BIODEGRADATION OF ENVIRONMENTAL SIGNIFICANT INORGANICS USING AEROBIC BACTERIA FOUND IN BLESBOKSPRUIT WETLAND, SOUTH AFRICA.

by

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DECLARATION

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Exact wording of the title of the dissertation as appearing on the electronic copy submitted for examination:

Biodegradation of environmental significant inorganics using aerobic bacteria found in Blesbokspruit Wetland, South Africa

I, Mpho Gift Kgabile, declare that the contents of this thesis represent my own single-handed work and that the thesis has not previously been submitted for academic examination towards any qualification. Additionally, it represents my own opinions and not necessarily those of the University of South Africa.

Signature

21/12/2022

Date

ABSTRACT

The Blesbokspruit wetland is known to be one of the imperative wetlands in Republic of South Africa (RSA), located in region of Ekurhuleni, Gauteng Province. A continuous discharge towards the Blesbokspruit wetland from effluents coming from the nearby Grootvlei mine, paper production company (South African Pulp and Paper Industries) and other anthropogenic activities accompanied with environmental impurities which include cyanide-residues has been witnessed within the wetland. Cyanide compounds depict distinct characteristics depending on chemical bindings with other elements which determines their severity and stability of the compound. Cyanides are either classified as either organic or inorganic cyanides and they are regarded as environmental significant contaminants. Biodegradation processes have proved to be an ideal tool to degrade environmental significant contaminants due to its cost effectiveness, eco-friendly and durability. The environmental significant inorganic placed under study is thiocyanate and its biodegradability using microorganisms isolated from soil, water and sludge within Blesbokspruit wetland. Among the most problematic inorganics found in wetlands are cyanides, particularly thiocyanate, which are toxicophores due to their cyanide content. Studies have shown that cyanides are present in effluents channelled by mining industries, which results in the deterioration of the Blesbokspruit wetland.

Microorganisms were isolated and identified using universal primers 16S-27F and 16S-1492R, targeting 16S rDNA sequence. Indicator plate technique was applied in order to detect microorganisms with thiocyanate biodegradation capabilities, where phenol red aided as an indicator to distinguish microorganisms that can degrade thiocyanate by observing a colour change from red to pink. Later, thiocyanate degrading isolates and mixed culture were inoculated in minimal media without addition of a carbon or nitrogen source and $1/10^{th}$ minimal medium containing a 24-hour starved culture was further inoculated in batch conical flasks containing minimal media with thiocyanate (SCN) either 150 mg SCN^-/L or 250 mg SCN^-/L. The sampling intervals were done every 24 hour-interval for a duration of 5 days, whereby the absorbance of microbial growth was measured at 600 nm and ammonium-nitrogen was measured with use of Merck Spectroquant Pharo 300.

The results have shown that only few bacterial isolates were more effective compared to the bacterial consortium with regards to biodegrading thiocyanate, whereas the highest biological thiocyanate removal efficiency achieved in this study was 97.44 % and 95.71 % under 150 mg SCN^-/L and 250 mg SCN^-/L by *Exiguobacterium sp*., respectively. Most of bacterial isolates gave less biological thiocyanate removal efficiency as compared to bacterial consortium which was dominantly comprised by *Pseudomonas sp*. It was concluded that aerobic bacteria obtained at the Blesbokspruit wetland were capable of biodegrading thiocyanate which is deemed as an environmental significant inorganic. The greater concentration amounts of thiocyanate, most bacteria seemed to be susceptible to exposure, although few bacteria exhibited some

form of resistance to some extent. Most of these bacteria were able nitrify the available ammonium-nitrogen.

Keywords: Inorganics; Thiocyanate; Biodegradation; Blesbokspruit wetland; Bacteria.

DEDICATION

Science is an absolute solution to humankind

This quote was incited by Karl Propper's quote stating that "**scientific knowledge is provisional**" which ignited realisation that everything we do as human beings is to improve ourselves to the most utter resolution. However, improvement will always continue as long as our energy and focus are channelled to one positive goal.

"**Every father should remember that one day his son will follow his example instead of his advice**"
Charles F. Kettering

This quote reminded me that I wasn't led by my father's advices only, however, his examples as well played a fundamental role in order to be inspired by taking this direction to become what I am today.

This work is dedicated to all those individuals who have innovative ideas not to doubt themselves because they could create a possible route to some new discoveries that can make people's lives better.
I dedicate this to my late father who wished to witnessed his son becoming a chemical engineer, so that he can be able to continue with his work and innovation to be enhanced to the next level. I dedicate this work to those who strongly believe in me as well to those who doubted me, to those who supported, encouraged, motivated, cultivated the drive of science and appointed the opportunity to me. To everyone who had an input to my work I thank you all for your gratitude and patience.

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RESEARCH OUTPUTS

The research outputs listed below are representation of scientific knowledge and development contributed by candidates who were directly and indirectly involved during his master's candidacy (January 2019-August 2022):

- **Presentation(s)**

Kgabile, M. G., Mosam, R. A., Mphehlo, D. T., Matambo, T. S. and Mekuto, L. 26-28 August 2019, Durban ICC, KwaZulu Natal, South Africa, Paper: Isolation of aerobic bacteria from compromised Blesbokspruit wetland with environmentally significant organics.

LAYOUT OF THESIS

The overall aim of the study was to biodegrade the detected inorganics of environmental significance that are in the Blesbokspruit wetland, which is situated in the Ekurhuleni region of the Gauteng Province.
The thesis is sectioned into 7 chapters that are arranged as follows:

Chapter 1: Introduction

The chapter entails of background, problem statement, hypothesis, aim and objectives as well as significance and delineation of the study.

Chapter 2: Literature review

This chapter comprises of reviews of this study discussed includes various sectors polluting water and wetlands, micropollutants affecting the wetlands, importance of Blesbokspruit wetlands and additional wetlands, environmentally significant inorganic impurities in Blesbokspruit and South African wetlands such as mineral acids, metal complex with organics, sulphates, inorganics salts, trace elements and cyanides. Furthermore, remediation methods of thiocyanate include biodegradation of thiocyanate which is either through inhibition of biodegradation or biodegradation by microorganisms.

Chapter 3: Methodology

This chapter consist of all the methods followed which includes collection of sludge, soil and water in the Blesbokspruit wetland. Additionally, microbial cultures identification isolation and characterisation of aerobic cultures, DNA Extraction using 16S RNA sequencing and cryopreservation for microbial isolates. Determination of thiocyanate degrading microorganisms, media preparation, screening plate method, biodegradation of thiocyanate using microorganism as well as analytical methods which involve thiocyanate determination and ammonium analysis as a product of thiocyanate biodegradation.

Chapter 4: Results and Discussion

This section includes results of the performed experimentation and discussion.

Chapter 5: Conclusion and Recommendations

Summarised outcomes and recommendation for possible future studies to be considered

Chapter 6: References

References of various articles in relation with this study

Chapter 7: Appendices

All the preparation calculations and obtained raw data of the experiments.

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LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
C_i	Initial concentration	mg/L
C_f	Final concentration	mg/L
R	Correlation coefficient	-
R^2	The determination coefficient	-
SCN_D^-	Biodegraded thiocyanate	mg/L
SCN_s^-	Initial thiocyanate	mg/L
SCN_R^-	Residual thiocyanate	mg/L
Y	The response variable	-

Subscripts

Symbol	Description	Units
-	pH	-
-	Temperature	℃
-	Concentration	mg/L or kg/L or mol/L

GLOSSARY

Abbreviation	Description
BTRE	Biological thiocyanate removal efficiency
CN_{SAD}	Strong acid dissociable cyanide
CN_{WAD}	Weak acid dissociable cyanide
F-CN	Free cyanide
$FeCl_3.6H_2O$	Ferric chloride hexahydrate
HCL	Hydrochloric acid
HCN	Hydrogen cyanide
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Monopotassium phosphate
KSCN	Potassium thiocyanate
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
MM	Minimal media
NH_3	Ammonia
NH_4^+	Ammonium ion
NH_4-N	Ammonium nitrogen
NO_3^-N	Nitrate nitrogen
PCR	Polymerase chain reaction
SCN	Thiocyanate
TDO	Thiocyanate degrading organisms

CHAPTER 1
INTRODUCTION

CHAPTER 1
1. INTRODUCTION

1.1. Background

South Africa is a semi-arid and water scarce country with a great demand for water (Donnenfeld *et al*., 2018; Odiyo and Makungo, 2012). There are about 114,000 wetlands that have been registered in South Africa and 48% of these wetlands are threatened by the impact of humans (Forest Peoples Programme, the International Indigenous Forum on Biodiversity and the Secretariat of the Convention on Biological Diversity, 2016). Wetlands are ecological vital systems since they are capable of recycling nutrients and have notable contribution to green- house gas emanations (Bodelier and Dedysh, 2013). As much as wetlands can be nutrient rich, they also harbour catalysts which aid in the biodegradation or treatment of effluents coming from sewage (commercial and domestic), acid mine drainage, industrial sectors, leaking sewers and faecal contaminants linked to poor infrastructure, littering and agricultural runoffs (McKay *et al*., 2018; Odiyo and Makungo, 2012). This is due to the occurrence of aerobic and anaerobic bioprocesses taking place within the wetlands. Bacteria play a vital role of keeping the streams healthy through biodegradation or bioprocessing of contaminants such as toxic inorganics and organics, heavy metals and other contaminants (Dixit *et al*., 2015; Sadowski *et al*., 2008).

These wetland systems have capacity to manipulate these toxic substances dumped within the environment via biodegradation which decreases the toxicity to manageable levels. Based on studies done by Dixit *et al*., (2015), the industries have become the major source of pollution to soil, streams and wetlands, with toxic chemicals which compromises the health of the wetland, the environment around it and the organisms thriving in these wetlands. However, there are factors that affect the microbial diversity by putting pressure into wetlands globally such as climate change and anthropogenic activities (Raja *et al*., 2014; Bodelier and Dedysh, 2013). Bodelier and Dedysh (2013) showed that microbial diversity and their function within wetland systems are being understudied as compared to aquatic and soil ecosystems. Therefore, future studies should focus on the function of microorganisms within wetlands.

Water pollution is problematic and poses a health risk to the communities that reside nearer to the contaminated sites and ecosystem, especially in places that have poor sanitation and/or no clean water supply (Roelke and Pierce, 2011). Communities living in such places are in greater risk since these communities rely on water from polluted rivers, lakes and dams for drinking and to maintain domestic agricultural activities in order to survive (Odiyo and Makungo, 2012). One of the major causes of surface water contamination is through anthropogenic sources such as mining and commercial industries that dump untreated waste and wastewater in surface waters. However, there have been methods which have been developed to treat such contaminants and they include chemical, physical and biological methods. Physical

processes are strenuous and expensive while chemical processes produce additional contaminants after the treatment process.

The eco-innovation models to treat environmental pollution have been slowly introduced. These models are found to add significant combination thus, providing with high value at low cost to the economy (Tyl *et al*., 2015). There are various methods that can be used to treat polluted water and these approaches may include chemical, physical, combination of physical and chemical and also biological treatment system. However, most of these systems are expensive, require high maintenance, usage of great amount of energy, lack of self-sustainability and some can only be effective for a short period of time. Biodegradation applications towards harmful waste have become progressively important due to its high effectiveness, eco-friendliness, ease of operation and low operational costs as compared to other alternative treatments (Ryan *et al*., 1991). For biodegradation or bioremediation to be achieved, environmental regulating factors such as nutrient availability, temperature, concentration of oxygen, pH, moisture content and concentration of toxic compounds, need to be accounted for (Abatenh *et al*., 2017).

1.2. Research problem statement

Industrial activities that are in close proximity to the Blesbokspruit wetland have been suspected of pumping effluents into the Blesbokspruit wetlands. The dumping of such toxic wastewater deteriorates the ecosystems within that wetland and poses a threat to human beings that utilise the wetland for a variety of purposes, which include agricultural activities that source irrigation water from this wetland. In addition, such wastes threaten drinking water supplies since this wetland eventually joins the Vaal Dam, which distributes drinking water in many residential areas across the Gauteng Province. Chemical contaminants such as thiocyanate, ammonia and nitrates are some of these contaminants. Some of the processes have been developed to treat these contaminants, which are mainly physicochemical processes. These processes are associated with additional contaminant production, excess sludge, high operational costs, ineffective for certain contaminants and energy intensive. Therefore, environmentally benign processes need to be developed such that the contaminants detected on this wetland can be removed from surface waters. Biological methods have been demonstrated to be robust, eco-friendly and cost effective, hence, a biological approach will be utilised in this study to treat noxious inorganics that are off environmental significance.

1.3. Hypothesis

It is hypothesised that bacteria that are isolated from the Blesbokspruit wetland will be able to biodegrade inorganics that are within the wetland. This hypothesis is based on the assumption that the organisms that

are present within the contaminated site have defence mechanisms against the contaminants and therefore, they can be able to degrade the thiocyanate.

1.4. Research objectives

The objectives of this study were:

- To collect samples from wet soil, sludge under wetland water and exposed sludge above wetland water.
- To isolate and characterise bacteria from sludge and soil samples collected from wetland
- To identify bacterial isolates using molecular diagnostic techniques.
- To evaluate the possible efficiency of bacterial isolates in biodegrading environmentally significant inorganics in minimal agar and broth that contains phenol red as an indicator.
- To evaluate which bacteria are more effective in biodegrading environmentally significant inorganics in the wetland.

1.5. Significance of the study

The study of microbial degradation of cyanide compounds holds significant promise for environmental biotechnology and industrial applications. Cyanide, a potent and toxic compound, is widely used in various industries, including mining, electroplating, and chemical manufacturing. Its release into the environment poses severe risks to ecosystems and human health. Traditional methods of cyanide removal, such as chemical oxidation and physical adsorption, often involve high costs and potential secondary pollution. In contrast, the use of microorganisms offers an eco-friendly, cost-effective, and sustainable alternative for cyanide detoxification.

Microorganisms, including bacteria, fungi, and algae, have evolved mechanisms to utilize cyanide as a nitrogen source, thereby neutralizing its toxicity. This bioremediation process not only reduces the environmental impact of cyanide but also transforms it into less harmful compounds, such as ammonia and carbon dioxide. The ability of certain microbial species to degrade cyanide efficiently can be harnessed to develop bioreactors and treatment systems for industrial effluents, thereby minimizing the ecological footprint of industrial activities.

The significance of this study extends to several key areas:

1. Environmental Protection: By employing microorganisms to degrade cyanide, the study contributes to the preservation of aquatic and terrestrial ecosystems. This biological approach mitigates the adverse effects of cyanide pollution, safeguarding biodiversity and promoting ecological balance.

2. Public Health: Cyanide exposure poses serious health risks, including respiratory failure and neurological damage. Microbial degradation reduces the concentration of cyanide in water bodies and soil, thereby decreasing the potential for human exposure and enhancing community health and safety.

3. Economic Benefits: The implementation of microbial degradation processes can lead to significant cost savings for industries. Unlike conventional methods, microbial treatment systems require lower energy inputs and maintenance costs, making them economically viable for large-scale applications.

4. Sustainability and Innovation: This study underscores the potential of harnessing natural biological processes for sustainable development. It encourages further research into the genetic and metabolic pathways of cyanide-degrading microorganisms, paving the way for innovative biotechnological solutions to environmental challenges.

5. Regulatory Compliance: As environmental regulations become increasingly stringent, industries are compelled to adopt cleaner technologies. Microbial degradation of cyanide aligns with regulatory frameworks aimed at reducing industrial pollution, facilitating compliance and enhancing corporate responsibility.

In conclusion, the study of microorganisms in the degradation of cyanide compounds represents a pivotal advancement in environmental science and industrial biotechnology. By leveraging the natural capabilities of microorganisms, this approach offers a promising pathway to address one of the most pressing environmental issues of our time. The integration of microbial degradation processes into industrial practices not only protects the environment but also promotes sustainable economic growth and public health.

1.6. Delineation of the study

The following listed factors were unconsidered in this study:

- Enzymology of studied thiocyanate degradation.
- Soil identification.
- Other inorganic contaminants were unconsidered even though detected to limit the scope of the study to the chosen thiocyanate.

- Study of sulphur/ sulphate, nitrates and nitrites

CHAPTER 2
LITERATURE REVIEW

CHAPTER 2
2. LITERATURE REVIEW

2.1. Sources of pollution for water and wetland environment in South Africa

Wetlands are naturally occurring, multifaceted wet environment that are interconnected to multiple streams such as lakes, dams and rivers which also may contain various substances that some are causing water pollution. Wetlands bring a transition of terrestrial and aquatic ecosystems, capable for averting eutrophication of inland and coastal waters through buffering for terrestrial run-off (Bodelier and Dedysh, 2013). Majority of water pollution is caused by mining industries, chemical companies, leakage of sewage, waste water and animal waste. Mining industries, they deposit waste such as raw acid-mine drainage (AMD) to the environment which end-ups in polluting water. The Blesbokspruit wetland, located in Republic of South Africa (RSA) intakes acid mine-water discharge coming from the nearby Grootvlei mine, other various pollutants coming from the paper production company (South African Pulp and Paper Industries) and close mine dumps (mine tailings), as well as the events that are done in urban and agricultural divisions (Mckay *et al*., 2018).

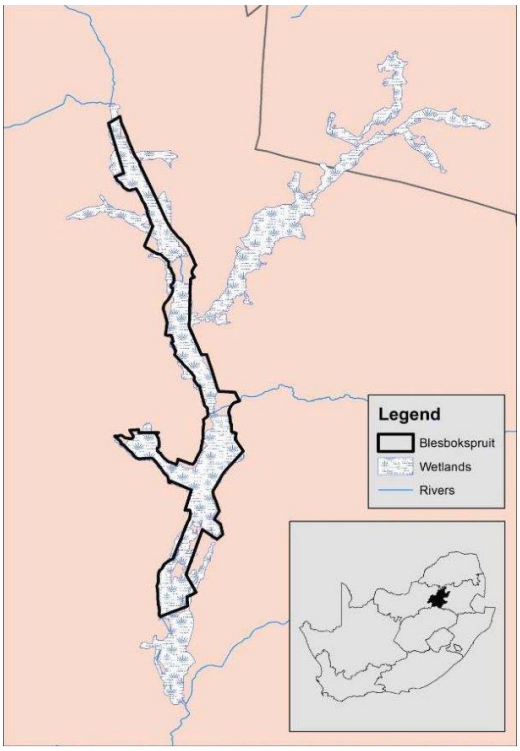

Figure 2.1: The Blesbokspruit wetland map in indication borders of the wetland, extended wetlands and external environment (Malherbe *et al.*, 2018)

Mining operation whether is done in small or large-scale, are naturally disruptive towards the environment and can generate large quantities of waste that will have harmful effects for periods of time (Kitula, 2006). Based on study done by Bakatula *et al. (2012),* shows that mines also generate waste that contains heavy metals which most are toxic in high concentrations. These heavy metals when washed down or mobilised may result into leaching to water on surface or underground water and they may require physical or conventional chemical removal methods which are expensive (Bakatula *et al.*, 2012). Additionally, mining performs cyanidation process, a type of anthropogenic activity done by mining industries during the extraction of valuable metals i.e., Silver (Ag) and Gold (Au) from refractory sulphur-based ores (Mekuto *et al.*, 2016b). Although, significant compounds like cyanide, their natural occurrence may contribute in environmental pollution, however, anthropogenic activities have greater influence in degrading the environment.

Agricultural activities, also pose a large threat towards the wetlands and underground water system with the use of pesticides, i.e., dichlobenil is utilised in treatment of weeds various agricultural sectors. However, dichlobenil is mostly degraded in soil into 2, 6-dichlorobenzamide, which is tenacious with a half-life ranging from 106 – 2079 days and with exceedingly mobility that allows it to easily contaminate groundwater as well as other natural water reservoirs (Martínková *et al.*, 2009). Some studies show that some wetlands are able to keep the environment healthy. Therefore, wetlands could possibly be used as an aid of biological treatment of polluted water.

There are industries that perform illegal dumping of waste of which may contain toxic substances which comes from last phase of the industrial activities (i.e. arsenic, copper, mercury, hydrocarbons arsenic, polychlorinated biphenyls) (Triassi *et al.*, 2015). The illegal disposal of waste has caused major deterioration of land, water (ground and surface) and also has an impact into air quality (Bakatula *et al.*, 2012; Triassi *et al.*, 2015). Such practice doesn't only cost the environment, but also cause health related risk to everyone living on the environment.

2.2. Micropollutants affecting the wetlands

Some microorganisms that are found in wetlands may produce lethal substances that may cause serious health risk. They may be either isolated from soil, sludges or water within the wetland. Algae has been one of the water contaminants due to their secretion of toxins when the cell raptures (Roelke and Pierce, 2011). Cyanobacteria (known as blue-green algae) is another type of algae which consist of many genera and species that are capable of producing a range of enormously potent inorganic and organic contaminants, which some encompassing hepato- and neurotoxic secondary metabolites (Harding and Paxton, 2001). Some higher plants, fungi and bacteria are also a contributing factor in contaminating the environment with compounds that are off environmentally significance as they are capable of biologically synthesising

cyanide (Ebbs, 2004; Baxter and Cummings, 2006). Groups organisms that are main source of producing cyanide shown in Table 2.1.

Table 2.1: Main sources of cyanide contributors to the environment (adopted from Mekuto, 2014).

Cyanogenic algae	Cyanogenic fungi	Cyanogenic bacteria	Cyanogenic crops
Anacystis nidulans	*Marasmius oreades*	*Chromobacterium*	*Malus domestica*
Chlorella vulgaris	*Stemphylium loti*	*violaceum*	*Manihot esculenta*
Plectonema boryanum		*Pseudomonas*	*Phaseolus lunatus*
		aeruginosa	
		Pseudomonas	
		fluorescens	

The study done by Mekuto (2014), demonstrate that bacteia produce cyanide as their defensive mechanism in order to inhibit predation, whereas in some cases it is used as an invasive mechanism. The common poisoning incidents that have been reported are the results of acute and sub-acute liver toxicity caused by microcystins (Harding and Paxton, 2001). Since wetlands differ, found in various environments and their flow rate differ, that also determines the formation of algae whether will be present or not. However, they are mostly formed within the edge or in a stagnant water where sunlight is present, since they are able to photosynthesise.

2.3. Importance of Blesbokspruit wetlands and other wetlands

Wetlands are known to perform a vital significant role within the environmental such as controlling or prevent flooding, serving as habitat for certain type of biodiversity and remediating or degrading pollutants to a certain extent (Ambani and Annegarn, 2016; McKay *et al.*, 2018). According to Ambani and Annegarn (2016), shows that the Blesbokspruit wetland is an important ecosystem within the urbanised economic focal point of the East Rand region due the fact it is surrounded by 5 towns (Benoni, Springs, Brakpan, Boksburg and Nigel) and also aids by buffering with water that enters the Vaal River (Ambani and Annegarn, 2016). Vaal River serves as main source of water for socio-economic activities occurring in the Gauteng province (Ambani and Annegarn, 2016). Additionally, East Rand region is huge in the practice of industrial agricultural activities, which the Blesbokspruit wetland benefits the surrounding communities with effective rainy weather that occurs at regular intervals. Most reports stated that the Blesbokspruit wetland has extensively or severely degraded due to acid mine-water and sewerage discharge, industrial effluents, agricultural runoffs and other polluting factors, however, the wetland seems to be capable of self-

managing and self-sustaining through biodegradation and bioremediation occurrence (Ambani and Annegarn, 2016; McKay *et al.*, 2018).

2.4. Environmentally significant inorganic impurities in Blesbokspruit and South African wetlands

Wetland environments constitutes water, soil, plants and sludge, whereby it may also contain pollutants which may classified as inorganic pollutants, organic pollutants, radioactive, thermal pollutants and other pollutants (Wasewar *et al.*, 2020). These pollutants are unnatural and found at high concentrations within the environment. Inorganic and organic pollutants have deemed to be environmentally significant impurities as they are primarily discharged through effluents channelled from industries and sewers to the water bodies that are used for water supplies for communities (Wasewar *et al.*, 2020). Inorganic impurities cannot be avoided and they are major significant compounds since they pose threat towards the environment. Mostly, these inorganic compounds are not toxic if properly handled. However, the issue arises when they interact with aqueous environment due to there are multifaceted systems that intermingle with the wetland environment. Some compounds within the aqueous environment formulate into complex toxic constituents that cannot be easily degraded, eradicated or inhibited through proven uncostly sufficient systems.

The general classification of environmental significant inorganics includes cyanides, metal complex organics, metals, mineral acids, sulphates, trace elements and mineral acids (Wasewar *et al.*, 2020). A list in Table 2.2. of different types of environmental significant inorganics found in contaminated environment and route cause.

Table 2.2: Common groups of environmental significant inorganics found in contaminated environment and their route source

Types of environmental significant inorganics		Examples	Remarks	Source	References
Cyanides	Inorganic	SCN^-, CNO^-	Cyanate unstable	Sewers, industry	(Mekuto, 2014)
	Free	HCN, CN^-	Weak acid and (pKa 9.24 at 25°C) equilibrium determined by pH		
	Simple	NaCN, KCN	pH below 8 exists as HCN, in aqueous solution ionisation occurs		
	Complex	CN_{SAD} and CN_{WAD}	CN_{SAD} strenuous ionisation process and CN_{WAD} ionises quickly		
Mineral acids		H_2SO_4, HNO_3, HCl	Strong acids, low pH ≤ 3 and they ionise completely	Industrial fumes	(Borah *et al.*, 2020)
Metal (M) and metal complex with organics (M-L)		M= Hg, Pb, As and M-L=	M is unstable and M-L most are stable due to the organic nature	Industrial activities	(Borah *et al.*, 2020)
Sulphates and sulphur-based compounds	Sulphates	$Na_2S_2O_3$, SO_4^{2-}, $CuSO_4$, $ZnSO_4$	Mostly are secondary pollutants, sulphate salts react in the air to form sulphuric acid	Volcanic activity	(Michalski, 2011; Borah *et al.*, 2020)

	Sulphur-based	H_2S, SO_2	Unstable gaseous compounds and some volatile. They form H_2SO_4	Industry, anaerobic fermentation and volcanic activities	(Borah *et al.*, 2020)
Inorganic salts		NH_4Cl, NH_4NO_3,	Ionises in aqueous solution	Farms/ agricultural activities, factories	(Borah *et al.*, 2020)
Trace elements		$ZnCl_2$, Zn, As,	Required in trace amounts	Industrial activities	(Borah *et al.*, 2020)

2.4.1. Mineral acids

Sulphuric acids (H_2SO_4), and nitric acid (HNO_3) are forms of mineral acid that are off environmental significant, which resulted from sulphur dioxide and nitrogen oxide (Borah *et al.,* 2020). Sulphur dioxide and nitrogen dissolve very easily in water, however, they can also react with oxygen as well as other chemicals to produce sulphuric and nitric acids (US EPA, 2021). HCl is also a main mineral acid, which is regarded as strong acid and easily ionise in water. Anthropogenic activities such as burning fossil fuels, mineral processing, chemical-based research laboratories, other industrial application, and illegal dumping are the main reason such environment impurities degrading the ecosystem. Acids in in general are less hazardous compounds due to they can be neutralised within the environment interaction, however, excessive amount in the environment will cause negative health impact towards communities surrounding that acid contaminated environment (Borah *et al.,* 2020).

2.4.2. Metals, metal complex with organics and sulphur-based compounds

Metals (M)/ heavy metals have forever been of environmentally problematic pollutants till this current era and they're continuously increasing in contaminating the environment. Acid mine drainage (AMD) has become the major hub for causing inorganic pollution towards the environment. Industrialisation of mining has caused a great significant impact towards the degradation of environment. AMD have great amounts of sulphur which react and results in sulphates. Later, those sulphates interact with other species. Hydrogen sulphide (H_2S) and sulphur dioxide (SO_2) are sulphur-based natural gases that are environmental contaminants that can both produce sulphuric acid (Borah *et al*., 2020).

2.4.3. Trace elements

Trace elements are crucial for biological processes and sustainability to organisms; however, they're required in minute concentration (Turdi and Yang, 2016). Lately, trace elements have become a global problem, since they are polluting the environment and several studies have proven detection of trace elements in high concentration within the environment. Trace element such as Zinc (Zn) is vital in diet of organisms (i.e. bacteria, plants, human beings) (NPI, 2021), which act as a cofactor for numerous enzymes (Borah *et al.,* 2020). However, Zn intake at high concentration forges an acute Zn intoxification (Plum *et al.,* 2010). Zinc is not the only problematic environmental factor, other trace elements include cadmium (Cd), chromium (Cr), etc. have negative health impact towards organisms in excessive amounts (Turdi and Yang, 2016).

2.4.4. Cyanides

Cyanide is a monovalent combining cyano group which its carbon triple bonded to the nitrogen atom, has a formula $-\mathrm{C} \equiv \mathrm{N}$ and miscible cyanide in aqueous environment present as cyanide ion that is negatively charged anion (Rabinowitz and Vogel, 2009; Mekuto, 2014). Cyanide compounds are the key major environmental significant inorganics, as to most environmental pollution-based studies,

cyanide is present and in great amounts. This group of compounds have contributed significantly in the environmental degradation and metallurgical industrial process are responsible (Mekuto *et al*., 2018). This is due to numerous sources of cyanide effluents that includes electroplating, refining of petrochemical, organic chemicals and plastics synthesis, former manufactured gas, aluminium process, mining and processing industries (Ebbs, 2004). For instance, South Africa is a hub for metal mining industries and cyanide environmental contamination occurrence is deemed to drastically increase. This leads to continuous increase of contamination of cyanide compounds in wetlands, river, dams and underground water around South Africa.

General categorisation of aqueous inorganic cyanide in industrial effluents exist in five categories which includes free cyanide (cyanide ion and hydrogen cyanide), simple cyanide (potassium cyanide and sodium cyanide), thiocyanate (SCN^-), weak acid dissociable cyanide (CN_{WAD}) and strong acid dissociable (CN_{SAD}) (Mekuto, 2014). CN_{WAD} are primarily cyanides infused with metal, also known as metal-complex cyanides which comprises of metals specie such as Zinc, Nickel, Copper, and others; whereas, CN_{SAD} are strong metal complexes containing metal species such as Gold, Silver, Cobalt and as well as Iron (Baxter and Cummings, 2006; Mekuto, 2014). Based on the study done by Mekuto (2014), stipulates that the toxicity strength levels of these inorganic cyanides categories, decreases as their ascending order, which is from free cyanides to CN_{SAD}.

In addition, free cyanides are the most toxic cyanide nature, consisting of compound that is very volatile (i.e., HCN) due to its slow boiling point (25.70°C) and HCN seepages as gas especially under favourable conditions (Mekuto, 2014). HCN is solvable particularly in alcohol and water, however, it doesn't ionise and considered as weak acid with a pKa value equals to 9.24 at room temperature (Mekuto, 2014). Ionisation of HCN is incomplete in water shown equation 2.1.

$$HCN_{(aq)} + H_2O_{(l)} \rightarrow H_3O^+ + CN^-_{(aq)} \qquad 2.1$$

Free cyanides have proven to be lethal at low concentration to both terrestrial and aquatic life, whereas to aquatic species are also highly sensitive towards cyanide (Donato *et al*., 2007; (Mekuto, 2014). Cyanide can be formed from nitriles, whereby ingestion of aliphatic nitrile by mammals, enzymatic metabolism process occurs and hydrogen cyanide (HCN) is produced shown in Equation 2.2 (National Research Council, 2014). R represents hydrocarbyl radical.

$$R - C \equiv N + P450_{\,(enzyme)} \rightarrow RR'OHC' - C \equiv N \;\rightarrow\; HC \equiv N + H_2O \qquad 2.2$$

Contaminated water with free cyanides poses threat to living organisms due to cyanide doesn't degrade overtime unless an effective cyanide-treatment systems is applied or presence of natural biodegrading microbes remediates it.

Simple cyanides encapsulate of cyanide salts where alkali metals i.e., potassium (K) and sodium (Na) are bonded to cyanide ion (CN^-) (Mekuto, 2014). Unlike free cyanides, simple cyanide can completely ionise and form ionic elements (Equation 2.3); thus, KCN in water (hydrolysis reaction) formulates free hydroxyl as well as hydrogen cyanide groups at pH ≥ 8 as shown in Equation 2.4 (Mekuto, 2014).

$$ACN \leftrightarrow A^+ + CN^- \quad 2.3$$

Where A and A^+ are representation of an alkali metal group.

$$K^+ + H_2O + \leftrightarrow K^+ + OH^- + HCN \quad 2.4$$

Complex cyanides include SCN^-, CN_{WAD} and CN_{SAD}, where these complex cyanides are formed due to the highly mercurial nature of free cyanide (Mekuto, 2014; Mekuto *et al.*, 2016a). Whereby, sulphur species reacts with present cyanide ions to formulate thiocyanate as shown in Equation 2.5 (Mekuto, 2014).

$$S^{2-} + CN^- \rightarrow SCN^- \quad 2.5$$

Since, thiocyanate is mostly found in wastewater of cyanide (Botz *et al.*, 2016), in presence of rhodanese, thiocyanate can be formed. Where, cyanide and thiosulphate react during the biological oxidation producing an end product of sulphite and thiocyanate under alkaline conditions (Gupta, 2010; Gould *et al*., 2012). It is also present in high quantity from gold leaching process and coal coking wastewater (Gould *et al*., 2012). Thiocyanate compounds are highly persistent due to it is non-volatile, stable and cannot be hydrolysed (Chaudhari and Kodam, 2010).

CN_{SAD} and CN_{WAD} are inorganic cyanide complexes that dissociate at lower pH condition (acidic conditions), whereby CN_{SAD} pH ≤ 1 and CN_{WAD} pH ≤ 4 (Mekuto, 2014). CN_{SAD} complexes includes $Ag(CN)_2^-$, $Au(CN)_2^-$, $Pt(CN)_4^{2-}$, $Co(CN)_6^{3-}$, $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ and CN_{WAD} complexes includes $Zn(CN)_4^{2-}$, $Cu(CN)_3^{2-}$, $Ni(CN)_4^{2-}$, $Hg(CN)_2$, and $Cd(CN)_4^{2-}$ (Mekuto, 2014).

Nitriles is another form of cyanide, also known as organic-cyano-group that constitutes a hydrocarbon group (represented as R) side group and a cyanide moiety (CN^-), whereby the general formula is $R-C\equiv N$. Nitriles are organic cyanides which can be either in aliphatic or aromatic form. Complex nitrile consists of metals, however, with less complications as compare to water due to their aprotic nature. As much as most nitriles are very useful and effective, they are highly toxic, mutagenic. teratogenic and carcinogenic due to presence of cyano-group attached in the organic compound, although most nitrile compounds are not nearly as toxic as simple cyanides (Martínková *et al.*, 2009; Mekuto, 2014; Sahu *et al.*, 2019). The nitrile substances that are most lethal metabolises quickly as compared those than slowly metabolises to form cyanide. Based on studies done by (Mekuto, 2014), cyanide specie's toxicity and reactivity are reliant on their structure, which influences their environmental fate and transport. Simplest

nitrile is acetonitrile (CH_3CN) which is soluble in water (Jim, 2004). Solubility of nitrile in water is listed in Table 2.3.

Table 2.3: Solubility in water of some aliphatic nitrile compound (Jim, 2004).

Nitrile compounds	**Solubility in water (20ºC)**
CH_3CN	Miscible
CH_3CH_2CN	10g / 100 ml
$CH_3CH_2CH_2CN$	3g / 100 ml

However, as the increase of backbone carbon chain bonded to the cyano-group, thus it loses the solubility in water and become immiscible. According to the studies done by (Jim, 2004), the reason for the solubility of some nitrile is due to nitrile molecules that cannot form hydrogen bonds with themselves, but they are able to hydrogen bond with water (H_2O) molecules. As illustrated in figure 2.2, hydrogen atom that is slightly positive in water molecule attracts the lone pair on the nitrogen atom in nitrile and hydrogen bond formed (Jim, 2004).

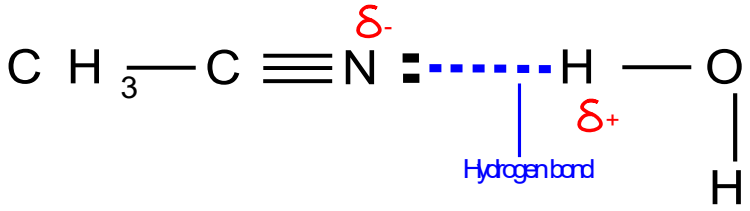

Figure 2.1. The formation of hydrogen bond between the slightly negative N and positive H atoms (Jim, 2004).

Aromatic nitriles are mostly immiscible in water due to the hydrocarbon ring which is nonpolar. They are miscible to nonpolar solvents and these aromatic nitriles consist of benzoyl bonded to the cyano-group. Occurrence of nitriles can be in two conformations within the environment, which is either in a solid or liquid form. They have an idiosyncratic odour and are colourless. Nitriles are harmful towards animals and humans, if ingested, dermally absorbed or inhaled (Mekuto, 2014). Nitriles can be inhaled during nitrile purification process; therefore, it is necessary for the process to be carried out inside an efficient fume due to their toxic nature (Armarego and Chai, 2013). Their occurrences in the environment are through natural synthesis and anthropogenic activities. The naturally occurring nitriles are formed by plants and microbes whereas the anthropogenic nitriles are formed through manufacturing industrial activities (Wyatt and Palmer, 1991; Mekuto, 2014). However, the nitriles detected in the environment mostly emanates from anthropogenic activities which discharges enormous amounts of nitrile contaminated waters to surface waters.

Based on various research, one of the problematics significant inorganics in wetlands includes cyanides particularly thiocyanate, is deemed to be toxicophores due to the cyanide nature they contain and studies have shown that these cyanides are present in the Blesbokspruit wetland as well as in other wetlands found in South Africa.

2.5. Remediation methods of thiocyanate

2.5.1. Biodegradation of thiocyanate

Wetlands are known to poses bacteria that use toxic compounds such as thiocyanate as their energy source, this therefore emphasises the significant role of these bacteria in ensuring safety of the wetland and those who use it as a water source for various activities. There are several types of bacterial species that have been proven to be capable of metabolising thiocyanate into carbonyl sulphide and cyanate products by using autotrophic and heterotrophic pathways. These microorganisms include; *Arthrobacter*, *Paracoccus*, *Pseudomonas*, and *Thiobacillus* and the equations by which biodegradation occurs are shown below respectively (2.6 and 2.7) (Gould *et al*., 2012; Kim *et al*., 2008; Lee *et al*., 2008).

$$SCN^- + H_2O \rightarrow HOCN + HS^- \quad (2.6)$$

$$HOCN + 2H_2O \rightarrow NH4^+ + HCO_3^- \quad (2.7)$$

Further oxidation of hydrogen sulphide results in sulphate and is represented by equation 2.8, while the overall equation is represented by equation 2.9 (Gould *et al*., 2012; Jeong and Chung, 2006).

$$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+ \quad (2.8)$$

$$SCN^- + 3H_2O + 2O_2 \rightarrow HCO_3^- + NH_4^+ + SO_4^{2-} + H^+ \quad (2.9)$$

The equations 2.8 and 2.9 show that sulphate is produced as the final product in the pathway when autotrophic bacteria such as *Thiobacillus thiocyanooxidans* are used, however when gram-negative heterotrophic bacteria are used, then tetrathionate is produced as the final product as shown in equation 2.10 (Gould *et al*., 2012).

$$4HSCN + \frac{9}{2}O_2 + 5H_2O \rightarrow S4O_6^{2-} + 4CO_2 + 4NH_3 + 2H^+ \quad (2.10)$$

Thiocyanate hydrolase is the enzyme that hydrolyses thiocyanate to ammonia and carbonyl sulphide in the carbonyl pathway, once this occurs, then carbonyl sulphide becomes oxidised into carbon dioxide and sulphate as shown in equation 2.11 and 2.12 below (Douglas Gould *et al*., 2012; Lee *et al*., 2008).

$$SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^- \quad (2.11)$$

$$COS + OH^- + 2O_2 \rightarrow CO_2 + SO_4^{2-} + H^+ \quad (2.12)$$

Bacteria such as nitrifiers and sulphur oxidising bacteria use the ammonia and sulphate products as a source of electron donors, nitrogen and energy (Douglas Gould *et al*., 2012; Lee *et al*., 2008).

2.5.1.1. Inhibition of biodegradation

High concentrations of ammonium inhibit microorganisms that are capable of biodegrading thiocyanate (Jeong and Chung, 2006; Kwon *et al*., 2002; Lay-Son and Drakides, 2008; Vázquez *et al*., 2006). According to an investigation and model by Vázquez *et al*. (2006) on biodegradation in coke wastewater, the efficiency of biodegradation decreased by 50% in an activated sludge system when ammonium concentrations increased to 260 mg/L in an activated sludge system with a hydraulic retention time (HRT) of 98 hours and degradation was inhibited once the ammonium concentration reached 360mg/L. Vázquez *et al*. (2006) also found out that the possible reason for biodegradation inhibition at 260 mg/L might be due to the use of real coke wastewater as it contains a higher amount of toxic chemicals that inhibit the activity of thiocyanate biodegrading bacteria, namely: phenol and cyanide.

An investigation on the effects of ammonium on thiocyanate biodegradation was done by Kwon *et al*. (2002) and he used the bacterium *Acremonium strictum* as his model microorganism, the results revealed that an increase in ammonium (about 3g/L) resulted in inhibition of biodegradation just as the findings of Vázquez *et al*. (2006). Mekuto *et al*. (2017), used a consortium of microorganisms in a continuous stirred tank bioreactor system to investigate the simultaneous biodegradation of thiocyanate and free cyanide, the results revealed that the microorganisms were capable of utilizing the ammonium that was produced by the biodegrading system. Thus, demonstrating the effectiveness and significance of using a consortium of microorganisms as a form of treatment against thiocyanate and ammonia.

2.5.1.2. Biodegradation by microorganisms

Microorganisms that are known to biodegrade cyanide-based compounds can yield cyanogenesis or degrade cyanide (Knowles, 2007). Bacteria that degrade thiocyanate are known to either utilise it for growth (nitrogen source) or for detoxification measures (Knowles, 2007). The washout of microorganisms in continuous systems decrease the efficiency of biological processes such as biodegradation over time and so in order to minimise this, biofilm bacteria are introduced and combined with biodegradation in order to minimise or prevent unwanted removal of biomass. In addition, biofilms are also capable of absorbing heavy metals in cyanidation waste water (Jeong and Chung, 2006; Lee *et al*., 2008; Huddy *et al*., 2015a). According to an investigation by Jeong and Chung (2006), the highest rate of biodegradation was 1.7 $kg.m^{-3}.day^{-1}$ using heterotrophic bacteria and even greater with a rate of 5.3 $kg.m^{-3}.day^{-1}$ when a biofilm system pack composed of pumice and zeolite was introduced, thus showing how the addition of biofilm systems improves the biological processes and their efficiency.

The robustness biofilm systems in biodegradation were further proven when Jeong and Chung (2006) demonstrated that autotrophs could biodegrade thiocyanate in an activated sludge biofilm system bed reactor (with 80% volume fluidised carriers) at a rate of 8.1 $kg.m^{-3}.day^{-1}$. The initial concentration of thiocyanate was 7000mg/L with an HRT of 36 hours and the results of the investigation showed that the degradation was efficiency of 99.9%.

CHAPTER 3
MATERIALS AND METHODS

CHAPTER 3
3. MATERIALS AND METHODS

3.1. Sampling of sludge, soil and water in the Blesbokspruit wetland

A random sample collection technique of the sludge (above and underneath water), water and wet soil was done at Blesbokspruit wetland which is located in the Gauteng Province, Republic of South Africa (RSA). Aseptically, soil and sludge were collected with a sterile scoop from sampling point, whereas water was collected directly using 500 mL Schott bottle (n = 3). All samples were labelled respectively, stored in sterile container and immediately transferred in the laboratory situated at the University of Johannesburg, Doornfontein Campus, South Africa. Samples were stored at 4 ℃, however, microorganisms from samples were isolated immediately (less than 4hrs) upon arrival at the laboratory as described in section 3.2.

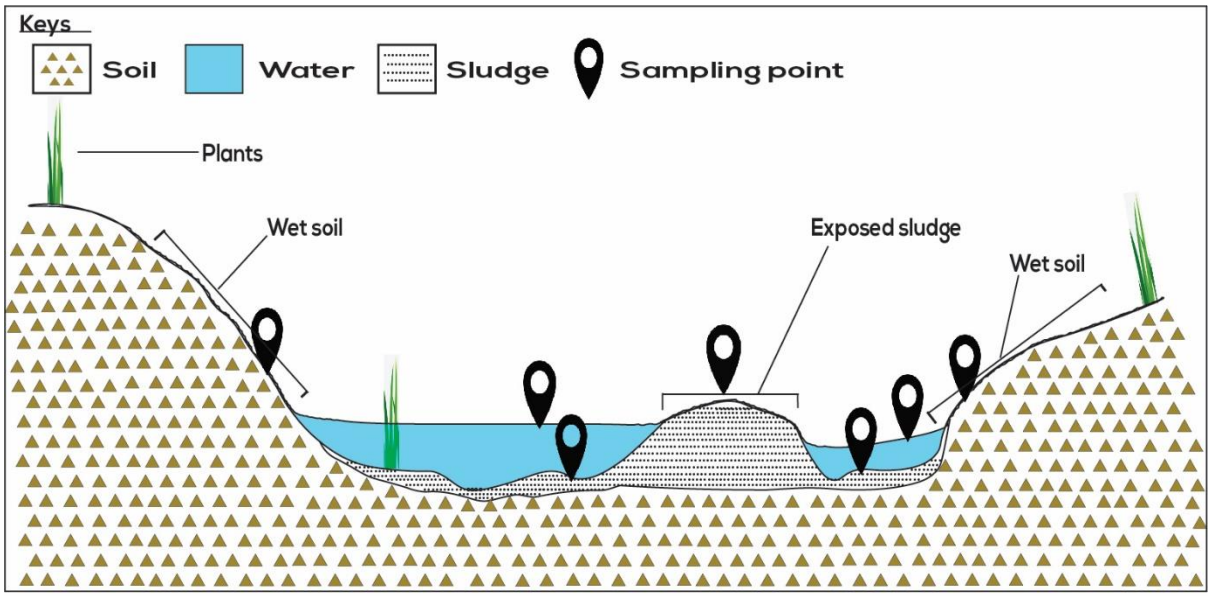

Figure 3.1: Cross-section of Blesbokspruit wetland at specific site illustrating the random sampling points, sludge (underneath and above surface water), water and soil.

3.2. Microbial cultures identification

3.2.1. Isolation and characterisation of aerobic cultures

Aerobic and facultative anaerobic bacteria were isolated from soil and sludge as follows: about ± 6 g of sludge and soil was weighed, then transferred into a falcon tube containing 10 mL of saline solution. The mixture was stirred in a vortex for approximately 30 seconds. Serial dilution was performed by transferring 1 mL of saline mix with soil and sludge separately under a series of dilutions before being spread plated on Nutrient Agar plates which contained antibiotics that suppress fungal development and the agar plates were incubated at 25 ℃ and 32 ℃. Different types of colonies were morphological characterised and isolated using the streak plating technique. The agar plates were incubated according

to their respective temperature (25℃ and 32℃) and then verified by performing Gram staining technique. Where there were mixed cultures, the streaking technique was repeated on fresh agar plates until pure isolates were successfully achieved.

3.2.2. DNA Extraction and 16S RNA sequencing

The microorganisms were streak plated in the Nutrient agar containing antibiotics for fungal suppression, incubated at 25 ℃ and 32 ℃ for 48-72 hours. The subsequent colonies were peaked using an inoculation loop and inoculated on a nutrient broth. This mixture was incubated at 25 °C and 32 °C at 120 rpm for 48 hours. After the cells have grown, 1 mL sample was concentrated by centrifugation at 8000 g for 15 minutes and DNA was extracted using the Bacterial DNA extraction kit (Zymo Research, California, USA), as per manufacturer's instruction. A DNA extract kit, Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005), was used to extract genomic DNA from the cultures. OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) was used to amplify the 16S rDNA sequence target region 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-CGGTTACCTTGTTACGACTT-3' with primers 16S-27F and 16S-1492R, targeting 16S rDNA sequence, respectively. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for every sample used in the study. CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI). This was followed by the Polymerase Chain Reaction (PCR) of the 16S rRNA.

3.2.3. Cryopreservation for microbial isolates

A modified method done by Mekuto (2014), microbial isolated cultures were persevered on a 50% v/v glycerol stock solution. Nutrient broth was used to grow microbial isolates for 24 hours at 35 ℃. After being diluted in a 1:1 (v/v) ratio with sterile glycerol in cryogenic labelled tubes, cells were stored at -80℃ until further use.

3.3. Determination of thiocyanate degrading microorganisms

3.3.1. Media preparation

Media (M1, M2, M3, M4 and M5) preparations were prepared using modified methods from Santoshkumar *et al.* (2010) and Mekuto *et al*. (2018). The composition of the mentioned media was as following in Table 3.1.

Table 3.1: Types of media that were prepared containing different composition

Acronym	Chemical composition
M1	Minimal media (MM) measured in (g) where it consisted of the following compounds; K_2HPO_4 (4.3), KH_2PO_4 (3.4), $MgCl.6H_2O$ (0.6) and transferred in 1 L of distilled water (dH_2O)
M2	M1 and bacteriological agar (2%) which was also supplemented with 100 mg SCN^-/L
M3	M1 which was supplemented with 100 mg SCN^-/L
M4	Enrichment media (Luria-Bertani broth) that was prepared by weighing 20 g of Luria Bertani broth and transferred in 1 L of dH_2O.
M5	Enrichment agar (Luria Bertani Agar) where it was dissolved in 1 L of dH_2O

M1, M2, M3, M4 and M5 were autoclaved at a temperature of 121 ℃ at 15psi for 15 minutes and thereafter, were allowed to cool to 40 ℃. Phenol red was initially prepared by weighing and transferring 0.2 g in 1 L of dH_2O. Then, 0.02% v/v of phenol red indicator was added to M2, M3 and M5 while 0.5% w/v glucose was added into M2. Aseptically, M2 and M5 were transferred into a petri dish and stored at 4℃, including M1, M2 and M4.

3.3.2. Screening plate method

A modified method done by Santoshkumar *et al*. (2010), all the microbial isolates stored in glycerol stock solution were transferred into sterile 50 mL falcon tubes containing M4 enrichment media and incubated for 24 to 48 hours at a shaking incubator speed of 120 rpm at 35 ℃. When growth was observed, pour plate technique was performed where 0.1 ml of bacterial isolates in falcon tubes was transferred into M2 indicator plate (containing of M2, glucose and phenol red). Thereafter, the plates were incubated for 24 to 72 hours after inoculation at 32℃. Microbial isolates that grew and showed colour change from red to pink, were further used for the thiocyanate biodegradation studies since the change in colour would have meant that the organisms were able to degrade thiocyanate to produce ammonium, which changes the colour of phenol red to pink.

3.4. Biodegradation of thiocyanate using microorganisms

Bacterial isolates that showed a colour change were used for the thiocyanate biodegradation study. Bacterial isolates were inoculated from glycerol stock solution and transferred into nutrient agar for

streaking technique, then incubated for 24 hours. These microbes were then inoculated and transferred in 20 mL of M1 and incubated for 24 hours at 32℃. Additionally,10 mL was transferred into conical flasks that contained 90 mL of minimal media that was supplemented with 150 and 250 mg SCN^-/L, which was later incubated on an orbital shaker at 180 rpm at 32℃. This experiment was done for a period of 5 days, where the sampling intervals were set at 24 hours. A total of 6 mL samples were taken from each flask and 2 mL was used for cell concentration study that was done using a spectrophotometer at wavelength of 600 nm. 4mL of those samples were kept for analytical studies that included ammonium (NH_4^+) and thiocyanate (SCN^-) analysis.

3.4.1. Analytical methods

3.4.1.1. Thiocyanate determination

All the samples were centrifuged at 14 000 g for 5 min and the thiocyanate concentration was determined using the ferric chloride method at a wavelength of 480 nm. In this method, 2M ferric chloride was prepared in a 1M HNO_3 solution. This solution was covered with aluminium foil to prevent the light from oxidising the solution. Equal volume of this solution and that of thiocyanate were mixed and the resultant mixture was measured using a spectrophotometer at 480 nm with reference to a blank solution.

3.4.1.2. Ammonium analysis

Ammonium concentration was measured using commercially available test kits from Merck Life Sciences, Germany. Merck ammonium (NH4+) (00683) was used to measure the concentration of ammonium in a Spectroquant Nova 60 Instrument. The ammonium test kit works on the Berthelot reaction between ammonium ions, chlorine and phenolic compounds to form indophenol dyes to form a dark blue-green complex which can be quantified spectrophotometrically.

3.4.2. Biological thiocyanate removal efficiency (BTRE)

A balanced mass equation for determined biological thiocyanate removal efficiency is shown in Equation 1

$$SCN_S^- - SCN_R^- = SCN_D^- \quad (1)$$

Calculation of residue percentage obtained from biological degraded thiocyanate concentration is shown Equation 2

$$SCN_R^-(\%) = \frac{SCN_R^-}{SCN_S^-} \times 100 \quad (2)$$

Biological thiocyanate removal efficiency was determined according to Equation 3 or 4.

$$BTRE\ (\%) = \frac{SCN_D^-}{SCN_S^-} \times 100 \quad (3)$$

Alternatively,

$$BTRE\ (\%) = 100 - SCN_R^-\ (\%) \quad (4)$$

Where SCN_D^- was the biological degraded thiocyanate (mg SCN^-/L), SCN_s^- was the initial thiocyanate concentration (mg SCN^-/L) and SCN_R^- was the residual thiocyanate concentration obtained in the inoculated medium (mg SCN^-/L).

CHAPTER 4
RESULTS AND DISCUSSIONS

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4. RESULTS AND DISCUSSIONS

4.1. Isolation and identification of thiocyanate degradation aerobic microorganisms

4.1.1. Introduction

Identification process of bacteria is critical, especially, when they pose cyanide degradation capability and have tolerance to thiocyanate. There are some bacterial isolates that cannot be taxonomically identified based on their phenotypic characteristics. The gel electrophoresis analysis alone cannot identify unknown environmental microorganisms as there are no defined specific targeted aerobic species and positive control of microorganisms. It is possible to characterise bacteria using 16S rDNA sequences by utilising the 27F and 1492R universal primers, where the 16S target region can be amplified and the amplicons be sequenced (Turner *et al.*, 1999).

4.1.2. Aims and objectives

The objectives of this study were to:

- Isolate microbe form soil, sludge and water from the wetland
- Identify microbes using phenotypic characteristics (characterisation) and molecular biology

4.1.3. Results and discussion

4.1.3.1. Isolation and identification of microbial culture

Microorganisms such as bacteria are abundant in nature and these places include normal and extreme environments (soil, oceans, hot springs, acidic conditions, etc.), living organisms (humans, animals and plants) as well as in toxic places (mine tailings, polluted wetlands, toxic waste substances etc.). Therefore, their isolation and identification are of great importance as they can either cause diseases and add more harm to living systems and health of organisms. They can also be useful in treating diseases and detoxifying toxic substances such as thiocyanate (Shafiei *et al*., 2020; Spurr *et al*., 2019; Willey *et al*., 2020). Bioremediation is a process that involves the use of plants, fungi and bacteria to alter, remove, degrade, detoxify or immobilize physical or chemical toxic waste from the environment (Madhavi and Mohini 2012). This process has gained a great importance as it not only acts as a cost-effective solution against pollution from toxic wastes especially from the mining sector. But, they also provide an eco-friendly mechanism against degradation of harmful substances, thus, increasing the need for the isolation and identification of microorganisms that can bio-remediate pollutants or toxic wastes (Abatenh *et al.*, 2017; Chandran *et al.*, 2020; Raghunandan *et al*., 2018).

Studies done by Li *et al*., (2020); Shafiei *et al*. (2020) and Spurr *et al*. (2019) observed that the bacteria that were commonly isolated from gold mines and surrounding areas such as mine effluents and

wetlands were similar to some of the microorganisms that were isolated and identified from the Blesbokspruit wetland (this study) and these organisms were X9C2 (*Exiguobacterium aurantiacum/ mexicanum*), X2A2 and X13A (*Pseudomonas sp./ veronii*), X13A2 (*Pseudomonas brassicacearum*), X6F2 (*Pseudomonas* sp./ *stutzeri*) and X4A2 (*Lysinibacillus xylanilyticus/ fusiformis, Bacillus* sp.). Although some bacteria were successfully cultured using nutrient agar and selective media, it is important to note that only a minor percentage of microorganisms can be cultured from environmental samples, which limits the number of microorganisms that have been isolated, identified and studied, leading to a gap in the research pool and diversity of unknown biodegrading bacteria that exist within the environment (Awasthi *et al*., 2020; Bursle and Robson, 2016; Dickson *et al*., 2014).

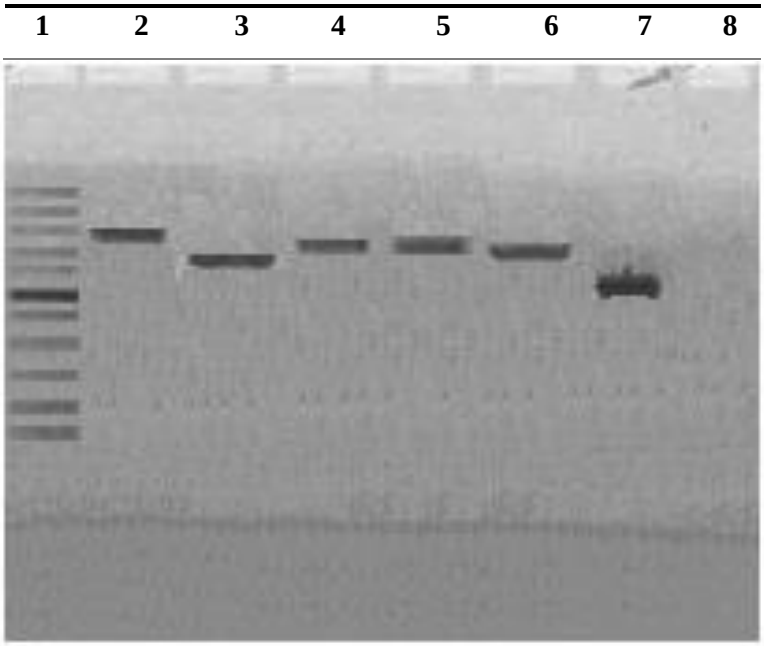


Figure 4.1. Agarose gel electrophoresis analysis of 16S rRNA gene amplified from six bacterial isolates. PCR amplified products were run on 1% agarose gel. Lane 1 indicates the DNA ladder (information of the ladder so that it easy to compare the DNA band sizes). Lanes 2 to 7 show the PCR amplified 16S rRNA gene of the bacterial isolates.

The amplification and sequencing of the 16S rRNA gene is relatively cost-effective and highly efficient, thus making it the method of choice when considering identification and phylogenetic grouping of microorganisms. A great variety of bioinformatics tools are able to work hand in hand with this technology, making the method more frequently used in the identification of microbial communities as well as phylogenetic similarities and microorganisms capable of bioremediation (Han *et al*., 2020; Lei *et al*., 2020; Lovley, 2003). The results of the study showed that the 16S rRNA sequences obtained from soil, sludge and water from the Blesbokspruit wetland were successfully extracted and amplified (see Figure 4.1). The identity of the isolated organisms is shown in Table 4.1 with their subsequent accession numbers, where *Pseudomonas* sp. was the predominant culturable bacteria that was isolated.

This observation is similar to that of Raper *et al.*, (2019) where the authors witnessed the dominance of *Pseudomonas stutzeri* in their samples.

Table 4.1: The identification of the isolated bacteria found in the Blesbokspruit wetland

Sample name	Requested ID	Predicted Organism	GenBank Accession no.
X2A2	DGRZPJTT01R	*Pseudomonas* sp., *Pseudomonas veronii*	CP026880.1, CP018420.1
X4A2	DGS0P5VD014	*Lysinibacillus xylanilyticus*, *Lysinibacillus fusiformis*	KX254351.1, KF475851.1
X6F2	DGT12X8B015	*Pseudomonas* sp., *Pseudomonas stutzeri*	FJ607427.1, EU652047.1
X9C2	DGT1N62C014	*Exiguobacterium aurantiacum*, *Exiguobacterium mexicanum*	KX008295.1, KT630833.1
X13A	DBY48S68013	*Pseudomonas* sp., *Pseudomonas veronii*	CP026880.1, CP018420.1
X13A2	DGT1N62C014	*Pseudomonas brassicacearum*	LT629713.1

4.2. Determination of microorganisms capable of biodegrading thiocyanate

4.2.1. Aims and objectives

The objectives of this study were to:

- Inoculate the identified isolates by using the spread-plating technique into solid media supplemented with thiocyanate in the presence of phenol red indicator.
- Observe which bacterial species were capable of transforming thiocyanate to ammonia (red colour to pink colour).

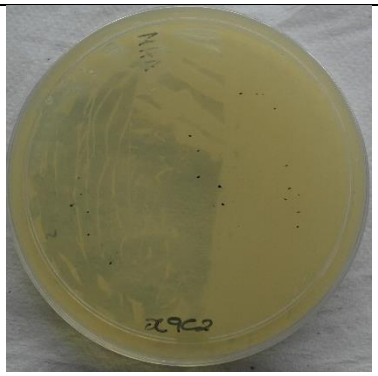
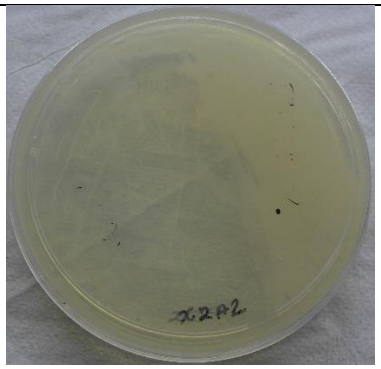
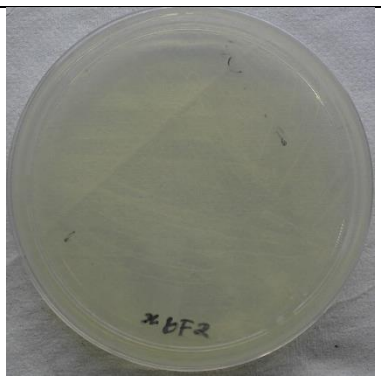
4.2.2. Results and discussion

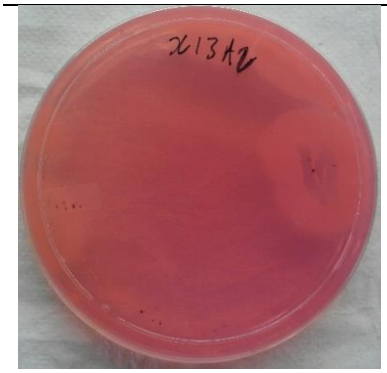
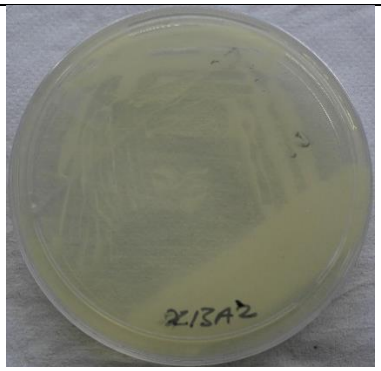
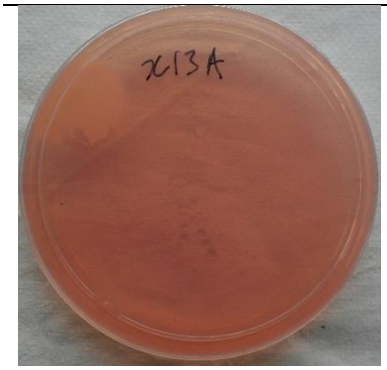
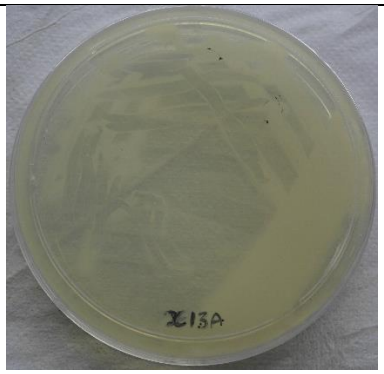
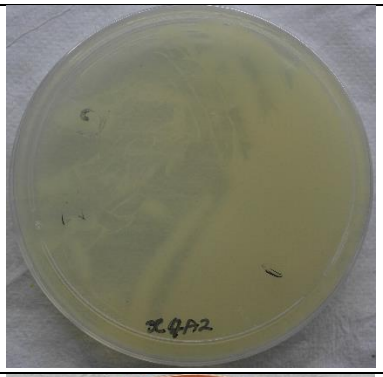
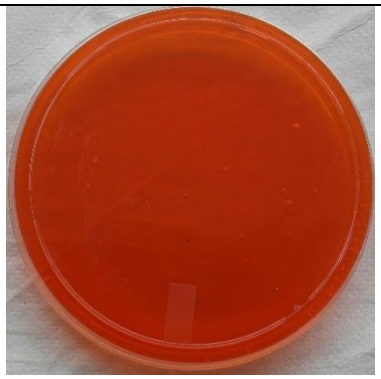
4.2.2.1. Screening microorganisms for thiocyanate biodegradation study

Most bacterial species are not capable of degrading thiocyanate due to the toxicity of thiocyanate to these organisms and the incapability of these organisms to secrete enzymes that can be able to hydrolyse thiocyanate. Therefore, it is ideal to identify bacterial species which are capable of biodegrading thiocyanate so that they can be used in subsequent studies and eliminate those which do not have the

capacity to biodegrade thiocyanate. The screening of capable biodegrading bacteria, whereby phenol red and thiocyanate were incorporated into the Mueller Hinton Agar (MHA) was done. Thiocyanate (100 mg/L) was mixed in equal proportion with the MHA after cooling to a temperature of 50 °C. The organisms which were previously grown were spread-plated on the surface of the MHA which also contained the indicator and incubated for 48 hours at 32 ℃. This process was done to examine if the microbial isolates were able to hydrolyse thiocyanate after a period of 48 hours. As indicated earlier, the degradation of thiocyanate results in the formation of ammonia, which in-turn results in the colour change of the indicator from red to pink (pH ≤ 8.1) (Santoshkumar *et al.*, 2010). This change of colour was used as a screening mechanism in this study to determine the organisms which are able to degrade thiocyanate. Table 4.2 demonstrates of indicator plate method forming pink colour after incubation.

Table 4.2: Indicator plate method demonstrating colour change from red to pink using the isolated bacteria.

Indicator plate	Control plates	Labels	Results
		*Exiguobacterium aurantiacum*, *Exiguobacterium mexicanum* (X9C2)	Intense pink colour was formed on screened plate. The positive control had growth, no pink colour and only slight yellow colour produced.
		*Pseudomonas* sp. *Pseudomonas veronii* (X2A2)	Intense pink colour was formed on screened plate. The positive control had growth and no indication of colour change developed.
		*Pseudomonas* sp., *Pseudomonas stutzeri* (X6F2)	Intense pink colour was formed on screened plate. The positive control had growth and no indication of colour change was formed.

		*Pseudomonas brassicacearum* (X13A2)	Pink colour was formed on screened plate. The positive control had growth and no indication of colour change was formed.
		*Pseudomonas* sp., *Pseudomonas veronii* (X13A)	Faint pink colour was formed on screened plate. The positive control had growth and no indication of colour change was formed.
		*Lysinibacillus xylanilyticus*, *Bacillus* sp., *Lysinibacillus fusiformis* (X4A2)	Intense pink colour was formed on screened plate. The positive control had growth and no indication of colour change was formed.
		Negative control PR+SCN$^-$	No growth was observed.
		Negative control no PR+SCN$^-$	No growth was observed.

		Negative control SCN^-	No growth was observed.

Bacteria indicator plate labelled X9C2, X2A2, X6F2 and X4A2 displayed intense pink colour indication while bacterium indicator plate labelled X13A2 produced a transparent pink and bacterium indicator plate labelled X13 had trace of faint pink. Thus, bacterium indicator plate labelled X13 demonstrated that the pH range was more skewed to neutral pH as compared to rest of the indicator plates. Bacteria indicator plate labelled X9C2, X2A2, X6F2 and X4A2 demonstrated that the ammonia that was produced was higher as compared to bacterium indicator plate labelled X13A2 due to the colour intensity, thus, the pH of the medium increased as the presence of ammonia increased (Santoshkumar *et al.*, 2010). Phenol red is categorised as an acid-base indicator, when acidic ($pH \leq 6.8$), it turns yellow, while at neutral pH is red and basic conditions ($pH \geq 7.4$) it turns to pink or fuchsia colour (Aryal, 2019). Aerobic thiocyanate biodegrading bacteria result in oxidation process where ammonia, sulphate and bicarbonate are formed (Hung and Pavlostathis, 1997). Therefore, the screening process by indicator plate method permits for the demonstration of aerobic bacterium isolates to indicate whether they are capable of degrading thiocyanate or not through the colour change from red to pink. Additionally, this screening process is robust, effective and time-saving since it can be done quickly. However, the bacterial conditional settings and growth or incubation duration contribute a vital role in this process. The principle behind the phenol red indicator demonstrates that the indication of colour change is determined by the presence of acidic or basic compounds, meaning the greater the amount of basic compound, the greater the colour intensity.

Exiguobacterium bacterial species have shown degrading capabilities of various compounds in order to exploit carbon or nitrogen as sole source and energy from thiocyanate. According to Mohanty and Mukherji (2008), *Exiguobacterium aurantiacum* has shown capabilities to biodegrade diesel oil as sole substrate by utilising carbon as main source. Furthermore, some *Exiguobacterium* bacteria proved to consist of co-metabolic abilities, where two or more substrates were metabolised by single type of *Exiguobacterium* strain at high concentration. *Exiguobacterium acetylicum* managed to co-metabolise thiocyanate and free cyanide under alkaline environment (Mekuto *et al.*, 2016b). *Exiguobacterium mexicanum* also demonstrated the ability to biodegrade ammonium, nitrite and nitrate in order to obtain nitrogen source under aerobic conditions (Cui *et al.*, 2021). *Exiguobacterium* sp. has been revealed to be capable of biodegrading N-methylated diaminotriphenylmethane at high concentrations while maintaining pH within basic conditions (Wang *et al.*, 2012). Thus, pink colour intensity is likely to be

observed since *Exiguobacterium* strains has shown great capabilities to biodegrade various environmental significant compounds.

Pseudomonas species have contributed significantly in the biodegradation of toxic compounds contaminating the environment and various research have proved that a wide range of *Pseudomonas* species can biodegrade various compounds. They also consist of multi-substrate catalytic proficiencies to degrade a broad range of compounds that are off environmental significance, i.e. *Pseudomonas brassicacearum* has been reported to have such robust abilities (Chen *et al.*, 2022) and based on the results observed herein, *Pseudomonas brassicacearum* has shown biodegrading capabilities towards thiocyanate. *Pseudomonas veronii* and *Pseudomonas sp*. have illustrated thiocyanate degradation potential due to the observed colour change to pink although no other previous studies have shown any successful thiocyanate biodegradation done by either of these species as compared to *Pseudomonas aeruginosa*. Based on studies done by Mekuto *et al.* (2018), about 78% and 98% biodegradation efficiency of thiocyanate through *Pseudomonas aeruginosa* was observed for the first time.

All the positive controls for growth did indicate growth of bacteria and also, they did not produce any colour change in the absence of the indicator although being grown in the presence of thiocyanate. All the negative controls demonstrated no growth, which proves that the sterilisation process and aseptic technique applied were effective.

4.3. Biodegradation of thiocyanate using capable microorganism

4.3.1. Aims and objectives:

The objectives of this study are to:

- Assess the pure and mixed cultures of the isolates for the biodegradation of thiocyanate in liquid media.
- Monitor the ammonium formation during thiocyanate biodegradation.
- Assess the interrelation between thiocyanate, ammonium and microorganisms.

4.3.2. Results and discussion

4.3.2.1. Thiocyanate biodegradation in MM.

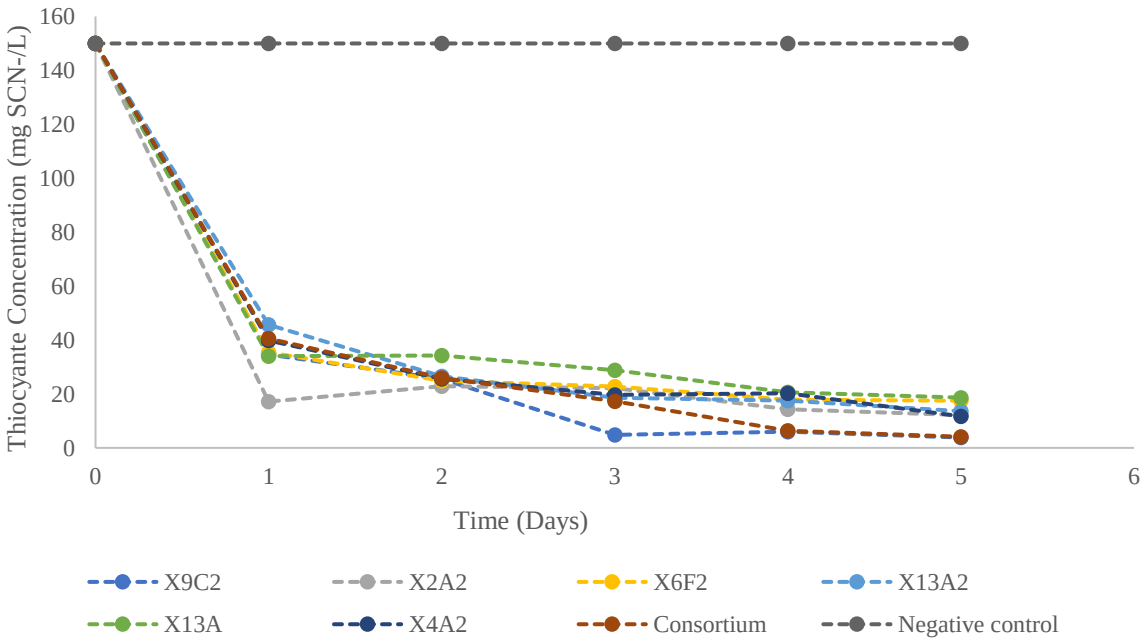


Figure 4.2. Thiocyanate biodegradation profile at a concentration at 150mg/L using the six bacterial isolates and their consortium.

The thiocyanate concentration was set at 150 mg/L at the beginning and after the 5-day period, the residual concentrations of thiocyanate for *Exiguobacterium aurantiacum/mexicanum*, *Pseudomonas* sp./*veronii* (X2A2), *Pseudomonas* sp./*stutzeri*, *Pseudomonas brassicacearum*, *Pseudomonas* sp./ *veronii* (X13A) and *Lysinibacillus xylanilyticus/ fusiformis* and the consortium were 3.841 mg SCN^-/L, 12.165 mg SCN^-/L, 17.477 mg SCN^-/L, 13.614 mg SCN^-/L, 18.642 mg SCN^-/L, 11.682 mg SCN^-/L and 4.125 mg SCN^-/L respectively (see Figure 4.2). A study which was conducted by Li *et al.* (2020) demonstrated the robustness of a mixed culture where the organisms were able to degrade thiocyanate from an initial concentration of 1818 mg SCN^-/L to a residual concentration of 0.68 mg SCN^-/L in a two-stage biological treatment process. In addition, Mekuto *et al*. (2017) utilised a microbial consortium to degrade increasing thiocyanate concentrations over a period of over 300 days where the authors achieved complete degradation over the mentioned period. These studies demonstrate the efficacy of using microbial consortiums in the degradation of thiocyanate.

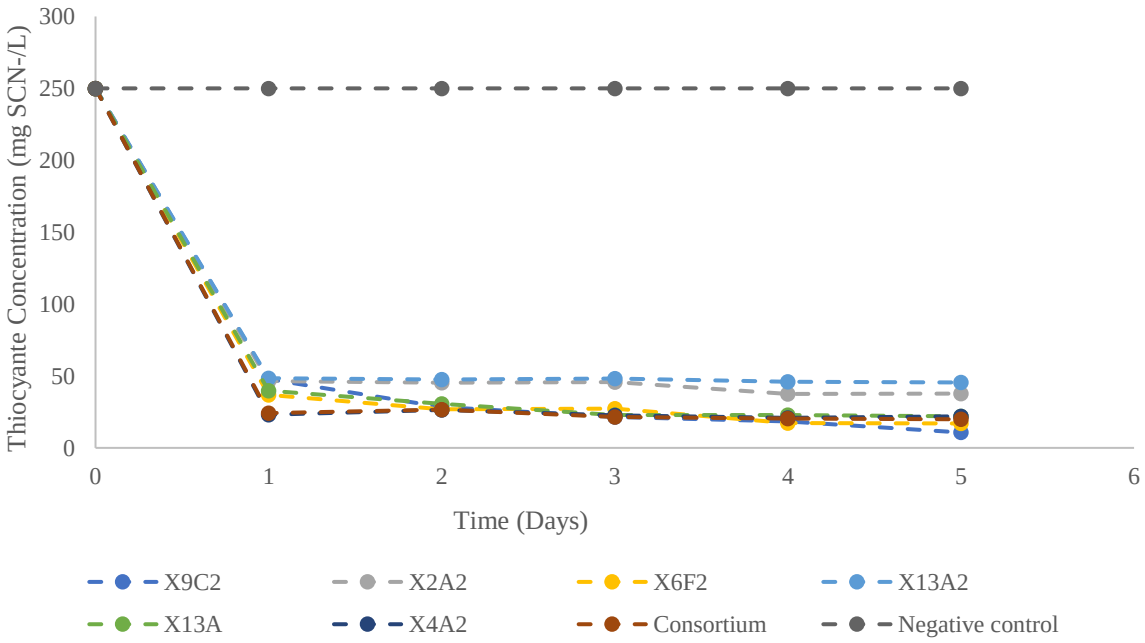


Figure 4.3. Thiocyanate biodegradation profile at a concentration at 250mg/L using the six bacterial isolates and their consortium.

The pure isolates and their consortium were tested for their biodegradative capacity at 250 mg SCN^-/L over a period of 5 days. The residual thiocyanate concentration from a culture of *Exiguobacterium aurantiacum/ mexicanum*, *Pseudomonas* sp./*veronii* (X2A2), *Pseudomonas* sp./*stutzeri*, *Pseudomonas brassicacearum*, *Pseudomonas* sp./*veronii* (X13A), *Lysinibacillus xylanilyticus/ fusiformis* and their consortium was 10.716 mg SCN^-/L, 37.818 mg SCN^-/L, 16.966 mg SCN^-/L, 45.489 mg SCN^-/L, 21.938 mg SCN^-/L, 21.909 mg SCN^-/L and 19.892 mg SCN^-/L respectively (see Figure 4.3). In a different study, thiocyanate degrading organisms were able to degrade 250 mg SCN^-/L completely over a 100 h period (Mekuto *et al*., 2018). Contrary to the observation that were observed when the thiocyanate concentration was set at 150 mg SCN^-/L, *Exiguobacterium aurantiacum/ mexicanum* achieved a higher degradation capacity compared to the consortium. This observation was not expected since the consortium is able to release multiple enzymes that can be able to rapidly degrade the thiocyanate. However, it is hypothesised that the competitive nature of the organisms might have resulted in the release of toxicants by certain organisms to eliminate competition. As a result, the biodegradative capacity of the consortium was lowered.

Table 4.3: Biodegradation efficiencies of the organisms at 150 mg SCN^-/L and 250 mg SCN^-/L over a 5-day period.

Organism	Batch experiment			
	SCN^- concentration at 150 mg/L		SCN^- concentration at 250 mg/L	
	Residual (mg/L)	Removal efficiency (%)	Residual (mg/L)	Removal efficiency (%)
Exiguobacterium aurantiacum/ mexicanum	2.56	97.44	4.29	95.71
Pseudomonas sp./ *veronii*	8.11	91.89	15.13	84.87
Pseudomonas sp./ *stutzeri*	11.65	88.35	6.79	93.21
Pseudomonas brassicacearum	9.08	90.92	18.20	81.8
Pseudomonas sp./ *veronii*	12.43	87.57	8.78	91.22
Lysinibacillus xylanilyticus/ fusiformis/ Bacillus sp.	7.79	92.21	8.76	91.24
Consortium	2.75	97.25	7.96	92.04

Exiguobacterium aurantiacum/mexicanum proved to have better SCN^- removal efficiency where the organism achieved 97.44 and 95.71% from initial concentration of 150 mg SCN^-/L and 250 mg SCN^-/L, respectively. The consortium of these organisms was expected to produce better SCN^- removal efficiency due to previous research studies have shown significant amounts of SCN^- removal efficiency obtained from mixed cultures, especially in high concentrations. A study demonstrated the SCN^- removal efficiency reaching 99.96% by decreasing 1818 mg SCN^-/L to 0.68 mg SCN^-/L in a two-stage unit using a mixed culture obtained from gold tailing wastewater (Li *et al.*, 2020). The mentioned study is one in many where the microbial consortium has shown its efficacy in treating contaminants. But at a higher concentration of 250 mg/L, the removal efficiency of the consortium was lowered, and it is hypothesised that this phenomenon was due to the competitiveness amongst the organisms within the culture.

4.3.2.2. Ammonium profiling during the biodegradation process

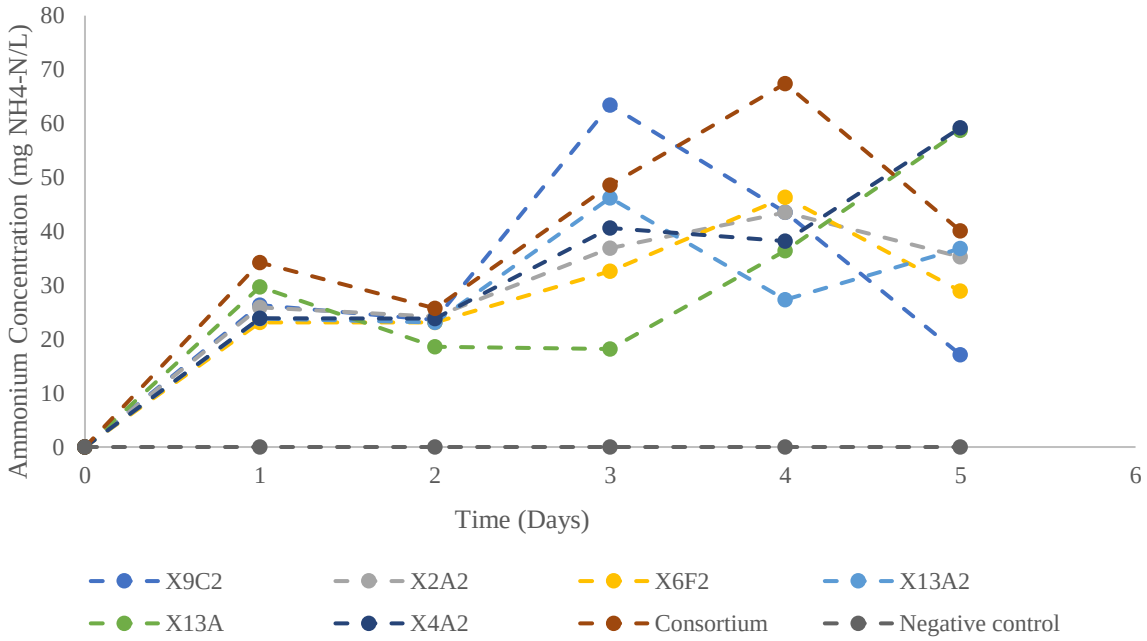


Figure 4.4. Ammonium concentration produced during biodegradation of thiocyanate in minimal media at 150mg SCN^-/L.

In the period of 120 hour (h) during thiocyanate biodegradation experiment at 150 mg SCN^-/L, the highest ammonium-nitrogen concentration observed was 67.4 mg NH_4-N/L which was obtained from the consortium of all 6 microorganisms (see Figure 4.3). In other experiments, the highest concentration was 63.4 mg NH_4-N/L obtained from *Exiguobacterium aurantiacum/mexicanum* and second highest concentration was 59.2 mg NH_4-N/L which was observed from *Lysinibacillus xylanilyticus/ fusiformis*. Other microbial isolates including *Pseudomonas* sp./*veronii* (X13A), *Pseudomonas* sp./*stutzeri*, *Pseudomonas brassicacearum* and *Pseudomonas* sp./*veronii* (X2A2), reached a maximum of 58.7 mg NH_4-N/L, 46.3 mg NH_4-N/L, 46.2 mg NH_4-N/L and 43.5 mg NH_4-N/L ammonium concentration, respectively. The culture with *Exiguobacterium aurantiacum/mexicanum* demonstrated the lowest residue of ammonium-nitrogen after 120 h. This validates that the *Exiguobacterium aurantiacum/mexicanum* has capabilities of nitrification as well as other samples have shown the decrease in ammonium-nitrogen. The ammonium concentration fluctuated, whereas in a theoretical perspective it's supposed to demonstrate an exponential increase. However, some thiocyanate degrading organisms are mostly known to utilise the n\ammonium as a nitrogen source. Thus, this constant decrease in ammonium signifies the utilisation of ammonium-nitrogen, which it also displays the effectiveness and robustness of these microorganisms.

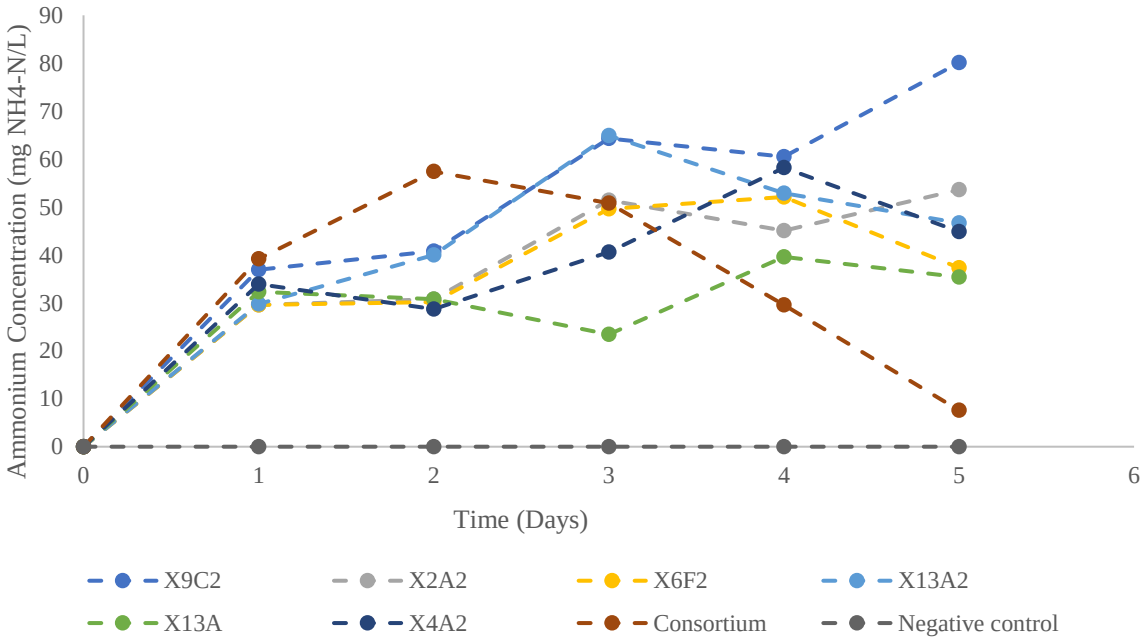


Figure 4.5. Ammonium concentration produced during biodegradation of thiocyanate in minimal media at 250 mg SCN^-/L.

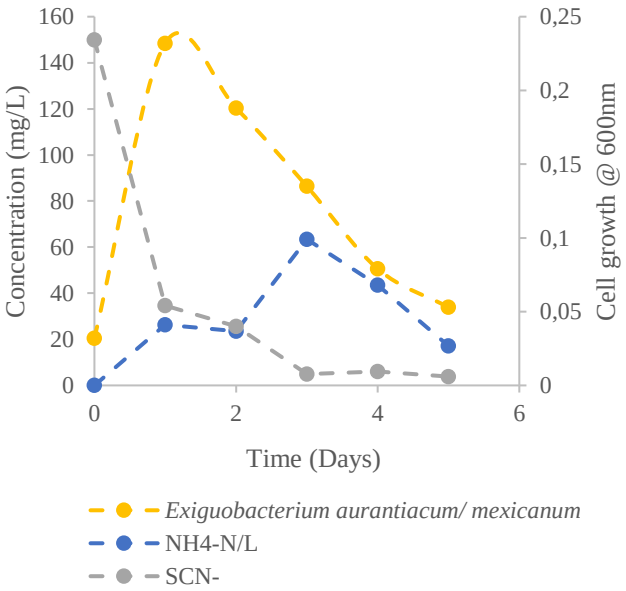
In the period of 120 hour (h) during thiocyanate biodegradation experiment at 250 mg SCN^-/L, the highest ammonium-nitrogen concentration was at 80.1 mg NH_4^+-N/L which was obtained by sample consisting *Exiguobacterium aurantiacum/mexicanum*. This was followed by the cultures containing *Pseudomonas brassicacearum*, *Lysinibacillus xylanilyticus*/ *fusiformis*, Consortium, *Pseudomonas* sp./ *veronii* (X2A2), *Pseudomonas sp./ stutzeri* and *Pseudomonas* sp./*veronii* (X13A) which obtained highest ammonium-nitrogen concentration of 64.9 mg NH_4^+-N/L at 72 h, 58.2 mg NH_4^+-N/L at 96 h, 57.4 mg NH_4^+-N/L at 48 h, 53.6 mg NH_4^+-N/L at 120 h, 52.1 mg NH_4^+-N/L at 96 h and 39.6 mg NH_4^+-N/L at 96 h period, respectively. Fluctuating trends of ammonium-nitrogen were observed in all the cultures and this is due to the concomitant production and utilisation of the ammonium-nitrogen throughout the operational period. The study that was done by Mekuto *et al.* (2018) observed that thiocyanate degrading organisms utilised ammonium-nitrogen which was produced from the thiocyanate biodegradation process. This observation occurred when organisms had completely utilised the thiocyanate and switched their metabolism to utilise the produced ammonium as a source of nitrogen. In addition, studies done by Li *et al.* (2020) revealed that the accumulation of ammonium-nitrogen of about 318 mg NH_4^+-N/L occurred from thiocyanate biodegradation of 1818 mg SCN^-/L, which later was utilised by the organisms. Therefore, this demonstrates that microorganisms used in this study were capable of utilising the ammonium-nitrogen as a nitrogen source especially in an environment with scarce nutrition. A study done by Spurr *et al.* (2019) illustrated that thiocyanate degrading organisms are able to use the ammonium which resulted from the degraded thiocyanate as

an essential nutrient source. Partially, the decrease of ammonium-nitrogen signifies nitrification, however, the monitoring of nitrites and nitrates was not carried out in this study. On the other hand, TDOs mostly were previously testified to possess nitrification and denitrification capabilities (Mekuto, *et al.*, 2016a; Mekuto *et al.*, 2018). Organisms of this study not only degrade thiocyanate; however, they further nitrify the produced ammonium-nitrogen. As a result of this, these microorganisms demonstrate the role that they play in their original habitat (Blesbokspruit wetland).

The maximum NH_4^+-N concentration obtained from this study (includes samples consist of 150 mg SCN^- / L and 250 mg SCN^-/ L) are lower as compared to other previous studies. A study conducted by Mekuto *et al.* (2018), as a result of thiocyanate degradation, ammonium was generated at a maximum concentration of 123 mg NH_4^+-N/L from an initial concentration of 250 mg SCN^-/L. Thiocyanate degrading organisms (TDO) usage of ammonium was evident after 51 hours when 115 mg NH_4^+-N/L was reached from initial thiocyanate concentration of 250 mg /L (Mekuto *et al.*, 2018). Additionally, a 100 mg/L dose of thiocyanate would theoretically release 24 mg/L of ammonium-nitrogen, of which about 10% could be converted into biomass (Li *et al.*, 2020). Conversely, ammonium nitrogen was inhibited from nitrification due to the presence of thiocyanate (Li *et al.*, 2020). Interestingly, all of the residual concentrations of NH_4^+-N obtained on all experiments in this study during day 5 were higher compared to studies done by Mekuto (2017), where after 170 h of incubation, the mixed culture utilized NH_4^+-N almost to zero from 250 mg SCN^-/L culture.

4.3.2.3. Thiocyanate and ammonium profile in relation with microbial growth

A



B

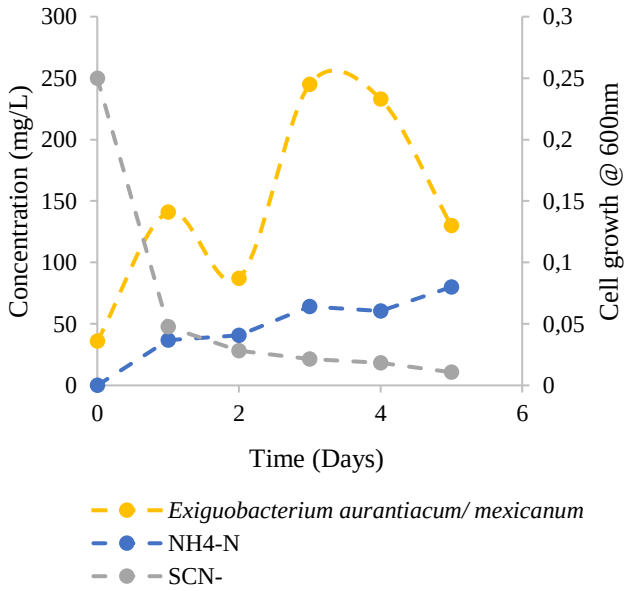


Figure 4.6. Thiocyanate and ammonium profile including cell growth of *Exiguobacterium aurantiacum/ mexicanum* measured absorbance at 600nm, where **A** consist of 150 mg SCN^-/L and **B** 250mg SCN^-/L.

Exiguobacterium aurantiacum/ mexicanum in Figure 4.6A depicted rapid growth during on day 1 as compared to Figure 4.6B, whereas the concentration of thiocyanate in both flasks decreased in an exponential manner. Additionally, the biodegradation efficiency in 4.6A and 4.6B has shown significant effectiveness as compared to most other thiocyanate degradation studies. In most research, microbial isolates (i.e., *Klebsiella sp.*) have shown up to 98.8% of thiocyanate degradation efficiency capabilities within a period of 7 days without nutritional supplementation (Ahn *et al.*, 2004). Whereas this overall thiocyanate biodegradation efficiency is achieved at the end of the investigation and exponential thiocyanate destruction occurs between day 2 to 4.

In Figure 4.6A, day 3 exhibited a drop in ammonium (NH_4^+-N) whereas in Figure 4.6B, it displayed fluctuations of NH_4^+-N. A study done by Cui *et al*. (2021) demonstrated that *Exiguobacterium mexicanum* under aerobic condition can degrade ammonium as a source of nitrogen. The thiocyanate levels reduced while the formed ammonium was utilised due to observed ammonium reduction occurrence after 72 hours in Figure 4.6A. This microorganism revealed to possess co-metabolic characteristics as it was able to biodegrade thiocyanate and the available NH_4^+-N while showing microbial growth. The growth of *Exiguobacterium aurantiacum/ mexicanum* in Figure 4.6A showed a death phase after day 1 until the end of the experimental period. This observation is quite peculiar since the biodegradation efficiency of this organism was high as compared to other tested microorganisms. In Figure 4.6B, *Exiguobacterium aurantiacum/ mexicanum* entered the death after day 4 and this might be attributed to the limited carbon sources within the medium even though the source of nitrogen was abundant in a form of ammonium-nitrogen.

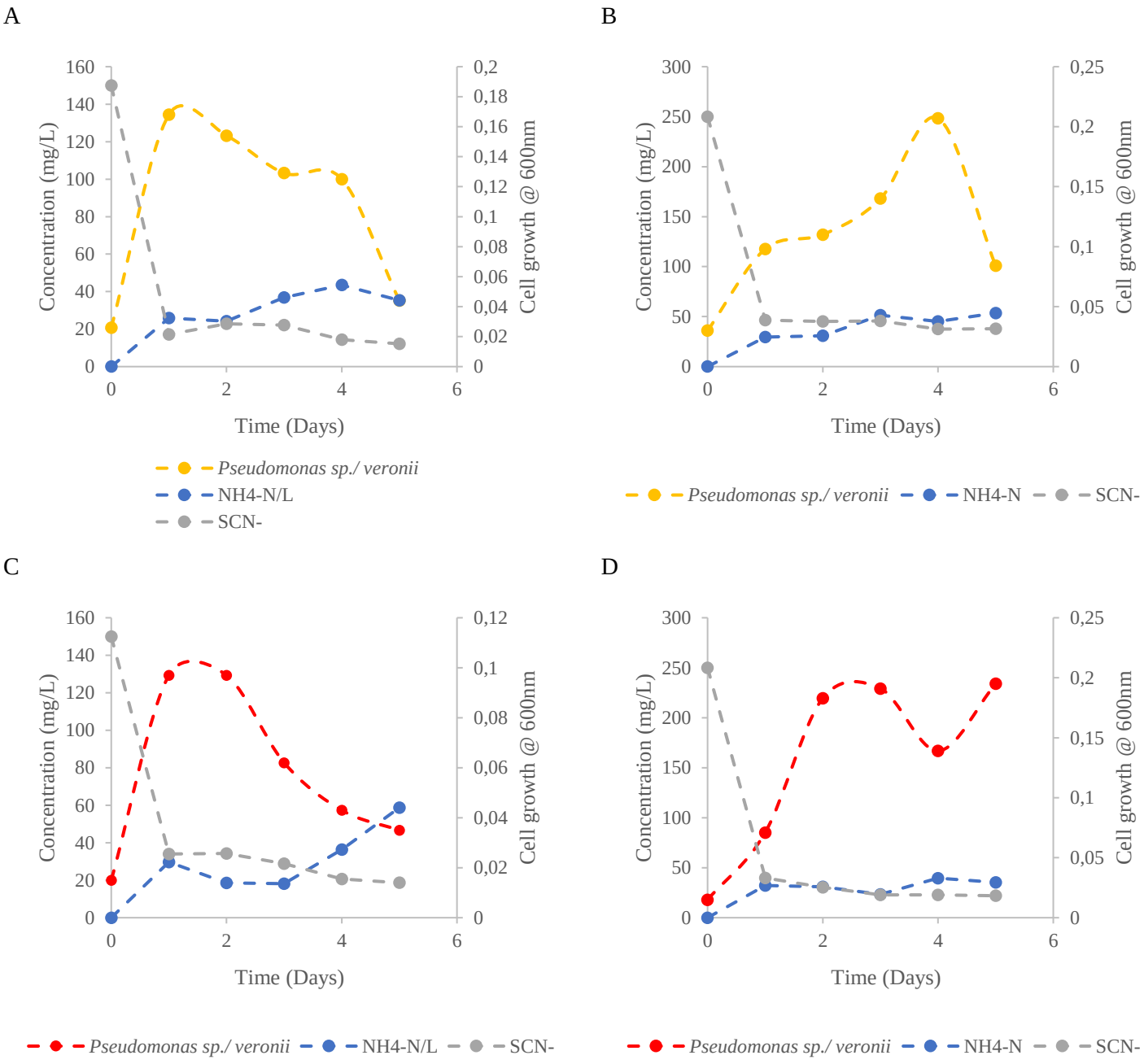


Figure 4.7. Thiocyanate and ammonium profile including cell growth of *Pseudomonas* sp./ *veronii* measured absorbance at 600 nm, where **A** and **C** consisted of 150 mg SCN^-/L, and **B** and **D** 250 mg SCN^-/L.

The *Pseudomonas* sp./ *veronii* used in Figure 4.7A and B (X2A2) was isolated and cultured separately from the ones in Figure 4.7C and D (X13A). However, based on sequencing report, they were identified as the same. Interestingly, when performing plate technique shown in (Table 4.2) and gel electrophoresis 16S rDNA shown in Figure 4.1, the results for their potential to biodegrade thiocyanate as well as migration point of the amplificated 16S gene were not the same. Additionally, their aerobic biological thiocyanate removal efficiency was also not the same, whereby, in Figure 4.7A, B, C and D

attained a biodegradation efficiency of 91.89 %, 84.87 %, 87.57 % and 91.22%, respectively. This signifies that the species could contain genomic content that is not 100 percent similar, even though the microorganisms share the same identity. Nevertheless, the phylogeny showed 100% of relation as shown in Figure 7.1 (Appendices). Growth patterns of Figure 4.7A and Figure 4.7C were not the same, also the highest microorganism absorbance of Figure 4.7A was higher compared to C. The ammonium-nitrogen residual on Figure 4.7C was greater than in Figure 4.7A after a 5-day period. Growth patterns observed in Figure 4.7B were not the same as that in Figure 4.7D, including highest absorbance of microorganism recorded in Figure 4.7B was more than that in Figure 4.7D. Ammonium-nitrogen residual concentration in Figure 4.7B was greater than that in Figure 4.7D.

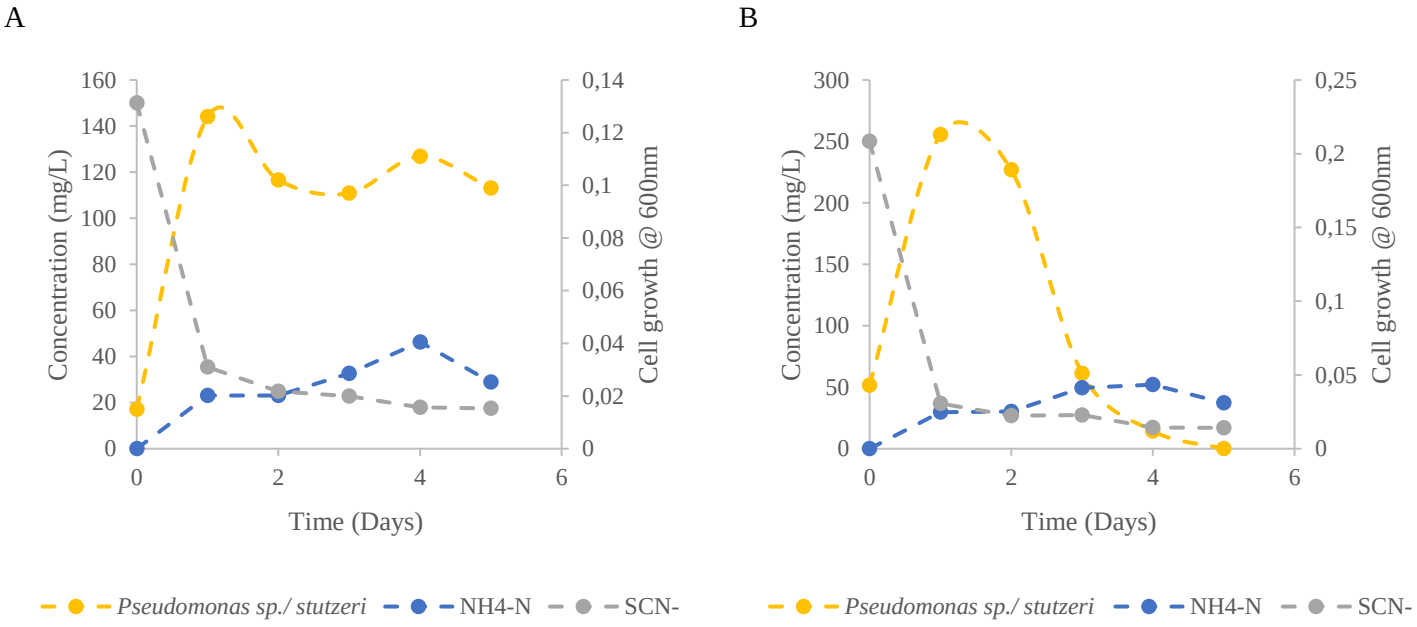


Figure 4.8. Thiocyanate and ammonium profile including cell growth of *Pseudomonas* sp./ *stutzeri* measured absorbance at 600 nm, where **A** consist of 150 mg SCN^-/L and **B** 250 mg SCN^-/L.

Pseudomonas sp./*stutzeri* demonstrated an exponential increase growth pattern, while SCN^- concentration exponentially decreased and ammonium-nitrogen concentration gradually increased from day 0 to 1 (see Figure 4.8A and B). Similar trends have been observed where thiocyanate with concentration of 7 mM SCN^- was biodegraded completely while an exponential increase of cell growth of *Pseudomonas stutzeri* was observed in less than 25 h (Grigor'eva *et al.*, 2006). The ammonium produced in Figure 4.8B is greater than ammonium produced in Figure 4.8A, due to the initial thiocyanate concentration levels in Figure 4.8B being higher than Figure 4.8A. However, similar trend of ammonium from Figure 4.8A and B were observed, whereby a gradual increase from day 0 to 4 was observed, then after day 4, depreciation in concentration was observed. Cell growth in Figure 4.8A has shown less growth as compared to Figure 4.8B. The biodegradation of SCN^- was not complete,

and this is due to presence of nitrogen source as shown in Figure 4.8A and B. Grigor'eva *et al.* (2006) studied thiocyanate degradation using *Pseudomonas stutzeri*, where no carbon source was present and the production of NH_4^+, NH_3 and $NO_3^-$was observed, however, inhibition of SCN^- consumption was observed due to the presence of these nitrogenous compounds since this organism preferred ammonium more than the thiocyanate as its nitrogen source.

In most studies, *Pseudomonas stutzeri* has been recognised as a thiocyanate degrading organism as well as nitrifiers and typically found in mining effluents. Studies done by Li *et al.* (2020) reaffirm this assertion, where *Pseudomonas stutzeri* was isolated from wastewater produced from a process of smelted gold and proved to degrade thiocyanate, as well as the study by Huddy *et al.* (2015b) proved *Pseudomonas stutzeri* has thiocyanate degradation capabilities and it was one of the dominant ASTER™ microorganisms.

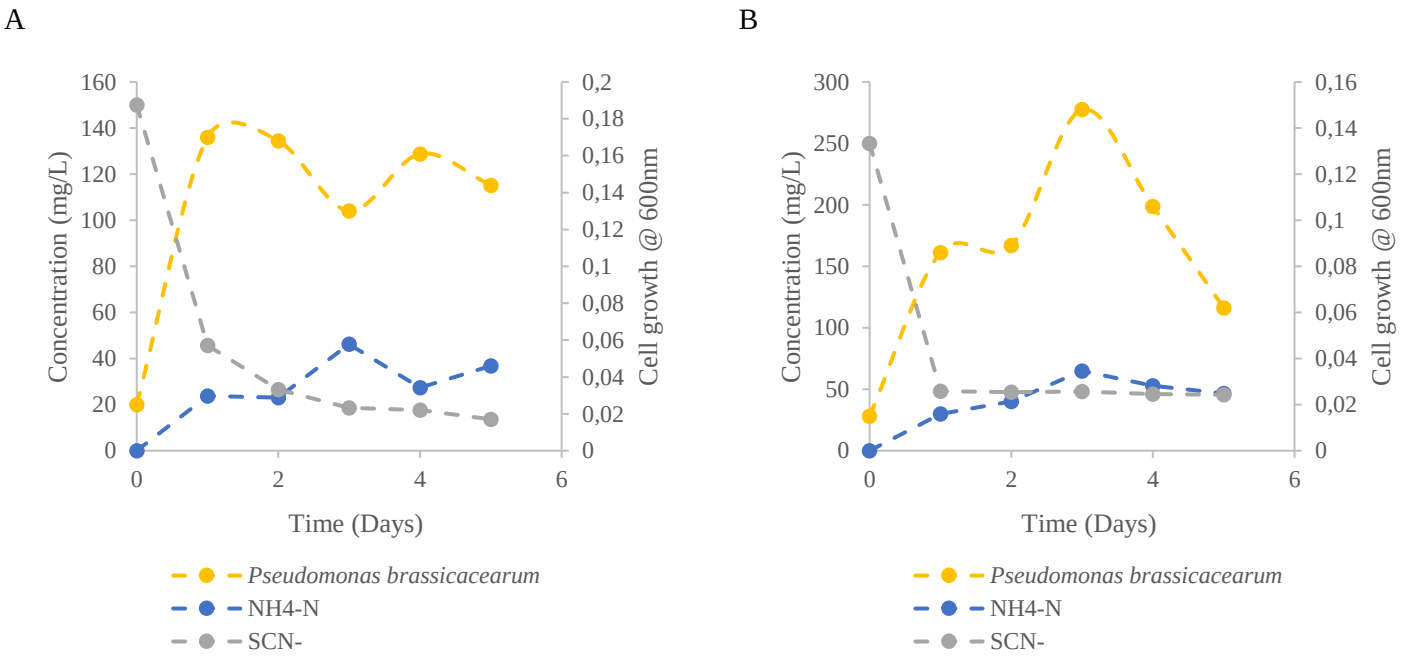


Figure 4.9. Thiocyanate and ammonium profile including cell growth of *Pseudomonas brassicacearum* measured absorbance at 600 nm, where **A** consist of 150 mg SCN^-/L and **B** 250 mg SCN^-/L.

An exponential decrease of thiocyanate, gradual increase of ammonium and exponential increase of microbial growth observed on both Figure 4.9A and B during the first 24h was witnessed. In Figure 4.9A, an exponential phase was witnessed from day 0 to 1 while in Figure 4.9B, the exponential phase was observed on day 2 to 3. Death phase in Figure 4.9A was observed after day 4 whereas in Figure 4.9B, it was observed from day 3 to 5. This illustrates that the microorganism was slightly sensitive towards greater concentrations of thiocyanate. Evidently, to support this observation, the thiocyanate removal efficiency of *Pseudomonas brassicacearum* illustrated that at 150 mg SCN^-/L was 90.92% and

at 250 mg SCN^-/L, it was 81.8%. This meant that the higher concentration of thiocyanate had a negative impact on this organism.

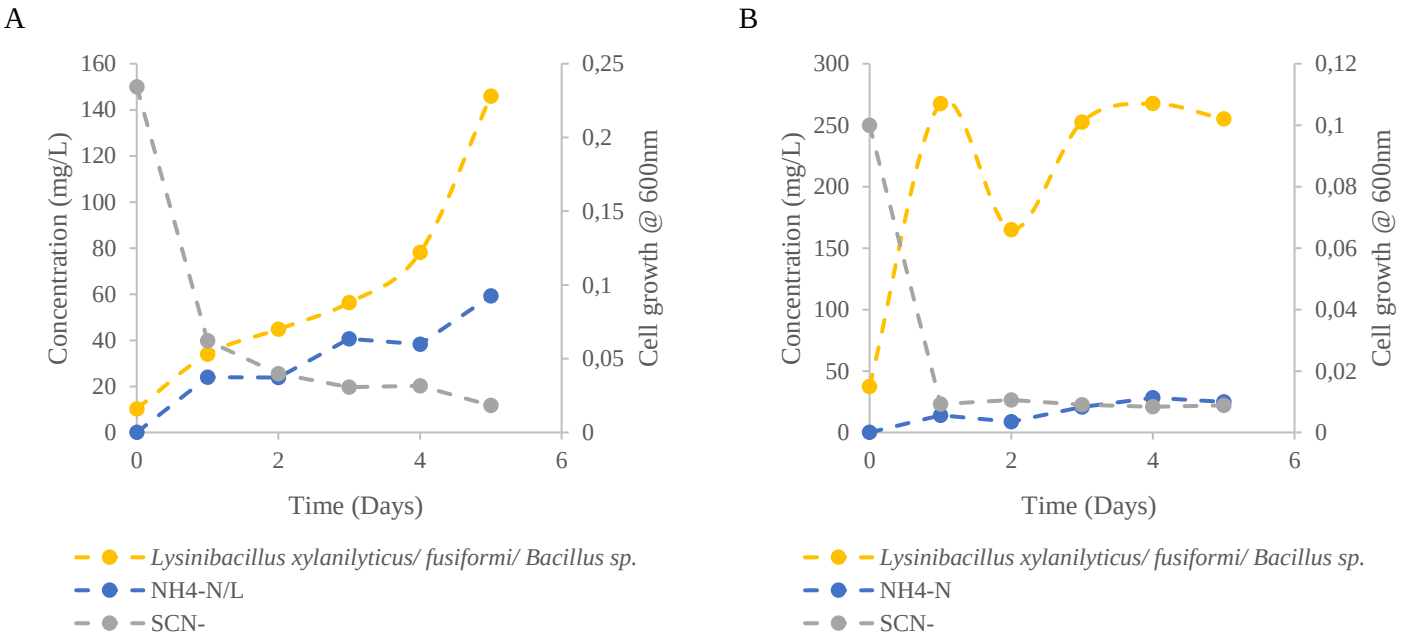
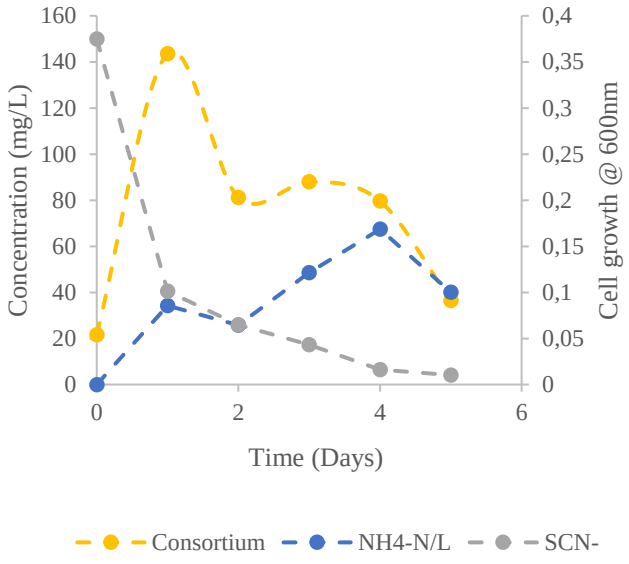


Figure 4.10. Thiocyanate and ammonium profile including cell growth of *Lysinibacillus xylanilyticus/ fusiformis/ Bacillus* sp. measured absorbance at 600 nm, where **A** consist of 150mg SCN^-/L and **B** 250mg SCN^-/L.

In Figure 4.10A, thiocyanate concentration trend was exponentially decreasing, whereas microorganism growth pattern was illustrating exponential phase throughout the 5-day period of study. There was a gradual increasing trend observed and slight decrease between day 1 to 2 and day 3 to 4 of ammonium-nitrogen in Figure 4.10A. In Figure 4.10B, thiocyanate concentration depreciated drastically during day 0 to 1 and a slight decrease of SCN^- concentration between day 1 to 5. Meanwhile, the microorganism demonstrated exponential phase day 0 to 1, day 2 to 3 and stationary phase from day 3 to 5. On the other hand, the produced ammonium concentration increased between day 0 to 1 as well as day 2 to 4 and slight decrease on day 1 to 2 and day 4 to 5. Figure 4.10A has shown greater amounts of ammonium produced compared to Figure 4.10B. The concentration of NH_4^+-N produced after a 5-day period in Figure 4.10A was higher than that observed in Figure 4.10B. This might be due to the lower biodegradation efficiency which was observed in Figure 4.10B.

A



B

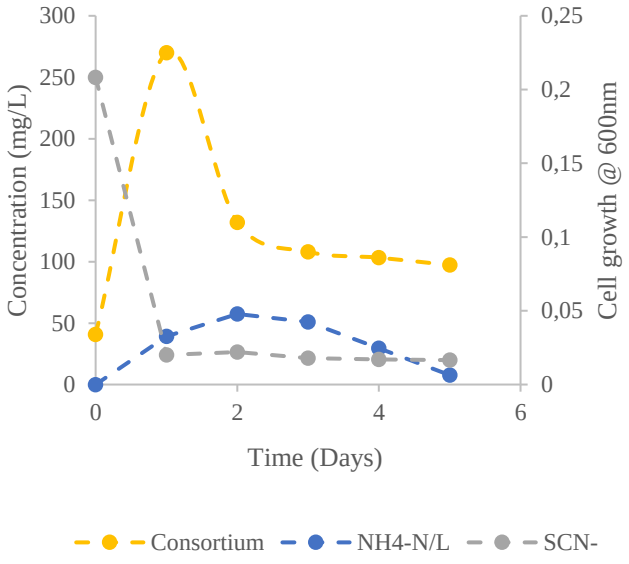


Figure 4.11. Thiocyanate and ammonium including cell growth of consortium measured absorbance at 600 nm, where **A** consist of 150 mg SCN^-/L and **B** 250 mg SCN^-/L.

Consortium of microbial culture observed in Figure 4.11A had higher thiocyanate removal efficiency and the microbial growth was higher compared to the culture in Figure 4.11B. Ammonium produced was to greater extent compared to consortium observed in Figure 4.11B. The mixture of microorganisms only fed in the sole carbon and nitrogen source obtained from thiocyanate without additional organic carbon source or nutrients. In previous research, microbial mixture has shown significancy of thiocyanate removal efficiency solely surviving on carbon obtained from thiocyanate. A consortium of *Enterobacter*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* was able to biodegrade 1800 mg SCN^-/L and reduced it to 0.02 mg SCN^-/L within 2 days (Belyi *et al.*, 2017). Bacteria degrade thiocyanate in various biochemical pathways in which some bacteria utilise autotrophic degradation route to acquire energy, whereas other bacteria exploit thiocyanate to obtain either nitrogen or sulphur as a growth source (Gould *et al.*, 2012). Furthermore, activated sludge obtained from steelwork studied in the period of 5 days was capable of biodegrading 120 mg SCN^-/ L (Raper *et al.*, 2019). It was expected that bacterial consortium will be capable of degrading thiocyanate without any nutritional supplements since bacterial isolates have shown independence of thiocyanate degrading capability. Consortium A has shown higher thiocyanate removal efficiency compared to consortium B due to the difference in concentration levels and the sensitivity of bacteria has shown to be slightly high to thiocyanate at 250 mg/L. However, it has been suggested that nitrification in consortium B is greater than consortium A due to the residual of NH_4^+-N is almost to zero (7,6 mg NH_4^+-N/L) during day 5. Activated sludge microorganisms use ammonium as a nitrogen source, but they are sensitive to the high concentration of thiocyanate, so nitrification rates increase after thiocyanate is removed (Hung and Pavlostathis, 1997).

Bacterial consortium A reached a greater number of microorganisms as compared to bacterial consortium B during the 5-day period. This is due to the highest amount of absorbance at 600 nm wavelength obtained by consortium A, which was 0.359 whereas consortium B was 0.125. This proves the sensitivity of bacterial consortium towards thiocyanate, as the concentration increases, it becomes challenging for these microorganisms to survive. Although, in most cases where there's mixed cultures, a symbiotic relationship is formed and biodegradation efficiency is achieved in great amounts, in some cases mixed cultures compete for available nutrients in numerous ways which leads to less biodegradation efficiency.

4.3.2.3.1. Summary

All the isolated bacteria proved to be capable of thiocyanate biodegradation although pure cultures proved to be more efficient in thiocyanate biodegradation as compared to the microbial consortium. Microorganism that had the similar identity proved to have different biodegradation efficiencies, and this might be attributed to the differences in strain types with a slight difference in the genetic make-up. Indicator plate technique was effective in the selection of thiocyanate degrading organisms.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

CHAPTER 5
5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusion

In this study, bacterial isolates were isolated successfully from the Blesbokspruit wetland and were assessed for the ability to biodegrade thiocyanate. These isolates were identified using the 16S rDNA gene followed Polymerase Chain Reaction and were BLASTED against the organisms in the NCBI Database for their identities. All the microorganisms which were isolated this study proved to be thiocyanate biodegrading organisms due to their capabilities of aerobically degrading thiocyanate successfully. The screening process using indicator plate method was highly effective for determination of thiocyanate biodegrading bacteria. This method determined that organisms which are capable of degrading thiocyanate.

Exiguobacterium aurantiacum/ mexicanum achieved the highest SCN^- removal efficiency of 97.44 % and 95.71 % under both thiocyanate concentration of 150 mg SCN^-/L and 250 mg SCN^-/L, respectively while the microbial consortium of the isolates demonstrated the second highest SCN^- removal efficiency of 97.25 % from 150 mg SCN^-/L (92.04 % from 250 mg SCN^-/L). *Pseudomonas* sp. / *stutzeri* achieved an SCN^- removal efficiency of 93.21% from 250 mg SCN^-/L while *Lysinibacillus xylanilyticus* / *fusiformis* obtained 92.21 % and 91.24 % from 150 and 250 mg SCN^-/L respectively. The other isolates achieved lower biodegradation which were above slightly > 90 %. Thiocyanate degrading organism of this study were dominated by *Pseudomonas* species and thiocyanate biodegradation was successful without the adjustment of pH. The bacterial consortium proved to have more biodegrading efficiency at 150 mg SCN^-/L of thiocyanate as compared to 250 mg SCN^-/L. It is then hypothesised that at higher concentrations of thiocyanate, the organisms competed which then led to the death of some key organisms thus resulting in lower degradation efficiencies.

5.2. Future research

The following recommendations are proposed for further research studies are as follows:

- Biodegradation of thiocyanate should be studied for a longer period than 5 days at higher concentrations.
- Enzymatic kinetic study should be undertaken for each organism to understand the biochemical pathway that each organism uses to degrade thiocyanate.
- Comparison of aerobic consortium obtained in soil, water and sludge from Blesbokspruit wetland to biodegrade thiocyanate at greater concentrations.

CHAPTER 6
REFERENCES

REFERENCES

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CHAPTER 7: APPENDICES

Appendix A

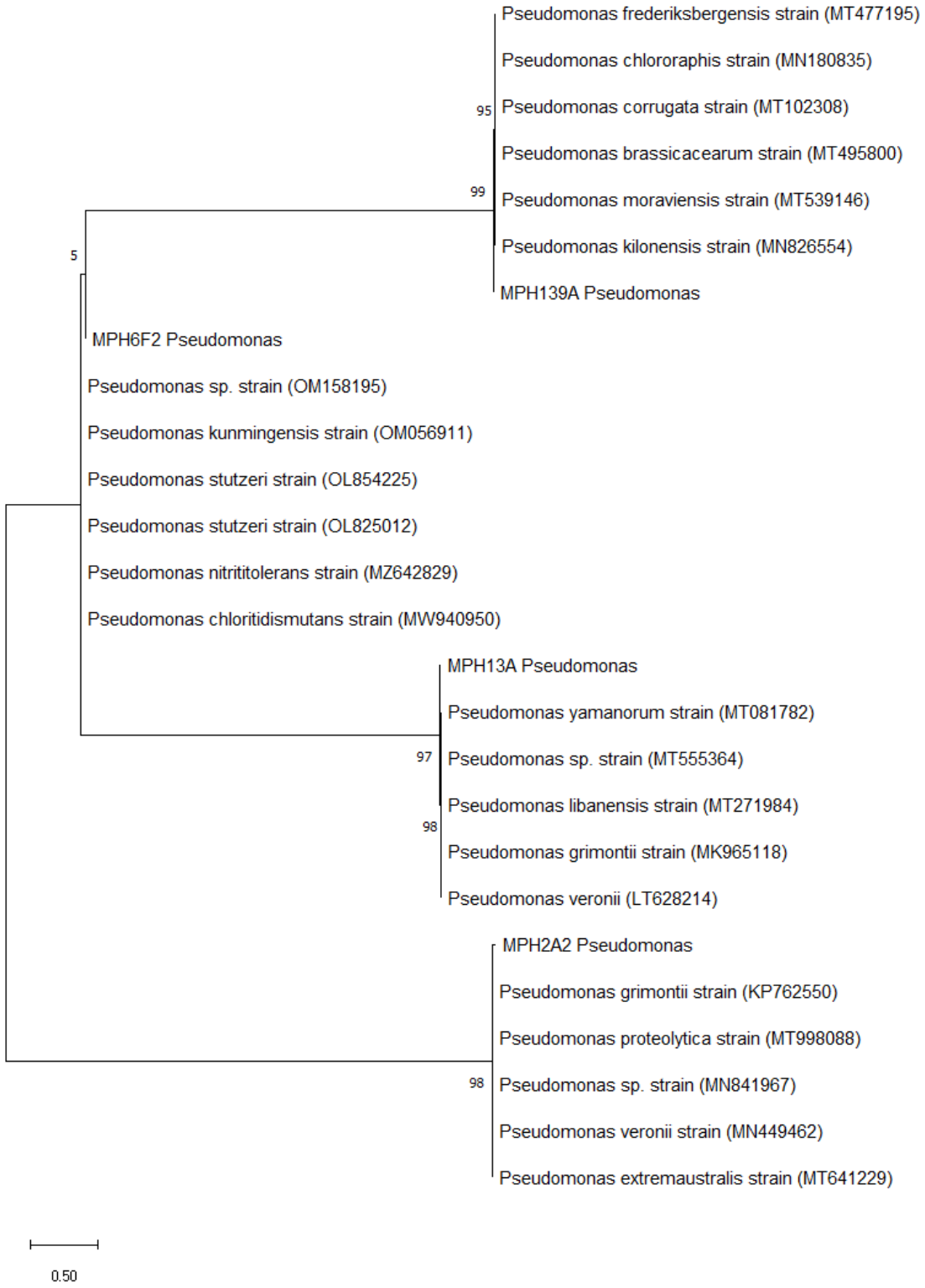

Figure 7.1. The phylogenetic tree of the *Pseudomonas* sp. which were isolated from the Blesbokspruit wetland

Conical flask microbial growth determination at 150mg/L and 250mg/L

Table 7.1: Determination of microbial growth under biodegradation of thiocyanate at 150mg/L

	Time (Days)	**Sample labels**							
		X9C2	X2A2	X6F2	X13A2	X13A	X4A2	Consortium	Negative control
Absorbance at 600nm	0	0.032	0.026	0.015	0.025	0.015	0.016	0.054	0.001
	1	0.232	0.168	0.126	0.170	0.097	0.053	0.359	0.000
	2	0.188	0.154	0.102	0.168	0.097	0.070	0.203	0.000
	3	0.135	0.129	0.097	0.130	0.062	0.088	0.220	0.002
	4	0.079	0.125	0.111	0.161	0.043	0.122	0.199	0.000
	5	0.053	0.044	0.099	0.144	0.035	0.228	0.091	0.000

Table 7.2. Determination of microbial growth under biodegradation of thiocyanate at 250mg/L

	Time (Days)	**Sample labels**							
		X9C2	X2A2	X6F2	X13A2	X13A	X4A2	Consortium	Negative control
Absorbance at 600nm	0	0.036	0.03	0.043	0.015	0.015	0.015	0.034	0.01
	1	0.141	0.098	0.213	0.086	0.071	0.107	0.225	0.00
	2	0.087	0.110	0.189	0.089	0.183	0.066	0.110	0.001
	3	0.245	0.140	0.051	0.148	0.191	0.101	0.090	0.001
	4	0.233	0.207	0.012	0.106	0.139	0.107	0.086	0.000
	5	0.130	0.084	0.000	0.113	0.195	0.102	0.081	0.000

Appendix B

Determination of thiocyanate using iron (III) chloride

Thiocyanate react with iron (III) ions to form an intense red colour complex ion according to equation 7.1. and 7.2.

$Fe^{3+}_{(aq)} + SCN^{-}_{(aq)} \rightarrow [FeSCN]^{2+}_{(aq)}$ 7.1

$[Fe(H_2O)_6]^{3+}_{(aq)} + SCN^{-}_{(aq)} \rightarrow [Fe(H_2O)_5SCN]^{2+}_{(aq)} + H_2O_{(l)}$ 7.2

Preparation of 0.41 mol/L Iron (III) Chloride in 500 mL 1 mol/L hydrochloric acid solution.

Chemical used.

- $FeCl_3.6H_2O$
- 32% HCl

$C = n/V$

$n = 0.41\ mol/L \times 0.5\ L$

$= 0.205$ mol of $FeCl_3$

Moles of H_2O in $FeCl_3.6H_2O = 6 \times 0.205 = 1.23$ moles.

$M(H_2O) = 2(1.008) + 15.999 = 18.02$ g/mol

$M(FeCl_3) = 55.845 + 3(35.45) = 162.195$ g/mol

$n = m/M$

Mass of $H_2O = 1.23\ mol \times 18.02\ g/mol = 22.165$ g

Mass of $FeCl_3 = 0.205\ mol \times 162.195\ g/mol = 33.25$ g

∴ Total mass of $FeCl_3.6H_2O$ in 500 mL 1M HCl = 22.165 + 33.25 = 55.2 g

Concentration of 37% and 1.19 kg/L HCl

M(HCl) = 1.008 + 35.45 = 35.5 g/mol

Concentration = 1.19 kg/L × 1000 g/1 kg × 0.37 × 1 mol/36.5 g = 12 mol/L

$C_iV_i = C_fV_f$

$12V_i = 1(500)$

$V_i = 500/12 = 41.\ 67 = 42$ mL

Therefore, to get 1M from 37% HCl, add 42 mL HCl to 458 mL H_2O

Appendix C

Preparation of 150 mg/L and 250 mg/L SCN^{-1}

M (KSCN)=39.09+32.06+12.011+14.007=97.168 g/mol

M(SCN)=32.06+12.011+14.007=58.078 g/mol

Fraction of SCN in KSCN = 58.078/97.168 = 0.597

1g SCN^- = 1/0.597 = 1.675 g KSCN

Mass of KSCN to prepare 150 mg/L SCN^- solution = 1.675g x 150 mg/L x 10^{-3} = 0,25125

Mass of KSCN to prepare 250 mg/L SCN^- solution = 1.675g x 250 mg/L x 10^{-3} = 0.41875

Table 7.3. Calibration curve of thiocyanate

Concentration (mg/L)	Absorbance @ 480 nm
0	0
10	0.653
20	0.995
40	1.667
60	2.318
80	2.940

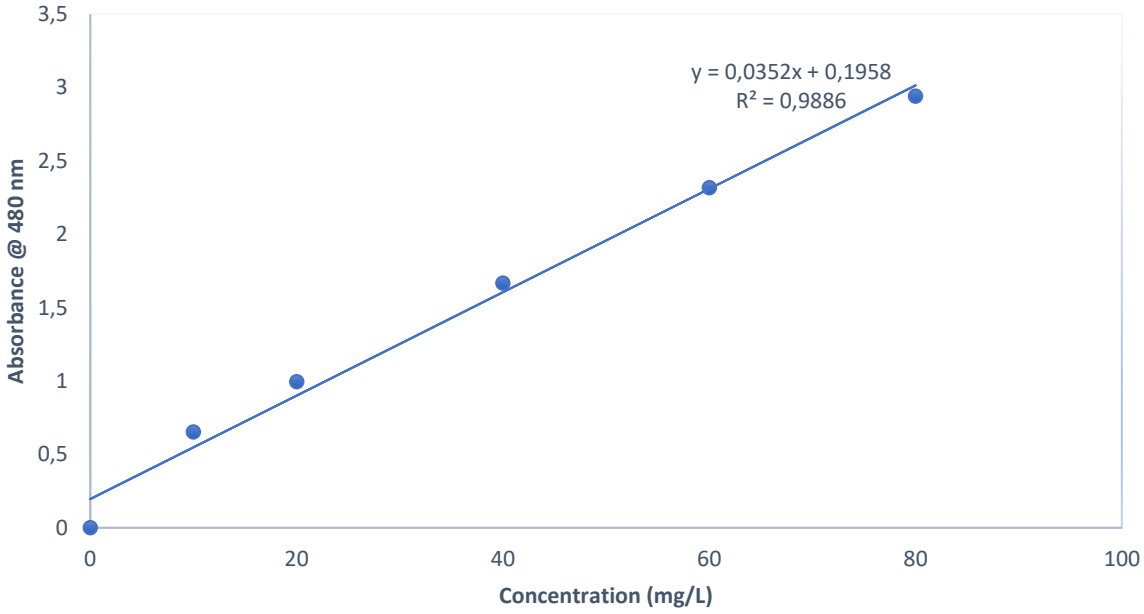

Figure 7.2. Graph representation of thiocyanate calibration curve