, NO_2-N and NO_3-N. Chen et al. (2012) also reported a similar occurrence with *Rhodococcus* sp. CPZ24 for which less than 2% and 7% for both NO_2-N and NO_3-N accumulated during their study.

Additionally, there was a correlation between NH_4-N disappearance and microbial growth (Fig. 4-8 C and 4-9), which suggested that the depletion of NH_4-N could be due to the utilisation of NH_4-N for cellular growth. The *Serratia* sp. used in this study had similar characteristics to those of *Pseudomonas aeruginosa*, which had a notable ability to oxidise nitrogen for respiration in cystic fibrosis patients and produce minute quantities of CN^-, which can facilitate a cyanide-resistant microorganism aerobic respiration even under cyanogenic conditions (Ray & Williams, 1997). Overall, yeast extract cultures resulted in microbial proliferation for the *Serratia* sp., although this did not translate to better TN removal.



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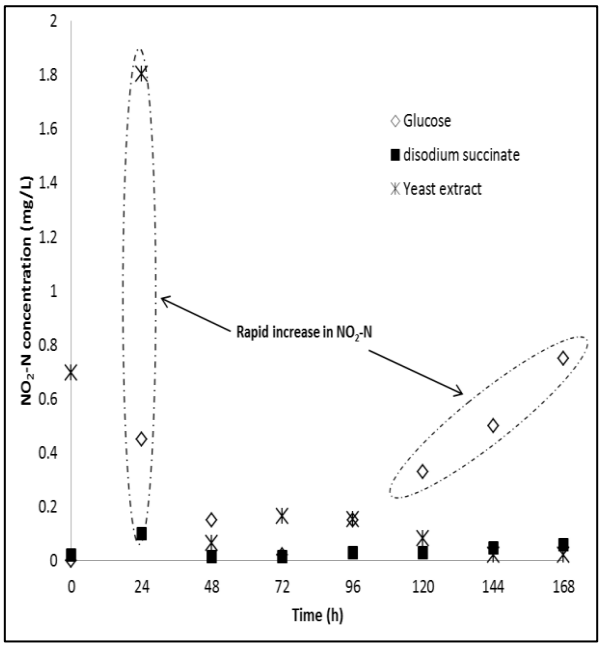

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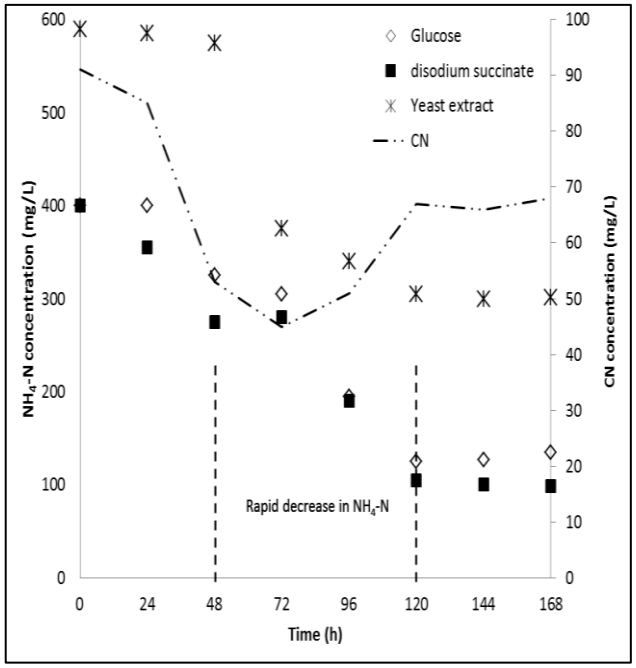

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Figure 4-8: Total nitrogen removal by *Serratia* sp. in batch cultures. Key: (A). NO_3-N conversion, (B). NO_2-N accumulation and/or conversion, (C). NH_4-N accumulation and/or conversion

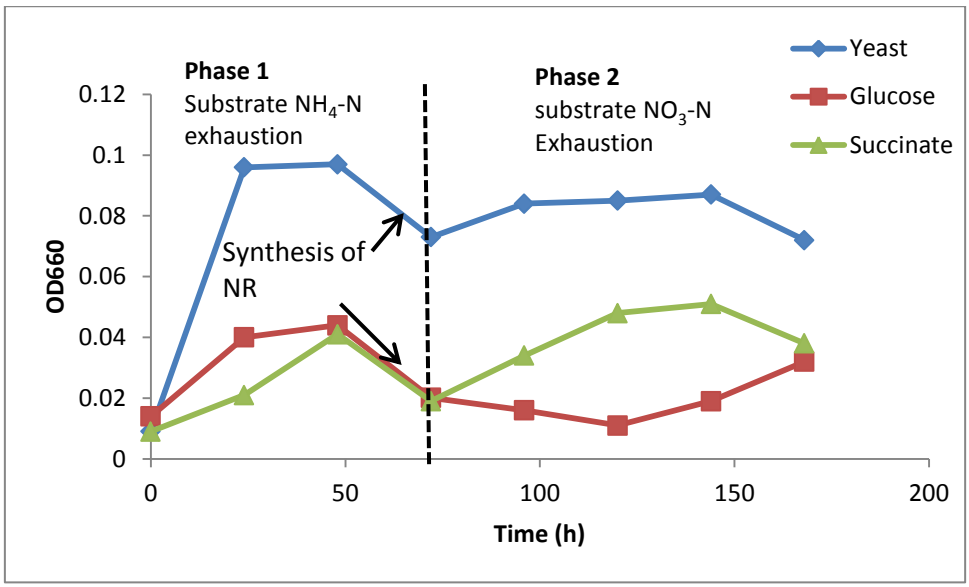


Figure 4-9: *Serratia* sp. growth in batch cultures

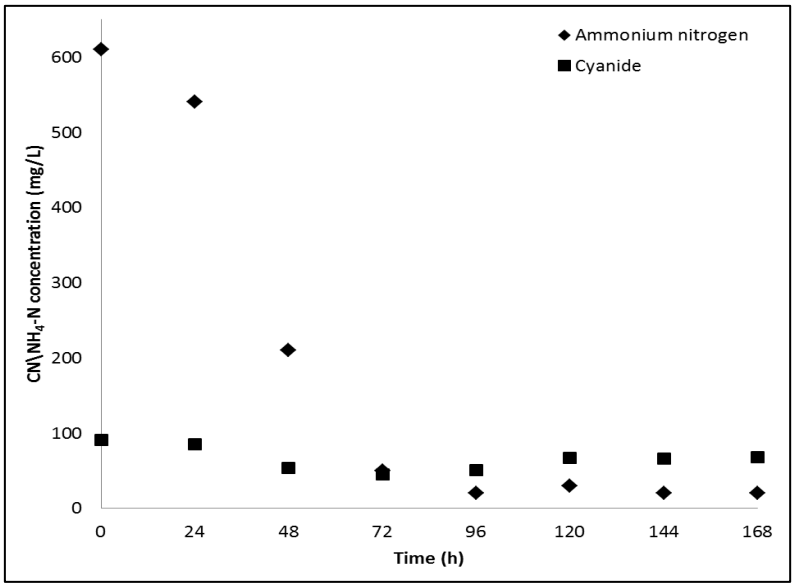
Figure 4-9 shows two phases in growth of *Serratia* sp., using the three different carbon sources. Secondary growth of *Serratia* sp. was observed. Two growth phases of the organism were observed, whereby Phase 1 (Fig. 4-9) of the microbial growth was from 0 to 75 h. The *Serratia* sp. metabolised NH_4-N as nitrogen source until it reached a concentration below 100 mg NH_4-N/L, thus indicating the depletion of NH_4-N (see Fig. 4-8C). Therefore, the synthesis of enzymes required to metabolise NO_3-N, which is a by-product of NH_4-N oxidation, was required.

In the second growth phase, referred to as Phase 2 (Fig. 4-9), the organisms began to utilise NO_3-N as a nitrogen source, thus the reduction of NO_3-N was observed from 96 h to 168 h (see Fig. 4-8B), particularly when succinate was used as the carbon source. However, the growth rate in Phase 2 was not high as that observed in Phase 1. This could be an indication that nitrate reductase is more sensitive to CN^- concentrations than AMO. Kim et al. (2011) also reported incomplete oxidation of NO_3 when CN^- loading was increased; it was concluded that denitrification is more sensitive to CN^- loading than nitrification. This required further assessment in a continuous system (MABR), as batch cultures are deemed too inefficient when processing a large quantity of wastewater, when compared with continuous systems.

4.3.4 Performance of the membrane-aerated biofilm reactors

Previously, nitrification and aerobic denitrification have been studied in batch cultures to obtain a single-stage nitrification and denitrification. There are limited studies reporting on the feasibility of MABR systems on the treatment of total nitrogen under cyanogenic conditions. In this study, MABR systems whereby the *Serratia* sp. biofilms were immobilised on hollow-fibre membranes were used for nitrification and aerobic denitrification of synthetic wastewater containing 610 mg NH_4-N/L in the presence of 100 mg CN^-/L, for which the nitrification efficiency by the *Serratia* sp. biofilms achieved was maintained at 97% after 96 h of bioreactor operation (see Fig. 4-10A). Hibiya et al. (2003) also reported 90% removal efficiency for TOC and total nitrogen from domestic modified wastewater using a similar MABR system.

To confirm nitrification and subsequent denitrification, both NO_2-N and NO_3-N were analysed in the effluent from the MABR systems (see Fig. 4-10B). In contrast to the results obtained in batch reactors, the formation of these by-products was observed to be greater in the MABR systems, although at reduced values than the theoretical values expected, which suggested that the hydraulic retention time used in the experiments was unsuitable and thus should be optimised. This also showed that the isolate used was capable of simultaneous nitrification and denitrification (SND) in continuous systems. The values observed were in the range of 0 mg NO_2-N/L to 13 mg NO_2-N/L, which abruptly decreased after the MABRs stabilised between 120 to 168 h of the bioreactor operation, with values thereafter remaining low as there was adequate NH_4-N/CN conversion. During this time, a maximum of 32 mg NO_3-N/L was also observed, an observation attributed to the presence and increased concentration of NO_2-N in the effluent from the MABRs. A decrease was also observed for NO_3-N after 120 h, which proved that aerobic denitrification was occurring as NO_3-N was being reduced.



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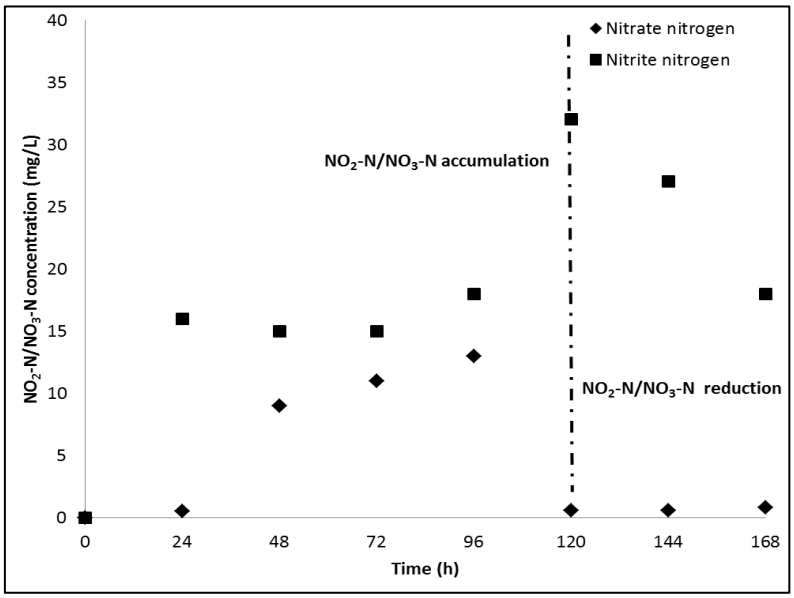


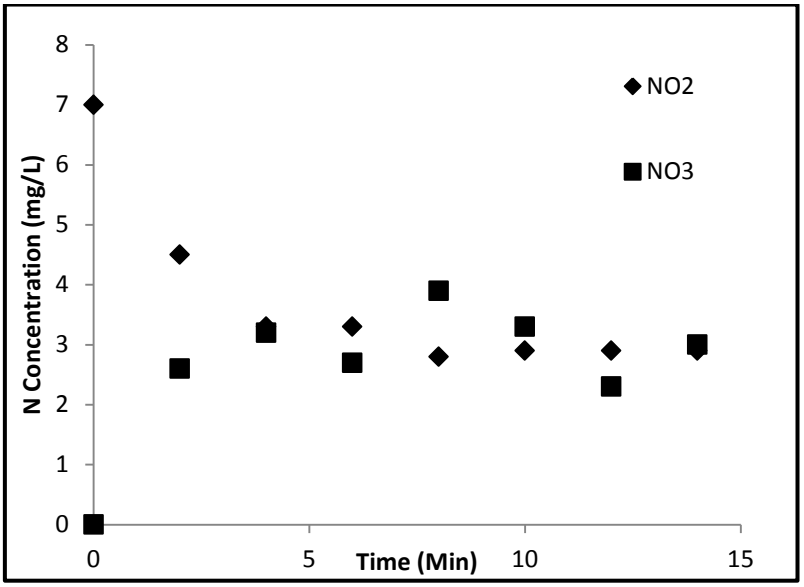
B

Figure 4-10: (A) NH_4-N biotransformation by *Serratia* sp. in a membrane-aerated biofilm reactor under cyanogenic conditions, (B) NO_2-N and NO_3-N in the effluent of the membrane-aerated biofilm reactors

4.3.5 Enzyme activity determination from harvested biofilms

To confirm the facilitation of total nitrogen removal by way of biotransformation to nitrogen gas using the immobilised *Serratia* sp. biofilms, enzyme activity assays for AMO, NaR and NiR were conducted using harvested biofilms. Free-cell extracts were obtained by using cell lysis in a buffer using glass beads. All the enzymes were successfully expressed (Fig. 4-11), showing the conversion of NO_2-N to NO_3-N, which is normally facilitated by NaR and NiR, which tentatively confirmed the presence of denitrification enzymes, while Figure 4-11B

indicates AMO activity that normally facilitates the biotransformation of NH_4-N. Overall, the results reported here require further elucidation, which requires rigorous and further investigation. This is an undertaking suitable for further research.



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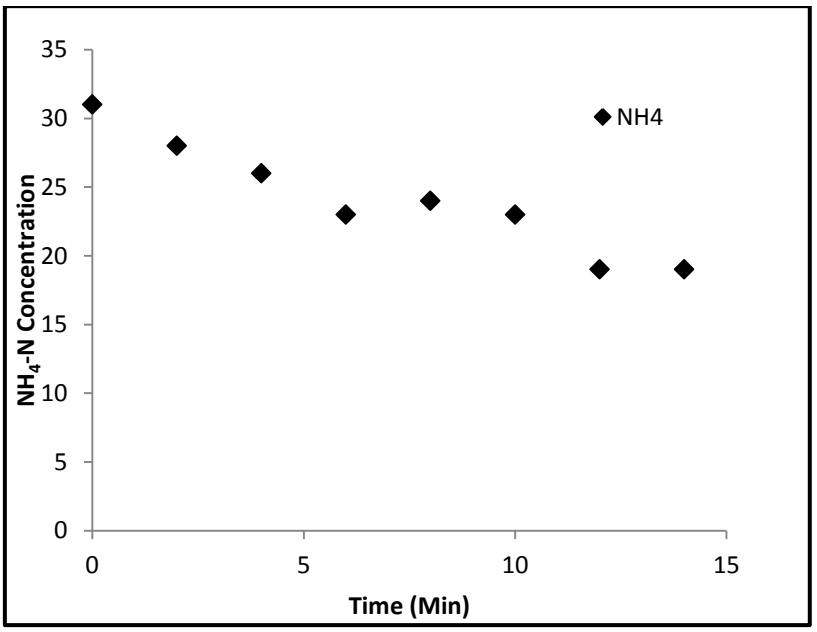

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Figure 4-11: Nitrification and denitrification enzyme activity experiments. A: NaR and NiR activity. B: AMO activity

4.3.6 Summary

In this part of the study, batch bioreactors were initially used to determine a suitable carbon source that can be used in the MABRs. Furthermore, MABRs were used to study

simultaneous nitrification and denitrification (SND) in the presence of CN^- using *Serratia* sp. biofilms to assess the viability of a continuous system, using succinate – a carbon source selected from the batch experiments. The generated results indicated that the simultaneous nitrification and denitrification occurred in the MABR and the enzyme activity tentatively confirmed the findings. This also indicated the presence of NaR, NiR and AMO in *Serratia* sp. used.

CHAPTER 5

CONCLUSIONS

AND

RECOMMENDATIONS

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Three strains denoted as I, H, and G were isolated from poultry slaughterhouse effluent for nitrification and aerobic denitrification in the presence of CN^- and NaCl. The 16S rDNA sequence of these strains revealed that I, H, and G are 95%, 93% and 97% similar to *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively. Response Surface Methodology was used to establish the appropriate concentrations for NH_4-N, CN^- and NaCl that have minimal effect on microbial growth for the isolates, for which 400 mg/L, 65.9 mg/L and 4.5% of NH_4-N, CN^- and NaCl were found to be the appropriate combination of these toxicants in which all three isolates could grow. Operational conditions such as pH and temperature were studied and the results showed that 34 ºC and a pH of 7 were the optimum conditions for total nitrogen reduction by *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. The three isolates were successfully utilised for total nitrogen removal in the presence of CN^- and NaCl, and the results indicated that *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp. were able to degrade up to 81%, 75%, and 71% of NH_4-N within 72 h, with trace accumulation of NO_2-N, NO_3-N.

Serratia sp. was used to further study the effect of carbon sources on nitrogen removal and the application of membrane aerated biofilm reactors for nitrification and aerobic denitrification in the presence of CN^-. Initially, succinate, glucose and yeast extract were compared for their suitability in nitrification by *Serratia* sp. in a batch reactor. *Serratia* sp. was found to be more effective in total nitrogen removal when succinate was used as a carbon source, with maximum removal efficiency of 74% being achieved, and least effective when a yeast extract was used, with an average removal efficiency of 24%. This resulted in the adoption of succinate-supplemented media for the assessment in continuous MABR systems, where a rapid decrease in NH_4-N concentration was observed, from 610 mg NH_4-N/L to 20 mg NH_4-N/L within 96 h, with an average removal efficiency of 97% after 120 h. Therefore these isolates demonstrated their potential for pilot-scale applications.

5.2 Recommendations

Recommendations for future studies are as follows:

- Study the performance of the isolates under different seasonal conditions to assess the impact of changes in seasons on nitrification and aerobic denitrification.
- Study the compatibility of these isolates with microbial strains found in sludge normally used with residential strains found in wastewater treatment plants.

- Study the effect of dissolved oxygen as it may influence the nitrifying ability of the employed microorganisms.
- Pay more attention to the inhibitory mechanism of CN^- and NaCl towards nitrification and aerobic denitrification to resolve the inhibition effects of these contaminants towards nitrification and aerobic denitrification.

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APPENDICES

APPENDIX A: MICROBIAL ISOLATION AND IDENTIFICATION

APPENDIX A.1 Gram staining procedure

- Add crystal violet stain over the fixed culture in the slide. Allow it to stand for 60 seconds
- Rinse the slide
- Add the iodine solution to the culture and allow it to stand for 60 seconds
- Rinse slide with running water
- Add a few drops of acetone
- Rinse it off with running water
- Add Safranin and allow it to stand for 60 seconds then rinse it off with running water and blot dry the slide.

APPENDIX A.2 Biochemical test results

Biochemical tests were performed in an overnight culture using Vitek compact system

Table A.2.1: Biochemical test results for G

Biochemical Details																	
1	BXYL	-	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	-	10	PyrA	+	11	AGAL	-	12	AlaA	(+)	13	TyrA	+	14	BNAG	+
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	+	22	INO	-	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	-	30	GlyA	-	31	dMAN	-
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	(-)	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	-	61	ESC	+	62	TTZ	(-)	63	POLYB_R	+						

Table A2.2.2: Biochemical test results for H

Biochemical Details																	
1	BXYL	-	3	LysA	-	4	AspA	(-)	5	LeuA	+	7	PheA	+	8	ProA	(-)
9	BGAL	+	10	PyrA	+	11	AGAL	-	12	AlaA	+	13	TyrA	+	14	BNAG	+
15	APPA	-	18	CDEX	-	19	dGAL	+	21	GLYG	-	22	INO	+	24	MdG	-
25	ELLM	(-)	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	-	31	dMAN	-
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	+	45	PVATE	-	46	AGLU	-	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

Table A2.2.3: Biochemical test results for I

Biochemical Details																
1	BXYL	-	3	LysA	+	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	+
15	APPA	-	18	CDEX	-	19	dGAL	+	21	GLYG	-	22	INO	-	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	+	31	dMAN	(+)
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	+	39	IRHA	+	41	BGLU	+
43	BMAN	+	44	PHC	-	45	PVATE	+	46	AGLU	-	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	-	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	-						

APPENDIX A.3 DNA extraction

1. Add 1 ml of an overnight culture to a 1.5 ml microcentrifuge tube.
2. Centrifuge at 13,000× g for 2 minutes. Discard the supernatant.
3. Re-suspend the cells into 480µl of 50mM EDTA.
4. Add 120 µl of lytic enzyme(s), and gently pipette to mix.
5. Incubate the sample at 37 °C for 60 minutes. Centrifuge for 2 minutes at 13,000× g and discard the supernatant.
6. Add 600 µl of Nuclei Lysis Solution.
7. Incubate at 80 °C for 5 minutes to lyse the cells, then cool to room temperature.
8. Add 3 µl of RNase solution to the cell lysate. Invert the tube 2–5 times to mix.
9. Incubate at 37 °C for 60 minutes and cool the sample to room temperature.
10. Add 200 µl of Protein Precipitation Solution to the RNase-treated cell lysate, and vortex.
11. Incubate the sample on ice for 5 minutes.
12. Centrifuge at 13,000× g for 3 minutes.
13. Transfer supernatant into a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol.
14. Gently mix by inversion until the thread-like strands of DNA form a visible mass.
15. Centrifuge at 13,000–16,000 × g for 2 minutes.
16. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600 µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.
17. Centrifuge at 13,000–16,000 × g for 2 minutes. Carefully remove the ethanol.
18. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.
19. Add 100 µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65 °C for 1 hour.

Table A.3.1: DNA concentration and purity

Strain	DNA Concentration (ng/µL)	Purity
I	139.4	1.74
H	183.7	1.94
G	106.8	1.69

APPENDIX B: FERMENTATION PREPARATION

APPENDIX B.1 Medium preparation

Basal medium

KH_2PO_4	1.5 g
Na_2HPO_4	7.9 g
$MgSO_4.7H_2O$	0.5 g

1 mL traces elemental per litre

Trace elemental solution

EDTA	50 g
$ZnSO_4.7H_2O$	2.2 g
$CaCl_2$	5.5 g
$MnCl_2.4H_2O$	5.06 g
$FeSO_4.7H_2O$	5.0 g
$(NH_4)_6Mo_7O_2.4H_2O$	1.1 g
$CuSO_4.5H_2O$	1.57 g
$CoCl_2.6H_2O$	1.61 g

Figure A3.1 Photograph of multiport Erlenmeyer flask design used in batch experiment to avoid CN^- volatilisation

APPENDIX C: ANALYTICAL PROCEDURES

APPENDIX C.1 Analytical methods

Pre-treatment of sample

Samples were collected

- Centrifuged 2 ml at 4000 rpm for 5 min to remove cells
- Pipette 1ml of sample into 10 ml volumetric flask and make up to the mark with distilled water

NH4+-N analysis

- Pipette 5ml of NH4-1 reagent into test tube
- Add 1 level blue micro spoon of NH4-2 reagent
- Add 0.10 ml of pre-treated sample
- Shake vigorously until reagent mix
- Incubate at room temperature for 15 min and analyse using Spectroquant®

CN-Test

Pipette 50 µl of pre-treated sample into test tube

Add 4950 µl of dH_2O into the tube

- Add 1 level green microspoon of CN-3
- Add 1 level blue microspoon of CN-4
- Shake vigorously until reagent mixes
- Incubate at room temperature for 10 min and measure in Spectroquant®
- Analyse immediately using Spectroquant®

NO3-N Test

- Pipette 5ml of NO3-2 into test tube
- Add 1 level blue microspoon of NO3-1
- Shake vigorously for 1 min until all the NO3-1 dissolves completely
- Pipette 1.5 ml of pretreated sample and mix briefly
- Incubate for 10 min at room temperature to allow reaction to take place
- Analyse immediately using Spectroquant®

NO2-N test

- Pipette 5 ml of pretreated sample into test tube
- Add 1 level microspoon of NO2-1
- Incubate for 10 min at room temperature to allow reaction to take place

COD test

- Pipette 2.20 ml of solution A into test tube
- Add 1.80 ml solution B into the test tube
- Add 1ml pretreated sample and vortex to mix
- Incubate in Spectroquant® TR420 at 148 ^{0}C for 2 hours
- Allow to cool for 10 min then vortex
- Analysed immediately using Spectroquant NOVA®

APPENDIX C.2 Preparation of phosphate buffer

Prepare:

0.1 M disodium hydrogen phosphate (14.2 g/l)

0.1 M HCl

0.1 M NaOH

Mix them according to the following table:

Phosphate buffer table

pH	Vol. of phosphate	Vol. of 0.1 M HCl	Vol. of 0.1 M NaOH
7	756.0 ml	244 ml	-

Figure C.1: Photograph of single-staged aerated membrane reactor set-up