Cape Peninsula University of Technology

A MIXED MICROBIAL COMMUNITY FOR THE TREATMENT OF FREE CYANIDE AND THIOCYANATE CONTAINING WASTEWATER

by

LUKHANYO MEKUTO

Thesis submitted in fulfilment of the requirements for the degree

Doctor of Engineering: Chemical Engineering

in the Faculty of
Engineering

at the

Cape Peninsula University of Technology

Supervisor: Prof S.K.O. Ntwampe

Cape Town

June 2017

Supervisor:

Prof. Seteno Karabo O. Ntwampe (EngD; HDHET*)

Associate Professor: Biotechnology

Acting HOD: Biotechnology and Consumer Science

Principal Investigator: Bioresource Engineering Research Group (*BioERG*)

Department of Biotechnology and Consumer Science

Cape Peninsula University of Technology

Cape Town

NtwampeS@cput.ac.za

DECLARATION

I, **LUKHANYO MEKUTO**, know the meaning of plagiarism and declare that all the work in this thesis, save for that which is properly acknowledged, is my own unaided work, both in concept and execution, apart from the normal guidance of my supervisor. Furthermore, the thesis represents my own opinions and not necessarily that of the National Research foundation of South Africa or that of the Cape Peninsula University of Technology and their sponsors.

Furthermore, this thesis has not been submitted for any degree or examination in any other university.

The intellectual concepts, theories, methodologies and mathematical derivations and model developments used in this thesis and published in various scientific journals were derived solely by the candidate and first author of the published manuscripts. Where appropriate, the intellectual property of others was acknowledged by using appropriate references.

8 May 2017

Signed **Date**

ABSTRACT

Industrial wastewater management pertaining to the mining industry has become increasingly stringent, with companies being required to develop environmentally benign wastewater management practices worldwide. The industries that utilise cyanide compounds for the recovery of precious and base metals in a process known as the cyanidation process, have contributed substantially to environmental deterioration and potable water reserve contamination due to the discharge of poorly treated, or untreated, cyanide containing wastewater. Hence, a biotechnological approach was undertaken in this study to remediate free cyanide (CN^-) and thiocyanate (SCN^-), which are the major chemical contaminants which are normally found in cyanidation wastewaters. Furthermore, this biotechnological approach was investigated to understand the fundamental aspects of using this approach such that the information gathered can be utilized in pilot plant studies. Therefore, bioprospecting of potential CN^- and SCN^--degrading organisms was undertaken using two approaches; (i) culture-dependent approach and (ii) culture-independent approach. Using the culture-dependent approach, *Pseudomonas aeruginosa* STK 03, *Exiguobacterium acetylicum* and *Bacillus marisflavi* were isolated from an oil spill site and river sediment samples, respectively. STK 03 was evaluated for the biodegradation of CN^- and SCN^- under alkaline conditions. The organism had a CN^- degradation efficiency of 80% and 32% from an initial concentration of 250 and 450 mg CN^-/L, respectively. Additionally, the organism was able to degrade SCN^-, achieving a degradation efficiency of 78% and 98% from non- and CN^- spiked cultures, respectively. Furthermore, the organism was capable of heterotrophic nitrification but was unable to denitrify aerobically, with the autotrophic degradation of CN^- by STK 03 being abortive.

Likewise, the capacity of *Exiguobacterium acetylicum* (GAN KT282229) and *Bacillus marisflavi* (GAN KR016603) to co-metabolise CN^- and SCN^- under alkaline conditions, was evaluated. *Exiguobacterium acetylicum* had a SCN^- degradation efficiency of 99,9% from an initial SCN^- concentration of 150 mg SCN^-/L, but the organism was unable to degrade CN^-. Consequently, *Bacillus marisflavi* had a CN^- degradation efficiency of 99% from an initial concentration of 200 mg CN^-/L. Similarly, this organism was unable to degrade SCN^-; hence, this resulted in the evaluation of co-metabolism of SCN^- and CN^- by the two microbial species in co-cultures subsequent to the optimisation of operational conditions using response surface methodology (RSM). A numeric optimisation technique was used to evaluate the optimisation of the input variables, i.e. pH, temperature, SCN^- and CN^- concentrations. The optimum conditions were found to be as follows: pH 9.0, temperature 34°C, 140 mg SCN^-/L and 205 mg CN^-/L, under which complete SCN^- and CN^- degradation would be achieved over a 168-hour period. Using the optimised data, co-metabolism of SCN^- and CN^- by both *E. acetylicum* and *B. marisflavi* was evaluated, achieving a combined degradation efficiency of ≥99,9%. The high biodegradative capacity of these organisms, including STK 03, resulted in their supplementation in a continuous bioreactor system to aid

in the biodegradation of SCN^- and CN^-, including biodegradation by-products; an approach suitable for industrial scale operations.

Although the role of the organisms that were isolated via the culture-dependent technique is important, this approach limited the research goals to the utilization of pure microbial cultures which are not representative of environmental settings and/or conditions. Hence, the isolation of mixed microbial communities was undertaken. CN^--degrading organisms (CDO's) were previously isolated in electroplating wastewater and maintained in a bioreactor while the SCN^--degrading organisms (TDO's) were isolated using a gravimetric technique since the CDO's were unable to degrade SCN^-. Similar to the first phase of the study, the CDO's and TDO's were identified using the culture-dependent and independent approaches. Cultivable microbial species were isolated from the CDO's (n = 13) and TDO's (n = 18), and the CDOs were largely dominated by *Bacillus* sp. while the TDOs were dominated by *Bacillus* sp., *Klebsiella oxytoca*, *Providencia* sp. and *Pseudomonas* sp. However, a metagenomic approach revealed the complexity and diversity of the microbial communities in contrast to the organisms that were detected using the culture-dependent techniques. Using the culture-independent technique, the organisms were mainly dominated by *Myroides odoratimimus* and *Proteus* sp. at 37.82% and 30.5% for CDOs, and 35.26% and 17.58% for TDOs, respectively. The co-culturing of the CDOs and TDOs resulted in biochemical changes of key metabolic enzymes, and this coincided with the complete degradation of CN^- and SCN^- simultaneously.

Since the biodegradation of the mentioned contaminants results in the production of ammonium (NH_4^+) and nitrates (NO_3^-), the capacity of the CDO's and TDO's separately and in co-cultures to conduct heterotrophic nitrification and aerobic denitrification was evaluated. Furthermore, the impact of CN^- and SCN^- on nitrification and denitrification was also assessed under alkaline conditions. The CDO's were able to nitrify under cyanogenic conditions, achieving NH_4^+-N removal rates above 1.66 mg NH_4^+-$N.L^{-1}.h^{-1}$, except when CN^- and SCN^- loading was 15 mg CN^-/L and 50 mg SCN^-/L respectively, which slightly inhibited nitrification. The TDO's were able to achieve a nitrification rate of 1.59 mg NH_4^+-$N.L^{-1}.h^{-1}$in the absence of both CN^- and SCN^-, while the presence of CN^- and SCN^- was inhibitory, with a nitrification rates of 1.14 mg NH_4^+-$N.L^{-1}.h^{-1}$. The CDO's and TDO's were able to denitrify aerobically, with the CDO's obtaining NO_3^--N removal rates above 0.67 mg NO_3^--$N.L^{-1}.h^{-1}$, irrespective of the tested CN^- and SCN^- concentration range. Denitrification by the TDO's was inhibited by CN^-, achieving a removal rate of 0.46 mg NO_3^--$N.L^{-1}.h^{-1}$ and 0.22 mg NO_3^--$N.L^{-1}.h^{-1}$ when CN^- concentration was 10 and 15 mg CN^-/L, respectively. However, when the CDO's and TDO's were co-cultured, the nitrification and aerobic denitrification removal rates were 1.78 mg NH_4^+-$N.L^{-1}.h^{-1}$ and 0.63 mg NO_3^--$N.L^{-1}.h^{-1}$ irrespective of CN^- and SCN^- concentrations. This proved the heterotrophic nitrification and aerobic denitrification capability of the CDO's and TDO's separately and in co-cultures. The co-culture of the CDO's and TDO's proved to be robust; hence, the co-culture was utilized for subsequent studies.

Due to the observed robustness of the CDO's and TDO's co-cultures, these cultures were mixed and served as an inoculum for the continuous biodegradation of SCN^- and CN^- in a dual-staged stirred tank bioreactor system. However, prior to the operation of the bioreactor system, the inoculum was identified using metagenomics and was observed to be largely dominated by *Thiobacillus* sp. and *Serratia* sp. The dominance of these organisms when the CDO's and TDO's were co-cultured is unclear, although these organisms were detected in low abundances in the CDO's and TDO's cultures. This microbial consortium was then evaluated for the biodegradation of SCN^- and CN^- under neutral to alkaline conditions, in a two-staged stirred tank bioreactor system operated in series. The bioreactors were operated across a range of residence times (7 d to 24 h), SCN^- (100-1000 mg SCN^-/L) and CN^- (200-450 mg CN^-/L) concentrations. The bioreactors were characterised by high SCN^- degradation efficiencies (>99.9%) throughout the experimental run except when the microorganisms were temporarily shocked by a pH increase and the introduction of CN^- within the system. Similarly, high CN^- biodegradation efficiencies (>99.9%) were observed subsequent to its introduction to the system. Planktonic microbial activity tests by organisms within the bioreactor system revealed high SCN^- and CN^- degradation efficiencies (>80%); a direct indication of high planktonic microbial activity within the bioreactor system. Furthermore, there was an observed total nitrogen removal by the organisms within the system, which demonstrated the nitrification and denitrification capacity of the organisms while the sulphate concentration increased as a result of SCN^- biodegradation, over a period of approximately 300 days. The results demonstrated the potential of the process to treat CN^- and SCN^- laden wastewaters.

Keywords: Aerobic denitrification, Biodegradation, Free cyanide, Heterotrophic nitrification, Thiocyanate.

DEDICATION

I dedicate this thesis to those who are willing to take up the challenge and embrace their ability to be free.

ACKNOWLEDGEMENTS

I acknowledge all those who have played a meaningful contribution in my life, from primary to tertiary education. I would like to specifically acknowledge the following people whose contribution made this doctoral research a success:

- **Professor S.K.O. Ntwampe**: You groomed me since 2009 when I was a second year student and instilled the desire and interest within me to love bioprocess engineering. In fact, it was through your inspiring lectures that I decided to focus on bioprocess engineering. Your ability to let me be a free thinker has resulted in me to attain this doctoral qualification. I appreciate all the things you have done for me, both personally and professionally. Keep on walking!
- **Professor Susan T.L. Harrison**: You took me in when I needed to do my internship and taught me the skills I have today. It was through your journal clubs that I learnt the writing skills that I have today and that is a gift that no one can ever take away. I would always appreciate and cherish all you have done for me, thank you!
- ***BioERG* members**: Though at times you have been an irritation, especially to the noise making ladies that I don't want to mention, such irritations were necessary. It kept me going.
- **My co-authors**, your contribution to the written manuscripts is greatly appreciated. Thank you.
- **Michael Tobin, Ndalo Katywa and Mmabatho Mobo:** Thank you for giving me the opportunity to use the resources that you have in your laboratory. Much appreciated!
- I have to mention **Mrs. Innocentia Erdogan** by name; you have been the greatest motivation to me. I might have disturbed you numerous times with my personal concerns, but you always made time for me. I appreciated the advices that you have given me and your wisdom.
- The **National Research Foundation** (grant no 95550): Thank you for the financial support.
- To **Family and Friends**, thank you for the support. The list is endless for me to mention everyone by name, your contribution is highly appreciated.

RESEARCH OUTPUTS

The following research outputs represent the contributions by the candidate to scientific knowledge and development during his doctoral candidacy (2015-2017):

Publications

Published

Mekuto, L., Ntwampe, S.K.O., Akcil, A. 2016. An integrated biological approach for treatment of cyanidation wastewater. *Science of the Total Environment*, 571:711-720.
DOI: 10.1016/j.scitotenv.2016.07.040

Mekuto, L., Ntwampe, S.K.O., Kena, M., Golela, M.T., Amodu, O.S. 2016. Free cyanide and thiocyanate biodegradation by *Pseudomonas aeruginosa* STK 03 capable of heterotrophic nitrification under alkaline conditions. *3 Biotech* 6:6. DOI: 10.1007/s13205-015-0317-2

Mekuto, L., Alegbeleye, O.O., Ntwampe, S.K.O., Ngongang, M.M., Mudumbi, J.B.N., Akinpelu, E.A. 2016. Co-metabolism of thiocyanate and free cyanide by *Exiguobacterium acetylicum* and *Bacillus marisflavi* under alkaline conditions. *3 Biotech* 6:173. DOI: 10.1007/s13205-015-0317-2

Mekuto, L., Ntwampe S.K.O., Utomi, C.E., Mobo, M., Mudumbi, J.B.N., Mewa-Ngongang, M., Akinpelu, E.A. 2017. Performance of a continuously stirred tank bioreactor system connected in series for the biodegradation of thiocyanate and free cyanide. *Journal of Environmental Chemical Engineering* 5: 1936-1945 DOI: 10.1016/j.jece.2017.03.038

Mekuto, L., Ntwampe, S.K.O., Mudumbi., J.B.N., Akinpelu, E.A., Mewa-Ngongang, M. Metagenomic data of free cyanide and thiocyanate degrading bacterial communities. *Data in Brief* 13:738-741 DOI: 10.1016/j.dib.2017.06.049

Submitted

Mekuto, L., Ntwampe, S.K.O., Mudumbi, J.B.N. Microbial communities associated with the co-metabolism of free cyanide and thiocyanate under alkaline conditions: biochemical reaction metamorphoses. Submitted to *Folia Microbiologica* [Manuscript ID:FOL-D-17-00159]

Mekuto, L., Ntwampe, S.K.O., Kim, Y.M., Ngongang, M.M., Mudumbi, J.B.N., Dlangamandla, N. & Akinpelu, E.A. Heterotrophic nitrification-aerobic denitrification potential of cyanide and thiocyanate degrading microbial communities under cyanogenic conditions. Submitted to the *Applied Water Science* (Manuscript ID: AWSC-D-17-00075)

Mekuto, L., Muchatibaya, G., Ntwampe, S.K.O. & Mudumbi, J.B.N. Sulphate production: An indirect technique for microbial growth, activity and thiocyanate biodegradation efficiency. Submitted to *3 Biotech* [Manuscript ID: BITC-D-17-01078]

Conference presentations

Mekuto, L., Ntwampe, S.K.O., Kena, M., Golela, T.M., Akinpelu, E.A., Amodu, O.S. Application of *Pseudomonas aeruginosa* STK 03 for the biodegradation of thiocyanate and free cyanide under alkaline conditions. 4th YWP-ZA Biennial and 1st African YWP Conference, 16-18 November 2015, Pretoria, South Africa.

LAYOUT OF THESIS

This research study was conducted at the Bioresource Engineering Research Group (*BioERG*), Department of Biotechnology and Consumer Science, Cape Peninsula University of Technology, South Africa. The references listed at the end of the thesis were losted in accordance with the Harvard method of referencing.

The thesis was divided into the following chapters:

- **Chapter 1**: This chapter covers the background of the research topic, the problem statement, the motivation for the execution of this study, the hypothesis and research questions that required an experimental response and the delineation of the study.
- **Chapter 2**: This chapter focuses on the literature surveyed whereby a detailed explanation of the background is explained with the assistance of published and unpublished research information. On this chapter, the research gaps and niches are highlighted from the existing available information, with a focused integration of these gaps and niches into the undertaken project.
- **Chapter 3**: This chapter reports on the materials and methods utilised for the accomplishment of the objectives of this study. Where appropriate, consulted methodologies are referenced.
- **Chapter 4 and 5**: These chapters focuses on the bioprospection of cyanide utilizing microorganisms and the capabilities of the isolated organism (s) to utilise the biodegradation by/end products (ammonium, nitrates, etc.) using a culture-dependent technique. Identification of the organisms using basic molecular biology techniques is also reported on these chapters.
- **Chapter 6**: This chapter focuses on the isolation of free cyanide and thiocyanate degrading organisms using a culture-independent technique. Identification of the organisms using basic and advanced molecular biology techniques is also reported.
- **Chapter 7**: This chapter evaluates the capacity of the microorganisms identified using culture-independent methods in the removal of total nitrogen heterotrophically and aerobically. Nitrogen compounds are produced from the CN^-/SCN^- biodegradation process and since these nitrogen compounds are environmental contaminants, the assessment of their removal using the isolated organisms was necessary.
- **Chapter 8**: This chapter is centred on the application of all the isolated organisms, identified using both the culture dependent and independent techniques for the continuous biodegradation of the free cyanide and thiocyanate over a range of varying operational parameters. This was done to assess the robustness and efficacy of the microorganisms.
- **Chapter 9**: The conclusions and future research recommendations are covered in this chapter. Furthermore, this chapter highlights the scientific advancements that were achieved on this study.

- **Chapter 10**: This chapter includes additional information culminating as a consequence of the undertaken research, which might be published in peer reviewed journals.
- **Chapter 11**: The list of consulted literature citations are listed on this chapter, in accordance with the Harvard method of referencing, as per university requirements.
- **Chapter 12**: The appendices are covered on this chapter. This includes the supplementary data on the chapters which form part of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
β_0	Constant	-
β_i	Linear coefficient	-
β_{ii}	Quadratic coefficient	-
β_{ij}	Interactive coefficient	-
CN_B^-	Biodegraded cyanide	mg/L
CN_R^-	Residual cyanide	mg/L
CN_S^-	Initial cyanide	mg/L
CN_V^-	Volatilized cyanide	mg/L
CN_{Vf}^-	Final cyanide in control	mg/L
CN_{Vo}^-	Initial cyanide in control	mg/L
R^2	Goodness of model fit	-
X_i	Coded independent variables	Units not defined
Y	Response variable	Units not defined

Greek symbols

Symbol	Description	Units
ε	Error	Units not defined

Subscripts

Symbol	Description	Units
A	pH	-
B	Temperature	° C
C	Free Cyanide	mg/L
D	Thiocyanate	mg/L

GLOSSARY

CCD	Central composite design
CDO	Cyanide degrading bacteria
CIP	Carbon-In-Pulp
CIL	Carbon-In-Leach
CN_{SAD}	Strong acid dissociable cyanide
CN_{WAD}	Weak acid dissociable cyanide
EPS	Extracellular Polymeric Substances
F-CN	Free cyanide
GAN	GenBank Accession number
HCN	Hydrogen cyanide
ICMC	International Cyanide Management Code
ICMI	International Cyanide Management Institute
KCN	Potassium cyanide
NaCN	Sodium Cyanide
NH_4^+	Ammonium
NO_3^-	Nitrate
NO_2^-	Nitrite
PCR	Polymerase chain reaction
RSM	Response surface methodology
SCN	Thiocyanate
TDO	Thiocyanate Degrading Organism

CHAPTER 1

INTRODUCTION

Introduction

1.1 Background

The cyanidation process has been, and still remains, a profitable and highly efficient process for the recovery of precious metals from a variety of sulphidic ores i.e. free milling, complex and refractory ores. Free milling ores are classified as high grade ores as they are non-refractory and can be directly sent for cyanidation without prior pretreatment whereas the complex ores are characterised by high cyanide and oxygen consumption due to the presence of interfering compounds that are present within the ores. Refractory sulphidic ores are ores whereby the gold particles are entrapped within the ore matrices, and hence, necessitates pretreatment to expose the gold particles for subsequent cyanidation (La Brooy et al., 1994). Refractory sulphidic ores are currently pretreated via a microbial process where iron and sulphur oxidising bacteria are utilised to expose the gold particles for subsequent cyanidation. The iron and sulphur oxidising organisms are chiefly responsible for the oxidation of the ferrous and sulphur moeties in which the gold particles are entrapped. However, the oxidation of the ores is rarely complete and during the cyanidation process, the cyanide solution reacts with un-oxidised sulphur species and metals to form elevated concentrations of thiocyanate (> 500 mg/L) (Adams, 2013, van Zyl et al., 2015) and a variety of metal-complexed cyanides, in a form of weak (CN_{WAD})and strong acid dissociable cyanides (CN_{SAD}), thus adding to the complexity of the solution matrix. The presence of free cyanide, thiocyanate and cyanide metal complexes from cyanidation wastewater culminates in the difficulty on the wastewater remediation using conventional methods. Thus, the wastewater is unable to be recycled back to the mineral bioleaching circuit since the bioleaching microorganisms are sensitive to the presence of cyanide. This has negative implications on water conservation and reuse (Adams, 2013, Adams and Lloyd, 2008, van Hille et al., 2015).

Numerous treatment techniques have been developed over the years and these include, chemical, physical and biological degradation processes. The chemical and physical methods are highly effective against free cyanide and CN_{WAD} but are unable to degrade CN_{SAD}. Additionally, these processes produce environmentally hazardous by- and/or end-products, and sludge, which requires licenced disposal, thus adding to operational costs. These techniques chemically oxidise cyanide compounds to less toxic compounds and/or cementation in soil to reduce permeability (Baxter and Cummings, 2006). The natural attenuation method involves the storage of the cyanidation effluent in storage ponds for an extended period of time, which can be decades. The mechanism of cyanide degradation in such a method is through biodegradation, adsorption, photo-decomposition and evaporation. However, storage ponds fail, especially during wet seasons, resulting in spillages which can directly contaminate the environment, surface and ground water reserves.

Biotechnological detoxification techniques have gained extensive attention and popularity over the years due to the environmental benignity, robustness, simplicity and economic viability of these processes. In addition, microbial-mediated cyanide degradation processes do not produce degradation products which are hazardous to the environment, but rather produce products which serve as sources of carbon and nitrogen to the organisms (Mekuto et al., 2015). In this process, microorganisms and/or crude lysates of the degrading organisms, are employed to degrade cyanide compounds via a series of enzymatic reactions which include hydrolytic, oxidative, reductive and substitution/transfer pathways (Ebbs, 2004, Gupta et al., 2010). The organisms are not only limited to the degradation process but are also responsible for the entrapment of metals through biosorption by excretion of extracellular polymeric substances (EPS), precipitation, complexation and oxidation-reduction reactions. However, extensive research has been directed at pure microbial cultures in mono substrates environments which do not represent environmental conditions. Contrarily, such studies provide fundamental information on the degradation pathways with a sole purpose of understanding the underlying degradation mechanisms. However, there has been minimal research interest on indigenous microbial communities either in batch or continuous systems. Furthermore, there has been no reports on the nitrogen removal capability (produced from cyanide degradation) of cyanide degrading organisms, which is paramount to the overall treatment performance such that the wastewater meets discharge levels and/or can be recycled to upstream circuits.

1.2 Problem statement

The utilization of the free cyanide for the recovery of precious metals from gold ores has resulted in environmental degradation, due to the discharge of poorly treated cyanide containing wastewater to potable and surface waters, resulting in health-related complications. The currently utilized processes are expensive and result in by or end-products which contribute to environmental deterioration. Additionally, these processes are unable to treat some of the cyanide complexes such as thiocyanate, hence, alternative and environmentally benign processes need to be developed. Biological treatment techniques offer an innovative, sustainable, robust and cost-effective alternative for the treatment of cyanide containing wastewaters. This process is advantageous since microorganisms are adaptable and can thus be manipulated to suit varying operational and environmental conditions. Microbial species are able to alter their metabolism at different environmental conditions, thus allowing for the uptake, treatment, sorption, and/or precipitation of thiocyanate, cyanide, ammonia, heavy metals and sulphates. Therefore, a biologically based technique is proposed in this study for the treatment of cyanide-based compounds using a microbial consortium, including their by or end-products.

1.3 Hypothesis

The cyanide and thiocyanate degrading microbial consortia will be able to degrade free cyanide and thiocyanate in mono and co-cultures irrespective of the tested contaminant concentration loading. Additionally, the organisms will be able to utilize the by and/or end products (i.e. NH_4^+, NO_3^-, SO_4^{2-})

resulting from the biodegradation process. However, the co-existence of both free cyanide and thiocyanate in the same media will have a slight negative influence on the microbial performance, depending on the initial contaminant concentrations. Furthermore, it is hypothesised that higher contaminant concentrations will negatively affect microbial performance both in batch and continuous systems.

1.4 Research questions

The following questions were addressed in this study:

- Are the isolated cultures able to degrade free cyanide and thiocyanate?
- Will the cultures be able to conduct nitrification and aerobic denitrification under non- or cyanogenic conditions?
- Will the biochemical properties of the TDOs and CDOs change when co-cultured?
- Will the co-existence of free cyanide and thiocyanate in the same media have a negative impact on the organisms?
- Will the co-culturing of the CDOs and TDOs increase the biodegradative strength of the consortium?
- Will the microbial consortium be able to degrade thiocyanate and free cyanide under varying operational and physicochemical conditions in a continuous system?

1.5 Objectives

The overall objective of this study was to provide the fundamental information necessary for the development of a biological process to treat cyanide and its related compounds.

Specific objectives:

- To isolate microbial cultures capable of free cyanide and thiocyanate biodegradation using culture-dependent and independent techniques.
- Identify the isolated organisms from culture dependent and independent techniques using molecular biology tools.
- To assess the nitrification and aerobic denitrification capability of the cyanide (CDO) and thiocyanate degrading organisms (TDO) in single and co-culture forms, under cyanogenic and non-cyanogenic conditions.
- Evaluate the biochemical changes of the CDOs and TDOs when cultured separately and in co-cultures to assess the up- or down-regulation of metabolic genes.
- Assess the biodegradation kinetics of the biodegradation process using the CDOs and TDOs when cultured separately and in co-cultures.
- To validate the robustness of the organisms in the biodegradation of thiocyanate and free cyanide in continuous system, using the co-culture of the CDOs and TDOs.

1.6 Delineation of the study

The following were not investigated in this study:

- The environmental impact of cyanide, although it is highlighted in the literature review section.
- Mathematical modelling of the biodegradation process, although some aspects are highlighted during optimization using RSM.
- The field application of the tested biodegradation process.
- Oxygen mass transfer characterisation within the bioreactor system.
- Bioreactor fouling.
- Enzymology associated with the biodegradation process.

CHAPTER 2

LITERATURE REVIEW

Published as: **Mekuto, L., Ntwampe, S.K.O. & Akcil, A**. 2016. An integrated biological approach for treatment of cyanidation wastewater. *Science of the Total Environment*, **571**:711-720.
DOI: 10.1016/j.scitotenv.2016.07.040

An integrated biological approach for treatment of cyanidation wastewater

2.1 Introduction

The leaching of gold bearing ores with cyanide has been reported since 1889 in New Zealand and South Africa (Dorr and Bosqui, 1950). Despite recent attempts to develop alternative extraction procedures such as the use of thiocyanate, ammonia, thiourea and thiosulphate (Hilson and Monhemius, 2006), the cyanidation process still remains the preferred method as it is economically viable and results in rapid extraction (Mudder and Botz, 2004). Elsner (1846) studied gold solubilisation in various cyanide concentrations and proposed the following complexation reaction:

$$\boldsymbol{2Au + 4NaCN + 2H_2O + O_2 \rightarrow 2NaAu(CN)_2 + 2NaOH + H_2O_2} \quad \text{(Eq. 2. 1)}$$

$$\boldsymbol{2Au + 4NaCN + H_2O_2 \rightarrow 2NaAu(CN)_2 + 2NaOH} \quad \text{(Eq. 2. 2)}$$

Overall equation:

$$\boldsymbol{4Au + 8NaCN + O_2 + 2H_2O \rightarrow 4NaAu(CN)_2 + 4NaOH} \quad \text{(Eq. 2. 3)}$$

Equations (2.1) and (2.2) were formulated based on the realisation that an oxidant was necessary for the process to proceed; hence, Equation 2.1 was formulated. The second realisation was that the gold ions combine with two cyanide anions to form a soluble gold complex, with hydrogen peroxide playing a crucial role both as a reactant and product (Johnson, 2015, Habashi, 1966).

Since the initial discovery of the cyanidation process, there have been major variations on the gold recovery process with various processes, which include amongst others, the introduction of carbon-in-leach (CIL), carbon-in-pulp (CIP) and heap leaching. Additionally, there has been extensive research and industrial application (e.g. BIOX process) on pre-treatment of refractory ores which are not amenable to leaching by direct cyanidation (van Aswegen et al., 2007). Commercialization of such pre-treatment technologies have resulted in direct financial gains for gold processes, as gold recovery rates increase from ores that were not amenable to direct cyanidation. However, calamities in cyanide storage facilities, which in the past resulted in environmental deterioration, have raised concern about the use of cyanide in the gold mining sector (Korte et al., 2000). In most cases, cyanide entered the environment by overflowing from storage ponds/tailings storage facilities, or through cracks/punctures in heap leaching liners (Hilson and Monhemius, 2006). Although free cyanide is not persistent in the environment, it does however react with chemical constituencies that are present in the contaminated environment to form a variety of complexes, which under certain environmental conditions dissociate to form free cyanide, which is highly toxic, thus resulting in health related issues. Additionally, cyanide

in soil has been found to form complexes with metallic species, forming stable cyanide compounds that are less toxic. This can result in prolonged presence of cyanide in soil. Although microbial flora contributes to cyanide degradation in soil, this however ensures gradual release of free cyanide over extended periods. There is limited information about the gradual release of free cyanide from its complexes resulting from microbial decomposition in contaminated soil. Therefore, the notion that cyanide is not persistent and does not result in environment deterioration and chronic health complications, is somewhat misleading (Logsdon et al., 1999).

Microbial species have low tolerance to free cyanide with a maximum threshold of 200 mg CN^-/L (Kuyucak and Akcil, 2013). However, in recent studies, there have been microorganisms that have been found to exceed the stipulated threshold (Luque-Almagro et al., 2005b, Mekuto et al., 2013). Because of the toxicity of cyanide, an international body that regulates the use of cyanide was established. The International Cyanide Management Code (ICMC) of the International Cyanide Management Institute (ICMI) (www.cyanide-code.org) deals with the proper management and approval of process certificates for cyanide-utilising industries. Additionally, the ICMC has enforced codes of practice that compel industries to implement alternative waste management practices in order to minimise environmental contamination by such industries (Gibbons, 2005, Akcil, 2002, Akcil, 2010).

Treatment methods have been developed to decontaminate cyanide containing process wastewater. These methods include (1) alkaline chlorination, (2) hydrogen peroxide, (3) the sulphur dioxide process (i.e. INCO process), (4) barren water rinse and (5) biological degradation. Processes (1) to (3) are highly expensive and produce excess sludge that requires suitable disposal measures, while processes such as the barren water rinse is best suited to climates that have an inexpensive source of fresh water and a positive water balance, making it limited to implement, especially in arid regions. Contrarily, biological degradation has proven to be a robust, environmentally benign and economically viable process, with miniscule process input requirements (Baxter and Cummings, 2006, Akcil and Mudder, 2003). The process makes use of microbial communities that utilise different biochemical pathways to degrade or transform cyanide and its by/end products, resulting in decontaminated wastewater that can be recycled back to mineral leaching circuits or disposed into surface waters.

There has been immense pressure on the mining industry to develop alternative environmentally friendly processes that would decontaminate cyanide-containing wastewaters. The main purpose of this review is to propose an integrated biological degradation process for cyanidation wastewater with the purpose of recycling process water to mineral bioleaching/bio-oxidation processes using cyanide insensitive microbial community in all the stages of the process. This would ensure improved process efficiency even during unfavourable conditions.

2.2 Types of the gold bearing ores

Gold bearing ores, which also contain significant quantities of silver and base metals, are classified as (1) free milling, (2) complex and (3) refractory, a classification that is based on the ores mineralogy. Free milling ores are milled such that 80% passes the 75-µm screen in order to achieve 80% gold recoveries by direct cyanidation. Complex ores are reagent-consuming ores due to the presence of reactive matter, thus resulting in higher reagent consumption, which ultimately results in low gold recoveries. Refractory ores are not amenable to direct cyanidation as the gold is trapped in the mineral matrix; hence, cyanide is unable to penetrate to leach out the gold, leading to low recoveries. Currently, refractory and complex gold ores are pre-treated via bio-oxidation, whereby iron- and sulphur-oxidising organisms are utilised to oxidise the iron and sulphur moieties within the ore, thus exposing the entrapped gold particles for subsequent cyanidation. Additionally, the process removes the carboneous matter and trace elements that are responsible for reagent consumption in complex ores. This in turn improves gold recoveries from ores.

2.3 Cyanidation process

Cyanidation is a process that utilizes cyanide as a leaching reagent for the extraction of precious metals (i.e. gold and silver) from ores. The industrial practice of this technique is conducted in three distinct ways: (1) heap leaching, (2) agitation leaching followed by carbon-in-pulp (CIP), and (3) agitated carbon-in-leach (CIL) (Akcil and Mudder, 2003). Heap leaching is generally used to beneficiate low-grade ores that contain low concentrations of gold (<0.04 ounce/ton) while tank leaching is used to beneficiate high-grade ores containing more than 0.04 ounce/ton.

2.3.1 Heap leaching

Heap leaching is advantageous because of its simple design, operational and capital cost, and shorter start up times. Heaps are constructed on lined pads with ore that is received directly from the mine. During heap leaching, ore is crushed and agglomerated prior to packing in the heap. This is done to increase permeability, allow finer particles to attach to bigger particles (Petersen and Dixon, 2007, Watling, 2006) and maintain high pH conditions which are necessary for the leaching process to succeed. During agglomeration, crushed ore is mixed with lime to ensure the maintenance of a higher pH within the heap. However, sulphidic refractory ores may need prior pre-treatment either by roasting, autoclaving or bio-oxidation, before being heap leached (Lopes and Johnson, 1988). During this process, cyanide is introduced from the top of the packed heap, in a downward irrigation direction with the pregnant leach solution (PLS) being recovered from the bottom of the heap.

The process typically has recovery efficiencies of 60 to 80% over a period of weeks or months, depending on the permeability and size of the heap. After the process has been completed, the heap is normally rinsed with water until the cyanide concentration is below the regulatory standard of that particular country. This form of treatment is unfavourable especially in semi-arid or arid countries and

for countries that have a negative water balance. The spent ore is discarded in designated disposal areas (Lopes and Johnson, 1988). The spent ore normally contains trace amounts of free cyanide but also contains elevated concentrations of weak and strong acid dissociable cyanides. These cyanides, under certain physicochemical conditions, dissociate to release free cyanide. Therefore, spent ore from the cyanidation process can exacerbate environmental contamination in a similar manner as cyanidation wastewater.

2.3.2 Agitated leaching

In this process, leaching takes place within a series of tanks where the ore is slurried with the leaching solution and the resulting gold-cyanide complex is adsorbed onto activated carbon. Leaching in a Carbon-In-Pulp (CIP) system takes place in interconnected series of tanks, while in a Carbon-In-Leach (CIL) system it is conducted in single tanks in a batch operational mode. Easily leachable ores such as oxide ores are milled ground to 210-µm and leached with a cyanide solution (100 mg CN^-/L) over a four- to twenty-four-hour period, with a 50% pulp density being appropriate for optimum leaching rates. However, sulphidic ores are processed by milling the ore to 44-µm and using a higher concentration of cyanide (200 mg CN^-/L) over a 72-hour period at a pulp density of 40% (Weiss, 1985, Cummins, 1973). This process has recovery efficiencies of ≥90% over a period of hours. Recently, higher cyanide concentrations (up to 400 mg CN^-/L) are utilised for higher recovery efficiency (≥ 98%).

During the cyanidation process, cyanide forms a variety of complexes with a number of chemical constituents that are present within gold bearing ores. These are classified using five categories: (1) Free cyanide, in the form of hydrogen cyanide (HCN) and cyanide ion (CN^-). (2) Simple cyanide in the form of sodium cyanide (NaCN) and potassium cyanide (KCN). (3) Thiocyanate (SCN). (4) Weak acid dissociable cyanide (CN_{WAD}) which is mainly metal-complexed cyanide (e.g. Zn, Cu, Ni, etc). (5) Strong acid dissociable cyanide (CN_{SAD}) (strong complexes with metals such as Fe, Co, Au and Ag). The toxicity of these cyanide compounds decreases from category (1) to (5). These categories are listed in Table 2.1.

Table 2. 1: Cyanide species with their relative toxicities

Type of cyanide	Species	Toxicity
Free cyanide	CN^-	High[1-2]
	HCN_{aq}	
	NaCN	
	KCN	
CN_{WAD}	$Zn(CN)_4^{2-}$	Intermediate to high (depends on environmental conditions)[4]
	$Cd(CN)_4^{2-}$	
	$Ag(CN)_2^-$	
	$Mn(CN)_6^{3-}$	
	$Ni(CN)_4^{2-}$	
	$Cu(CN)_3^{2-}$	
	$Cr(CN)_6^{3-}$	
CN_{SAD}	$Fe(CN)_6^{3-}$	Low[5]
	$Fe(CN)_6^{4-}$	
	$Co(CN)_6^{4-}$	
	$Au(CN)_2^-$	
	Pt group metal complexes	
Other species	SCN^-	Low[3]
	CNO^-	Low[3]
	C_2N_2[a]	High[1-2]
	CNCl[a]	High [1-2]

[a] Presence of these species in leach solutions is unclear.

[1-5]Categorization of cyanide species

2.4 Toxicity of cyanide and its impact on active biological processes

Due to the high reactivity of the cyanide anion, it is able to form complexes with a variety of metallic species that form part of enzymatic structures, thus inhibiting their role and cellular growth. There are three main inhibition mechanisms: (1) Formation of cyanohydrin derivatives through reaction with keto-compounds. (2) Formation of nitrile derivatives through reaction with Schiff-base intermediates. (3) Chelation of di- and trivalent metals in metalloenzymes. A typical example of cyanide inhibition is the irreversible reaction of cyanide to ferric heme iron within the structure of the cytochrome c oxidase, thus inhibiting oxidative phosphorylation. Oxygen utilization will therefore be impaired, thus resulting in cessation of aerobic metabolic activity (van Buuren et al., 1972, Jones et al., 1984, Massey and Edmondson, 1970).

Cyanide contamination in surface and ground water reserves has been proven to be detrimental to the lives of the organisms that feed from such water reserves. Additionally, the transport of cyanide and related complexes from contaminated matrices into active biological processes such as those used in municipal wastewater treatment plants (WWTPs), may lead to low efficiencies within such processes because of cyanide susceptibility of the microbial communities that are employed in such systems. Numerous studies on the inhibition of nitrification by low concentrations of cyanide have been studied elsewhere (Han et al., 2014, Kim et al., 2011b, Sharma and Philip, 2014). The presence of free cyanide above 0.2 mg CN^-/L has been found to completely inhibit the nitrification process while thiocyanate and ferric cyanide inhibited nitrification at concentrations of 200 and 100 mg/L respectively (Kim et al., 2008). Additionally, Neufeld et al. (1986) observed a maximum tolerance threshold of 0.11 mg CN^-/L while in a separate study, the presence of free cyanide above a concentration of 2.5 mg CN^-/L prolonged the biodegradation of phenols and aromatic hydrocarbons (Sharma and Philip, 2014). In contrast, the *Nitrobacter* and *Nitrospira* strains that were detected in an activated sludge process treating coking wastewater were able to tolerate up to 50 mg CN^-/L, where the microbial community changed as a result of cyanide loading and sensitivity of some of the microbial organisms to free cyanide, resulting in the dominance of *Nitrobacter* and *Nitrospira* strains (Kim et al., 2011c). In a separate study, the activity of *Leptospirillum ferriphillum* and *Acidithiobacillus caldus*, key iron and sulphur oxidising organisms during bio-oxidation of gold ores, were inhibited by the presence of thiocyanate at a concentration of 1.25 mg/L. However, the adapted *A. caldus* tolerated up to a concentration of 7 mg/L, thus demonstrating the adaptability of the organism to changing environmental conditions, although with low sulphur oxidation rates (van Hille et al., 2015).

The inability of microbial communities/species to perform their respective functions as a result of cyanide presence and sensitivity proves to be disastrous. In WWTP's, the presence of cyanide results in limited or no nutrient removal in secondary treatment stages of the WWTP's, where autotrophic nitrifying and denitrifying microbial communities are employed, resulting in the failure of such systems. Furthermore, the presence of cyanide compounds in bio-oxidation processes results in poor refractory and complex ores pre-treatment due to the sensitivity of the organisms employed in such systems, thus resulting in low gold recoveries during cyanidation. Therefore, adequate, environmentally benign and cost-effective processes need to be developed to equip cyanide utilising industries with an alternative technology for treatment of their cyanide containing effluent and residue.

2.5 Treatment methods

Cyanide treatment from mining and metallurgical operations is required due to the potential toxicity of cyanide to the environment. Cyanide treatment methods are classified as destruction-based processes or recovery-based methods (Botz et al., 2005a, Botz et al., 2015). This review focuses on destruction-based processes where cyanide is oxidised to less toxic compounds. These destruction-based treatment

methods may include treatment or removal of cyanide from the following options: (1) Supernatant solutions from tailings ponds. (2) Spent slurry tailings from milling operations. (3) Seepage from ponds or tailings. (4) Solutions from heap leaching operations (Botz and Mudder, 2002b). The treatment of cyanide-containing solutions is achieved through destruction or recovery processes, with a focus on the degradation of cyanide-containing residue or wastewater. The selection of an appropriate treatment process requires consideration of numerous factors which are based on the chemical characteristics of the waste, the volume of the waste that is to be treated, the environmental setting of the treatment site, the desired effluent quality, the availability of reagents or suitable process waters and the regulations that are in place to ensure that the discharge meets the regulatory guidelines (Akcil, 2003, Botz et al., 2015). Cyanide treatment options are classified as (1) Natural, (2) Physical, (3) Chemical and (4) Biological degradation.

2.5.1 Natural degradation

Natural degradation of cyanide is achieved by natural processes such as biodegradation, adsorption, photodecomposition, oxidation and volatilization without human interference. However, the mechanism of degradation is affected by physicochemical parameters such as temperature, pH, water chemistry and dissolved oxygen concentration. The solution pH plays a major role in the volatilization of cyanide. HCN gas has a pKa value of 9.24 at 25°C (Johnson, 2015, Kuyucak and Akcil, 2013), meaning that below a pH of 9.24, cyanide is available as hydrocyanic acid, which volatilises as hydrogen cyanide gas – since cyanide has a high vapour pressure. An increase in temperature, reduced liquid-depth to surface-area ratios and turbulence increase the rate of volatilization significantly while solution contact with carbon dioxide results in a decrease in pH, which in turn results in increased hydrogen cyanide gas volatilization (Nsimba, 2009, Johnson, 2015).

Strong acid dissociable cyanides such as iron cyanide complexes are decomposed via ultraviolet rays' exposure from the sun, thus resulting in the dissociation of the complex to its original constituents: free cyanide and metal ion. However, the wastewater needs direct exposure to sunlight and this is facilitated by pneumatic mixing of the wastewater, ensuring exposure of water to sunlight (ultraviolet light) (Oudjehani et al., 2002). However, during natural attenuation processes, the wastewater is normally found in stagnant form, thus limiting exposure of the water to sunlight (Botz and Mudder, 2000) and resulting in the restriction of cyanide degradation. Furthermore, natural attenuation processes are extremely slow due to the stagnant nature of the wastewater and can take years and sometimes, decades for the process water to meet discharge regulations.

Thiocyanate, ferricyanide and ferrocyanide were found to be the major contaminants in ground water reserves, and remedial actions of the ground water was achieved through natural attenuation from a period of ten years where the mechanism of degradation was proposed to be biodegradation and

adsorption. Strong acid dissociable cyanides were detected at high concentrations after the ten-year period, suggesting that they were not degraded (Gagnon et al., 2004). In a separate study, natural attenuation via the biodegradation mechanism was assessed in fresh (three months), and old (about nine-year-old) tailings. Results reported free cyanide biodegradation by heterotrophic bacterial species, while strong acid dissociable were not degraded over a period of 96 days (Oudjehani et al., 2002). Due to slow degradation rates in natural attenuation process, alternative treatment methods were developed and these include chemical and biological treatment methods.

2.5.2 Physical methods

Physical processes that are currently utilised are mainly based on the dilution of the cyanide containing wastewaters to meet the discharge requirements, without the utilization of any chemical reagents. The Barren/fresh water rinse is such a method. In this method, fresh water is utilised to rinse the heap from barren ponds and the water is also utilised to reduce evaporative losses. The mechanism of cyanide reduction is mainly through dilution and volatilization, and slight, microbial degradation and complexation (Mosher and Figueroa, 1996). The engineering costs associated with the process are, amongst others; pumping capacity, line installation, instrumentation, irrigation and equipment residence time. The practicality of such a process is virtually impossible, especially in arid or semi-arid regions.

2.5.3 Chemical treatment

The following chemical treatment methods are utilised in the mining industry: the alkaline chlorination method; ozonation; copper catalysed hydrogen peroxide; acidification-volatilization-reneutralization (AVR) process; the sulphur dioxide process; sulphidization-acidification-recycling-thickening (SART) process, Caro's acid treatment methods and gas filled membrane absorption process (GFMA). These chemical treatment methods are thoroughly discussed in a number of reviews (Baxter and Cummings, 2006, Botz and Mudder, 2002a, Adams, 2013, Botz et al., 2005b, Mosher and Figueroa, 1996, Estay et al., 2013). However, a comparison of the different chemical treatment methods with their advantages and disadvantages has been summarized in Table 2.2.

Table 2.2: Advantages and disadvantages of cyanide treatment methods (Adopted from Mosher and Figueroa (1996)).

Treatment method	Advantages	Disadvantages
Alkaline chlorination	Well established.	CN_{SAD}, ammonia and chlorides are not removed. Adds hazardous metals to water. Poor process control leads to toxic intermediates. Reacts preferentially with SCN.
Ozonation	-	Unable to treat CN_{SAD} and ammonia. Poor treatment performance. Very expensive process.
Hydrogen peroxide	Simple to operate. High efficiencies.	Unable to treat SCN and ammonia. Excess precipitate accumulation. Expensive reagent. Requires removal of catalyst after process completion.
AVR process	Highly efficient process.	Very expensive process. HCN gas might escape and cause life-threatening situations. Sludge handling problems.
SART	High cyanide recoveries. Recovers cyanide from $CN_{WAD.}$ Cyanide can be regenerated. Economically viable.	Difficulties with sizing of solid-liquid separations equipment. Use of hazardous chemicals.
GFMA	Highly efficient process. Safe, HCN escape is restricted. Applied in cyanide wastewaters from different industries. No secondary metabolites produced.	Reduced efficiency with solutions containing solids (>50 mg/L).

	Simple operation.	
Sulphur dioxide	Inexpensive reagent use. Faster degradation rates. Effective at treating slurries.	Reagent use requires licence payments. Process adds sulphates to treated water. Excess precipitate accumulation.
Caro's acid	Faster degradation rates.	Expensive reagents. Sludge accumulation problems.

2.5.4 Biological treatment

Biological degradation is generally categorised into: (1) Phytoremediation and (2) Microbial remediation. The mechanism of cyanide degradation is similar in both methods. These processes make use of enzymes that degrade or convert cyanide species to less harmful by/end products.

2.5.5 Phytoremediation of cyanide

The remediation of cyanide by plant species has been under intense research. To date, the application of this process has been limited to laboratory studies. A variety of plant species have been reported to have a potential for removing cyanide in soil and solutions. These plants include *Eichhornia crassipes* (Ebel et al., 2007), *Hordeum vulgare*, *Avena sativa*, *Sorghum bicolor* (Samiotakis and Ebbs, 2004), *Salix babylonica*, *Sambucus chinensis* (Yu et al., 2005b), amongst other plants species. These plant species metabolise free cyanide via two pathways; the cyanoalanine pathway and the sulphur transferase pathways (Samiotakis and Ebbs, 2004). However, the cyanoalanine pathway is the most common pathway due to asparagine formation – an amino acid which is critical for nitrogen supply in most plants (Yu et al., 2012, Machingura and Ebbs, 2010, Machingura et al., 2013). The metabolism of cyanide by *Sambucus chinensis* and *Torilis japonica* was investigated. *S. chinesis* was observed to possess the highest removal rate of 8.8 mg CN^- kg^{-1} h^{-1}, followed by *T. japonica* at 7.5 mg CN^- kg^{-1} h^{-1} (Yu et al., 2004); while in a separate study, *Sorghum bicolor* and *Linum usatassimum* converted the radiolabelled Prussian blue, a CN_{SAD}, to carbon dioxide and accumulated the majority of cyanide within the plants (± 140 mg CN^-/kg plant)(Kang et al., 2007). However, this process has been hindered by the levels of phytotoxicity: a phenomenon that is attributed to the toxicity levels of cyanide to the plant species that carry out phytoremediation. Yu et al. (2005a) investigated the phytotoxicity of cyanide in *Salix babylonica* and made the following observations: Cyanide levels below 1 mg CN^-/L were not toxic, while severe signs of toxicity were witnessed when cyanide levels were above 9.3 mg CN^-/L. While Larsen et al. (2004) observed that *Salix viminalis* that were exposed to 0.4 mg CN^-/L demonstrated minimal signs of transpiration depression while doses of 8 to 20 mg CN^-/L were lethal to *S. viminalis*.

Although some of the *S. bicolor* species have been reported to tolerate up to 50 mg CN^-/L (Trapp et al., 2003), the majority of the reported plant species have been reported to have low cyanide tolerance and the degradation rates are extremely low. Additionally, these process are extremely slow and would require vast amounts of land if commercialised, as proposed by Trapp et al. (2003). The proposed commercialisation of the process would include, among others, the administration of the cyanide dosage to the plants. This means, that cyanide would have to be diluted to concentrations that can be metabolisable by the plants, which is, below a concentration of 10 mg CN^-/L. The practicability of such a process is minimal especially in arid and semi-arid regions. Additionally, the growth of some of the proposed plants may be minimal or nonexistent in some regions. Furthermore, the genetic and physiological traits of these plants may change in different regions due to differences in soil type and nutrition. Therefore, this makes the commercialization of this process almost impossible due to the reasons mentioned previously.

2.5.6 Microbial remediation of cyanide

Over the past twenty years, the application of microbial species in the form of bacteria, fungi, protozoa, algae and yeasts has gained considerable attention due to the robustness and environmentally friendliness of the process. These organisms use a variety of enzymatic pathways to destruct or convert cyanide to less hazardous products. This is, however, dependent on the type of enzyme that a particular microorganism possesses. These enzymatic pathways are published elsewhere (Ebbs, 2004, Gupta et al., 2010, Dash et al., 2009). Briefly, these include (1) hydrolytic, (2) oxidative, (3) reductive, (4) substitution or transfer, and (5) synthesis pathways. The first three pathways are degradative pathways where the microbial species destruct cyanide to form ammonia, formic acid, carbon dioxide, methane and carboxylic acid, while the last two pathways are assimilative. The type of enzymatic pathway that a particular organism employs is dictated by the environmental conditions (pH, temperature and presence of toxins) and gene structure. These organisms have been observed to work efficiently in biofilms, where they are able to micro-manage their environment to suit their needs. Within these biofilm structures, organisms are able to destruct or convert cyanide successfully and are able to entrap cationic metals (i.e. Cu, Ni, Fe, Ni, etc) within their structure due to the anionic properties associated with biofilms (Singh et al., 2006). A number of studies of free cyanide, metal-complexed cyanides and thiocyanate have been reported elsewhere with successful operational efficiencies (Jeong and Chung, 2006, Patil and Paknikar, 2000, Mekuto et al., 2015, van Zyl et al., 2011, van Hille et al., 2015).

The Homestake Mine has proven that this process is robust and economically viable when the mine commissioned a biological process that would destruct cyanidation wastewater in 1984 (Stott et al., 2001). However, the challenge with this process is that traditional cyanide-sensitive nitrifying and denitrifying organisms were employed and often, this resulted in process failures when cyanide compounds overflowed to the secondary stages. More recently, Gold Fields Limited has developed a

pilot plant that treats thiocyanate containing wastewater through the Activated Sludge Tailings Effluent Remediation (ASTER™) process with the overall aim of recycling the process water to the BIOX® process (van Buuren et al., 2011). However, this process is mainly limited to the destruction of thiocyanate and relies on chemical methods to remediate the remaining contaminants.

These industrial applications demonstrate that biological processes are sustainable and economically viable. These processes can be operated for longer periods since the microbial species are adaptable and can be manipulated to fit under different operational and environmental conditions, thus allowing for uptake, treatment, sorption, and/or precipitation of thiocyanate, cyanide, ammonia, heavy metals and sulphates. Although this process has been proven to be economically viable, industry still prefers the use of chemical methods instead of the biological technique. This may be attributed to the rapid degradation rates that are observed in such processes and the development of biological processes can be time consuming and requires additional research on wastewaters generated from different ore types (Mosher and Figueroa, 1996).

2.6 Integrated biological treatment of cyanide

Typical industrial bioprocesses that focus on treatment of cyanidation wastewater, such as that employed at the Homestake Mine, consist of two separate but integrated treatment stages. The first stage consists of cyanide and/or thiocyanate degrading microorganisms where the biodegradation products are generally dominated by the presence of bicarbonate/formate/carboxylic acid (depending on the degradation pathway that a microbe employs), ammonia (Eq. 2.4) and sulphates (Eq. 2.5). However, in order for these microorganisms to propagate and degrade cyanide, they need nutrients in the form of carbon, phosphorus and nitrogen (in this case, cyanide/thiocyanate).

$$CN^- + O_2 + H_2O \rightarrow HCO_3^- + NH_3 \qquad \text{Eq. 2. 4}$$

$$SCN^- + 2O_2 + 3H_2O \rightarrow HCO_3^- + NH_4^+ + SO_4^{2-} + H^+ \qquad \text{Eq. 2. 5}$$

Due to the toxicity and eutrophication promotion by the presence of ammoniated compounds, the second stage of the treatment process is normally incorporated with an autotrophic nitrification and anaerobic denitrification system, where the ammonia and nitrates are oxidised to nitrogen gas. Ammonia is oxidised to nitrate in a two-step reaction (Eq. 6 and 7) where it is firstly converted to nitrite and thereafter to nitrate

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad \text{Eq. 2. 6}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \qquad \text{Eq. 2. 7}$$

The formed nitrates are immediately reduced to nitrogen gas through the action of nitrate reductase enzymes. The process is normally anaerobic and consists of a diverse microbial community that are either strict anaerobes or facultative anaerobes (Knowles, 1982, Awolusi et al., 2015b, Awolusi et al., 2015a, Zumft, 1997). The nitrifying and denitrifying organisms are non-competitive; hence, the wastewater can be remediated successfully. However, nitrification and denitrification using traditional microbial communities is very slow and highly sensitive to process shifts and the presence of cyanide compounds. The treatment of cyanide containing wastewater is hardly 100%, especially during cold seasons, hence cyanide degradation efficiencies decrease, thus resulting in the overflow of cyanide to secondary stages: nitrification and denitrification stages. The nitrifying and denitrifying organisms are highly sensitive to the presence of cyanide, thus the presence of this compound on these systems would have deleterious effects, causing decrease in effluent water quality (Wild et al., 1994, Kim et al., 2011b). Some of the consulted literature on the effects of cyanide inhibition on nitrification and denitrification has been discussed in Section 2.4.

Recently, research has focused on microorganisms that are able to conduct both nitrification and denitrification aerobically and under heterotrophic conditions, replacing the traditional autotrophic nitrification and anaerobic denitrification organisms. Research on these organisms gained popularity in the late 1980s and early 1990s. Earlier studies revealed that these organisms were characterised by the absence of nitrites and/or nitrates accumulation (Robertson and Kuenen, 1990), which contradicted the autotrophic nitrification system that relied on the nitrites and nitrates accumulation as proof of successful nitrification. This is due to the fact that most heterotrophic nitrifiers are also denitrifiers, and the nitrites or nitrates produced from the nitrification process may be simultaneously converted to nitrogen gas or one of the volatile nitrogen oxides (van Niel et al., 1992). Hence, the concentration of oxidised ammonia may not necessarily reflect the concentration of the oxidation products that are accumulating in the medium. However, it has been observed that these organisms share similar enzymological characteristics and activity with the traditional nitrifiers and denitrifiers. It has, however, been the aerobic denitrifying enzymes that have been associated with controversy (Gupta, 1997). Although the technology received criticism in the past, it has been accepted by the scientific community as one of the viable alternatives to traditional systems.

To date, a number of organisms that are capable of heterotrophic nitrification and aerobic denitrification have been isolated and identified. These organisms include *Pseudomonas stutzeri* T13 (Sun et al., 2015), *Alcaligenes faecalis* C16 (Liu et al., 2015), *Providencia rettgeri* YL (Taylor et al., 2009), *Agrobacterium sp*. LAD9 (Chen and Ni, 2012), *Rhodococcus sp*. CPZ24 (Chen et al., 2012), *Bacillus methylotriphicus* L7 (Zhang et al., 2012) . During heterotrophic nitrification and aerobic denitrification, alkalinity is produced during aerobic denitrification, which can be used to neutralise the acidity that is

generated through the heterotrophic nitrification, thus reducing the costs of pH-adjustment (He et al., 2016).

However, due to the susceptibility of these organisms to cyanide compounds, recent studies have focused on the application of the same cyanide degrading organisms for heterotrophic nitrification and aerobic denitrification. Mekuto et al. (2015) observed simultaneous nitrification and aerobic denitrification in a cyanide degradation process using the same microbial species while in a separate study, a cyanide-insensitive microbial community was able to nitrify and aerobically denitrify in the presence of cyanide (Mpongwana et al., 2016), thus demonstrating the effectiveness of such microbial communities in a cyanide biodegradation process. Hence, an overall cyanide-insensitive biological process is proposed where all the stages, *viz* cyanide biodegradation stages, and nitrification and aerobic denitrification stages, are inoculated with the same microbial community for successful cyanide degradation as demonstrated in Fig. 2.1. Nitrification and denitrification stages can be expanded by incorporating a pre-denitrification stage, to reduce the toxicity of the produced ammonium and nitrates to the nitrification and denitrification stages. This would ensure maximum utilisation of these contaminants in the nitrification and aerobic denitrification stages. This phenomenon has been observed elsewhere (Kim et al., 2011a, Villemur et al., 2015), with successful operational efficiencies.

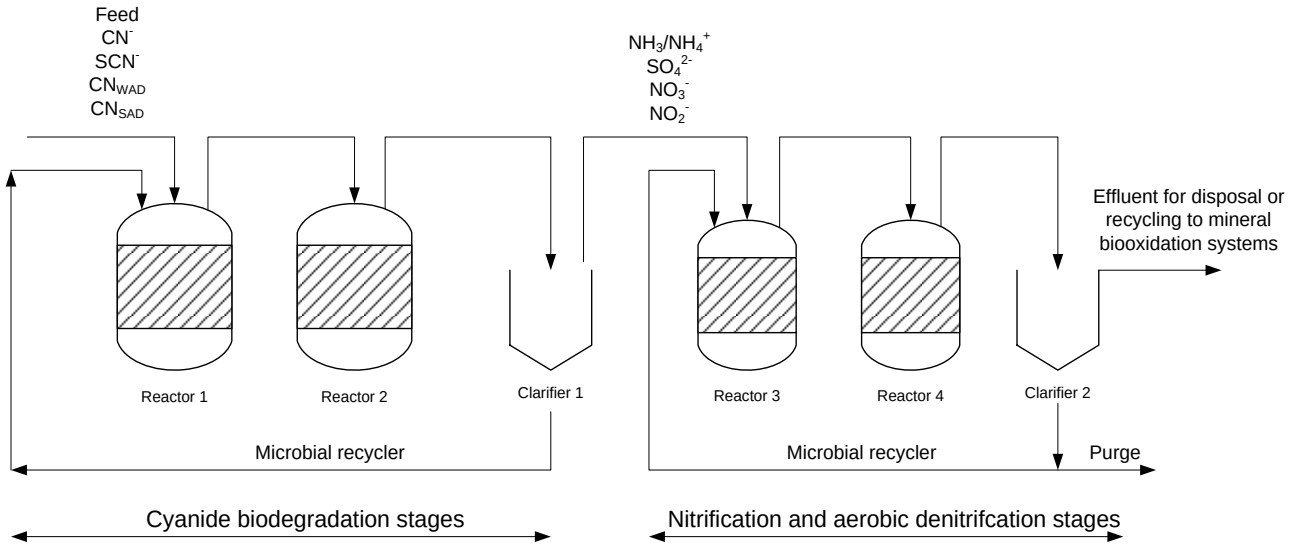


Fig. 2. 1: A proposed cyanide degradation system.

However, the successful operation of such a process will require a thorough knowledge and understanding of the fundamental and operational aspects of the proposed process. One of the key fundamentals is a thorough understanding and characterisation of the microbial community that contributes to the success of the bioprocess. This information would enable development of predictive models (Stott et al., 2001, Whitlock, 1990). The success of the Homestake Mine in treating cyanide-

containing wastewater was based on the understanding of the microbial communities that were involved in the process. The initial assessment that was employed using continuous stirred tank reactors (CSTRs) showed unfavourable results, owing to limited knowledge and understanding of the microbial population used. Subsequent work using CSTRs and rotating biological contactors (RBCs) with a different microbial consortium, showed encouraging results for cyanide degradation. Recently, the microbial population that is involved in the ASTER™ process was observed to be far more complex than initially reported by van Buuren et al. (2011), and is comprised of a diverse microbial species such as bacteria, motile eukaryotes, filamentous fungi and algae (Huddy et al., 2015)

Some of the identified culturable microbial organisms that are capable of cyanide degradation are listed in Table 2.3. However, it is well known that only 1% of the environmental microbial species are able to be cultured in a laboratory, hence the detection of these organisms through culture-based techniques may not truly represent the microbial organisms that contribute to cyanide degradation. Therefore, there is a need for robust and reliable techniques such as the clone library approach (Huddy et al., 2015) and/or metagenomic sequencing for accurate identification of microbial populations and their metabolic roles within the process (Handelsman, 2004, Schloss and Handelsman, 2003, Cowan et al., 2005). The microbial communities involved in the ASTER™ process and their intrinsic metabolic contributions to the process, have been recently elucidated elsewhere (Kantor et al., 2015). The authors observed the dominance of *Thiobacillus* sp. whose genomes harbour unreported operon for thiocyanate degradation. Furthermore, the microbial community was observed to be largely autotrophic through genome-based metabolic predictions, with a smaller portion of the community being heterotrophic. Such fundamental knowledge of bioprocesses adds value to the effectiveness of the process.

The operational research is equally imperative as is the fundamental research, as it also determines the success of the bioprocess. Desirable operational parameters such as temperature, influent cyanide concentration and aeration should be considered, as these external factors contribute to the type of degradation pathway employed by the microorganisms (Dash et al., 2009). For example, the presence of cyanide, either as free cyanide or as complexed cyanide, can result in the activation of one or two enzymatic pathways for the degradation of cyanide – depending on the microbial species employed in such a process and in such environmental conditions (Ebbs, 2004). In addition, the solubility and bioavailability of cyanide in soil-water systems also influences the selection of a desired pathway by the microorganisms (Dash et al., 2009). Overall, the combination of both fundamental and operational research would ensure sustainability, reliance and robustness of the process that would effectively eliminate cyanide presence in cyanidation wastewater through an integrated approach of using the same microbial population for cyanide biodegradation, nitrification and aerobic denitrification. Thus, these organisms can be referred to as cyanide degraders, nitrifiers and aerobic denitrifiers. The generated waste sludge can be digested anaerobically to produce methane, which can assist in the energy inputs

of the process. Alternatively, the waste sludge can be utilised as a source of manure for agricultural activities.

.

Table 2. 3: Culturable microbial species capable of degrading cyanide compounds.

Microorganism	Operation	C-source	N-source	Product(s)	Temp (°C)	pH	Reference
Pseudomonas sp.	Batch	Whey	CN_{WAD}	NH_4, CO_2	30	9.2-11.4	(Akcil et al., 2003)
Bacillus pumilus C1	Batch/fed-batch	Nutrient broth	NaCN	-	25	10.5	(Meyers et al., 1991)
Fusarium solani	Batch	Glucose	$K_2Ni(CN)_4$, KCN	NH_4, HCOOH	25	7.0	(Barclay et al., 1998)
Scenedesmus obliquus	Batch	NaCN	NaCN	NH4,CO2	-	10.3	(Gurbuz et al., 2009)
Burkholderia cepacia C-3	Batch	Glucose, Fructose	KCN, KSCN, Nitriles	NH_3, HCOOH	30	10	(Adjei and Ohta, 1999)
Azotobacter vinelandii	Continuous	Cassava	NaCN	NH_3, CH_4	30	7-8.5	(Kaewkannetra et al., 2009)
Klebsiella oxytoca	Batch	Glucose	CN, SCN	NH_4, CH_4	30	7.0	(Kao et al., 2003)
Pseudomonas fluorescens	Batch	Glucose	$Fe(CN)_6$	NH_4, CO_2	25	5.0	(Dursun et al., 1999)
Trametes versicolor	Batch	Citrate	KCN	NH4,CO2	30	10.5	(Cabuk et al., 2006)
Trichoderma ssp.	Batch	Glucose	CN-	NH4,CO2	25	6.5	(Ezzi and Lynch, 2005)
Aspergillus awamori	Continuous	Citrus sinensis waste	KCN	NH4,HCOOH	40	8.84	(Santos et al., 2013)
Thiobacillus sp.	Batch	CO_2	SCN	NH_4, CO_2, SO_4	20-40	6-9.3	(Stott et al., 2001)
Klebsiella sp.	Batch	SCN	SCN	NH_4, SO_4,	38	7.0	(Ahn et al., 2005)
Halomonas sp.	Batch	Glucose, Fructose,CO_2, Acetate	SCN	NH_4,CO_2, SO_4	20-40	6-9.3	(Stott et al., 2001)
Pseudomonas pseudoalcaligenes	Batch	Acetate	NaCN	NH_4, CO_2	30	9.5-10.0	(Huertas et al., 2010)
Rhodococcus sp.	Batch	KCN	KCN	NH_4, HCOOH	30		(Maniyam et al., 2013)

Pseudomonas stutzeri, Pseudomonas putida	Batch	Lactate, Sucrose	KCN, KSCN	NH_4, SO_4^{2-}, CO_2	28-30	9.0-9.2	(Karavaiko et al., 2000)
Fusarium oxysporum CCMI 876	Continuous	Czampek broth	CN-	NH4, HCOOH	30	8.0	(Campos et al., 2006)
Halothiobacillus halophilus/hydrothermalis	Batch	SCN	SCN	-	35	7.0-8.0	(Sorokin et al., 2014)
Pseudomonas sp.	Continuous	Glucose	$[Cu(CN)_4]^{2-}$	-	35	7.5	(Patil and Paknikar, 1999)
Citrobacter sp.		Sugarcane mollasses	$[Zn(CN)_4]^{2-}$				
Pseudomonas putida	Batch	Glucose	$K_2[Ni(CN)_4]$	NH_4, CO_2	30	7.0	(Silva-Avalos et al., 1990)
Acremonium strictum	Batch	SCN	SCN	NH_4, SO_4^{2-}	25	-	(Kwon et al., 2002a)
Cryptococcus humicolus	Batch	Glucose	$K_2[Ni(CN)_4]$	NH_4, HCOOH	25	-	(Kwon et al., 2002b)
Methylobacterium thiocyanatum	Batch	Glucose	SCN	NH_3, CO_2, KOH	30	-	(Wood et al., 1998)
Klebsiella pneumoniae Ralstonia sp.	Batch	Glucose	SCN	NH_4, SO_4^2	37	6.0	(Chaudhari and Kodam, 2010)
Thiobacillus thioparus	Batch	CO_2	SCN	NH_3, COS*	30-40	7.0	(Katayama et al., 1992)
Acinetobacter johnsonii Pseudomonas diminuta	Batch		SCN	NH_4, SO_4^2	28	7.6	(Boucabeille et al., 1994)
Paracoccus thiocyanatus	-	SCN	SCN	-	30-37	7.5-8.0	(Katayama et al., 1995)
Burkholderia phytofirmans	Batch	Acetate	SCN	NH_4, SO_4^2	25	6.5	(Vu et al., 2013)
Micractinium sp.	Batch	$NaHCO_3$	SCN	NH_4, SO_4^2	-	8.2	(Ryu et al., 2015)
Thiohalobacter thiocyanaticus	Batch	$NaHCO_3$	SCN	COS, NH_3	30	7.3-7.5	(Sorokin et al., 2010)

Thialkalivibrio thiocyanodenitrificans	Batch	$NaHCO_3$	SCN	COS, NH_3	30	9.9	(Sorokin et al., 2004)
Thiohalophilus thiocyanoxidans	Batch	$NaHCO_3$	SCN	COS, NH_3	37	7.5	(Bezsudnova et al., 2007)

*COS – Carbonyl Sulfide

2.7 Summary

The currently utilised physical and chemical treatments for the treatment of cyanidation wastewater present disadvantages, such as high costs and the additional environmental burden that these processes pose. Although these processes are characterised by rapid degradation rates, however they are unable to treat certain cyanide complexes. A biological treatment process is found to be the most effective, robust, environmentally benign and cost effective method for the destruction of all cyanide related compounds. However, this process has been hindered by the use of cyanide sensitive microbial species in secondary stages that carry out the nitrification and denitrification processes, thus rendering the process ineffective, especially during cold seasons. Hence, this review proposes the utilisation of the same cyanide-insensitive microbial consortium throughout the process for the destruction of cyanide compounds and its biodegradation by-products heterotrophically. This would improve process efficiencies even in cases where cyanide compounds overflow to secondary stages. Additionally, this would ensure process water re-use to upstream bioleaching circuits, thus preserving the use of excessive water. This type of process would be beneficial for operations that are in areas with negative water balances. The recent full-scale industrial projects on cyanide biodegradation has have demonstrated the robustness and competitiveness of this process in comparison to the existing chemical and physical treatment technologies, and in future, the process might add economic value, as some cyanide degrading organisms produce biogas from cyanide biodegradation.

The research gaps which will be addressed in this study are highlighted as follows:

- There has been minimal research that focuses on the microbial co-metabolism of both CN^- and SCN^- in the same wastewater, especially under alkaline conditions.
- Furthermore, there has been minimal research of biodegradation of CN^- and SCN^- in continuous bioreactor systems under varying physicochemical parameters. Most studies have been restricted to batch culture systems.
- The microbial community characterization of the organisms that are associated with the biodegradation of CN^- and SCN^- has been mostly undertaken using culture-dependent techniques, which misrepresents the microbial composition since it focuses on cultivable organisms. Hence, this study focused on both culture dependent and independent techniques for accurate diagnosis of microbial diversity.
- An assessment on the microbial biochemical changes after co-culturing has never been evaluated.
- The ability of CN^- and SCN^- degrading organisms to conduct heterotrophic nitrification and aerobic denitrification for the utilization of the biodegradation by-products has never been investigated, either under cyanogenic or non-cyanogenic conditions. In most cases, CN^- and SCN^- sensitive organisms have been utilized and this has led to process failures.

CHAPTER 3

MATERIALS AND METHODS

Materials and methods

3.1 Materials

3.1.1 Microorganisms

3.1.1.1 Pure cultures

Pseudomonas aeruginosa STK 03 (AN KR011154), *Bacillus marisflavi* (AN KT282229) and *Exiguobacterium acetylicum* (AN KR016603) are cyanide and thiocyanate degrading organisms isolated from an oil spill site contaminated with cyano groups and in Diep River, Cape Town, South Africa respectively. The organisms were continuously maintained on nutrient agar slants and stored at 4 °C. For long term storage, the organisms were cryopreserved in 40 % v/v glycerol solution at -80 °C.

3.1.2 Isolation procedures

3.1.2.1 *Pseudomonas aeruginosa* STK 03

A bacterium that was able to grow on CN^- and SCN^- containing media was isolated from a site contaminated with cyano group containing compounds including poly aromatic hydrocarbons. The isolate was denoted as STK 03. The organism was isolated using a culture-dependent technique. A serial dilution on sterile saline solution (0.9% v/v NaCl) was performed on the original sample and plated on nutrient agar plates containing 100 mg CN^-/L and 50 mg SCN^-/L at 30 °C for 48 h. This was done to selectively isolate CN^- and SCN^- tolerant organisms. Identification of the organism was performed using the 16S rDNA sequencing technique followed by Polymerase Chain Reaction (PCR) as discussed in Section 3.2.2.

3.1.2.2 *Bacillus marisflavi* and *Exiguobacterium acetylicum*

Bacterial species able to grow on media containing CN^- and SCN^- were isolated from the Diep River, Cape Town, South Africa. A culture-dependent technique was employed to isolate the organisms subsequent to serial dilutions of the original sample in saline solutions. This was followed by plating on nutrient agar containing 100 mg CN^-/L and 100 mg SCN^-/L, and incubation at 30 C for 48 h, with an intention of isolating CN^- and SCN^--tolerant organisms. Two microbial organisms were selectively isolated, each from CN^- and SCN^--containing media. The identification of the isolated organisms was performed using the 16S rDNA sequencing technique followed by Polymerase Chain Reaction (PCR) as discussed in Section 3.2.2

3.1.2.3 Microbial communities

The cyanide degrading organisms (CDO) and thiocyanate degrading organisms (TDO) were isolated from electroplating wastewater containing elevated free cyanide (CN^-) concentrations (≤150 mg CN^-/L) (Mekuto, 2014) and in thiocyanate (SCN^-) containing wastewater respectively. The CDOs were observed to be unable to utilise SCN^- and this led to the isolation of thiocyanate utilising organisms using the gravimetric method. In this technique, a synthetic wastewater solution (500 mL) containing (g/L); K_2HPO_4 (3.4), KH_2PO_4 (4.3), Glucose (0.01), SCN^- (0.2) and CN^- (0.2), at a pH of 10 (±0.05),

was used to isolate the TDOs. The solution was left outside the laboratory for two months to allow airborne microorganisms to settle in the media. After the two month period, the culture was transferred, maintained and agitated (200 rpm) in a 1L stirred tank bioreactor (height of 250 mm and a diameter of 104 mm) driving 2-x-four bladed Rushton-type impellers, operated at a 48 h hydraulic retention time using the aforementioned media. Aeration was set at 400 mL/min and the reactor was operated at room temperature (21 °C to 25 °C). Similarly, the CDO's were maintained in a separate bioreactor operated at similar conditions to that of the TDO's. However, the aeration was set at 200 mL/min and the SCN^- concentration was set at 5 mg/L, using the aforementioned media.

3.1.2.4 Isolation of culturable organisms

A fraction of the biofilm containing media from Section 3.1.2.3 was filter sterilised in a 0.22 µm Millipore membrane and thereafter, the filter was washed with sterile distilled water. This was done to isolate culturable organisms from the enrichment culture. Serial dilutions on sterile distilled water were performed subsequent to plating on nutrient agar plates that contained 100 mg SCN^-/L. The plates were incubated at 30 °C for a period of 120 h. The colonies were selected based on their morphological appearance and purified by re-streaking on freshly-prepared media containing 100 mg SCN^-/L. Gram reaction of the isolates was performed to confirm the purity of the isolates and the organisms were subsequently cryopreserved.

3.2 Methods and procedures

3.2.1 Inoculum preparation

3.2.1.1 Pure cultures

Initially, the inoculum was prepared by inoculating single colonies of the isolates (*P. aeruginosa* STK 03, *B. marisflavi* and *E. acetylicum*) in Oxoid nutrient broth and agitated at 180 rpm in a shaking incubator set at 30 °C, for a period of 48 h. For subsequent experiments, the inoculum was prepared in minimal media (MM), without the presence of a nitrogen source. The MM contained (g/L): K_2HPO_4 (4.3), KH_2PO_4 (3.4), $MgCl_2.6H_2O$ (0.4) and Glucose (0.1). The inoculum concentration was set at 10 % (v/v) with an exception of Chapter 9 where these organisms were supplemented at 1 %(v/v), to aid in the biodegradation of SCN^- and CN^-.

3.2.1.2 CDO's AND TDO's

A 20 mL solution of the CDO and TDO samples were withdrawn from the bioreactor system and centrifuged at 14,000 rpm for 5 min to concentrate the microbial cells. The cells were washed twice with a phosphate buffer solution (pH = 7.0) and thereafter, the cells were re-suspended in sterile distilled water subsequent to inoculation (1% v/v) in a nitrogen-free minimal media (MM) (pH = 9.9) as described Section 3.2.1.1. The cells were grown for a period of 48 h at a temperature of 30 ºC in 250 mL Erlenmeyer flasks containing 200 mL of MM and inoculated at a concentration of 10 %v/v.

3.2.2 DNA extraction and PCR amplification

The genomic DNA of the isolated microorganisms, either as pure or mixed cultures, was extracted using a commercial DNA extraction kit (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The presence of the genomic DNA was assessed using a 1% (w/v) molecular grade agarose gel containing 0.5 µg/mL ethidium bromide (EtBr), using 1X Tris-acetate-ethylenediamine tetra-acetic acid (TAE) electrophoresis buffer at 100V for 1 h. PCR was performed using a GeneAmp PCR 9700 System (Applied Biosystems, USA). Target DNA was amplified using PCR in a total reaction volume of 25 µL containing 5 µL (± 50 ng/µL) of the purified gDNA, 0.5µL of 10 µM of the forward and reverse primers, 12.5 µL of a 2X KapaTaq Readymix solution (KapaBiosystems, South Africa) and made up using sterile distilled water. Bacterial specific primers used were the forward 8F primer 5'-AGAGTTTGATCCTGGCTCAG-'3 and reverse primer 1492R 5'-GGTTACCTTGTTACGACTT-'3. The amplification process included an initial denaturing step at 94°C for 10 min, followed by 36 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 1 min. The reaction was completed with a final extension period of 7 min at 72 °C followed by cooling and storage at 4 °C. PCR amplicons (2 µL) were electrophoretically analysed on a 1% (w/v) molecular grade agarose gel that was stained with ethidium bromide, using 1X TAE electrophoresis buffer at 100V for 1 h. The sequences were blasted against the NCBI GenBank database (www.ncbi.nlm.nih.gov).

3.2.3 16S rRNA amplicon gene sequencing

Total genomic DNA was extracted directly from the CDOs and TDOs, using commercially available extraction kits (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. The presence of the genomic DNA was assessed as per section 2.2.2. The purified DNA was PCR amplified using the 16S rRNA forward bacterial primers 27F-16S-5'-AGAGTTTGATCMTGGCTCAG-'3 and reverse primers 518R-16S-5'-ATTACCGCGGCTGCTGG-'3 (Satokari et al., 2001) that targeted the V1 and V3 regions of the 16s rRNA. The PCR amplicons were sent for sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa), a commercial NGS service provider. Briefly, the PCR amplicons were gel purified, end repaired and illumina® specific adapter sequence were ligated to each amplicon. Following quantification, the samples were individually indexed, followed by a purification step. Amplicons were then sequenced using the illumina® MiSeq-2000, using a MiSeq V3 (600 cycle) kit. 20Mb of the data (2x300 bp long paired end reads) were produced for each sample. The Basic Local Alignment Search Tool (BLAST)-based data analysis was performed using an Inqaba in-house developed data analysis pipeline.

3.2.4 Microbial community structure

Microbial samples were concentrated by centrifugation at 10 000 *g* for 5 min at 4 °C and were fixed in 2.5% glutaraldehyde overnight at 4 °C. The glutaraldehyde solution was discarded and the fixed organisms were washed twice with phosphate buffer (pH 7.0), followed by microbial dehydration in an alcohol series, i.e. 50%, 70 % and 100% (v/v) ethanol, for a period 12 h at 4°C. The samples were dried

using hexamethyldisilazane (HMDS) solution and were visualised using a scanning electron microscope (Nova NanoSEM 230) after the samples were mounted on a stub that was later coated with carbon.

3.2.5 VITEK® 2 Compact system

3.2.5.1 Pure culture biochemical identity

The culturable CDOs and TDOs were cultured on nutrient agar for a period of 16 h at 30 °C prior to analysis. The isolates were inoculated on the VITEK GN (Gram negative) and GP (Gram positive) colorimetric strips using the VITEK® 2 Compact system (BioMérieux, France). Microbial suspensions were adjusted to a McFarland standard of 0.50 to 0.63 in 0.5% (w/v) sodium chloride using a DensiLameter (BioMérieux, France). The VITEK® 2 Compact system detects biochemical reactions at 15 min intervals and the generated data was analysed using the VITEK 2 software version VT-R03.1, according to the manufacturer's instructions.

3.2.5.2 Mixed cultures

The biochemical properties of the CDOs, TDOs and a mixed culture of both CDOs and TDOs (referred to as CDO + TDO) were evaluated using the VITEK® 2 Compact system. The cells were grown for 16 to18 h at 30 °C in MM and thereafter, recovered by centrifugation at 10 000 g for 5 min at 4 °C. The cultures were inoculated into GN, GP, YST (Yeast or moulds) and BCL (Bacilli) colorimetric cards. Microbial suspensions were adjusted to a McFarland standard of 0.50 to 0.63 (GN and GP) and 1.8 to 2.2 (YST and BCL), in 0.5% (w/v) sodium chloride using a DensiLameter. This was done to assess the biochemical reaction changes prior and post co-culturing of the CDOs and TDOs.

3.2.6 Biological cyanide removal efficiency

A mass balance equation for the determination of the biologically degraded cyanide, taking into account cyanide volatilisation, is shown in Eq. 1 and 2.

$$\mathbf{CN_S^-} - (\mathbf{CN_R^-} + \mathbf{CN_V^-}) = \mathbf{CN_B^-} \qquad \text{Eq. 3. 1}$$

Where

$$\mathbf{CN_V^-} = (\mathbf{CN_{Vo}^-} - \mathbf{CN_{Vf}^-}) \qquad \text{Eq. 3. 2}$$

Biological removal efficiency (BRE) was determined according to Eq. 3

$$\text{BRE (\%)} = \frac{\boldsymbol{CN_B^-}}{\boldsymbol{CN_S^-}} \times \mathbf{100} \qquad \text{Eq. 3. 3}$$

Where CN_B^- is the biologically degraded cyanide (mg CN^-/L), CN_S^- is the initial free cyanide concentration in the media (mg CN^-/L), CN_R^- is the residual free cyanide measured in the inoculated media (mg CN^-/L), CN_V^- is the cyanide that volatilised during culture incubation (mg CN^-/L), CN_{Vo}^- is the initial cyanide concentration in the control cultures (mg CN^-/L), and CN_{Vf}^- is the final cyanide concentration in the control cultures (mg CN^-/L).

3.2.7 Statistical analysis

The experimental error was calculated as the standard error of mean using the standard deviation obtained from the multiple sets of data (n = 2), as demonstrated on Equation 4:

$$\text{SEM} = \frac{\textbf{Standard deviation}}{\sqrt{\textbf{number of samples tested}}} \qquad \text{Eq. 3. 4}$$

3.3 Analytical methods

Merck ammonium (NH_4^+) (00683), cyanide (CN^-) (09701), nitrate (NO_3^-) (14773), nitrite (NO_2^-) (and sulphate (00617) test kits were used to quantify the concentration of free cyanide, ammonium, and nitrates using a Merck Spectroquant Nova 60 instrument. Briefly, the cyanide test kit works on the reaction of cyanide with chloramine-T and pyridine-barbituric acid (Lambert et al., 1975). The ammonium test kit works on the Berthelot reaction between ammonium ions, chlorine and phenolic compounds to form indophenol dyes (Patton and Crouch, 1977). The nitrate test kit makes use of concentrated sulphuric acid in the presence of a benzoic acid derivative while the sulphate test kit makes use of the reaction between sulphates and barium ions and the sulphates are measured turbidimetrically (Kolmert et al., 2000). Nitrites were determined according to the method of Rider and Mellon (1946). In this technique, nitrite ions react with 4-aminobenzenesulfonic acid and 1-aminonaphtalene to form a reddish-pink colour which absorbs at a wavelength of 520 nm. The pH was measured using a Crison Basic20 pH meter which was calibrated daily. The microbial population was quantified using a Jenway 6715 UV/visible spectrophotometer at a wavelength of 600 nm (pure cultures) and counted directly using the Thoma counting chamber (mixed cultures). Thiocyanate was quantified using the ferric method (Katayama et al., 1992). In this technique, SCN^- reacts with ferric iron under acidic conditions to form a red-orange colour that absorbs at 420 nm.

CHAPTER 4

RESULTS

Published as: **Mekuto, L.,** Ntwampe, S.K.O., Kena, M., Golela, M.T., Amodu, O.S. 2016. Free cyanide and thiocyanate biodegradation by *Pseudomonas aeruginosa* STK 03 capable of heterotrophic nitrification under alkaline conditions. *3 Biotech* 6:6. DOI: 10.1007/s13205-015-0317-2

Free cyanide and thiocyanate biodegradation by *Pseudomonas aeruginosa* STK 03 capable of heterotrophic nitrification under alkaline conditions

4.1 Introduction

Natural and anthropogenic activities contribute to CN^- and thiocyanate (SCN^-) contamination in the environment. However, a significant source of cyanide contamination is through anthropogenic activities such as the cyanidation process, resulting in the formation of high CN^- and SCN^- laden wastewaters. The environmental deteriorative nature of the traditional chemical remediating methodologies, such as alkaline chlorination, hydrogen peroxide, etc. has necessitated for development of environmentally friendly processes which would not add to environmental destruction (Botz et al., 2005b, Mudder and Botz, 2004). More attention has been devoted to biotechnological approaches for the degradation of CN^- and SCN^- as it is cost effective, environmentally benign and does not produce end-products which are hazardous to the environment (Akcil and Mudder, 2003, Patil and Paknikar, 1999). The existence of CN/SCN resistant, tolerant and degrading bacterial and fungal organisms, has contributed significantly to the development of an effective degradation process, through understanding the microbiological contributions of individual organisms such that accurate predictive models can be developed (Stott et al., 2001).

A number of studies have been reported on bacterial decomposition of CN^- and SCN^-, and organisms such as *Bacillus pumilus*, *Klebsiella oxytoca*, *Burkholderia cepacia*, *Rhodococcus* sp., *Thiobacillus* sp., *Halomonas ssp* and many other organisms have potential to degrade CN^- and SCN^- (Adjei and Ohta, 2000; Kao et al., 2003; Meyers et al., 1991; Stott et al., 2001; Maniyam et al, 2011). Organisms belonging to the Pseudomonadaceae family have also been reported to degrade CN^-, SCN^- and metal-complexed cyanides. Organisms such as *Pseudomonas stutzeri*, *Pseudomonas putida*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas flourescens* have been observed as CN^- and SCN^- degraders (Grigor'eva et al., 2006, Grigor'eva et al., 2009, Grigor'eva et al., 2008, Karavaiko et al., 2000, Luque-Almagro et al., 2005a). However, CN^- and SCN^- degradation by a *Pseudomonas aeruginosa* strain, has never been reported. In addition, the effect of CN^- on SCN^- biodegradation by *P. aeruginosa* has never been reported, especially under alkaline conditions.

4.2 Objectives

The objectives of the present study were:

- To isolate and identify an organism capable of CN^- and SCN^- degradation under alkaline conditions.
- To assess the capability of the isolate(s) to conduct heterotrophic nitrification and aerobic denitrification.
- To assess the effect of CN^- spiking on SCN^- biodegradation by the isolate.

4.3 Materials and methods

4.3.1 Microorganism and inoculum preparation

Pseudomonas aeruginosa STK 03 was isolated and identified as described in Chapter 3. The organism was maintained on agar slants at 4 °C and sub-cultured every week. The sub-culturing media was spiked with minute concentration of CN^- and SCN^-, to maintain and adapt the organism in an environment containing CN^- and SCN^-. The inoculum was prepared as stipulated in Chapter 3 at 10% (v/v) of the total volume used for the biodegradation studies. However, the pH of the media was adjusted to an initial pH of 10 for CN^- studies and 8.5 for SCN^- studies, since the organism was unable to degrade thiocyanate above a pH of 8.5. The pH was not controlled during the duration of the study.

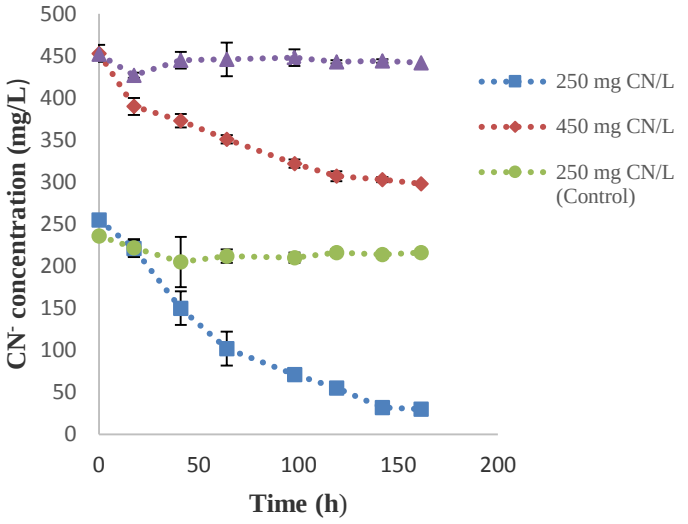
The organism was inoculated in MM which was supplemented with CN^- (as KCN) at concentrations of 250 and 450 mg CN^-/L, and SCN^- (as KSCN) at 250 mg SCN^-/L, in a total working volume of 200 mL. The uninoculated bioreactors served as controls. The bioreactors were incubated in an orbital shaker at 180 rpm and 30 °C. CN^- and SCN^- studies were run separately. CN^- studies were ran in airtight shake flasks fitted with sampling ports while SCN^- studies were ran in Erlenmeyer flasks. The use of airtight flasks was utilised to minimise CN^- volatilisation. To demonstrate nitrification and aerobic denitrification capability, the isolate was inoculated onto 200 mL Erlenmeyer flasks with MM medium, containing an initial concentration of NH_4^+ (as NH_4Cl) and NO_3^- (as $NaNO_3$) of 300 mg NH_4^+/L and 100 mg NO_3^--N/L, respectively. The initial pH was set at a pH of 10 for both the nitrification and denitrification studies. Aliquots (2 mL) were periodically withdrawn from the flasks and analysed for CN^-, SCN^-, NH_4^+, NO_3^- and SO_4^{2-} as described in Chapter 3.

4.4 Results and discussion

In this study, a CN^- and SCN^- tolerant bacterium was isolated and identified as *Pseudomonas aeruginosa* STK 03. CN^- biodegradation by *Pseudomonas aeruginosa* STK 03 and growth patterns in MM are shown in Fig 4.1A and 4.1B, respectively. The organism was able to degrade 250 and 450 mg CN^-/L, achieving a BRE of 80% and 32% within 150 h, respectively. Recently, it has been reported that an active aerobic degradation process has a maximum CN^- threshold concentration of 200 mg CN^-/L (Kuyucak and Akcil, 2013). However, in this study, *Pseudomonas aeruginosa* STK 03 was able to degrade CN^- in cultures containing CN^- concentrations above 200 mg CN^-/L. CN^- degradation was accompanied by growth of the organism, with the initial CN^- having a negative impact on the growth of the organism. The cultures that had low CN^- concentrations showed a shorter lag phase while the cultures with a higher concentration demonstrated a prolonged lag phase. This phenomenon was observed elsewhere (Mekuto et al., 2013), where a *Bacillus* consortia showed varying lag phases with respect to different initial CN^- concentrations, with cultures with the higher concentrations showing a prolonged lag phase. The prolonged lag phase with an increase in CN^-concentration was a result of cyanide inhibition on microbial growth. The degradation of CN^- resulted in the accumulation of NH_4^+ in the

medium, which suggested a possible hydrolytic mechanism of CN^- degradation (Ebbs, 2004, Akcil et al., 2003). The maximum NH_4^+ concentration from the cultures that had an initial CN^- concentration of 250 and 450 mg CN^-/L was 86 and 61 mg NH_4^+-N/L respectively. Subsequently, the NO_3^- concentration accumulated in the media, with an observed maximum nitrate nitrogen concentration of 31.2 and 62.4 mg NO_3^--N/L being observed, respectively (see Fig 4.2).

A



B

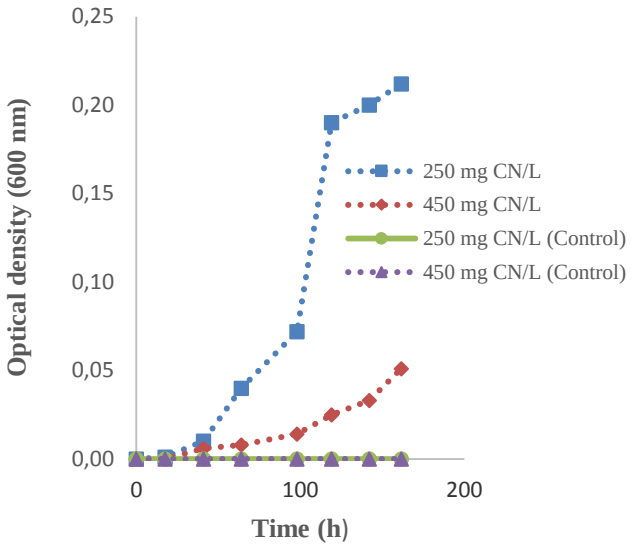


Fig. 4.1: CN^- degradation profile at different concentrations and growth profile of *Pseudomonas aeruginosa* STK 03 (A) and growth patterns of the organism (B). Error bars represent deviations.

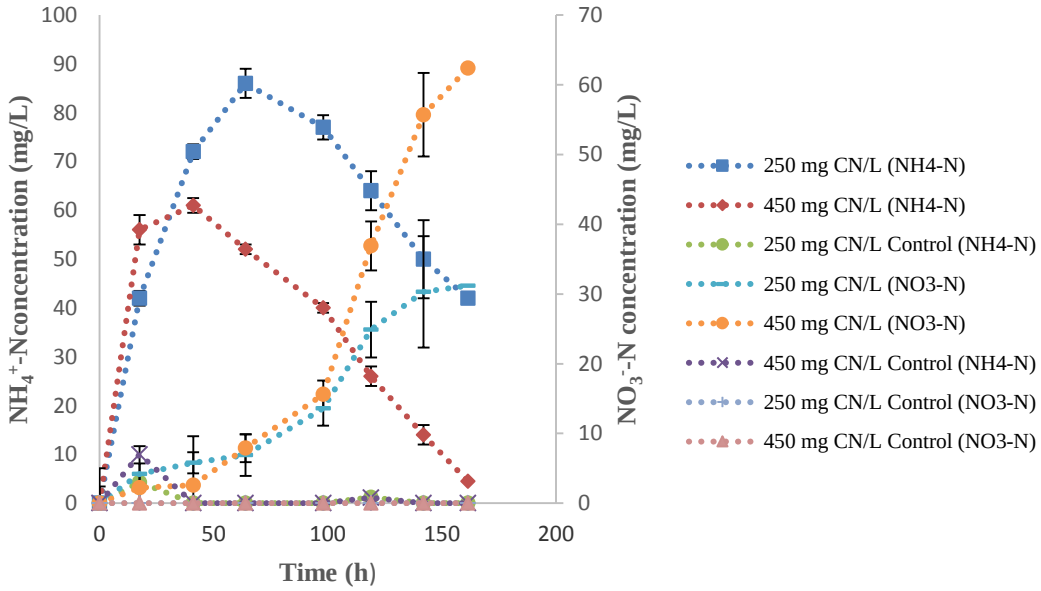


Fig. 4.2: NH_4^+ and nitrate nitrogen profiles as a function of time. Error bars represent deviations.

The NH_4^+ concentration decreased after 64 and 41 h from both cultures, with the residual NH_4^+ concentration being 42 and 4.5 mg NH_4^+-N/L from the cultures that contained an initial CN^- concentration of 250 and 450 mg CN^-/L, respectively. This showed heterotrophic nitrification capability of *Pseudomonas aeruginosa* STK 03. However, the organism was unable to remove NO_3^-, thus demonstrating the incapability of the organism to carry out aerobic denitrification. However, *Pseudomonas stutzeri* C3 was found to be able to carry out aerobic denitrification but was unable to carry out heterotrophic nitrification (Ji et al., 2015), while in a separate study *Pseudomonas stutzeri* YZN-001 was able to carry out nitrification and aerobic denitrification (Zhang et al., 2011); a suggestion that isolate STK 03 does not possess denitrification characteristics that are responsible for total nitrogen removal in cyanide contaminated effluent. To prove heterotrophic nitrification and aerobic denitrification, both NH_4^+ (as NH_4Cl) and NO_3^- (as $NaNO_3$) were used as nitrogen sources, in separate studies. STK 03 was able to carry out nitrification (see Fig 4.3), achieving a nitrification rate of 1.56 mg NH_4^+-N.L^{-1}.h^{-1} with subsequent production and accumulation of NO_3^- and NO_2^- while NH_4^+ stripping was determined to amount to 15%. Both nitrates and nitrites increased during the nitrification stage; however, the concentration of NO_2^- decreased to 1.75 mg NO_2^--N/L after 150 h, with the accumulation of nitrates being observed (Table 4.1).

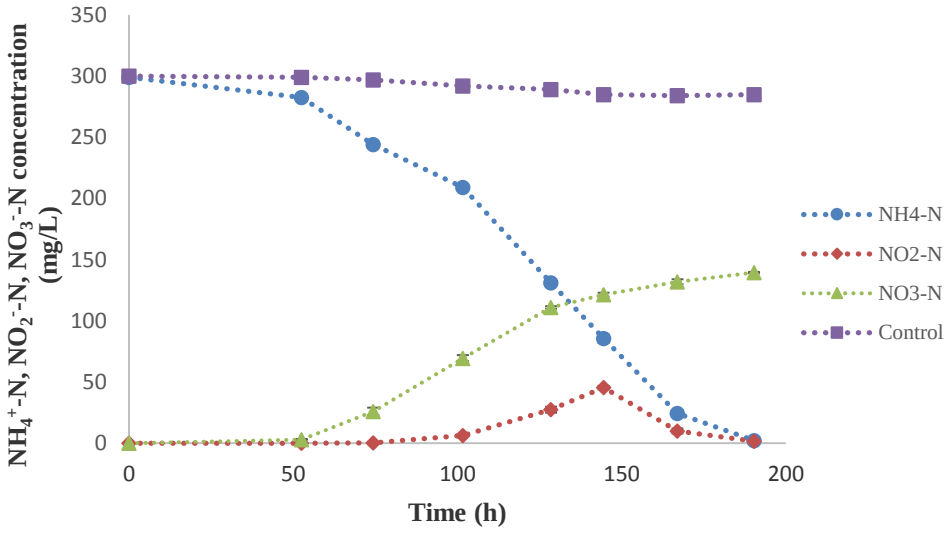


Fig. 4.3: Heterotrophic nitrification profile as a function of time. Error bars represent deviations.

Table 4.1: Aerobic denitrification by *Pseudomonas aeruginosa* STK 03.

Time (h)	NO_3^--N (mg/L)	NO_2^--N (mg/L)
0	100	0
24	97	0
72	96	0
96	96	0

Pseudomonas aeruginosa STK 03 was unable to degrade CN^- without the presence of a carbon source, i.e. whey waste (Fig 4.4). In the presence of a carbon source, there was a logarithmic increase of ammonium nitrogen from 0 to 40 h and thereafter, the ammonium concentration reached a plateau. The detection of NH_4^+ in the media was due to cell death or disruption and subsequent release of NH_4^+ related compounds due to CN^- toxicity. This meant that STK 03 was unable to use CN^- as a carbon and nitrogen source, and therefore an external carbon source was necessary to meet the carbon source requirements of the organism.

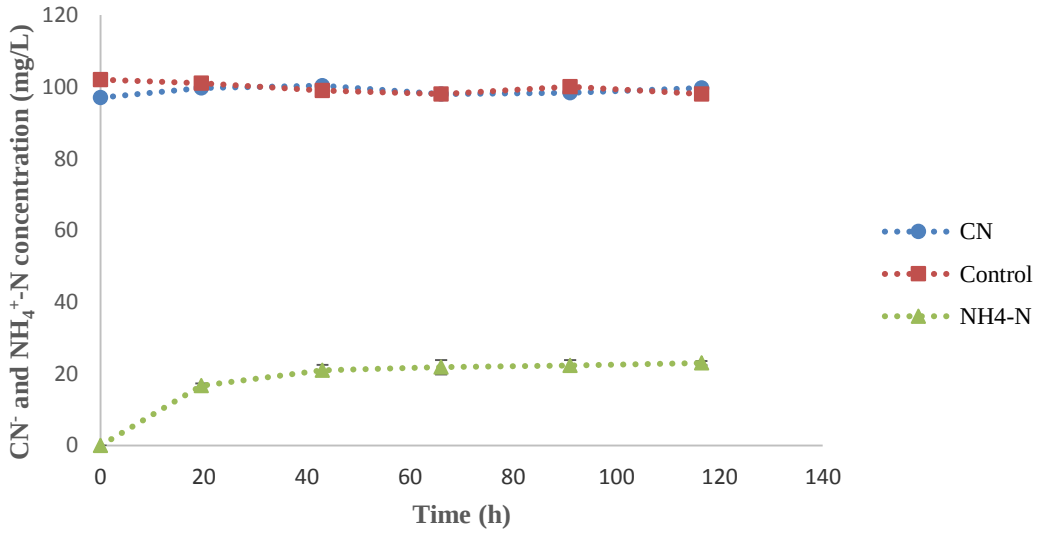


Fig. 4.4: Autotrophic degradation of CN^- and NH_4^+ formation profile. Error bars represent deviations.

The capability of the isolate to degrade SCN^- was evaluated in batch cultures and the organism was able to degrade 250 mg SCN^-/L to 55.5 mg SCN^-/L over a period of 200 h (Fig 4.5). This is equivalent to a degradation efficiency of 78%. SCN^- degradation resulted in the accumulation of SO_4^{2-}, with the maximum residual concentration of 90 mg SO_4^{2-}-S/L being observed. Maximum NH_4^+ and NO_3^- nitrogen was 120 mg NH_4^+-N/L and 90 mg NO_3^--N/L respectively, with observed nitrification after 120 h

resulting in residual NH_4^+ concentration of 53 mg NH_4^+-N/L after 200 h. Denitrification of the nitrates was not observed thus demonstrating incapacity of STK 03 to denitrify.

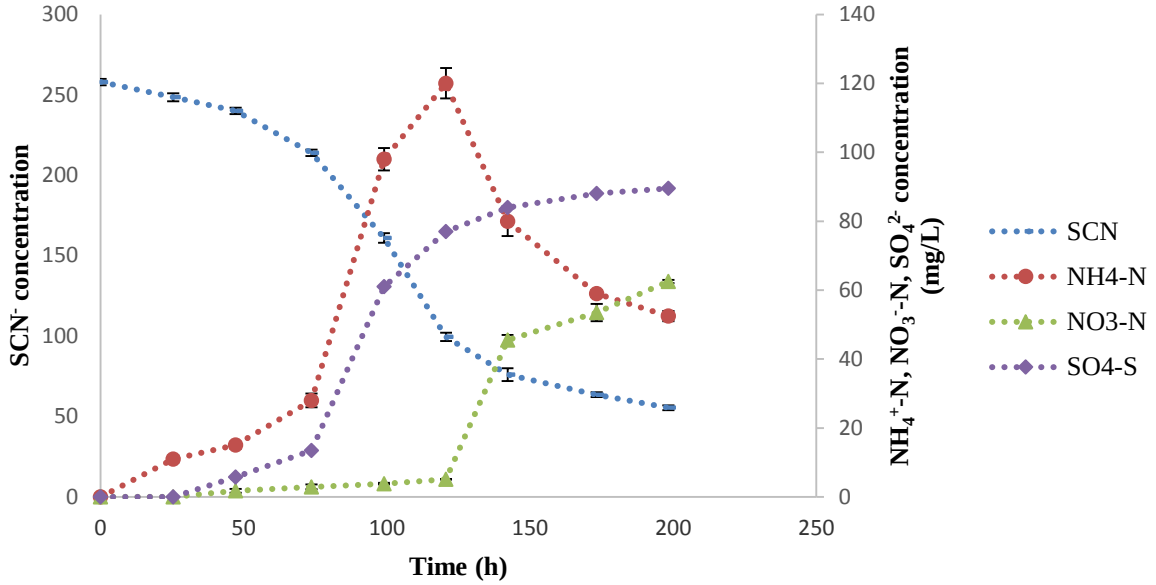


Fig. 4.5: SCN^- degradation profile and formation of degradation products without presence of CN^-. Error bars represent deviations

SCN^- degradation under the influence of CN^- spiking was also evaluated (Fig 4.6). CN^- spiking was carried out at 25 h and 100 h. Under these conditions, STK 03 had a degradation efficiency increase to 98% from an initial concentration of 250 mg SCN^-/L, meaning that the presence of CN^- propagated SCN^- degradation. It was hypothesised that this observation might be due to a metabolic shock response that might have triggered or up-regulated the expression of SCN^- degrading enzymes. The residual SCN^- concentration was observed to be 4.7 mg SCN^-/L. SO_4^{2-} and NO_3^- accumulated throughout the experiments reached a maximum SO_4^{2-} and NO_3^- concentration of 144.5 mg SO_4^{2-}-S/L and 55 mg NO_3^--N/L. SCN^- degradation was accompanied by NH_4^+ generation, resulting in a maximum NH_4^+ concentration of 123 mg NH_4^+-N/L. Ammonium oxidation from 120 h was observed with a sudden increase in nitrates thereafter; although denitrification was not observed.

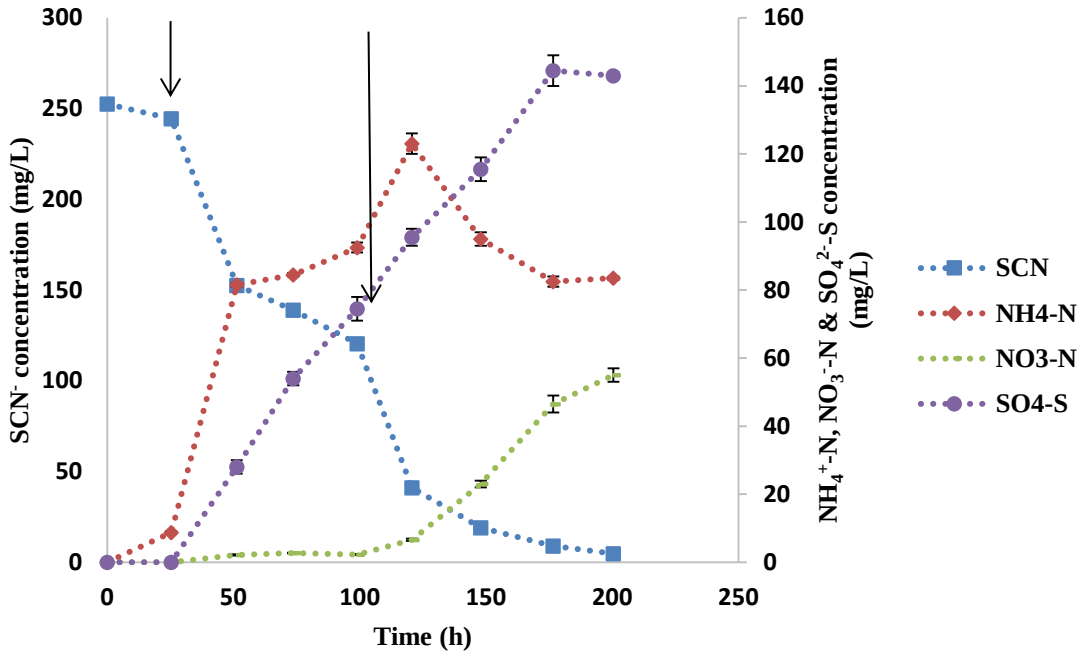


Fig. 4.6: SCN^- degradation profile and formation of degradation products with presence of CN^-. Error bars represent deviations. The arrows represent cyanide spiking intervals.

Pseudomonas stutzeri 18 and *putida* 21 were able to degrade SCN^- from an initial concentration of 60 mg SCN^-/L with the terminal sulphur products from SCN^- degradation being thiosulfate and tetrathionate, respectively (Grigor'eva et al., 2006). In this study, the terminal sulphur product from SCN^- degradation were SO_4^{2-}. This suggested that these organisms employ a different biochemical pathway for the degradation of SCN^-.

4.5 Summary

This study demonstrated the ability of *Pseudomonas aeruginosa* STK 03, which was originally isolated from an oil spill site contaminated with compounds containing cyano groups, was able to degrade CN^- and SCN^- under alkaline conditions, achieving a BRE of 80% and 32% from 250 mg CN^-/L and 450 mg CN^-/L respectively. Additionally, the SCN^- degradation efficiency was 78% and 98% from non- and CN^--spiked cultures, respectively. This was a first study on SCN^- degradation under alkaline conditions by an organism belonging to the Pseudomonadaceae family. Additionally, STK 03 surpassed the stipulated CN^- tolerance threshold of 200 mg CN^-/L, making this organism valuable for application in large scale wastewater treatment applications, particularly for wastewater containing CN^- and SCN^-. Furthermore, this study demonstrated that the presence of CN^- accelerated SCN^- degradation rates. This information is valuable in constituting a microbial consortium for the degradation of cyanide containing wastewater.

Consequently, the information gathered in this chapter was expanded by bioprospection of additional CN^- and SCN^- utilizing organisms which would also would result in the construction of a microbial consortium that is robust and effective, and that information is presented in the next chapter.

CHAPTER 5

RESULTS

Published as: **Mekuto, L.,** Alegbeleye, O.O., Ntwampe, S.K.O., Ngongang, M.M., Mudumbi, J.B., Akinpelu, E.A. 2016. Co-metabolism of thiocyanate and free cyanide by *Exiguobacterium acetylicum* and *Bacillus marisflavi* under alkaline conditions. *3 Biotech* 6:173. DOI: 10.1007/s13205-015-0317-2

Co-metabolism of thiocyanate and free cyanide by *Exiguobacterium acetylicum* and *Bacillus marisflavi* under alkaline conditions

5.1 Introduction

Cyanide is a naturally occurring compound which is produced by a variety of living organisms such as fungi, plants, bacteria and algae (Luque-Almagro et al., 2005a), and is also existent in the stratosphere and non-urban troposphere as a result of natural activities such as gases from volcanoes and burning of biomass. The contribution of these natural activities to cyanide contamination in the environment is insignificant as compared to anthropogenic activities. Anthropogenic activities contribute significantly to environmental deterioration as a result of the vast utilization of cyanide compounds, which in-turn generate extensive cyanide containing wastewaters. The mining industry accounts for 90% of cyanide utilization, making it the largest cyanide utilizing industry (Hu et al., 2015, Kitis et al., 2005, Kuyucak and Akcil, 2013).

Biological methods have been proven as the most sustainable and efficient process for the treatment of cyanide compounds. However, the success of such a process lies in the ability of the microbial species to be applied *in-situ*, under both aerobic and anaerobic conditions, in active and passive systems, suspended and attached growth systems (Akcil and Mudder, 2003, Akcil et al., 2003). Furthermore, the microbial species can be manipulated to handle large influent flows and tolerate high CN^- and SCN^- concentrations, allowing for the uptake, catalysis, sorption and/or precipitation of CN^-, SCN^-, weak and strong acid dissociable cyanide complexes. However, this method has been hindered by the lack of thorough understanding of the individual microbial species, including the detoxification mechanism, which contribute to the successful degradation of CN^- and SCN^- (Huddy et al., 2015). The understanding of the intrinsic metabolic contributions of individual microbial species within a CN^- and SCN^- degrading consortia is paramount, and to achieve this, the metabolic activities of individual species needs to be understood as these species employ different metabolic pathways for the degradation of CN^- and SCN^-. These metabolic pathways include the hydrolytic, oxidative, reduction and substitution/transfer pathways (Gupta et al., 2010, Ebbs, 2004, Dash et al., 2009). This information would add to the design of a high-strength microbial consortium that would ensure the maximum degradation of cyano-containing compounds. Hence, it is necessary to investigate the co-metabolism of the major cyanidation constituents, i.e. CN^- and SCN^-, under alkaline conditions.

5.2 Objectives

The objectives of this study were:

- To isolate organism(s) capable of degrading CN^- and SCN^- under alkaline conditions to augment the cultures used in Chapter 4.
- To assess the co-metabolism of CN^- and SCN^- using the isolated organisms in co-cultures.
- To optimize the culture conditions for successful co-metabolism of CN^- and SCN^-.

5.3 Materials and methods

5.3.1 Microorganisms

Bacterial species that were able to grow on media containing CN^- and SCN^- were isolated from the Diep River, Cape Town, South Africa. A culture-dependent technique was employed to isolate the organisms subsequent to serial dilutions of the original sample in saline solutions. This was followed by plating on nutrient agar containing 100 mg CN^-/L and 100 mg SCN^-/L, and incubation at 30 °C for 48 h, with an intention of isolating $CN^-$and SCN^--tolerant organisms. Two microbial organisms were selectively isolated; each from CN^- and SCN^- containing media.

The inoculum preparation for the isolated organisms and their identification was done as stipulated in Chapter 3. For the optimisation studies, the cultures were grown at the conditions determined by Response Surface Methodology (see Table 5.2), for a period of 48 h prior to optimization. The inoculum concentration was 10% (v/v). After the consensus sequences were deposited on the NCBI database, the isolates were assigned the following accession numbers: KT282229 (*E. acetylicum*) and KR016603 (*B. marisflavi*).

5.3.2 Experimental plan

The initial degradation experiments were conducted using nutrient broth as media, to assess the biodegradative capacity of *E. acetylicum* and *B. marisflavi* at a CN^- and SCN^- concentrations of 200 mg CN^-/L and 200 mg SCN^-/L, respectively. The organisms were inoculated in MM that was supplemented with CN^- (as KCN) and SCN^- (as KSCN), in nutrient broth. *Exiguobacterium acetylicum* and *Bacillus mariflavi* were both evaluated for the biodegradation of both CN^- and SCN^- separately. For the CN^- degradation studies, the pH of the media was at 9.5 since free cyanide is available in anionic state at this pH (Johnson, 2015). The SCN^- were conducted at a pH of 8.0. Due to the high degradation efficiencies that were obtained from MM, this media was utilized as growth media for the subsequent experiments. The effect of carbon source supplementation on the biodegradation process was assessed using glucose, fructose, acetate, starch and sucrose as sources of carbon. These experiments were run over a period of 72 h, at CN^- and SCN^- concentrations of 100 mg CN^-/L and 100 mg SCN^-/L, respectively.

5.3.3 Response Surface Methodology: Central composite design

A mathematical and statistical optimisation methodology, referred to as Response Surface Methodology (RSM), was employed in this study to optimise the operational parameters that influence the CN^-/SCN^- biodegradation process. This was achieved using the Design-Expert® software (version 6.0.8, Stat-Ease Inc., Minneapolis, USA). The chosen operational parameters, i.e. pH, Temperature, SCN^- and CN^- concentrations were evaluated to determine the optimum operational conditions which would result in complete degradation of SCN^- and CN^- in the same media. A 25-run experimental design was constructed using central composite design (CCD) at three levels: low (-2), medium (0) and high (+2) (see Table 5.1). The experimental design and their corresponding responses are tabulated in Table 5.2.

All the experiments were conducted in 250 mL multiport airtight Erlenmeyer flasks and these flasks were used to minimise free cyanide volatilisation. The working volume was set at 100 mL using MM for growth and the experiment was conducted at 180 rpm at an inoculum concentration of 10% (v/v) for a period of 168 h. The inoculum was prepared as described in Chapter 3, while the uninoculated flasks served as controls. The generated results from the biodegradation experiments served as a response (Y), as described in Equation 5.1.

$$\boldsymbol{Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon} \quad \text{(Eq 5.1)}$$

where Y is the predicted response [degradation efficiency (%)] , β_0 is the interception coefficient, β_i , $\beta_{ii}$and β_{ij} are the linear effect, quadratic and interaction coefficients, respectively. X_i and X_j are input variables that influence the degradation efficiency (Y), while ε represents the random error.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The analysis included the overall model significance (F-test), correlation coefficient R, and determination coefficient R^2, which measures the goodness of fit for the regression model. The response surface plots and contour plots were generated using Eq. 5.1.

Table 5.1: Independent variables and levels used for central composite design.

Variables	**Code**	**Range and levels**				
		+2	**+1**	**0**	**+1**	**-2**
pH	A	10.00	11	9.00	7.0	8.00
Temperature (°C)	B	37.0	40.5	33.50	26.5	30.0
Free cyanide (mg/L)	C	300	400	200	0.0	100
Thiocyanate (mg/L)	D	300	0.0	200	400	100

α = 2.0

Table 5.2: Central composite design using 3 variables and the corresponding response

Run	**A**	**B**	**C**	**D**	**Degradation (%)**
1	9.0	40.5	200	200	62
2	9.0	26.5	200	200	98
3	9.0	33.50	200	200	97
4	8.0	30.0	300	300	61
5	9.0	33.50	200	200	97
6	8.0	30.0	300	100	95
7	9.0	33.5	400	200	92

8	11.0	33.5	200	200	52
9	9.0	33.5	200	400	89
10	7.0	33.5	200	200	94
11	10.0	30.0	300	300	59
12	10.0	30.0	100	300	42
13	10.0	37.0	300	100	79
14	9.0	33.5	0.0	200	99
15	8.0	37.0	300	300	82
16	9.0	33.50	200	200	97
17	9.0	33.5	200	0.0	99
18	10.0	30.0	100	100	76
19	8.0	37.0	300	100	88
20	10.0	37.0	100	100	78
21	8.0	30.0	100	100	98
22	8.0	37.0	100	300	95
23	9.0	33.50	200	200	97
24	9.0	33.50	200	200	97
25	8.0	30.0	100	300	92
26	10.0	37.0	100	300	61
27	10.0	37.0	300	300	73
28	9.0	33.50	200	200	97
29	10.0	30.0	300	100	82
30	8.0	37.0	100	100	98

A, B and C represent the coded level of variables while α represents the axial point with coded level of 2.0

5.3.4 Model validation

The accuracy of the predicted optimum conditions, which would ultimately result in over 99% degradation efficiency as predicted by RSM, was assessed by evaluating the co-metabolism of SCN^- and CN^- by *E.acetylicum* and *B. marisflavi* at the optimised conditions. This was done to validate the predicted degradation efficiencies compared to the actual experimental data.

5.4 Results and discussion

5.4.1 Isolation and identification of the CN^- and SCN^- degrading bacteria

A culture-dependent approach was utilized for the isolation of organisms that are able to tolerate and/or degrade CN^- and SCN^- separately, and two bacterial species were isolated and identified. *Bacillus mariflavi* (accession number KR016603) grew only in CN^- containing media while *Exiguobacterium acetylicum* (accession number KT282229) grew in SCN^- containing media. The ability of these

organisms to degrade CN^- and SCN^- was evaluated where nutrient broth was used as the growth media. SCN^- degradation by *E. acetylicum* and its growth pattern in nutrient broth is shown in Fig. 5.1. The organism was able to achieve complete degradation of SCN^- from an initial concentration of 200 mg SCN^-/L over a period of 144 h under slightly alkaline conditions, but was unable to degrade free cyanide (Appendix A Fig. A1). On the other hand, CN^- degradation by *B. marisflavi* was evaluated under alkaline conditions, where the organism was able to degrade CN^- completely over a period of 144 h (Fig. 5.1). However, *B. marisflavi* was observed to be unable to degrade SCN^- (Appendix A Fig. A1).

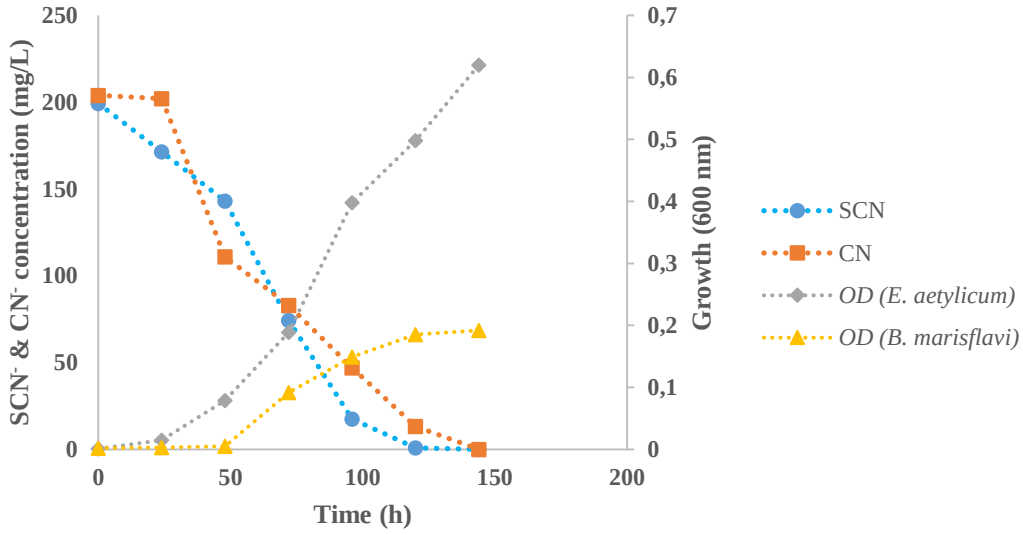
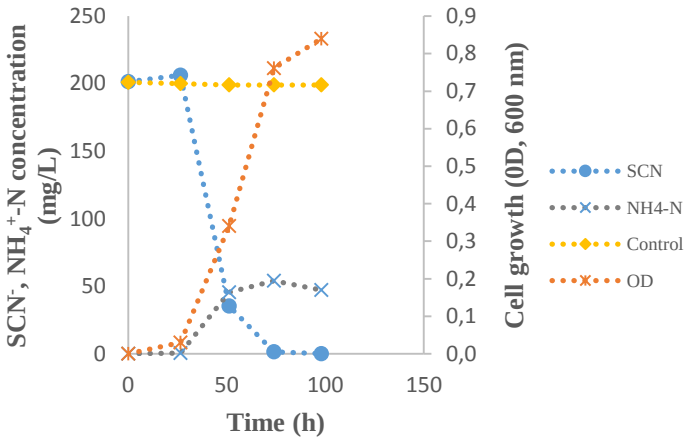


Fig. 5.1: SCN^- and CN^- degradation profile in nutrient broth media.

Subsequent to the evaluation of these organisms' ability to degrade CN^-/SCN^- in nutrient broth, minimal media (MM) was evaluated as the potential growth media for the two organisms. These media was utilized as it contains minimal nutrient supplementation, thus ensuring minimal costs that are associated with nutrient supply. When MM was utilized as the growth media, *E. acetylicum* achieved complete degradation of SCN^- over a period of 98 h (Fig. 5.2a), while *B. marisflavi* achieved complete CN^- degradation over a 98 h period (Fig. 5.2b).

A



B

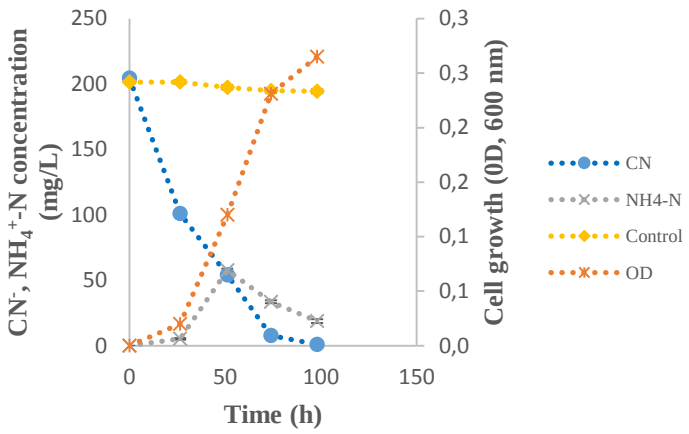
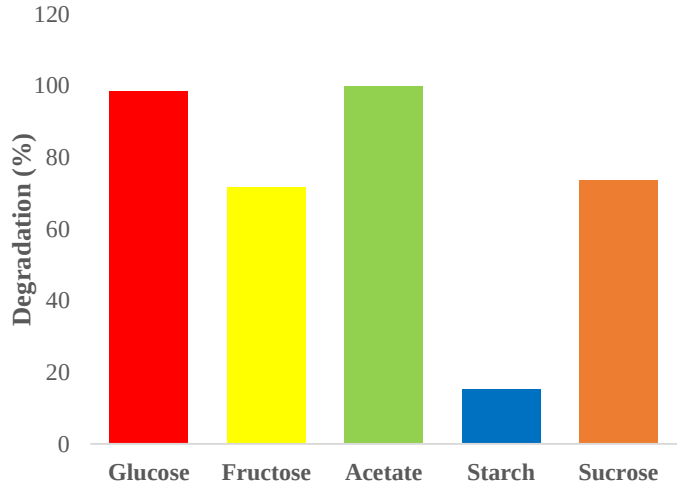


Fig. 5.2: The SCN^- and CN^- degradation profiles by (a) *Exiguobacterium acetylicum* and (b) *Bacillus mariflavi* in minimal media.

A



B

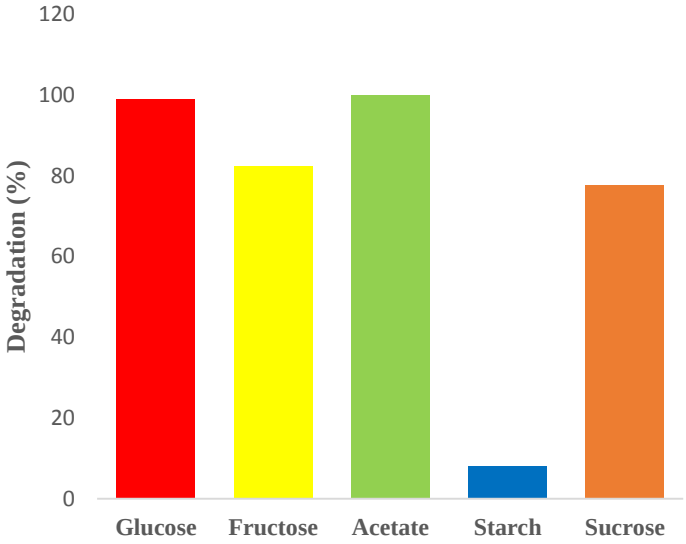


Fig. 5.3: The effect of carbon source on SCN^- and CN^- degradation by (a) *Exiguobacterium acetylicum* and (b) *Bacillus mariflavi*.

A variety of carbon sources were evaluated, to assess the best carbon source for the two organisms and, glucose and acetate was observed to be the preferred carbon source as it resulted in ≥99.9% CN^- and 99.8% SCN^- degradation efficiencies by *E. acetylicum* and *B. marisflavi* respectively (Fig. 5.3a & b). Glucose was chosen as a preferred carbon source in-line with the previous experimental setups that were undertaken in this study.

5.4.2 Response Surface Methology: central composite design (CCD)

The relationship amongst the independent variables and their optimum conditions were evaluated using CCD. Parameters such as pH (A), operating temperature (B), initial CN^- concentration (C) and initial SCN^- concentration (D) were selected as the important input variables that can affect cyanide and thiocyanate biodegradation. Through the CCD method, the combined impact of these parameters were studied from the generated 30 run experimentation (see Table 5.2).The model equation, which is a second-order polynomial equation for CN^- and SCN^- degradation efficiency was obtained by performing regression analysis (Eq. 5.2).

$$Y = 97.00 - 10.13A - 0.96B - 1.46C - 6.21D - 7.16A^2 - 5.41B^2 - 1.53C^2 - 1.91D^2 + 0.94AB + 5.81AC - 1.94AD + 0.063BC + 4.06BD - 0.56CD \quad \text{(Eq 5.2)}$$

Table 5.3: Analysis of variance (ANOVA) for the quadratic model

Source	**Sum of squares**	**DF**	**Mean square**	***F* value**	**Prob > *F***
Model	6288.55	14	449.18	4.55	0.0031
A	2460.38	1	2460.38	24.90	0.0002
B	22.04	1	22.04	0.22	0.6435
C	51.04	1	51.04	0.52	0.4834
D	925.04		925.04	9.36	0.0079
A^2	1404.67	1	1404.67	14.21	0.0019
B^2	801.67	1	801.67	8.11	0.0122
C^2	64.31	1	64.31	0.65	0.4324
D^2	99.67		99.67	1.01	0.3312
AB	14.06	1	14.06	0.14	0.7113
AC	540.56	1	540.56	5.47	0.0336
AD	60.06		60.06	0.61	0.4477
BC	0.062	1	0.062	0.000632	0.9803
BD	264.06		264.06	2.67	0.1229
CD	5.06		5.06	0.051	0.8240
Residual	1482.25	15	98.82	-	-
Lack of fit	1482.25	10	148.22	-	-

R^2 = 0.90, CV = 11.81, Adj. R^2 = 80.93

Table 5.4: Observed and predicted responses obtained using CCD.

Run no.	Observed (mg/L)	Predicted (mg/L)
1	98.00	108.13
2	76.00	78.25
3	98.00	96.08
4	78.00	69.96
5	95.00	94.58
6	82.00	87.96
7	88.00	82.79
8	79.00	79.92
9	92.00	92.58
10	42.00	54.96
11	95.00	96.79
12	61.00	62.92
13	61.00	76.79
14	59.00	62.42
15	82.00	81.25
16	73.00	70.63
17	94.00	88.63
18	52.00	48.13
19	98.00	77.29
20	62.00	73.46
21	99.00	93.79
22	92.00	87.96
23	99.00	101.79
24	89.00	76.96
25	97.00	97.00
26	97.00	97.00
27	97.00	97.00
28	97.00	97.00
29	97.00	97.00
30	97.00	97.00

The R^2 of the regression equation was 0.90, indicating that the model was suitable for describing the co-metabolism of CN^- and SCN^- as a function of the selected factors (Fig. 5.4a). The regression model also revealed that the model explained 90% of the experimental results. The high value of the adjusted determination coefficient indicated the significance of the model. The Fisher's *F*-test was used to assess

the adequacy of the model and the results are shown in Table 5.3. The model *F* value of 4.55 implied that the model used was significant and the *P* values showed the significance of the coefficients, thus highlighting interactions between the variables. The adequate precision ratio of 8.536 obtained indicated an adequate signal while the lower value of the coefficient of variance (CV = 11.81) indicated better precision and reliability of the experiments. The confirmation of the adequacy and reliability of the generated model is paramount as it ensures sufficient representation of the actual test. Satisfactory normal probability plot of the residuals was achieved as the plots approximated along a straight line (Fig. 5.4b). The observed and predicted results based on the generated quadratic model (Eq. 5.2) are shown in Table 5.4.

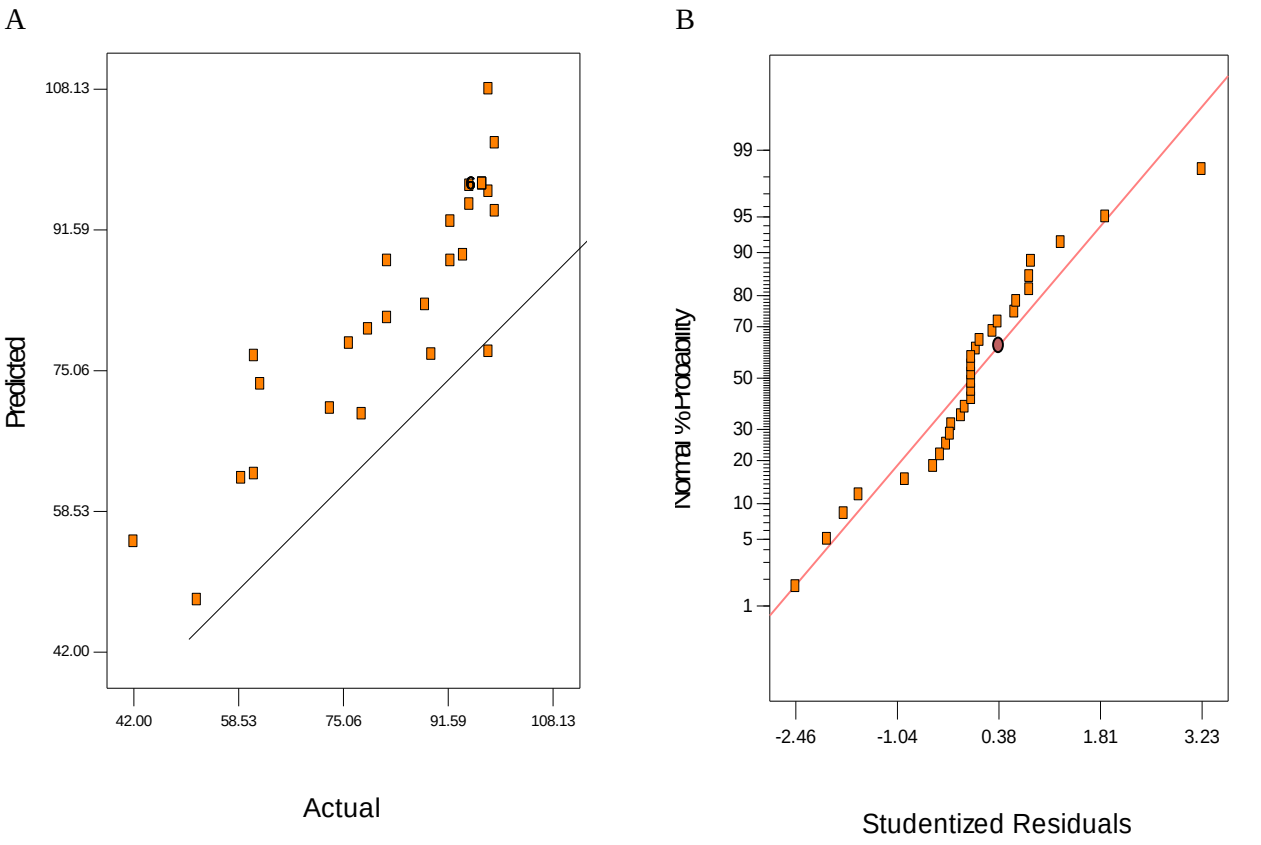


Fig. 5.4: Graphical profiles representing (a) the predicted and the actual values of CN^-/SCN^- degradation efficiency, and (b) the normal probability of the studentized residuals.

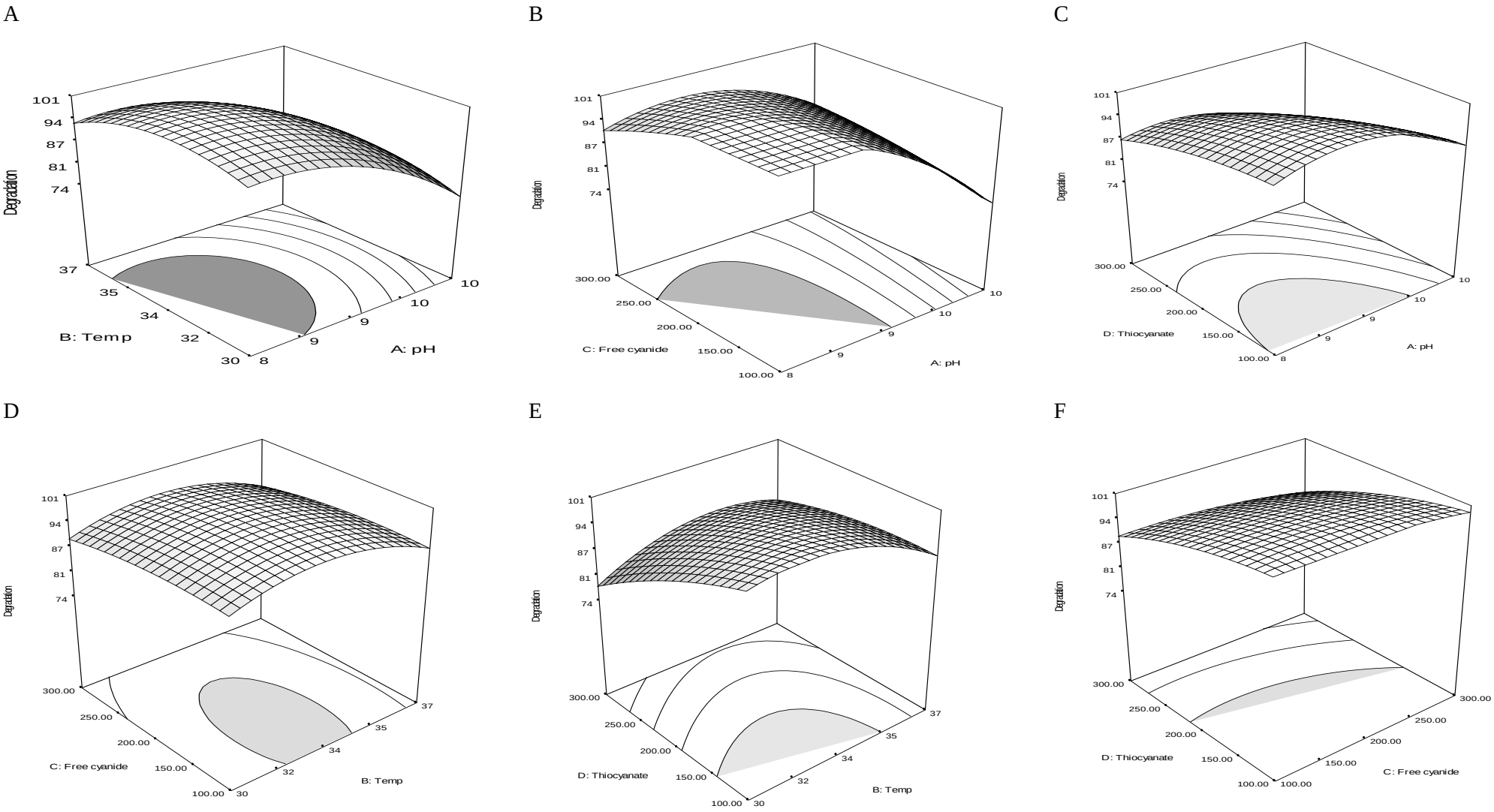


Fig. 5.5: The response surface and contour plots of the interactions of (a) temperature and pH, (b) Free cyanide and pH, (c) thiocyanate and pH, (d) Free cyanide and temperature, (e) thiocyanate and temperature and, (f) thiocyanate and free cyanide

The interaction between the tested variables were determined by plotting the response surface curves. 3D response surface and the 2D contour plots are the graphical demonstrations of the regression equation, and both are represented in Figs. 5.5a - f. The main goal of RSM is to obtain optimum conditions of the tested variables such that the response is maximized. The resulting optimal responses for pH, temperature, CN^- and SCN^- concentrations were found to be 9.0, 34 °C, 140 mg SCN^-/L and 205 mg CN^-/L, respectively, with a desirability of 0.96, where an overall degradation efficiency of over 99% can be achieved over an incubation period of 168 h.

5.4.3 Model validation

The accuracy of the predicted model was evaluated. Using the optimum conditions, the co-culture of *B. marisflavi* and *E. acetylicum* was assessed for the co-metabolism of CN^- and SCN^- in a batch system. The organisms completely degraded CN^- and SCN^- over a period of 170 h (Fig. 5.6). The degradation of these compounds was accompanied by the production of ammonium, nitrates and sulphates. There was an observed decrease in the concentrations of ammonium and nitrates after 120 h, suggesting the utilization of these compounds by the microbial species, while the sulphates accumulated throughout the experimental run, producing the maximum sulphate concentration of 93.5 mg SO_4^{2-}-S/L. These results confirmed the accuracy and reliability of the generated model.

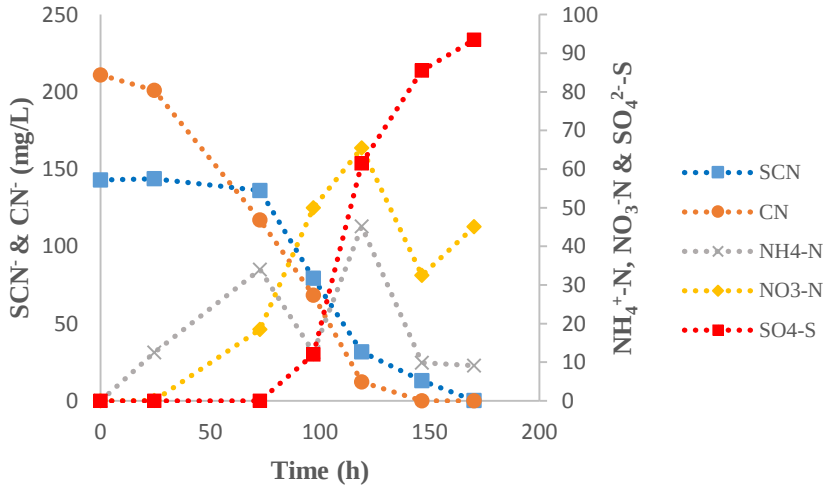


Fig. 5.6: Model validation graphical profile on the co-metabolism of SCN^- and CN^-.

This is the first report on the cyanide degrading capacity of *Bacillus marisflavi* and thiocyanate biodegradation by *Exiguobacterium acetylicum*. Since *B. marisflavi* was unable to degrade SCN^- and the incapacity of *E. acetylicum* to degrade CN^-, a co-metabolism study was undertaken to assess the effectiveness of co-culturing for the overall biodegradation of both CN^- and SCN^- in the same media. The co-cultures effectively degraded CN^- and SCN^- under alkaline conditions. Chaudhari and Kodam (2010) have shown that a co-culture of *Klebsiella pneumoniae* and *Ralstonia* sp. was able to degrade high SCN^- concentrations, achieving degradation rates of 500 mg. $L^{-1}.h^{-1}$. Consequently, 34 °C, pH 9.0,

CN^- concentration of 140 mg CN^-/L and SCN^- concentration of 205 mg SCN^-/L were determined to be the appropriate physicochemical conditions for the maximum co-metabolism of CN^- and SCN^-. Through RSM, *Bacillus* sp. CN-22 was observed to have optimum conditions at pH 10.3 and temperature of 31 °C, and CN-22 was able to tolerate up to 700 mg CN^-/L. *Bacillus* species have been observed to be highly tolerant to high CN^- concentrations in batch and continuous systems. Mekuto et al. (2015) assessed a consortium of *Bacillus* sp. in a continuous mode using a packed bed reactor under RSM-optimised conditions of: pH 9.88 and temperature of 33.8 °C. The authors observed a degradation efficiency of over 99% when CN^- concentration was gradually increased from 100 to 500 mg CN^-/L over a period of 80 days. *E. acetylicum* and *B. marisflavi* have been observed to be tolerant to CN^- and SCN^- concentrations of 400 mg CN^-/L and 400 mg SCN^-/L in the same media (see Table 5.2), making these organisms one of the few that tolerate the co-existence of CN^- and SCN^- at high concentrations. Furthermore, there are limited studies on SCN^- degradation under alkaline conditions. A symbiotic relationship was observed between the two organisms. This maybe as a result of the close genetic relatedness of the species belonging to the *Exiguobacterium* and *Bacillus* genera (Farrow et al., 1992, Farrow et al., 1994, Yumoto et al., 2004), thus resulting in the observed compatibility of the two organisms.

5.5 Summary

This study focused on the co-metabolism of the CN^- and SCN^- by the isolated *Bacillus marisflavi* and *Exiguobacterium acetylicum*. Analysis of the generated data revealed that the organisms successfully degraded both CN^- and SCN^-, achieving over 99% degradation efficiencies. This demonstrated a symbiotic relationship between the two organisms. Using Response Surface Methodology, the optimum pH, temperature, CN^- and SCN^- concentrations were found to be 9.0, 34 °C, 140 mg CN^-/L and 205 mg SCN^-/L, respectively. Using this data, the generated model was validated through batch experiments, and the organisms completely degraded CN^- and SCN^- over 170 h, under alkaline conditions. This confirmed that the generated model is accurate and reliable. This is the first report on the co-metabolism of CN^- and SCN^- under alkaline conditions. Furthermore, this is the first report on CN^- and SCN^- by *Exiguobacterium acetylicum* and *Bacillus marisflavi*.

The successful assessment of the organisms isolated in Chapter 4 and in this chapter, affirmed the suitability of these cultivable organisms for the biodegradation of CN^- and SCN^- under alkaline conditions. Although the contributions of these organisms is paramount, there was, however, a realisation that the employment of these three organisms would not fully represent environmental conditions. Hence, a further microbiological prospection for the isolation of microbial communities, which have the capacity to degrade the aforementioned contaminants, was necessary. This was the focus of the next chapter.

CHAPTER 6

RESULTS

Mekuto, L. & Ntwampe, S.K.O. Microbial communities associated with the co-metabolism of free cyanide and thiocyanate under alkaline conditions: biochemical reaction metamorphoses. Submitted to *Folia Microbiologica* [Manuscript ID:FOL-D-17-00159]

Data article (supplementary data of the aforementioned article)

Mekuto, L., Ntwampe, S.K.O., Mudumbi., J.B.N., Akinpelu, E.A. & Mewa-Ngongang, M. Metagenomic data of free cyanide and thiocyanate degrading bacterial communities. Accepted in *Data in Brief* [Manuscript ID: DIB-S-17-00430] – see page 63 and Appendix B Table B1 and 2.

Microbial communities associated with the co-metabolism of free cyanide and thiocyanate under alkaline conditions: biochemical reaction metamorphoses

6.1 Introduction

Cyanide containing wastes, which are produced from various industries, have been determined to be detrimental to living organisms and the environment, thus necessitating environmental benign detoxification techniques. Biotechnological approaches for the treatment of cyanide containing wastewaters, have gained popularity over the years due the robustness, cost-effectiveness and environmental friendliness of this process. In addition, microbial-mediated cyanide degradation does not produce degradation products which are hazardous to the environment, but rather produce products which serve as sources of carbon and nitrogen to the employed organisms (Mekuto et al., 2015). This is achieved through diverse enzymatic reactions that the cyanide degrading organisms possess, which have been documented elsewhere (Ebbs, 2004, Gupta et al., 2010).

The first microbial-mediated commercial cyanide degradation system was reported at the Homestake Gold mine; a process that was dominated by *Pseudomonas paucimobilis* which was able to treat 4 million gallons of cyanide containing wastewater per day. This process remained successful throughout the operational years until mine closure (Whitlock, 1990). This demonstrated the effectiveness of utilising microorganisms in the treatment of cyanide containing wastewaters. However, this process was hampered by the utilisation of traditional autotrophic nitrifying and denitrifying organisms downstream of the cyanide degradation system, and this led to process failures due to the susceptibility of such organisms to CN^- (Akcil, 2003). Additionally, there was a lack of a thorough understanding of the microbial constituents contributing to that process. The characterisation of the organisms that contribute to the process is imperative, as it dictates the performance of the system and, in cases where the system fails, the characterisation would enable the determination of the organisms that would be necessary for re-inoculation within the system, to regain successful process performance. Therefore, there is a need for further characterization of CN^- and SCN^- degrading organisms, which would ultimately assist in the construction of an effective inoculum for the treatment of CN^-/SCN^--laden wastewaters.

6.2 Objectives

The objectives of the present study were:

- To isolate thiocyanate degrading organisms (TDO) using a gravimetric technique.
- To assess the ability of the TDO's, including the previously isolated cyanide degrading organisms (CDO's) to degrade CN^- and SCN^- under alkaline conditions.
- To identify and characterize CN^- and SCN^- degrading organisms using a culture-dependent and independent approach.

- To evaluate the co-metabolism of CN^- and SCN^- by the CDO's and TDO's separately and when co-cultured.
- To assess the phenotypical changes after the co-culturing of the CDO's and TDO's.

6.3 Materials and methods

The TDO's and CDO's were employed in this study to evaluate their ability to degrade CN^- and SCN^- in batch cultures. The CDO's were previously isolated in electroplating wastewater (Mekuto et al., 2013) while the TDO's were isolated using a gravimetric method which is described in Chapter 3, Section 3.1.2.3. The inoculum preparation was conducted as described in Chapter 3, Section 3.2.1.2.

6.3.1 Batch biodegradation experiments

6.3.1.1 CN^- and SCN^- biodegradation experiments

CN^- biodegradation experiments were performed in 250 mL airtight multiport flasks with a working volume of 100 mL. These flasks were utilised to minimise CN^- volatilisation. Minimal media (MM) was utilised as growth media and the chemical composition of this media has been described in Chapter 3. The pH of the media was set at 9.9. The CDO's and TDO's were inoculated separately, on MM containing 250 mg CN^-/L and 250 mg SCN^-/L, respectively, at 10% (v/v) from previously grown cultures. The flasks were incubated in an orbital shaker at 30 °C and 180 rpm. Samples were collected at various time intervals and centrifuged at 10 000 *g* for 5 min prior to the analysis of CN^-, SCN^-, NH_4^+-N, NO_3^--N, NO_2^--N and SO_4^{2-}-S. Uninoculated flasks served as a control. A triplicate set of experiments were used to obtain averaged data.

6.3.1.2 Co-metabolism of CN^- and SCN^- experiments

This study was conducted in 250 mL airtight multiport flasks with a working volume of 100 mL. Previously grown CDO's and TDO's were mixed and inoculated on MM supplemented with both CN^- and SCN^- in the same media. This was done to evaluate the co-metabolism of CN^- and SCN^-. The pH of the media was set at 9.9 and the concentrations were set 250 mg/L for both free cyanide and thiocyanate. The incubation and sampling procedure was performed as described in Section 3.5.1.

6.4 Identification of organisms using a culture-dependent and independent approach

The culturable CDO's and TDO's were cultured on nutrient agar for a period of 16 h at 30 °C and were identified using VITEK® 2 Compact system and a genetic approach as described in Chapter 3, Section 3.2.5.1 and 3.2.2 respectively. A culture-independent approach was undertaken to identify the microbial communities. In this technique, total DNA was extracted directly from the microbial communities and was followed by PCR amplification that targeted the V1 and V3 regions of the 16S rRNA gene. The sequencing and the microbial community structure analysis were conducted as described in Chapter 3.

6.5 Evaluation of biochemical reactions

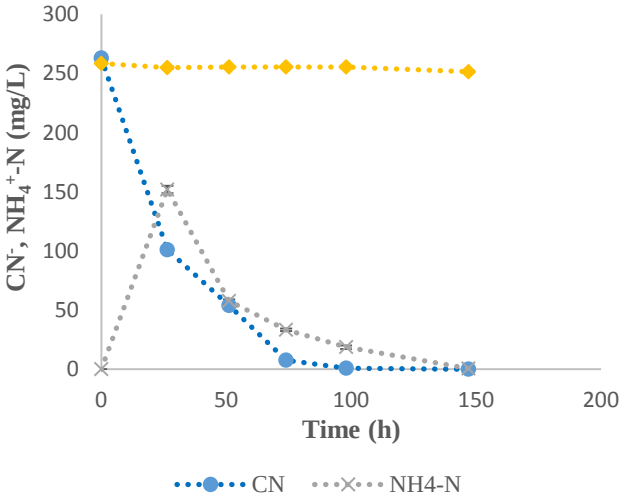
The biochemical properties of the CDO's, TDO's and a mixed culture of both CDO's and TDO's (referred to as CDO + TDO) were evaluated using the VITEK® 2 Compact system. The cells were grown for 16 to18 h at 30 °C in MM and thereafter, recovered by centrifugation at 10 000 *g* for 5 min at 4 °C. The cultures were inoculated into GN, GP, YST (Yeast or moulds) and BCL (Bacilli) colorimetric cards. Microbial suspensions were adjusted to a McFarland standard of 0.50 to 0.63 (GN and GP) and 1.8 to 2.2 (YST and BCL), in 0.5% (w/v) sodium chloride using a DensiLameter. This was done to assess the biochemical reaction changes prior and post co-culturing of the CDOs and TDOs.

6.6 Results and discussion

6.6.1 CN^- and SCN^- biodegradation experiments

In the present study, the biodegradation of CN^- by the CDO was evaluated in batch cultures, where the initial free cyanide concentration was set at 250 mg CN^-/L. The CDO completely degraded CN^-, achieving a BRE of 97.3% (see Fig 6.1a) but were unable to degrade SCN^- (see Fig. 6.2). The inability of the CDO's to degrade SCN^- is unclear, although microorganisms that possess SCN^- biodegradation potential such as *Burkholderia cepacia* (Adjei and Ohta, 1999), *Pseudomonas putida* (Chapatwala et al., 1998) and other organisms were detected within the CDO's. The biodegradation of CN^- was accompanied by the production of NH_4^+. The NH_4^+ concentration increased between 0 and 26 h to a concentration of 152 mg NH_4^+-N/L and thereafter, the concentration decreased to 0.7 mg NH_4^+-N/L. Similarly, the ability of the TDOs to degrade SCN^- was assessed under alkaline conditions and these organisms achieved a degradation efficiency exceeding 99.9% (see Fig 6.1b). In comparison to the CDO's, the TDO's were also observed to have CN^- degradation potential as they were able to degrade CN^-, achieving a BRE of 89% (see Fig 6.2). Similarly, the degradation of SCN^- resulted in the presence and accumulation of NH_4^+ and SO_4^{2-} within the medium. The NH_4^+ reached 115 mg NH_4^+-N/L after 51 h and thereafter decreased, suggesting NH_4^+ utilisation by the TDO. The SO_4^{2-} accumulated throughout the experimental run, with the maximum concentration of 90 mg SO_4^{2-}-S/L after 100 h.

A



B

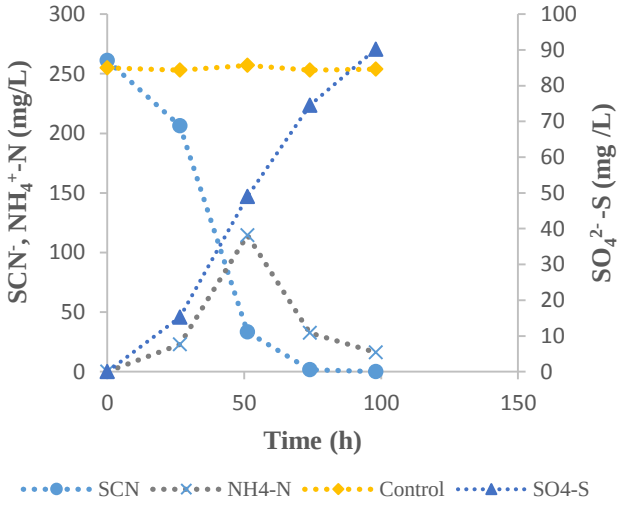


Fig. 6.1: Biodegradation of CN^- and SCN^- by (a) cyanide degrading organisms (CDO) and (b) thiocyanate degrading organisms (TDO) Error bars represent deviations

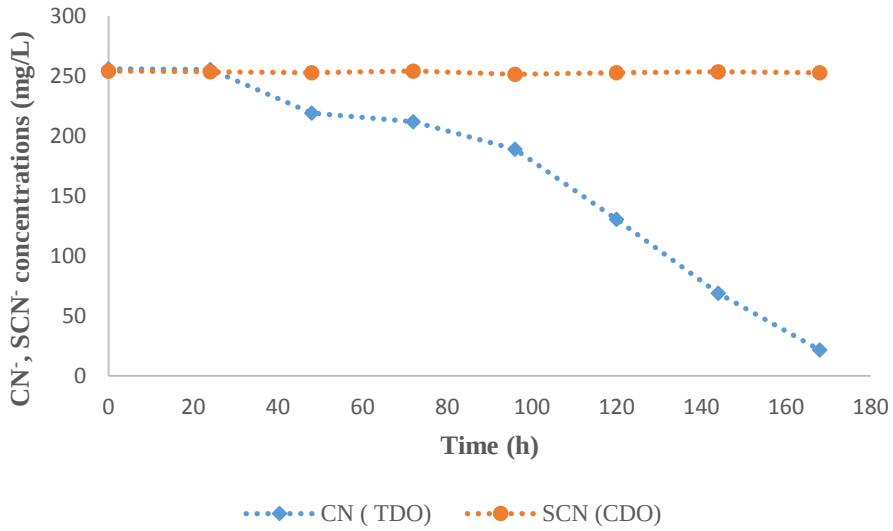


Fig. 6.2: CN^- and SCN^- degradation profiles by TDO and CDO respectively.

6.6.2 Co-metabolism of CN^- and SCN^-

The co-metabolism of CN^- and SCN^- by a mixed culture of the CDO's and TDO's was assessed in batch cultures, under alkaline conditions. This was done to evaluate the relationship between the utilised microbial communities, where complete degradation of CN^- and SCN^- was achieved within 170 h (see Fig 6.3). The degradation of CN^- and SCN^- occurred simultaneously, demonstrating the importance of co-culturing for effective treatment of CN^-/SCN^- wastewaters. Successful co-metabolism of high CN^- concentrations (up to 2000 mg CN^-/L) by a mixed culture of *Trichoderma* sp. and *Fusarium* sp. was achieved in a separate study (Ezzi and Lynch, 2005), while accelerated co-metabolism of SCN^- and CN^-

under alkaline conditions was achieved in the previous chapter (Chapter 5). This is one of the few studies reporting on the degradation of SCN^- under alkaline conditions. Most studies are focused mainly on the degradation of SCN^- under neutral pH conditions (van Zyl et al., 2011, Jeong and Chung, 2006). Wastewaters generated from metallurgical processes are mostly alkaline (Akcil, 2003); hence, studies undertaken in such conditions are vital. Sorokin et al. (2001) demonstrated the capacity of the *Halomonas* sp. and *Thioalkalivibrio* sp. to biodegrade under alkaline conditions and observed that these microbial species were able to degrade SCN, producing cyanate, which was subsequently utilised by the organisms. Furthermore, *Thioalkalivibrio thiocyanodenitrificans* degraded SCN^- aerobically and anaerobically under alkaline conditions, demonstrating the effectiveness of the applied microbial organisms (Sorokin et al., 2004). The degradation process was followed by the production of NH_4^+- and NO_3^-, with maximum concentrations of 246.5 mg NH_4^+-N/L and 114 mg NO_3^--N/L respectively. The produced NH_4^+ and NO_3^- were utilised by the organisms after 72 h (for NH_4^+-N) and 100 h (for NO_3^--N).

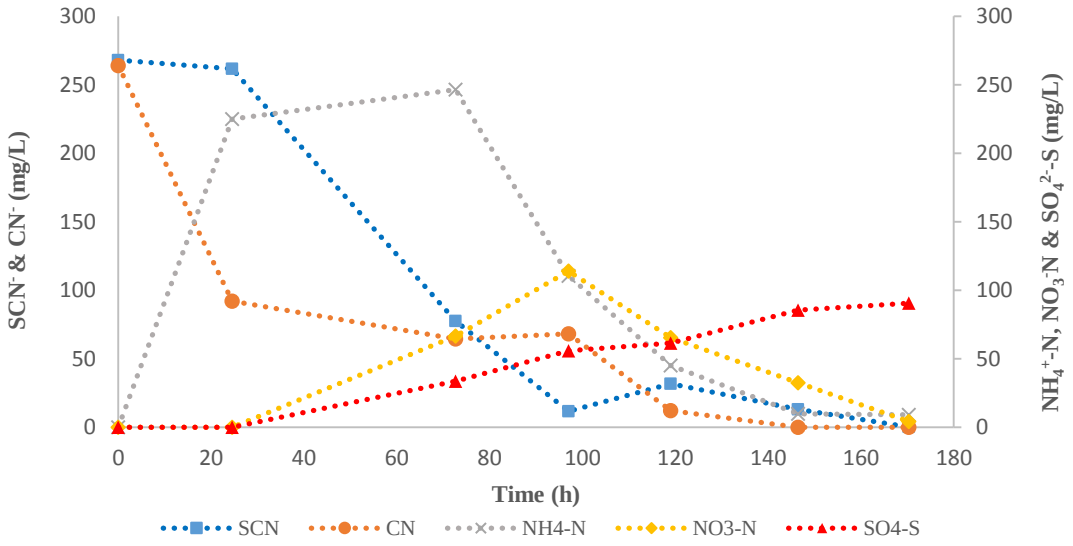


Fig. 6.3: Co-metabolism of CN^- and SCN^- by the CDO and TDO co-cultures. Error bars represent deviations

The utilisation of the produced NH_4^+ by the CDO and TDO has demonstrated the effectiveness of these organisms in the overall treatment of cyanide containing wastewaters, including its degradation of the by/end products. This phenomenon was observed in Chapter 4 where *Pseudomonas aeruginosa* STK 03 was able to conduct heterotrophic nitrification under cyanide containing conditions. Similarly, Mpongwana et al. (2016) observed heterotrophic nitrification and aerobic denitrification by *Enterobacter*, *Yersinia* and *Serratia* sp. in the presence of CN^-. Microbial species such as *Alcaligenes faecalis*, which were detected in the microbial cultures, were previously reported to possess heterotrophic nitrification and aerobic denitrification potential (Papen et al., 1989, Joo et al., 2005). In addition, denitrifying organisms such as *Alicycliphilus denitrificans* (Mechichi et al., 2003),

Thiobacillus denitrificans (Yu et al., 2015), *Pseudomonas denitrificans* (Kornaros and Lyberatos, 1998) and other organisms were detected from the CDO's and TDO's. These organisms were previously reported to possess denitrification capabilities. The ability of these organisms to conduct nitrification and denitrification aerobically and heterotrophically under cyanide-laden conditions, signifies the robustness and effectiveness of these organisms, a necessary characteristic for microorganisms that could be utilised for the treatment of cyanidation wastewater as proposed in Chapter 2.

6.6.3 Culture-dependent approach

The culturable CDO's and TDO's were isolated from cyanide and thiocyanate-laden habitats, and were identified comparatively using the Vitek® 2 Compact system and a DNA-based approach. The Vitek® 2 Compact system assigned the thirteen culturable CDO's to *Bacillus* sp. with a probability of 99% (see Table 6.1). The CDO's were further confirmed using a DNA-based approach, confirming the *Bacillus* sp. identity that the Vitek® 2 Compact system detected. Similarly, identification of the TDO's using the Vitek® 2 Compact system achieved similar results compared to the DNA-based approach (see Table 6 2). This observation demonstrated the accuracy of the Vitek® 2 Compact system for identification of culturable organisms which are available on the system's database. This observation was confirmed in a separate study, where an organism with an atypical phenotype (mucous morphology) was identified successfully via the DNA-based approach and Vitek® 2 Compact system as *Escherichia coli* whereas the MALDI-TOF technique characterised the organism as *Citrobacter* sp. (Książczyk et al., 2016). Furthermore, the Vitek® 2 system also allows for the rapid detection of antibiotic or antimicrobial resistance, especially from pathogenic organisms (Barry et al., 2003); an important microbiological characteristic. Due to the limitations associated with phenotypic techniques, some of the isolated organisms were not accurately identified by the Vitek® 2 Compact system i.e. sample 2, 4, 7, 10, 11, 12, 14 & 16. The inaccuracy in the detection of the afore-mentioned organisms might be due the limitations on the Vitek® 2 Compact system database and the similarities of the biochemical properties of organisms, thus resulting in inaccurate diagnosis.

Table 6.1: Identification of the CDO using the Vitek® 2 Compact system and PCR-based technique.

Sample	Vitek® 2 Compact	PCR	Accession number
A	*Bacillus pumilus*	*Bacillus pumilus/safensis*	-
B	*Bacillus pumilus*	*Bacillus licheniformis*	-
C	*Unidentified*	*Bacillus pumilus/safensis*	-
D	*Unidentified*	*Bacillus pumilus/safensis*	-
E	*Bacillus pumilus*	*Bacillus pumilus/safensis*	-
F	*Bacillus sp*	*Bacillus licheniformis*	-
G	*Bacillus pumilus*	*Bacillus pumilus/safensis*	-
H	*Bacillus pumilus*	*Bacillus pumilus/safensis*	
I	*Bacillus pumilus*	*Bacillus pumilus/safensis*	-
J	*Bacillus sp*	*Bacillus pumilus/safensis*	-
K	*Bacillus sp*	*Bacillus subtilis*	-
L	Unidentified	*Bacillus pumilus/safensis*	-
M	Unidentified	*Bacillus pumilus/safensis*	-

Table 6.2: Identification of the TDO using the Vitek® 2 Compact system and PCR-based technique.

Sample	Vitek® 2 Compact	PCR	Accession number
1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	KR011154
2	Unidentified organism	MS	-
3	*Bacillus pumilus*	*Alcaligenes faecalis*	KX185712
4	*Achromobacter xylosoxidans*	MS	-
5	*Providencia rettgeri*	*Providencia rettgeri*	-
6	*Klebsiella oxytoca*	*Klebsiella oxytoca*	KT767971
7	*Klebsiella 61neumonia/ oxytoca*	MS	-
8	*Klebsiella 61neumonia/ oxytoca*	*Klebsiella oxytoca*	KT825748
9	*Bacillus cereus/thuringiensis/mycoides*	*Bacillus cereus/subtilis*	FJ696633/JF706263
10	*Acinetobacter sp*	*Bacillus pumilus/safensis*	HQ003450/KM054689
11	*Raoultella ornithinolytica*	*Enterobacter sp.*	KJ806392
12	*Brucella melitensis*	MS	-
13	*Providencia rettgeri*	*Providencia vermicola/rettgeri*	KJ909024/KJ909021
14	*Sphingomonas paucimobilis*	*Pseudomonas stutzeri*	KX344913
15	*Providencia rettgeri*	*Providencia vermicola/rettgeri*	NR_042415/KC456564
16	*Sphingomonas paucimobilis/ Acinetobacter haemolyticus*	*Bacillus pumilus/safensis*	KR780583/KU921072

17	*Bacillus cereus/thuringiensis/mycoides*	*Bacillus cereus/subtilis*	DQ289055/JX188065
18	*Providencia rettgeri*	*Providencia vermicola/rettgeri*	KC456564/NR_042415

Phenotypic techniques are generally characterised by their inability to differentiate between related or fastidious organisms (Amor et al., 2007, Houpikian and Raoult, 2002). This phenomenon was observed by Schröttner et al. (2014), where the Vitek® 2 system reliably identified pathogenic species belonging to *Myroides* genus but was unable to differentiate between *Myroides odoratus* and *Myroides odoratimimus*. The database of the Vitek® 2 Compact system may lack the existence of novel organisms; hence, resulting in the mis-identification of organisms, whereas the DNA-based techniques are able to detect novel organisms that were not previously identified using constantly updated databases and search engines (Amor et al., 2007, Emerson et al., 2008, Houpikian and Raoult, 2002). However, phenotypic techniques are necessary for the preliminary identification of organisms, especially through the Vitek® 2 Compact system, which provides physiological traits of the organisms.

6.6.4 Culture-independent approach

The culturable organisms represent a minute representation (1%) of the total microbial populations (Amann et al., 1995) in a particular ecological setting. Since the identification of microbial communities via the culturable technique has been reported to misrepresent the microbial composition of a particular habitat, a culture-independent approach was undertaken to fully elucidate the microbial composition within the CDO's and TDO's, using 16S rRNA amplicon gene sequencing. This technique revealed the complexity of the microbial diversity within the CDO's and TDO's. The CDO's were dominated by bacteria (98.63%) (Fig 6.4a), with the detected organisms that belong to *Proteobacteria* (46.25%), *Bacteriodetes* (44.79%), including unidentified organisms (8.35%) (Fig 6.4b), with their related family profiles (Fig 6.4c). Similarly, the TDO's were dominated by bacteria (98.56%) (Fig 6.4d), with the detected organisms that belong to *Proteobacteria* (46.94%), *Bacteriodetes* (42.41%) and unidentified organisms (9.96%) (Fig 6.4e), with their respective families (Fig 6.4f). The structure of the CDOs was dominated by congested, slimy, rod-shaped organisms (Fig 6.5a), while the TDO's contained scattered, rod-shaped organisms (Fig 6.5b).

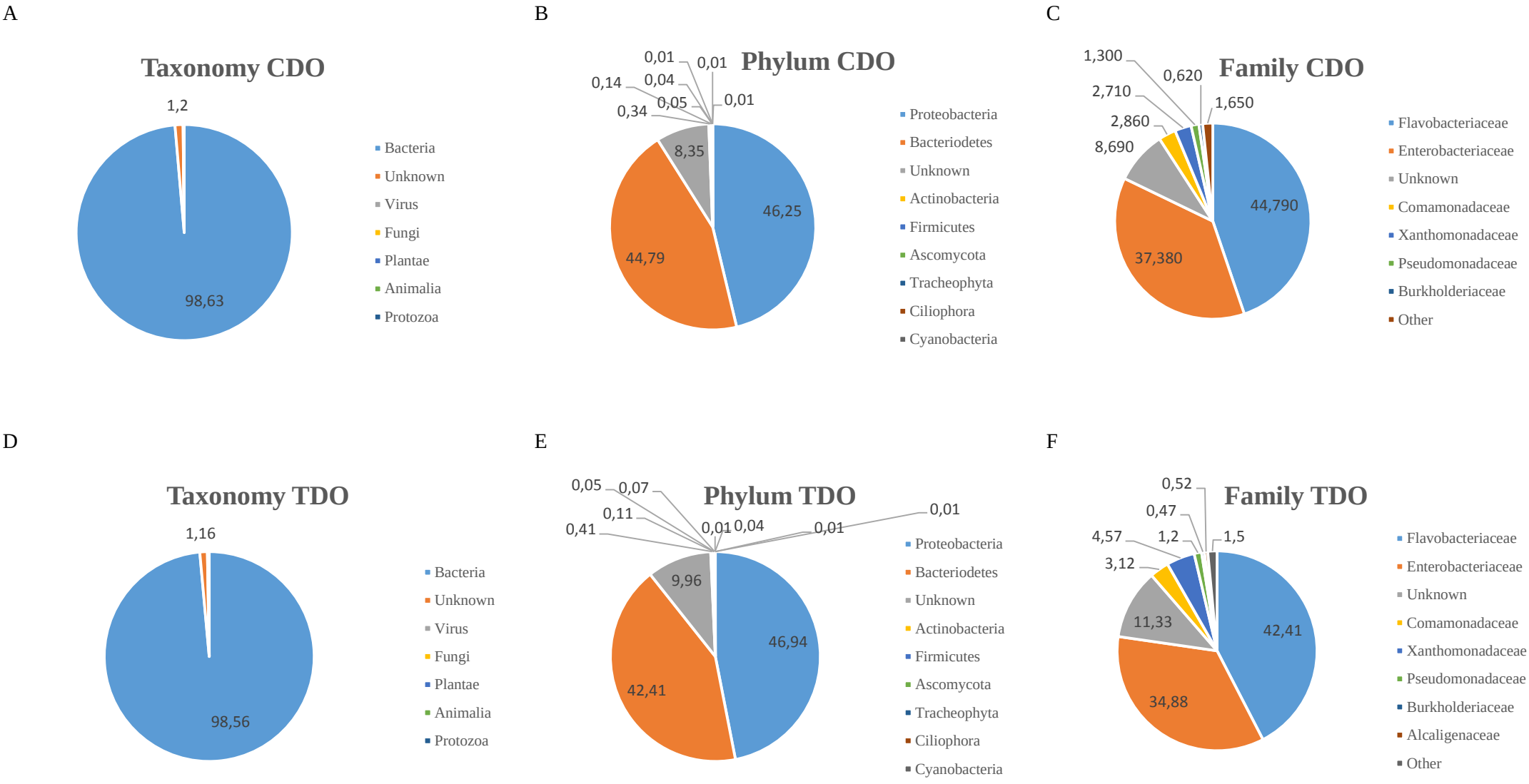
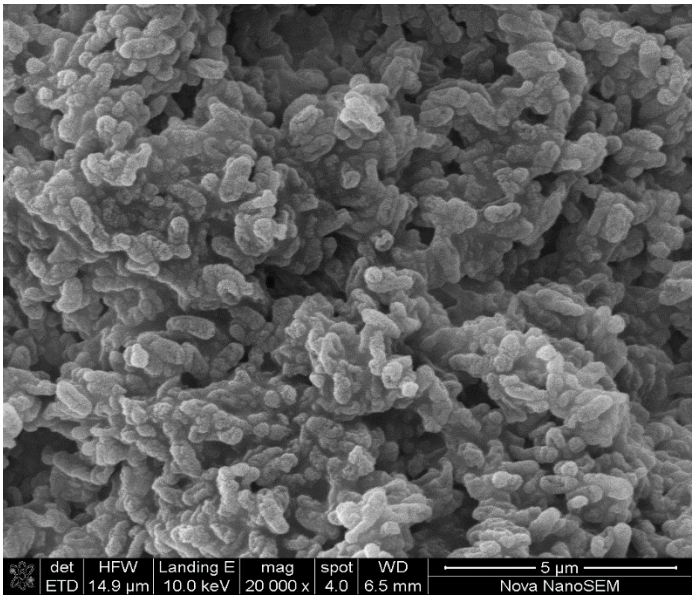


Fig. 6.4: A microbial characterization profile demonstrating, CDOs taxonomy (A), phylum (B) and family of the inoculum (C), and TDOs taxonomy (D), phylum (E) and family of the inoculum (F).

A



B

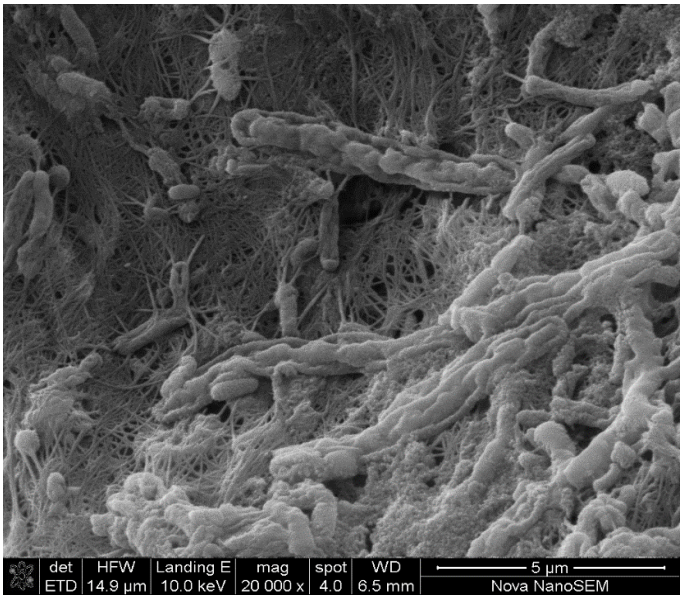


Fig. 6.5: Scanning electron microscopic images demonstrating the structure of the (a) CDO's and (b) TDO's.

The bacterial composition of the CDO's and TDO's was determined to be diverse and complex (see Appendix B Table B1 & 2) and were majorly dominated by *Myroides odoratimimus* and *Proteus* sp. at 37.82% and 30.5% for CDO's; including 35.26% and 17.58% for TDO's respectively (see Fig 6.4a & b). The dominance of these organisms within the CDO's and TDO's was hypothesised to be opportunistic since there are no reports on their ability to degrade CN^- and SCN^-. Recent research has reported on the pathogenicity of these organisms, where they were associated with urinary tract infections, meningitis, pneumonia and other diseases (Coker et al., 2000, Maraki et al., 2012, Schröttner et al., 2014). Organisms belonging to *Ralstonia, Thiobacillus, Bacillus, Pseudomonas, Klebsiella, Burkholderia* and other organisms have been reported to possess CN^- and SCN^- biodegradation capabilities were detected within the CDOs and TDOs (see Appendix B Table B1 & 2). These organisms and their biodegradative functions were evaluated in numerous studies and are summarised in Chapter 2. Recent culture-independent studies on the ASTER™ process organisms revealed the complexity and diversity of the ASTER™ process organisms (Huddy et al., 2015) compared to the culture-dependent approach which was reported earlier (van Buuren et al., 2011). The study detected the presence of *Ralstonia, Thiobacillus, Bacillus, Pseudomonas* and *Sphingomonas* sp.; organisms which detected in the current study. Kantor et al. (2015) further investigated the microbial composition and metabolic functions of the ASTER™ process organisms and demonstrated that the bulk fraction of the ASTER™ organisms were autotrophic, dominated by *Thiobacillus* species which comprised of operons which harboured functions that were not previously reported. Therefore, the detected microbial species in this

study would aid in a better understanding of the microbial composition found in CN^- and SCN^- containing wastewaters and would likely contribute to a pool of identified organisms possessing the capacity to degrade CN^- and SCN^-, which will ultimately result in improved process performance.

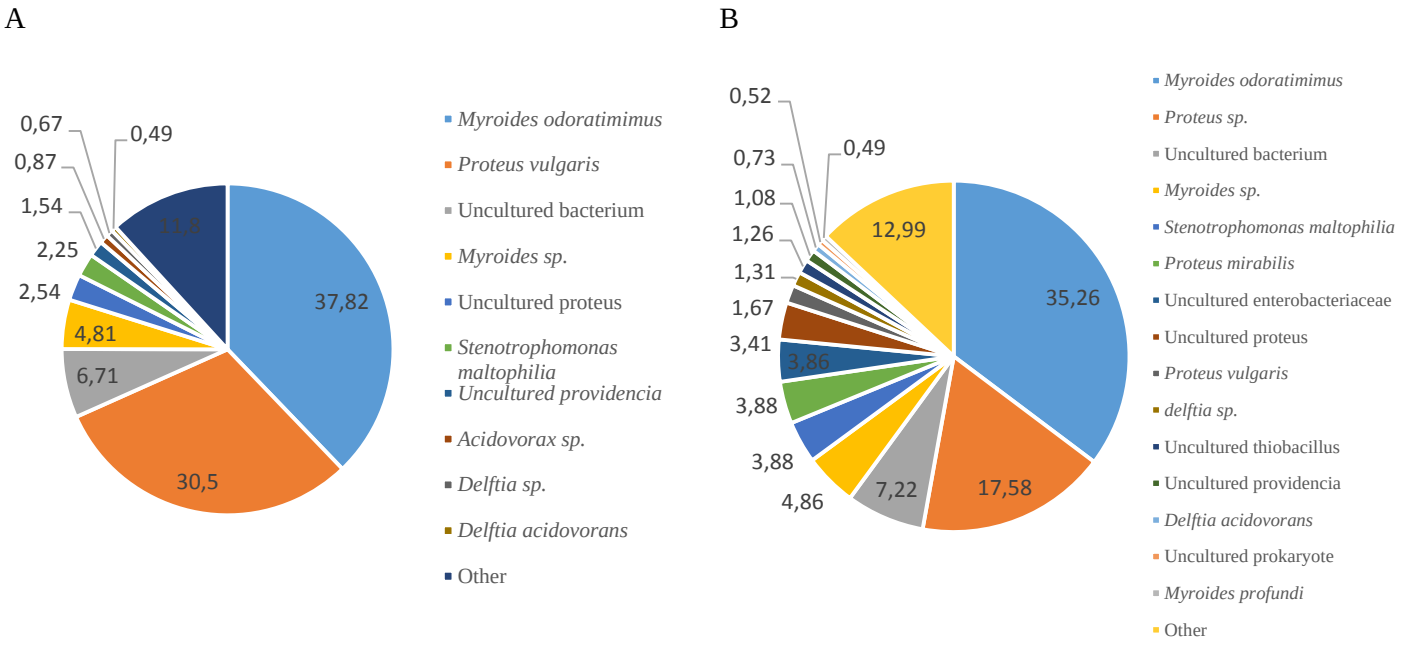


Fig. 6.6: The microbial composition profiles of the identified CDO's (A) and TDO's (B).

6.6.5 Biochemical reactions

The changes in the biochemical properties of the CDO's and TDO's prior and post co-culturing were ascertained via the Vitek® 2 Compact System. The CDO's and TDO's were inoculated on GN, GP, YST and BCL colorimetric Vitek® 2 Compact System cards, separately and as mixed cultures. The data demonstrated negative phenotypic changes with respect to α-Glucosidase and β-Xylosidase activities (Appendix B Table B4), and Amigdalin assimilation (Appendix B Table B5), Pyruvate assimilation and L-Pyrrolydonyl-arylamidase (Appendix B Table B6), after co-culturing. The metamorphoses in the biochemical properties of the consortiums when co-cultured is unclear. However, this phenomenon may be attributed mainly to competition and contaminant toxicity. Although there was an observed biochemical change in key metabolic enzymes, the simultaneous biodegradative capacity of the consortiums' co-culture increased, demonstrating the efficacy of co-culturing.

6.7 Summary

The isolated microbial communities demonstrated effective CN^- and SCN^- biodegradation capacity, achieving complete degradation efficiency from the CDO's and TDO's. Additionally, the reported research data demonstrated the biasness associated with the culture-dependent techniques, for the identification of microbial communities associated with co-metabolism of CN^- and SCN^-. This is due to

the inability of most microbial communities to grow on solid media (Amann et al., 1995), resulting in inaccurate characterization of the microbial communities. However, culture-independent techniques revealed diverse microbial communities that were majorly dominated by *Myroides odoratimimus and Proteus* sp. In addition, the biochemical properties of the CDO's and TDO's changed when these communities were co-cultured, which led to the rapid co-metabolism of CN^- and SCN^-.

The high CN^- and SCN^- biodegradation efficiencies achieved by the CDO's and TDO's resulted in the production of ammonium and nitrates. Hence, the next chapter focused on the heterotrophic nitrification and aerobic denitrification capabilities of these microbial communities under non- and cyanogenic conditions.

CHAPTER 7

RESULTS

Mekuto, L., Ntwampe, S.K.O., Kim, Y.M., Ngongang, M.M., Mudumbi, J.B.N., Dlangamandla, N. & Akinepelu, E.A. Heterotrophic nitrification-aerobic denitrification potential of cyanide and thiocyanate degrading microbial communities under cyanogenic conditions. Submitted to the *Applied Water Science* (Manuscript ID: AWSC-D-17-00075)

Heterotrophic nitrification-aerobic denitrification potential of cyanide and thiocyanate degrading microbial communities under cyanogenic conditions

7.1 Introduction

Biological nitrogen removal (BNR) is commonly utilised for the effective removal of nitrogenous compounds in wastewater. Traditional BNR systems consist of two steps: nitrification by autotrophs under aerobic conditions, followed by denitrification by heterotrophs under anoxic conditions (Yao et al., 2013). However, this system is associated with the following shortcomings: (i) the nitrification step is slow due to the low growth rate of autotrophs; (ii) autotrophs are vulnerable to high concentrations of ammonium and organic matter; and (iii) nitrification and denitrification reactors are separated due to the aerobic and anaerobic nature of the organisms employed in nitrification and denitrification, respectively (Joo et al., 2005, Chen and Ni, 2011). However, recently, heterotrophic organisms capable of nitrification and denitrification under aerobic conditions have been reported. Novel bacterial species such as *Bacillus methylotrophicus* L7 (Zhang et al., 2012), *Pseudomonas stutzeri* YZN-001 (Zhang et al., 2011), *Rhodococcus* sp. CPZ24 (Chen et al., 2012), *Alcaligenes faecalis* C16 (Chen et al., 2012) and other species have been reported to undergo heterotrophic nitrification and aerobic denitrification. These systems are associated with high nitrogen removal efficiencies due to the high growth rate of the microorganisms used in such systems. Utilising these microorganisms, in comparison to traditional autotrophic nitrifying and anoxic denitrifying organisms, has the following advantages: (i) Heterotrophs grow rapidly, therefore microbial recycling is not required, (ii) Nitrification and denitrification can be achieved simultaneously, (iii) Minimal acclimation problems are observed, (iv) There is no need for pH-control, as the acidity that is generated during heterotrophic nitrification would be offset by the alkalinity produced during aerobic denitrification, (v) Heterotrophic nitrifiers are tolerant to low temperatures as compared to autotrophic organisms (Yao et al., 2013, Chen and Ni, 2011, Liu et al., 2015).

However, limited research has been conducted to evaluate the susceptibility of heterotrophic nitrifiers and aerobic denitrifiers to CN^- and SCN^- compounds in relation to the overall treatment of cyanide species found in cyanidation wastewater. A similar system where the presence of CN^-, SCN^- and phenol on autotrophic nitrifying and denitrifying organisms has been reported; however such a system was related to the remediation of cokes and gasification wastewaters (Kim et al., 2008, Guo et al., 2017, Kapoor et al., 2016), which have disparate contaminants compared to cyanidation wastewater. Therefore, there is a need for the evaluation of CN^- and SCN^- degrading organisms to conduct heterotrophic nitrification and aerobic denitrification under cyanogenic conditions since the biodegradation of CN^- and SCN^- is rarely complete, especially during cold seasons. Hence, this study was aimed at evaluating the heterotrophic nitrification and aerobic denitrification propensity of CN^- and SCN degrading organisms, with the overall aim of applying the same organisms in the biodegradation

of CN^- or SCN and its related complexes – such that the resultant effluent can meet the discharge standards and/or be recycled to mineral bio/processing units.

7.2 Objectives

The objectives of this study were:

- To evaluate the capability of the CDO's and TDO's to conduct nitrification and aerobic denitrification with and without the presence of CN^- and SCN^-.
- To assess the co-culturing of the CDO's and TDO's to conduct nitrification and aerobic denitrification with and without the presence of CN^- and SCN^-.

7.3 Materials and methods

7.3.1 Heterotrophic nitrification and aerobic denitrification by CDO and TDO: Effect of CN^- and SCN^-

To assess the capacity of TDO's and CDO's to conduct heterotrophic nitrification and aerobic denitrification, the cultures were inoculated in 200 mL flasks with MM, containing an initial concentration of ammonium (as NH_4Cl) and nitrate (as $NaNO_3$) of 250 mg NH_4^+-N/L and 100 mg NO_3^--N/L, respectively. The effect of SCN^- and CN^- on nitrification and denitrification was assessed by supplementing the required concentrations of SCN^- (25 and 50 mg SCN^-/L) (as KSCN) and CN^- (5, 10 and 15 mg CN^-/L) (as NaCN) (Merck, Germany). The dual effect of both SCN^- and CN^- was also assessed at 25 mg SCN^-/L, while CN^- was varied as stated previously. The effect of CN^- and SCN^- on CN^- and SCN^- degraders was evaluated separately and in co-cultures. Oxygen was not pumped into the media, thus the organisms relied solely on the dissolved oxygen in the wastewater, which was equivalent to ~5 mg/L (BANTE820 portable dissolved oxygen meter, BANTE instruments, China). The pH of the media was set at 9.9 and temperature at 30ºC for both nitrification and denitrification studies. These chosen conditions were based on (i) the fact that cyanide containing wastewaters are mostly alkaline (Santos et al., 2013, Akcil and Mudder, 2003) and (ii) previous optimization studies on cyanide degrading organisms observed these conditions as being most suitable for successful cyanide biodegradability (Mekuto et al., 2015); hence, these conditions were chosen on a pragmatic basis. The nitrification and denitrification rates were reported as averaged rates. Uninoculated flasks served as controls while the experimental error was calculated as the standard error of mean using the standard deviation obtained from a duplicate set of data (n = 2).

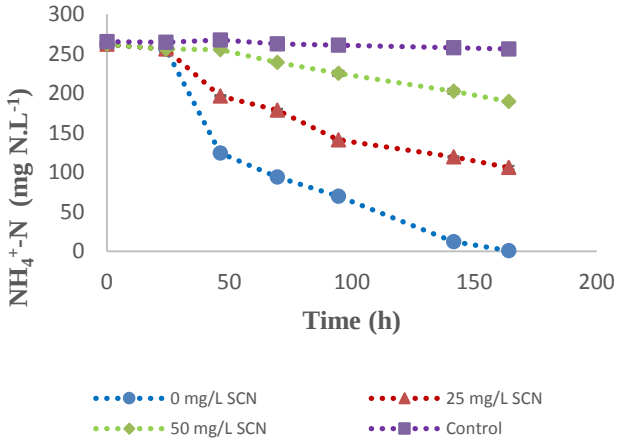
7.4 Results and discussion

7.4.1 Effect of SCN^- and CN^- on heterotrophic nitrification by TDO and CDO

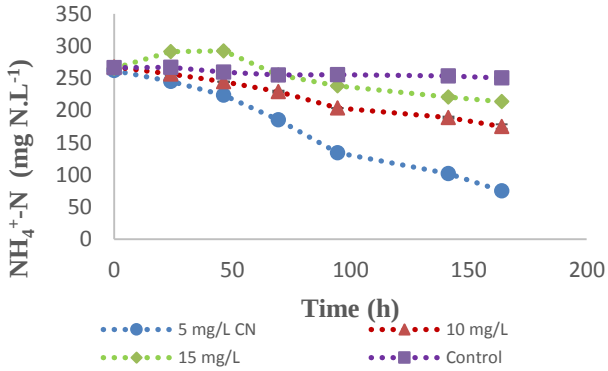
Heterotrophic nitrification under non-SCN^-containing conditions by the members of the TDO community resulted in a nitrification rate of 1.59 mg NH_4^+-N. $L^{-1}.h^{-1}$ while there was an observed inhibition on heterotrophic nitrification, as the nitrification rate decreased to 0.95 and 0.44 mg NH_4^+-N.

$L^{-1}.h^{-1}$ in media containing 25 and 50 mg SCN^-/L, respectively (Fig. 7.1a). After nitrification, SCN^- was not detected in the media (data not shown), suggesting its complete biodegradation. Nitrification rates under CN^- concentration of 5, 10 and 15 mg CN^-/L were observed to be 1.14, 0.57 and 0.32 NH_4^+-N. $L^{-1}.h^{-1}$, respectively (Fig. 7.1b). This demonstrated the acute toxicity and inhibition of nitrification by CN^-. This phenomena was observed elsewhere (Neufeld et al., 1986), where 0.11 mg CN^-/L was suggested to be an accepted concentration for nitrification to transpire. The presence of both CN^- (varying concentration) and SCN^- (25 mg SCN^-/L) proved to be detrimental on nitrification by TDO's as the nitrification rates decreased drastically. The observed nitrification rates were 1.0, 0.51 and 0.301 NH_4^+-N. $L^{-1}.h^{-1}$ from the initial concentrations of 5, 10 and 15 mg CN^-/L while the SCN concentration was kept at 25 mg SCN^-/L, respectively (Fig. 7.1c).

A



B



C

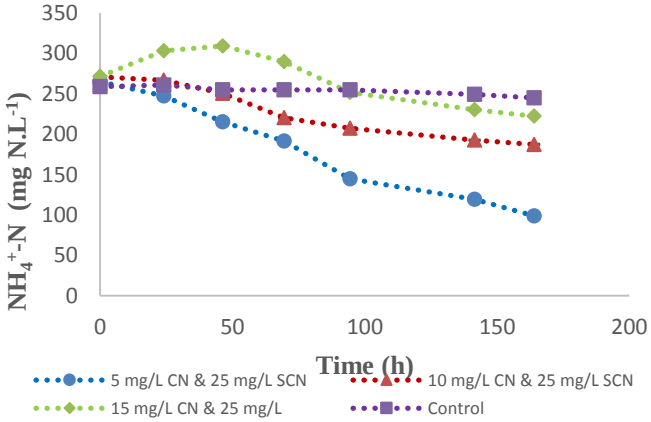


Fig. 7.1: Heterotrophic nitrification profiles by TDO under, (a) varying SCN^- concentration, (b) varying CN^- concentrations and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

The utilisation of NH_4^+ by the TDO's resulted in the production of both NO_3^- and NO_2^- within the media. The maximum concentration of NO_3^- produced from the media supplemented with 0, 25, 50 mg SCN^-/L and 5, 10, 15 mg CN^-/L was 136, 44, 1.65 (Fig 7.2a) and 68, 32.4 and 24.7 mg NO_3^--N/L respectively (Fig. 7.2b). Similarly, the maximum NO_3^- concentration from the dual impact of SCN^- and CN^- was

46.5, 37 and 15 mg NO_3^--N/L respectively (Fig. 7.2c). The NO_2^- concentration in all the tested media was less than 20 mg NO_2^--N/L. In this study, it was observed that the NO_3^- formation rate from NH_4^+ oxidation was not in a 1:1 ratio as defined by Equation 2.15 and 2.16. This was due to the consortia utilising the NH_4^+ as a nitrogen source; hence, a portion of the NH_4^+ was used intracellularly by the employed microbial organisms, resulting in low NO_3^- detection in the media. This was also observed when *Alcaligenes faecalis* No.4 heterotrophically oxidised NH_4^+, resulting in lower detection of NO_3^- within the media (Joo et al., 2005). The authors observed that 50% of the NO_3^- was utilised intracellularly by *A. faecalis* No.4 and almost 39 to 48% was denitrified simultaneously and/or immediately after formation. It is imperative to note that the percentage of NH_4^+ that is utilised intracellularly varies between species and strains. Additionally, heterotrophic nitrifying organisms have been observed to have the ability to simultaneously nitrify and denitrify aerobically in the same media (Robertson et al., 1988, Gupta, 1997, Chen et al., 2012). Therefore, the low detection of NO_3^- in the media can be attributed to its assimilation by the microbial species; an observation which was witnessed in Pseudomonas stutzeri T13 (Sun et al., 2017).

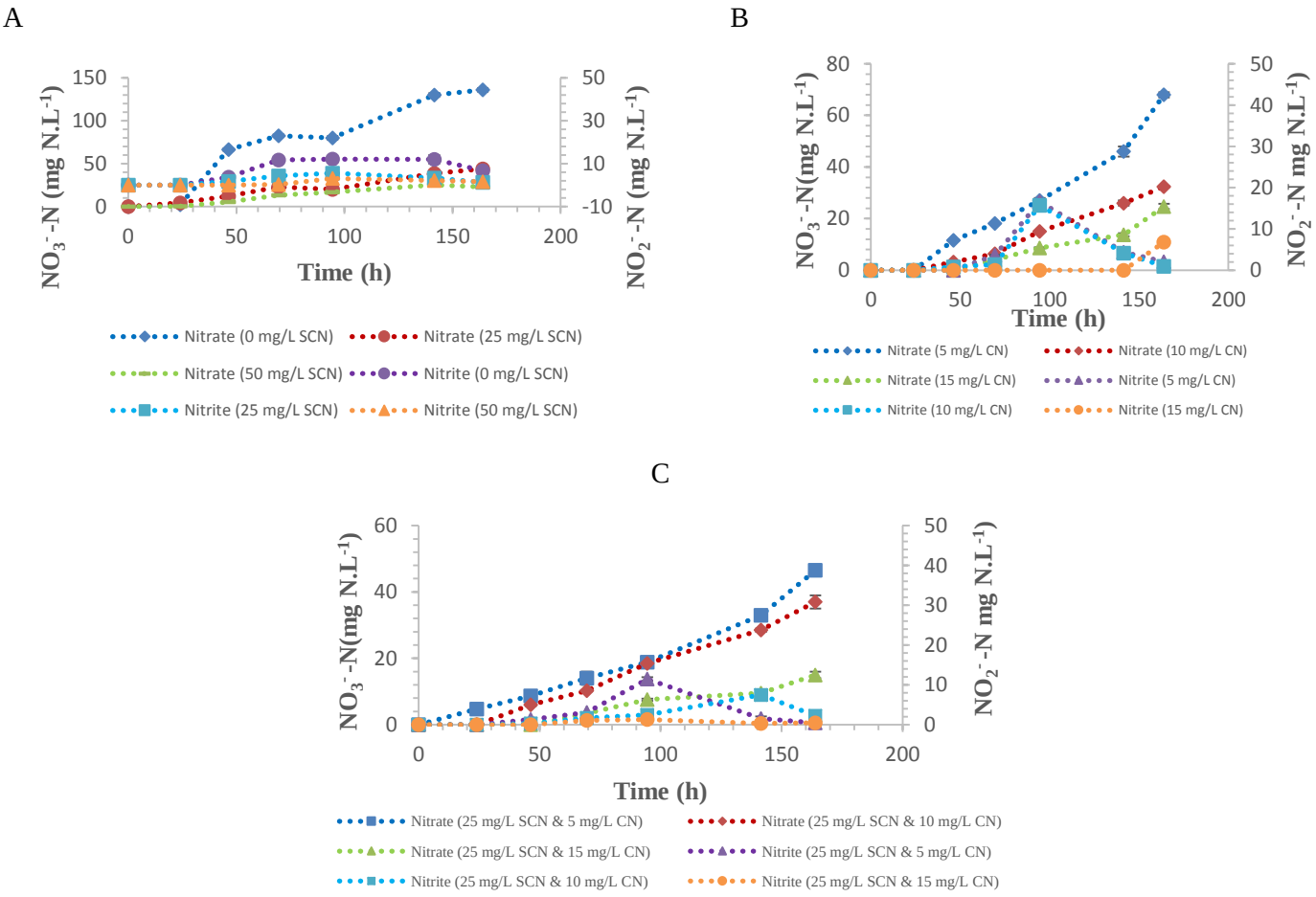


Fig. 7.2: NO_3^- and NO_2^- formation profiles from heterotrophic nitrification by TDO under, (a) varying SCN^- concentration, (b) varying CN^- concentration and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

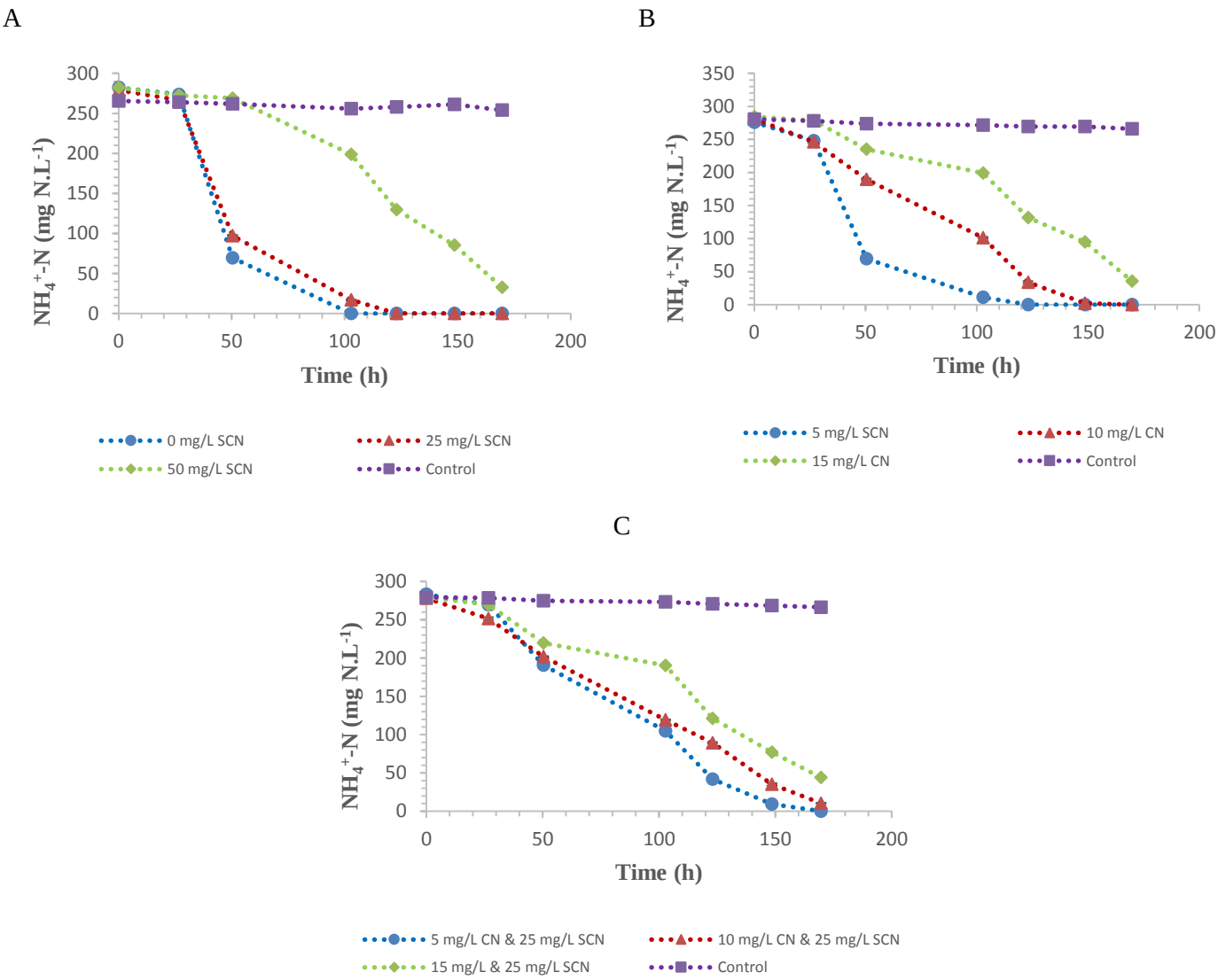


Fig. 7.3: Heterotrophic nitrification profiles by CDO under, (a) varying SCN^- concentration, (b) varying CN^- concentration and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

Heterotrophic nitrification by the CDO's proved to be robust as these species were unaffected by the presence of both SCN^- and CN^-. These organisms demonstrated high nitrification rates of 1.66, 1.67 and 1.47 NH_4^+-N. $L^{-1}.h^{-1}$ in media spiked with 0, 25 and 50 mg SCN^-/L, respectively (Fig. 7.3a). Similarly, CN^- concentration did not have any inhibitory effect on CDO's as they were able to achieve nitrification rates of 1.63, 1.66 and 1.47 mg NH_4^+-N. $L^{-1}.h^{-1}$ from media spiked with 5, 10 and 15 mg CN^-/L, respectively (Fig. 7.3b). Furthermore the organisms demonstrated their effectiveness – as these species were not inhibited by the dual presence of SCN^- and CN^- achieving nitrification rates of 1.67, 1.58 and 1.39 mg NH_4^+-N. $L^{-1}.h^{-1}$ from the initial concentrations of CN^- and SCN^- of 5, 10 and 15 mg CN^-/L while the SCN concentration was kept at 25 mg SCN^-/L, respectively (Fig. 7.3c). Although there is a scarcity of information on alkaline nitrification, it has been observed that traditional nitrifying organisms are unable to perform nitrification efficiently under highly alkaline conditions (Ruiz et al., 2003, Strous et

al., 1997) with nitrification efficiency being at pH ranges of 7.0 to 8.5 (Koren et al., 2000). This study has shown that both the TDO's and CDO's were able to nitrify under alkaline conditions. The maximum concentrations of NO_3^- produced from the media supplemented with 0, 25, 50 mg SCN^-/L and 5, 10, 15 mg CN^-/L were 135, 126.5, 107.7 (Fig 7.4a) and 115.9, 111.2 and 88.7 mg NO_3^--N/L, respectively (Fig. 7.4b). Similarly, the maximum NO_3^- concentrations in culture containing both SCN and CN^- were100.95, 98.3 and 68.9 mg NO_3^--N/L (Fig. 7.4c). The NO_2^- concentration in all the tested media was less than 20 mg NO_2^--N/L. These organisms, i.e. CDO's, were also observed to carry out nitrification and aerobic denitrification in a continuous CN^- biodegradation process elsewhere (Mekuto et al., 2015), demonstrating their effectiveness as cyanide degraders, nitrifiers and denitrifiers.

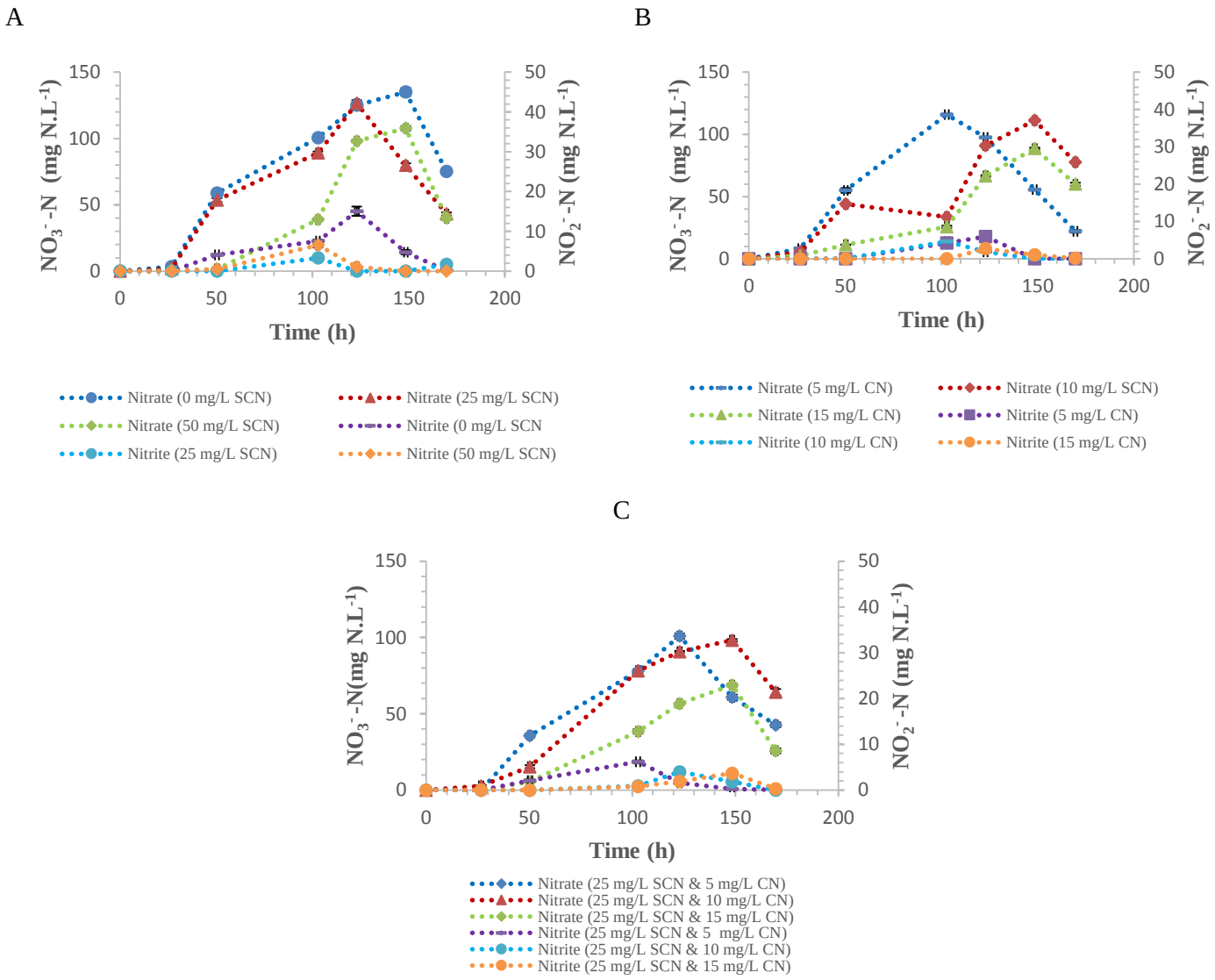


Fig. 7.4: Nitrate and nitrite formation profiles from heterotrophic nitrification by CDO under, (a) varying SCN^- concentration, (b) varying CN^- concentration and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

7.4.2 Effect of SCN^- and CN^- on aerobic denitrification by TDO and CDO

The aerobic denitrification potential of the TDO's and CDO's was evaluated under cyanogenic conditions, from an initial NO_3^- concentration of 100 mg NO_3^--N/L. The TDO demonstrated the ability to denitrify aerobically as these species were able to achieve a complete denitrification rate of 0.66 mg NO_3^--$N.L^{-1}.h^{-1}$ in media supplemented with 0, 25, 50 mg SCN^-/L, respectively (Fig. 7.5a) with minimal detection of NO_2^- within the media (≤0.04 mg NO_2^--N/L). Similarly, 5 mg CN^-/L did not inhibit aerobic denitrification by these species since the denitrification rate exceeded 0.64 mg NO_3^--$N.L^{-1}.h^{-1}$. However, denitrification was inhibited by the presence of 10 and 15 mg CN^-/L, resulting in denitrification rates of 0.46 and 0.22 mg NO_3^--$N.L^{-1}.h^{-1}$, respectively (Fig 7.5b). Additionally, the dual effect of CN^- and SCN^- proved to be detrimental, especially when the media was spiked with 25 mg SCN^-/L and 10 mg CN^-/L, as well as 25 mg SCN^-/L and 15 mg CN^-/L, achieving denitrification rates of 0.39 and 0.11 mg NO_3^--$N.L^{-1}.h^{-1}$, respectively, while denitrification was unaffected by the presence of 25 mg SCN^-/L and 5 mg CN^-/L (Fig. 7.5c). The denitrification trends illustrated in Fig. 7.5c were similar to those observed in Fig. 7.5b, which demonstrated the inhibitory effect that CN^- had on the denitrification by the TDO's.

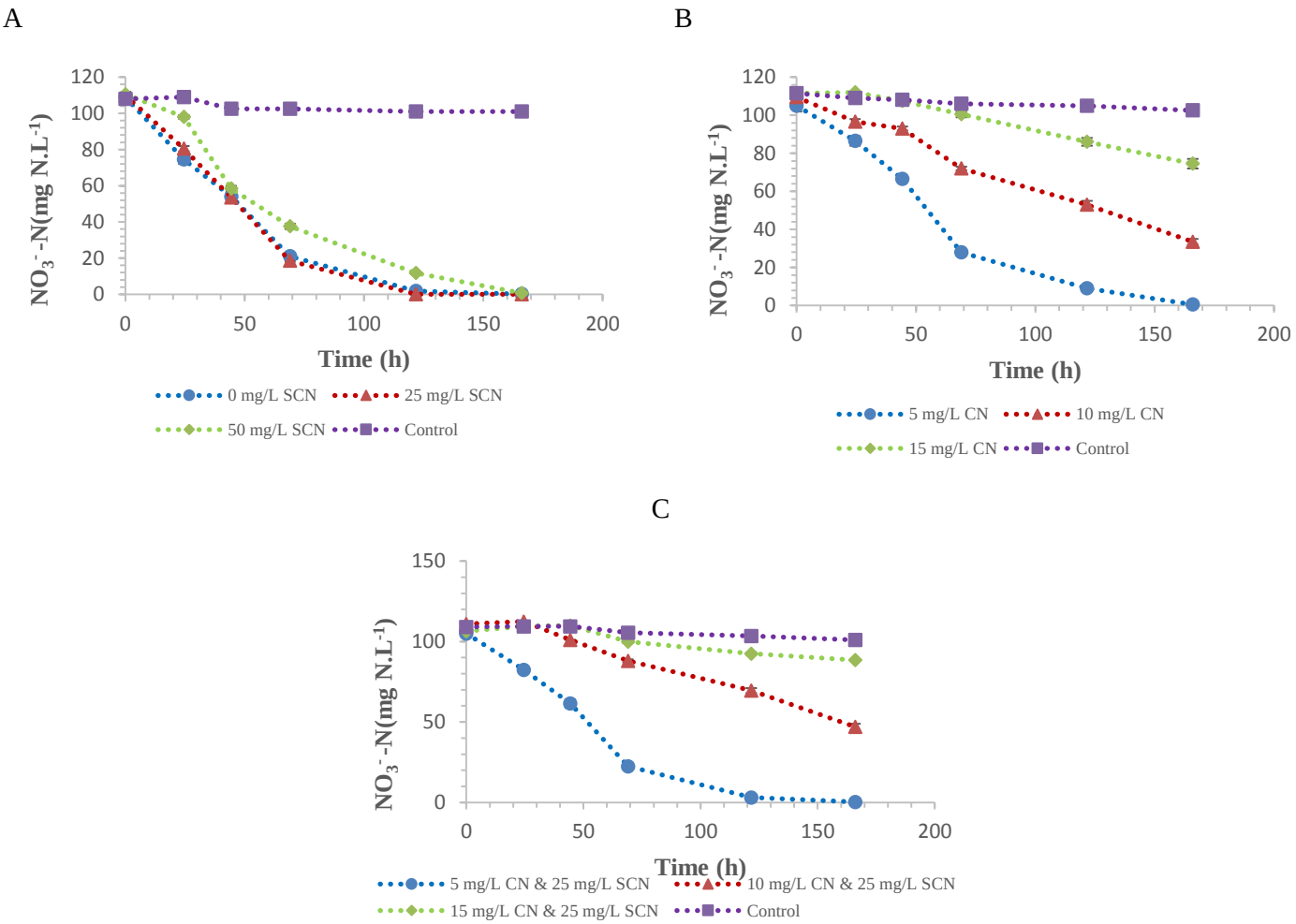


Fig. 7.5: Aerobic denitrification profiles by TDO under (a) varying SCN^- concentration, (b) varying CN^- concentration and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

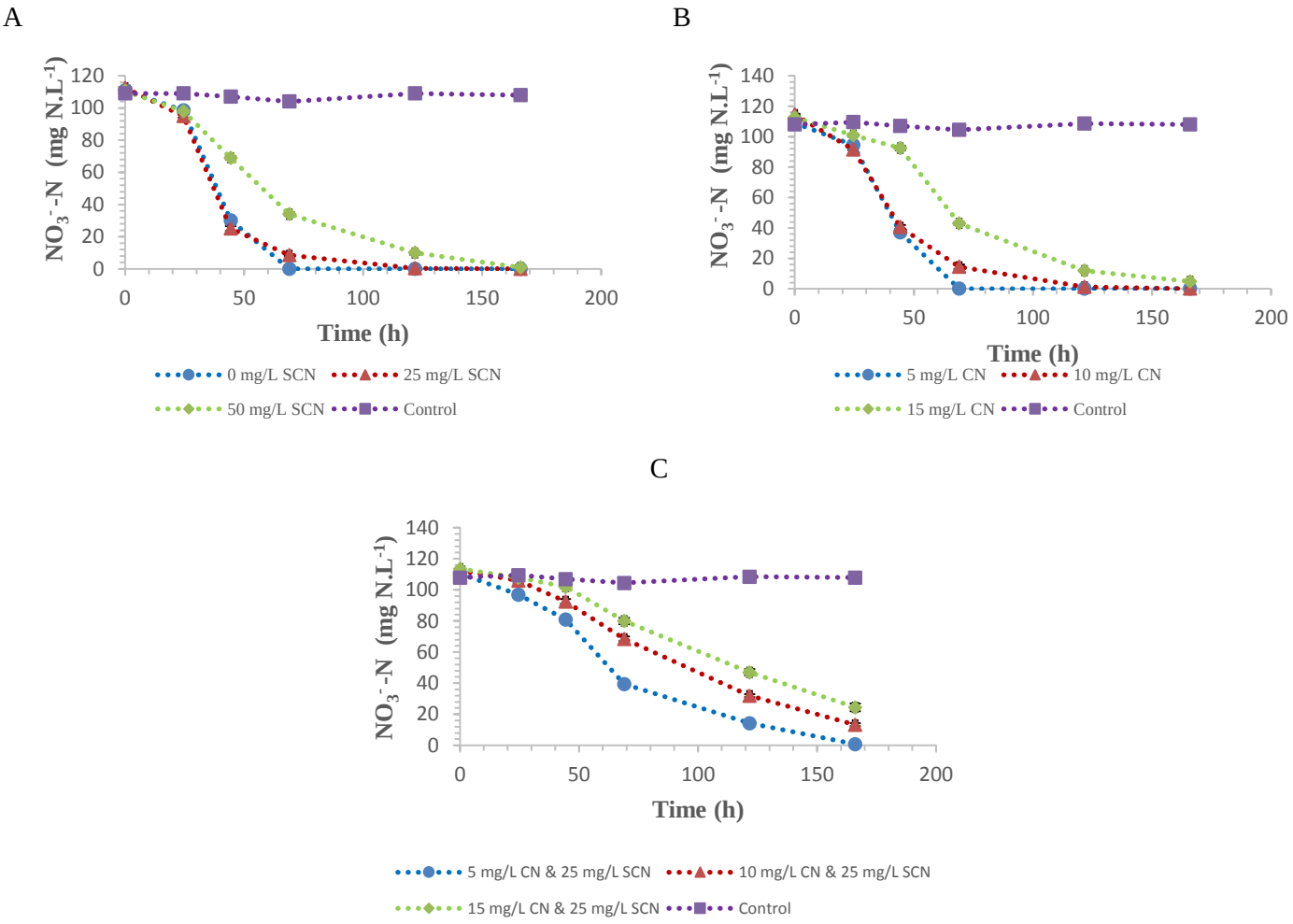


Fig. 7.6: Aerobic denitrification profiles by TDO under (a) varying SCN^- concentration, (b) varying CN^- concentration and (c) a combination of SCN^- and CN^-. Error bars represent deviations.

However, the CDO's demonstrated a high denitrification potential, as these species were not affected by the presence of SCN^-, CN^- or the dual effect of SCN^- and CN^-. The denitrification rates were in excess of 0.67 mg NO_3^--N.L^{-1}.h^{-1} irrespective of toxicant concentration (Fig. 7.6a & b). Aerobic denitrification was slightly inhibited when the media was spiked with 25 mg SCN^-/L and 10 mg CN^-/L, and 25 mg SCN^-/L and 15 mg CN^-/L, achieving denitrification rates of 0.6 and 0.54 mg NO_3^--N.L^{-1}.h^{-1} (Fig. 7.6c). Aerobic denitrification has been previously reported to produce nitrous oxide as an intermediate prior to the formation of nitrogen. The enzymology involved in this process was explained elsewhere (Robertson and Kuenen, 1984). However, the production of nitrous oxide from aerobic denitrification systems has been largely investigated in axenic cultures (Chen et al., 2003, Takaya et al., 2003), which is not representative of microbial populations. The description of pathways and the enzymology associated with the production of nitrous oxide from aerobic denitrification systems in microbial populations has been reported to be challenging, since individual organisms within the population utilise different biochemical pathways for the utilisation of nitrates (Ishii et al., 2014).

7.4.3 Simultaneous heterotrophic nitrification and aerobic denitrification by a mixed culture of TDO and CDO

CDO's were co-cultured with the TDO's and evaluated for simultaneous nitrification and aerobic denitrification in the presence of 25 mg SCN^-/L and 15 mg CN^-/L. The co-culture was able to simultaneously nitrify and aerobically denitrify in the presence of SCN^- and CN^-, achieving nitrification and denitrification efficiencies of 100% (1.83 mg NH_4^+-N/L) (Fig. 7.7). There was an observed nitrate nitrogen increase to 104 mg NO_3^--N/L between 20 h to 100 h; thereafter, the nitrate nitrogen concentration decreased.

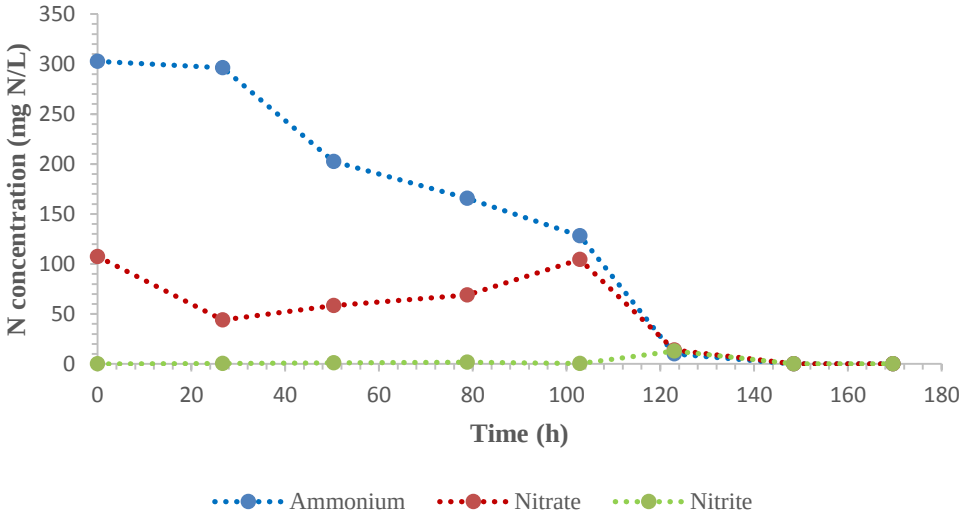


Fig. 7.7: Graphical representation for simultaneous nitrification and denitrification by a mixed cultured of TDO and CDO's. Error bars represent deviations.

Simultaneous nitrification and aerobic denitrification has been reported from various studies (Zhang et al., 2011, He et al., 2016). However, these studies were undertaken under SCN^- and CN^--free systems. Nitrification under CN^--laden conditions was investigated using activated sludge obtained from a coking wastewater treatment plant. In this study, nitrification was inhibited when the media was supplemented with 0.2 mg CN^-/L (Kim et al., 2008), with SCN^- having a minimal inhibitory effect on nitrification. The high CN^- tolerance is due to the differences in composition, strength and overall quality of the microbial consortia used (Kim et al., 2011c). This study demonstrated the robustness of heterotrophic nitrification and aerobic denitrification under CN^- and SCN^- laden conditions, when subjected to a mixed culture of TDO's and CDO's.

7.5 Summary

Heterotrophic nitrification and aerobic denitrification under SCN^- and CN^--laden conditions by thiocyanate and free cyanide degrading organisms were successfully demonstrated, particularly in co-cultures. CN^-(≥10 mg CN^-/L) inhibited nitrification and denitrification in media inoculated with

thiocyanate degrading organisms, while the cyanide degrading organisms were not inhibited by SCN^- nor CN^-, irrespective of the concentration load. Additionally, the presence of both CN^- (≥10 mg CN^-/L) and SCN^- (25 mg SCN^-/L) inhibited nitrification and aerobic denitrification by the thiocyanate degraders while the cyanide degraders were not inhibited. Mixed culture containing both SCN^- and CN^- degraders was able to nitrify and aerobically denitrify without being inhibited by the presence of both SCN^- and CN^-, irrespective of the toxicant concentration. This demonstrated the robustness and effectiveness of these organisms to carry out heterotrophic nitrification and aerobic denitrification under cyanogenic conditions – a process which can be applied to the nitrification and denitrification of ammonium nitrogen and nitrates formed from CN^- and SCN^- biodegradation systems. The influx of these toxicants into secondary processes (nitrification and denitrification) will not have an inhibitory effect on nitrification and denitrification since these organisms are able to degrade, nitrify and denitrify.

Following the milestones achieved from the previous chapters and in this chapter, the efficacy of the isolated microorganisms needed to be evaluated under varying physicochemical parameters in a continuous system, and this information is covered in the next chapter.

CHAPTER 8

RESULTS

Mekuto, L., Ntwampe, S.K.O., Utomi, C.E., Mobo, M., Mudumbi, J.B.N. & Ngongang, M.M. Biodegradation of thiocyanate and free cyanide in a dual stage continuous stirred tank bioreactor. *Journal of Environmental Chemical Engineering* 5: 1936-1945 DOI: 10.1016/j.jece.2017.03.038

Biodegradation of thiocyanate and free cyanide in a dual stage continuous stirred tank bioreactor

8.1 Introduction

Cyanide is produced naturally by a variety of micro- and/or macrobiotic species in minute concentrations as compared to the cyanide wastes which are largely generated from the mining industry (Luque-Almagro et al., 2005a). The utilization of CN^- in the mining industry results in a variety of chemical reactions with gold ore constituents, forming chemical complexes which vary widely in their stability and solubility (Adams, 2013). The major chemical constituents resulting from the cyanidation process are CN^-, SCN^- and metal-complexed cyanides, as weak acid (Cu, Cd, Ni, Zn, etc.) and strong acid dissociable cyanides (Au, Ag, Fe, Co, etc.) (Mudder and Botz, 2004, Johnson, 2015). The prevalence and co-existence of these chemical compounds is harmful to both the environment and living organisms. This effect has been observed elsewhere, where artisanal small scale gold mining (ASGM) operations in West African countries such as Burkina Faso, where the effluents of such mining activities directly affected the lives of living organisms, including humans, that reside in close proximity to these ASGMs'. Such activities have contributed immensely to environmental destruction (Razanamahandry et al., 2016, Carling et al., 2013).

The co-existence of SCN^- and CN^-, as the major pollutants, can result in the contamination of the receiving water bodies, which in-turn reduces the availability of potable and usable water reserves (Akcil and Mudder, 2003). Therefore, this necessitates the development of robust and environmentally benign processes that would reduce the CN^- and SCN^- concentrations to acceptable levels which are suitable for discharge, without hampering ecological settings. The development of such methods has been enforced by the International Cyanide Management Code (ICMC), a code which was established by the International Cyanide Management Institute (ICMI) (www.cyanide-code.org) (Akcil, 2010). Biological destruction of CN^- and SCN^- in wastewaters has gained popularity due to the robustness, environmentally friendliness and economic viability of the process. In this process, microorganisms are employed to degrade cyanide compounds via a series of enzymatic reactions which have been widely documented (Ebbs, 2004, Gupta et al., 2010, Dash et al., 2009). Industrial processes such as the ASTER™ process and the operations at the Homestake and LaRonde gold mines in Canada (Villemeur et al., 2015), have demonstrated the feasibility and robustness of a biological process for treating cyanide-laden wastewaters. In most cases, the biodegradation process produces bicarbonate alkalinity which neutralises the acidity that is produced from the biodegradation process. The involved chemical reactions have been previously discussed in Section 2.6.

However, recent research has mainly focused on the utilisation of pure microbial cultures that are mainly operated in batch systems (Huertas et al., 2010, Kao et al., 2003, Karamba et al., 2016). Such studies provide fundamental research as they seek to understand the fundamental roles that pure cultures play in the biodegradation process, with a purpose of constructing a high-strength microbial community that

would effectively degrade these compounds. Numerous studies have focused on the biodegradation of CN^- and SCN^- separately and in batch cultures, with limited reports on the co-metabolism of these contaminants in continuous systems.

8.2 Objectives

The objectives of this study were to:

- Evaluate the capability of the CDO's and TDO's as mixed microbial enrichments for the biodegradation of SCN^- and CN^- in a dual-stage continuously stirred tank bioreactor system.
- Evaluate the impact of SCN^- and CN^- concentration escalations on the performance of the bioreactor system.
- Examine the microbial activity of the mixed cultures with respect to changes in physicochemical conditions.

8.3 Materials and methods

8.3.1 Microbial culture

For the continuous biodegradation of SCN^- and CN^-, the TDO's and CDO's were mixed at a 1:1 ratio and the total DNA of the mixed microbial cultures was extracted, followed by PCR amplification of the 16S rDNA gene as described in Chapter 3. The DNA was sequenced as described in Chapter 3, section 3.2.3. The microbial community structure analysis of the mixed culture was analysed as described in Chapter 3.

8.3.2 Experimental design

The bioreactors, with a 1L working volume, were arranged in a dual-stage mode, using the New Brunswick BioFlo 110 reactors (New Brunswick Scientific Co., INC, New York, USA), which were operated in series (denoted as R1 and R2) (see Fig 9.1). The effluent from R1 was recycled back to R1 relative to the set hydraulic retention time (HRT). Hence, R1 operated at half of the set HRT. Mixing was achieved using overhead stirrers driving Rushton impellers, for continuous mixing at 250 rpm. Air was introduced from the bottom of the reactors at a flow rate of 400 mL/min and the reactors were operated at room temperature (21 °C to 25 °C) with no temperature control. The feed solution contained CN^- (200 – 450 mg CN^-/L), SCN^- (100 – 1000 mg SCN^-/L), phosphate sources (as 3.4 g/L KH_2PO_4 and 4.3 g/L K_2HPO_4), glucose (0.1 g/L) and magnesium (as 0.6 g/L $MgCl_2.6H_2O$). The growth media was pumped into the reactor system using a peristaltic pump at different feed flow rates, depending on the desired hydraulic retention time. In this system, nutrient recycling to R1 was evident as demonstrated in Fig. 8.1.

During the start-up period, the initial pH of the feed solution was not controlled and it ranged from 6.9 to 7.1. The reactors were inoculated with a seed culture at a concentration of 10 % (v/v) (equivalent to 100 mL of culture in 900 mL of media), in media containing 150 mg SCN^-/L only.

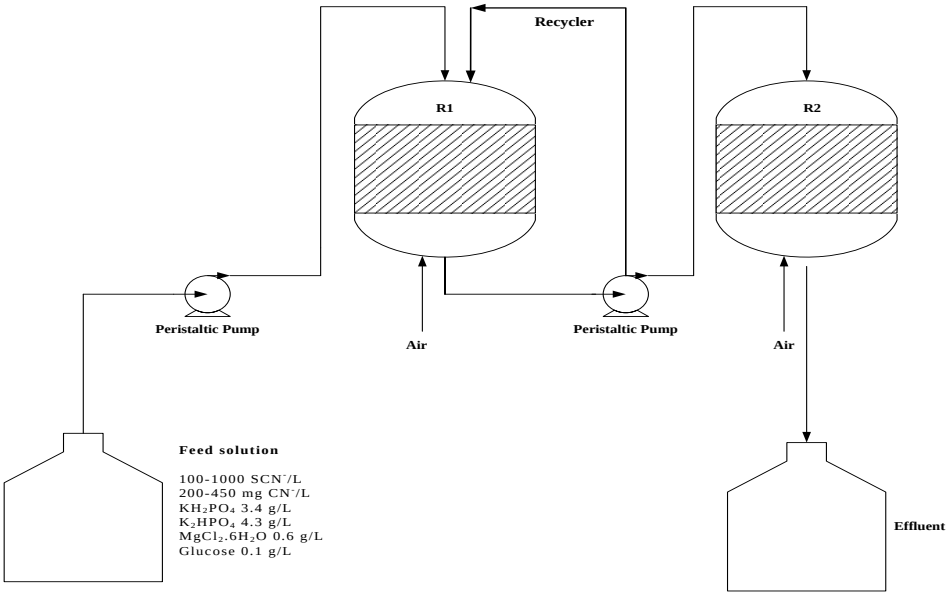


Fig. 8.1: A schematic demonstration of the dual stage continuous stirred tank reactor system.

The reactors were initially ran in batch mode until complete degradation of SCN^- (after 10 days) and thereafter, the system was allowed to stabilise for an additional 5 days to allow the consortium to utilise the produced total nitrogen prior to the bioreactors being operated in a continuous mode where the hydraulic residence time (HRT) (from 7 day - 1 day), SCN^- concentration (100 – 1000 mg SCN^-/L) and CN^- concentrations (200 – 450 mg CN^-/L) were varied.

The system was switched to continuous mode on day 15 and operated at a 7 day HRT at a feed SCN^- concentration of 100 mg SCN^-/L. On day 82, the HRT was changed to 5 days with constant feed SCN^- concentration of 100 mg SCN^-/L. On day 119, the HRT was changed to 2.5 days and on day 137, the HRT was adjusted to 1 day; thereafter, this HRT was maintained throughout the rest of the experimental run. On day 156, the SCN^- concentration was adjusted to 150 mg SCN^-/L subsequent to SCN^- feed increment to 250 mg SCN^-/L on day 178. Prior to SCN^- increment to 500 mg SCN^-/L on day 189, the reactors were inoculated with *Pseudomonas aeruginosa* STK 03, *Exiguobacterium acetylicum* and *Bacillus marisflavi* at a concentration of 1 %(v/v) from a previously grown culture at 30 °C in nitrogen-free MM described in Chapter 4 and 5. The reactors were temporarily operated in batch mode over a 24 hour period, to allow the organisms to acclimatize to the system and thereafter, the SCN^- concentration was increased to 500 mg SCN^-/L on day 189. These organisms were introduced to the system to counter the TN concentration build-up within the system and to aid in the removal of SCN^-, CN^- and TN. On day 204, the SCN^- feed concentration was further increased to 1000 mg SCN^-/L, which was maintained until the end of the experiment. On day 224, the pH of the feed solution was adjusted to 9.9 and CN^- was introduced at a concentration of 200 mg CN^-/L; a CN^- threshold tolerance for most microorganisms (Kuyucak and Akcil, 2013). The CN^- concentration was further increased to 450 mg CN^-/L on day 261

and the reactors were operated under these conditions (450 mg CN^-/L, 1000 mg SCN^-/L and pH 9.9) until the end of the experiment (day 308). On day 280, microbial samples were withdrawn from R1 and R2 for SEM analysis as described in Chapter 3, Section 3.2.4.

8.3.3 Microbial activity tests

Microbial activity tests were conducted on planktonic microbial species, when the operational parameters were changed. This was done to assess the activity of the planktonic organisms within the system when the operational parameters were changed. The media contained 3.4 g/L KH_2PO_4, 4.3 g/L K_2HPO_4, glucose (0.1 g/L), 0.6 g/L $MgCl_2.6H_2O$ and 100 mg SCN-/L (for SCN^- degradation) and 100 mg CN^-/L (for CN^- degradation). For SCN^- activity tests, the pH was adjusted to 7.0 while for CN^- activity tests, the pH was adjusted to 9.9.

8.4 Results and discussion

8.4.1 Microbial culture characterisation and identification

Prior to the inoculation of the reactors, the inoculum was identified and characterised using the 16S rDNA amplicon gene sequencing approach. This culture-independent technique provides direct and more meaningful insights on the microbial composition of a consortium without prior culturing. This is advantageous as researchers are able to fully elucidate the microbial composition of a particular ecological habitat, as compared to the biased culture-dependent techniques which only focuses on the culturable organisms. This is disadvantageous since 1% of the total microbial composition that exists can be cultured on suitable growth media (Amann et al., 1995); hence, the detected organisms would not truly represent the actual consortium composition within the reactor. The inoculum was mainly dominated by bacteria (67%) (Fig 8.2a) which was majorly constituted by bacteria belonging to the Pretobacteria (23.95%) and unknown/non assigned (57.52%) phylum (Fig 8.2b). In this study, non-assigned and/or unknown organisms constituted the majority of the organisms within the inoculum and this was also confirmed by the family profile (see Fig 8.2c), and this was later attributed to the prevalence of uncultured bacteria (Fig 8.2d). The dominant bacterial species which were detected in the inoculum was majorly constituted with *Thiobacillus* sp and *Serratia* sp. *Thiobacillus* species have been largely documented as autotrophic SCN^- degrading organisms (Katayama et al., 1992) while *Serratia* sp. have been observed to possess CN^- degradation capabilities (Karamba et al., 2016, Mpongwana et al., 2016), ensuring maximum degradation of both SCN^- and CN^-.

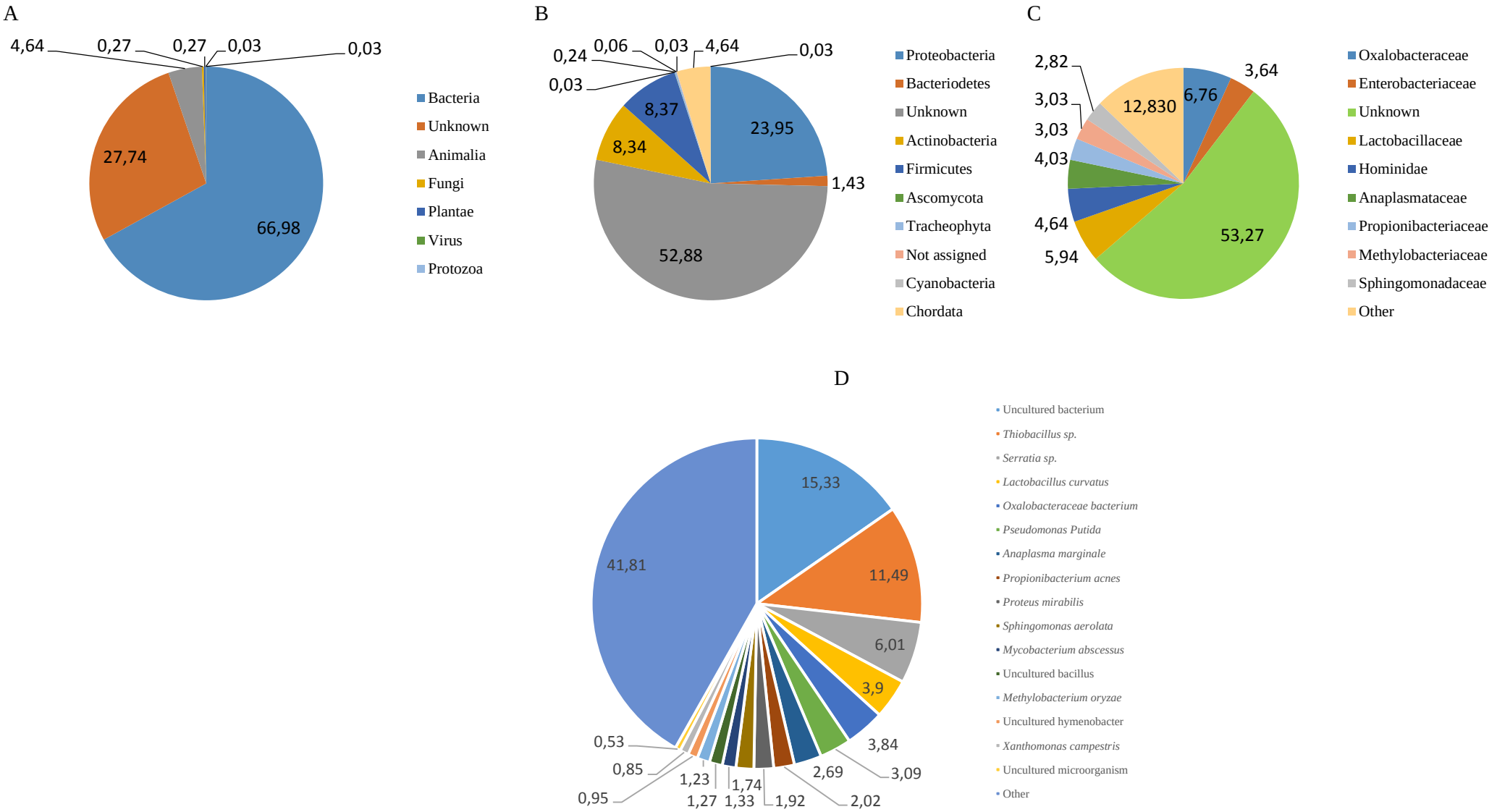


Fig. 8. 2: A microbial characterization profile demonstrating the (a) taxonomy, (b) phylum, (c) family and (d) Genus of the inoculum

Amongst the 41.81% of the unclassified organisms, referred to as "other" in Fig 8.2d, are organisms which belong to the *Microbacterium, Sphingomonas, Methylobacterium, Burkholderia, Pseudomonas, Raoultella, Klebsiella, Serratia, Acinetobacter, Cyanobacter, Bacillus* and *Corynebacterium* genus (see Appendix C Table C1). The organisms belonging to these genus have been observed to possess CN^- and SCN^- biodegradation capacity (see Chapter 2). *Microbacterium* sp. and *Sphingomonas* sp. were previously identified in the ASTER^{TM} process as the dominant organisms for the biodegradation of SCN^- (van Buuren et al., 2011, van Zyl et al., 2015), while in a separate studies, *Burkholderia* sp. have been determined to be effective SCN^- and CN^- degraders (Vu et al., 2013). Furthermore, *Klebsiella* sp. were identified and characterised as having high SCN^- and CN^- biodegradation rates (Ahn et al., 2004, Chaudhari and Kodam, 2010, Chen et al., 2008), including nitriles (Chen *et al*., 2010). These organisms are of economic importance as they are able to produce methane from the biodegradation process (Kao *et al*., 2003). Similarly, *Serratia* sp., *Bacillus* sp., *Pseudomonas* sp. and *Methylobacterium* sp. have been observed to be highly effective in the degradation of CN^- and SCN^- (Karamba et al., 2016, Mekuto et al., 2015, Grigor'eva et al., 2009, Wood et al., 1998) and are also able to utilise the produced biodegradation end-products. The presence of these complex microorganisms reflect the high-strength nature of the consortium for effective biodegradation of CN^- and SCN^-.

8.4.2 Microbial community structure using SEM

The inoculum was majorly constituted of rod shaped organisms in combination with yeast-like organisms (Fig 8.3a). On day 52, the community structured changed in R1 as this reactor was dominated by filamentous organisms and minimal rod shaped organisms (Fig 8.3b), and on day 273, biofilm formation was evident which comprised of filamentous, rod-shaped and yeast-like organisms (Fig 8.3c). This demonstrated microbial growth within the system as a result of nutrient uptake by the organisms. Similarly, the community structure in R2 on day 52 was mainly colonised by rod-shaped organisms (see Fig 8.3d) which later transformed into a slimy-like structure (Fig 8.3e), with observed rod-shaped organisms that were embedded within the slimy biofilm structure. Furthermore, the image also demonstrated the minute apertures within the structure of the biofilm, which provided channels for solute transport and uptake within the inner structure of the biofilm.

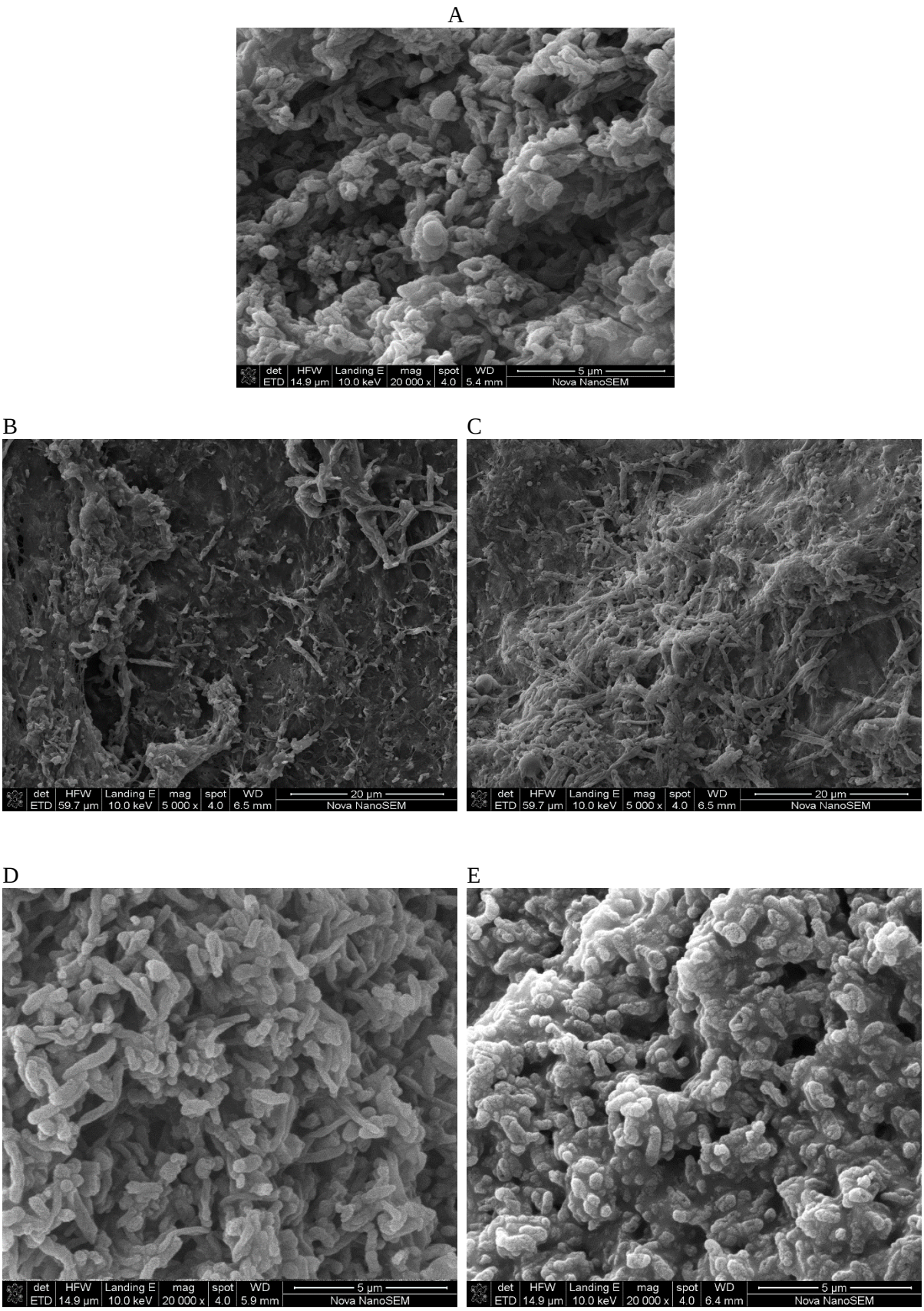


Fig. 8.3: Scanning electron microscopy images demonstrating microbial structure of the (a) inoculum, (b,c) microorganisms in reactor 1 at 52 and 273 days respectively, (c,d) microorganisms in reactor 2 at 52 and 273 days respectively.

8.4.3 Performance of bioreactor system

A dual staged bioreactor system was established and operated at room temperature (22 to 27 °C) for a period of approximately 300 days. During the start-up period, the concentration of SCN^- was set a 150 mg SCN^-/L and complete biodegradation of SCN^- was observed after 22 days with an initial pH range of 6.9 to 7.1 (Fig 8.4). After the start-up period, it was evident that the organisms had acclimatized and this was verified by high degradation efficiencies (> 90%), in both R1 and R2, when the overall HRT was set at 7 days. The residual SCN^- concentration was below 10 mg SCN^-/L. Similarly, the biodegradation efficiencies increased drastically when the HRT was decreased from 7 days to 2.5 days, with an observed degradation efficiency of >99.9% from day 82 to day 135. This was followed by a further decrease in residence time (to 1 day), where the high degradation efficiencies were maintained. Due to this, the concentration of SCN^- was increased to 150 mg SCN^-/L (day 156) and subsequently to 250 mg SCN^-/L (day 178) which was accompanied by high degradation efficiencies of > 99.9%.

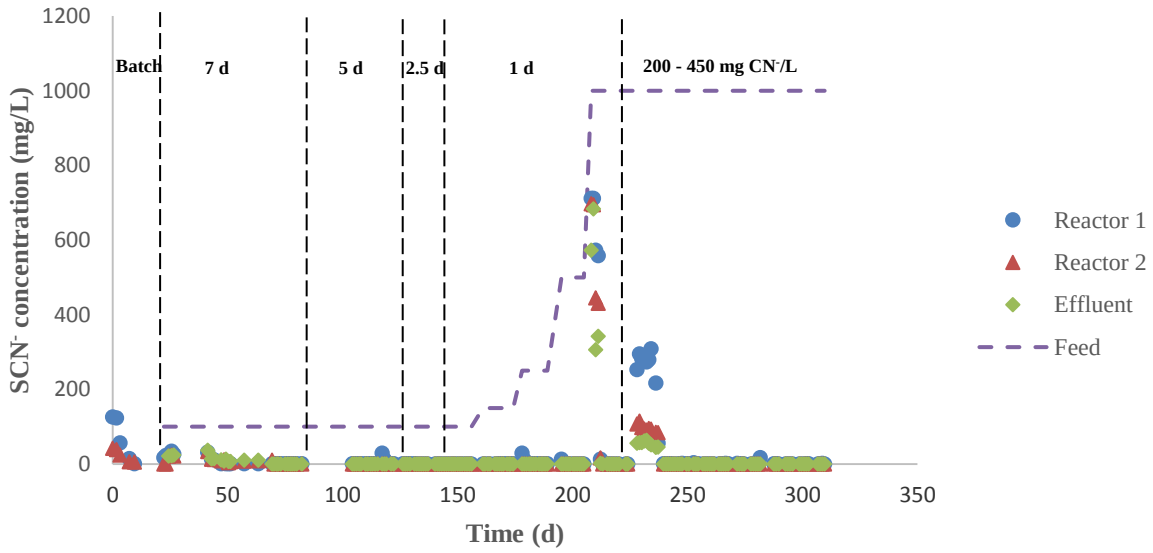


Fig. 8.4: Residual thiocyanate and free cyanide concentrations as a function of feed concentration and time.

On day 189, prior to the SCN^- increment to 500 mg SCN^-/L, the reactors were inoculated with *Pseudomonas aeruginosa* STK 03 (Chapter 4), *Exiguobacterium acetylicum* and *Bacillus marisflavi* (Chapter 5). These organisms were previously observed to biodegrade CN^- and SCN^- under alkaline conditions, and would hence, assist in accelerated biodetoxification of these contaminants. At this period, biofilm development was noticed on the walls, impellers and stainless steel piping system of the reactors. Furthermore, the growth of these organisms resulted in the blockage of the air sparging system, thus resulting in microaerobic conditions. The introduction of these organisms to the bioreactor system further bolstered and ensured satisfactory degradation efficiencies, resulting in the degradation efficiencies exceeding 99%. When the SCN^- concentration was increased to 1000 mg SCN^-/L on day 204, there was an observed inhibition on microbial activity. This was confirmed by the microbial activity tests where the biodegradation efficiency was below 90% (Fig 8.5). The activity was later regained after

18 days and this was followed by a deliberate pH increase to 9.9 and the introduction of CN^- at a concentration of 200 mg CN^-/L on day 224. This proved to be detrimental to the performance of the organisms as the residual SCN^- concentration averaged 280 (R1), 155 (R2) and 50 mg SCN^-/L in the effluent respectively, from day 224 to day 240. However, after this period, the complete degradation of SCN^- was observed. During the stress period, complete degradation of CN^- was observed (Fig 8.6), and this demonstrated the adequacy of the biofilm for effective degradation of both CN^- and SCN^-. This was denoted from the regained high degradation efficiencies observed (>99.9%), which were maintained until the end of the experimental run.

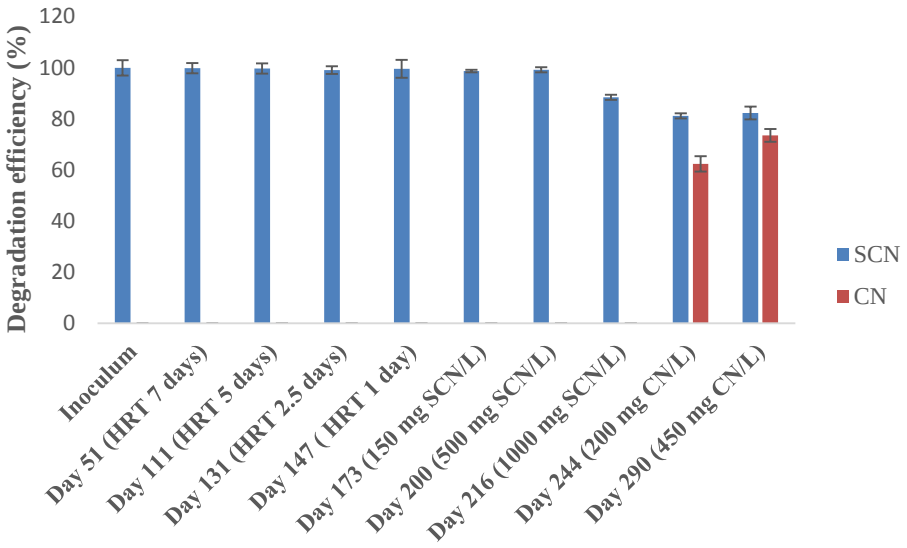


Fig. 8.5: Graphical profile of microbial activity tests.

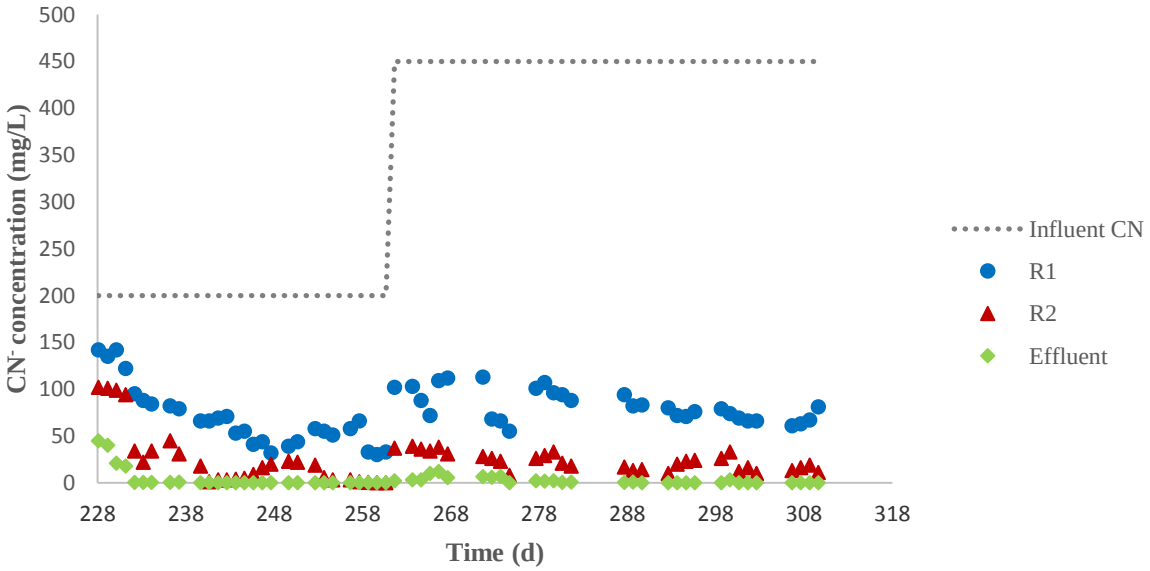


Fig. 8.6: Residual free cyanide concentration as a function of feed concentration and time.

The biodegradation of CN^- and SCN^- results in the production of nitrogenous compounds in the form of NH_4^+ (from CN^- and SCN^- degradation), NO_2^- and NO_3^- (from NH_4^+ metabolism). The combinations of these nitrogenous compounds were denoted as total nitrogen (TN). The biodegradation of SCN^- from day 0 to day 179 resulted in the fluctuation of TN which was below 100 mg N/L (Fig 8.7). However, from day 188 to day 224, there was an observed increase and accumulation of TN within the reactor systems, suggesting reduced temporal microbial activity due to the increase in SCN^- concentration. In addition, the simultaneous increase in pH and the introduction of CN^- within the system stressed the organisms, thus leading to reduced microbial activity and reduced TN removal. Conversely, after the stress period, the organisms were able to remove TN, with an average effluent concentration of approximately 17 mg N/L. This suggested that the organisms were able to conduct heterotrophic nitrification and aerobic denitrification under cyanide and thiocyanate laden conditions. This phenomenon was observed in Chapter 7 where the microbial communities consisting of the TDOs and CDOs were observed to nitrify and aerobically denitrify under cyanogenic conditions. In addition, Razanamahandry *et al*. (2016) observed nitrification potential of organisms that were able to degrade CN^-, while Mekuto et al. (2015) observed simultaneous nitrification and aerobic denitrification in a continuous CN^- biodegradation system. These studies demonstrated the ability of SCN^- and CN^- degrading organisms to conduct heterotrophic nitrification and aerobic denitrification. However, complete TN removal is desired and this can be achieved by incorporating an additional TN removal bioreactor and/or by introducing a pre-aerobic denitrification stage. In a separate study, the incorporation of a pre-denitrification stage resulted in the optimised TN removal from SCN^- biodegradation system; an operational strategy that can be taken into account for reactor configuration directed at industrial application (Villemur et al., 2015).

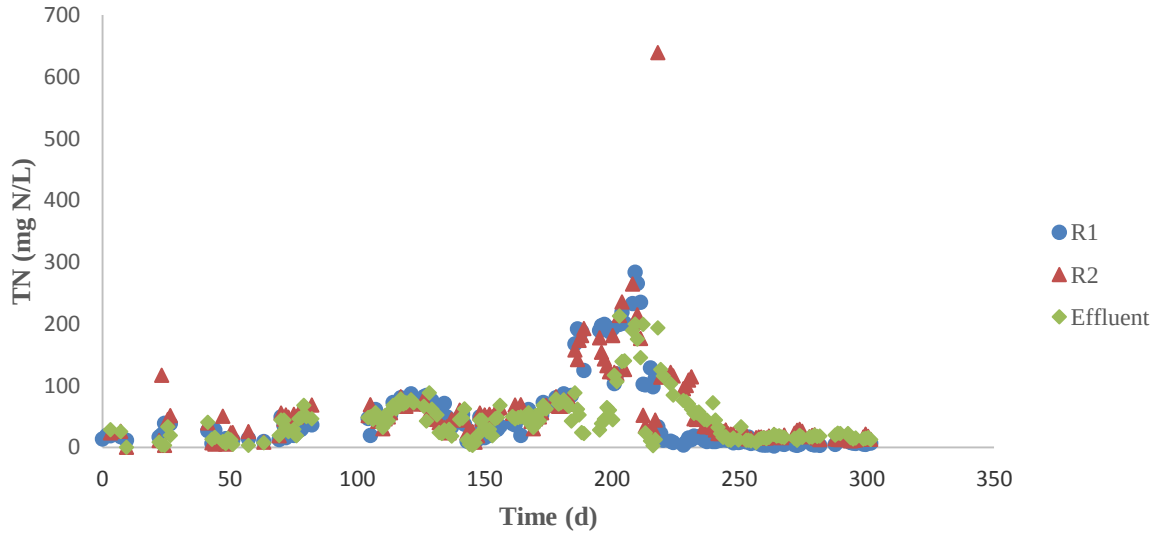


Fig. 8.7: Total nitrogen profile produced from SCN^- and CN^- biodegradation.

The biodegradation of SCN^- results in the production of sulphates. The sulphate concentrations were monitored throughout the experimental run (see Fig 8.8). The concentrations of the produced SO_4^{2-} increased with an increase in SCN^- loading as a result of the biodegradation of SCN^-. The maximum

SO_4^{2-} concentration was observed when the SCN^- loading was increased to 1000 mg SCN^-/L which averaged approximately 1600 mg SO_4^{2-}-S/L. In a separate study, the production of sulphates was indirectly correlated with microbial growth and SCN^- biodegradation efficiencies (Supplementary file A). It was suggested that the production of SO_4^{2-} from SCN^- biodegradation systems can be used as an indirect technique for microbial proliferation and colonisation since most SCN^- degraders are unable to utilise sulphates as a source of sulphur.

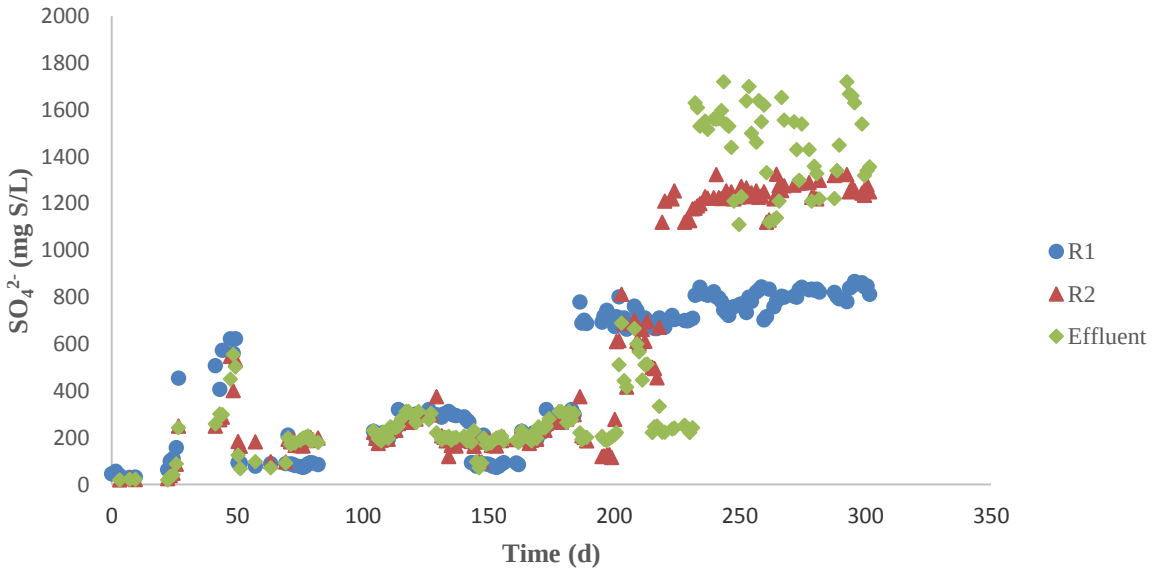


Fig. 8.8: Sulphate concentration profile as a function of time.

The idiosyncrancies in pH were assessed during the experimental run (Fig 8.9). During the start-up period, the pH decreased sharply to an average of 5 across the bioreactors. The pH decrease was due to the acidity produced by the biodegradation process which might have superseded the bicarbonate alkalinity and/or the autotrophic organisms within the reactors rapidly utilised the bicarbonate as an alternative carbon source as seen in a study by (Hung and Pavlostathis, 1999). However, the pH increased when the system was switched into continuous mode and the pH plateaued along neutrality after day 76. The pH was increased after day 224 to 9.9 but the measured pH in R1, R2 and the effluent averaged at 8.6, 7.1 and 7.0, respectively. Similarly, this observation was attributed to the acidity generated from the biodegradation of SCN^- which led to a pH decrease.

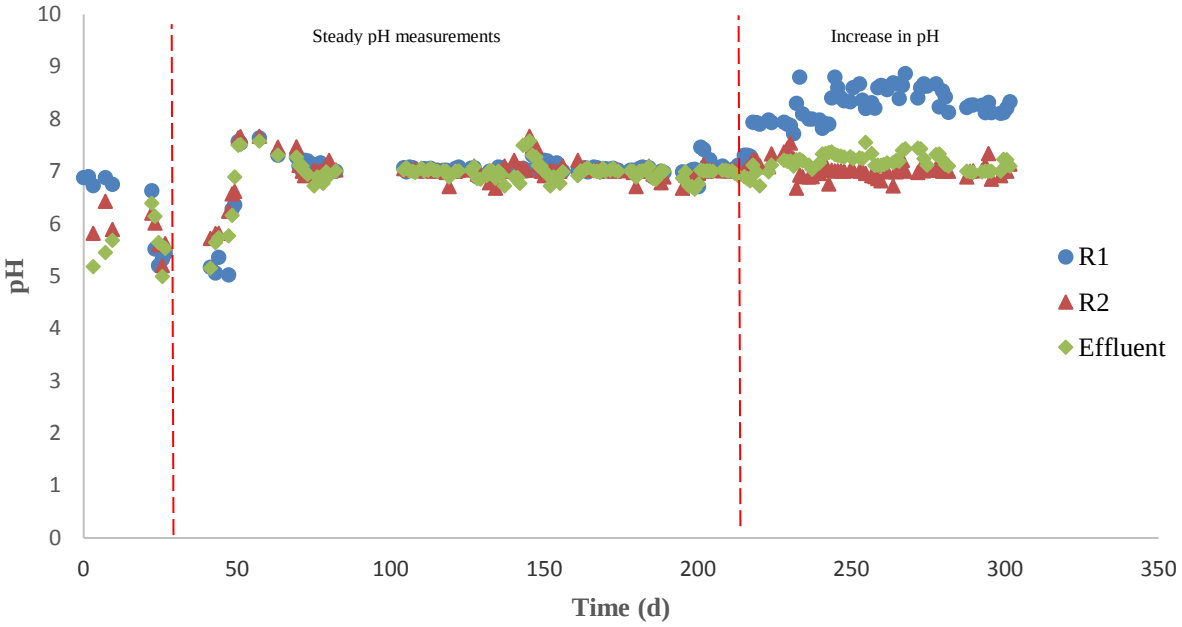


Fig 9.9: Graphical representation of pH as a function of time.

8.5 Summary

It was evident from this study that SCN^- and CN^- loading did not have a dire impact on the performance of the microbial communities that were present within the bioreactor system. Furthermore, changes in residence time had minimal impact on process performance as the organisms maintained an overall biodegradation efficiency of >99.9%, irrespective of the SCN^- and CN^- loading, a demonstration of microbial adaptability. However, the simultaneous changes in pH and the introduction of CN^- had a slight effect on the performance of the system, with an observed recuperation of process performance thereafter. This was confirmed through planktonic microbial activity tests which revealed high SCN^- and CN^- biodegradation efficiencies. Furthermore, the organisms were able to utilise the biodegradation by- and end-products, i.e. NH_4^+, NO_3^- and NO_2^-; an indication of nitrification and denitrification potential of the active microorganisms within the system.

This study provided the fundamental research aspects which are necessary for the successful operation of a pilot scale plant. The scientific advancements achieved in this study and the conclusions, including the recommendations for future research, are highlighted in the next chapter.

CHAPTER 9

SUMMARY AND CONCLUSIONS

Summary and conclusions

9.1 Summary and conclusions

Cyanide (CN^-) compounds are produced from natural and anthropogenic sources in different forms. Cynogenic plants, bacteria, fungi and algae are capable of producing CN^- compounds, as a defence mechanism against predation and/or invasion of a particular ecological habitat in search for nutrient sources. However, these sources of CN^- don't contribute significantly to the presence of CN^- in the environment. The major sources of CN^- contamination in the environment are due to anthropogenic activities such as the metal mining, metal processing and finishing processes, chemical production processes and petroleum processing. These processes produce CN^- compounds of varying stabilities and reactivity, making their treatment complex. The different forms of cyanide determines their environmental fate and transport in soil and water bodies. Due to the high toxicity of cyanide compounds, mainly as hydrogen cyanide (HCN), stringent water quality regulations have been enforced such that acceptable cyanide concentrations in water and soil are at a minimum. However, there are vast challenges concerning cyanide contamination in water and soil, which amongst others include: (i) the chemistry and speciation of cyanide, (ii) the differentiation in their reactivity, toxicity and treatability of various cyanide forms and the challenges in the analytical techniques used to detect these compounds.

Owing to the toxicity and high reactivity of CN^- compounds, an array of remediation techniques have been developed to allow for treatment of surface, ground and wastewaters, including contaminated soils. The successful operation of the biological process at the Homestake mine (Québec, Canada), used for the treatment of cyanidation wastewater using rotating biological contactors (RBC), prompted an interest amongst researchers to develop biotechnological processes for CN^-/SCN^- laden wastewaters. From thereon, substantial research has been undertaken for the assessment of microbial organisms to degrade a variety of CN^- compounds using pure, co-cultures and mixed culture systems from different reactor configurations. Recent enzymatic degradation studies have contributed to a thorough understanding of the biochemistry that underlies the biodegradation processes. Additionally, the bioprospecting and characterization of contributing microbial species has been proven to be key in the success of the degradation process.

Therefore, this study began with the bioprospecting of organisms capable of degrading the major CN^- compounds (i.e. CN^- and SCN^-) which are prevalent in cyanidation wastewaters. This incorporated the isolation of *Pseudomonas aeruginosa* STK 03, *Bacillus marisflavi* and *Exiguobacterium acetylicum*, from petroleum waste and river sediments, respectively (Chapter 3). The ability of these organisms to degrade CN^- and SCN^- was evaluated in batch cultures and *Pseudomonas aeruginosa* STK 03 achieved a BRE of 80% and 32% from 250 mg CN^-/L and 450 mg CN^-/L, respectively. In addition, CN^- spiking at specified intervals increased the biodegradation capacity of STK 03 for the biodegradation of SCN^-,

achieving degradation efficiencies of 78% and 98% from non- and cyanide spiked cultures, respectively. This was ascribed to the toxicity of CN^- to STK 03 which led to the rapid release SCN^--degrading enzymes. Furthermore, the co-metabolism of CN^- and SCN^- by *Bacillus marisflavi* and *Exiguobacterium acetylicum* was successful since the generated data revealed that the organisms successfully degraded both CN^- and SCN^-, achieving over 99% degradation efficiencies (Chapter 5). Response Surface Methodology determined the effective operating window of these organisms in terms of pH, temperature, CN^- and SCN^- concentrations and these were observed to be 9.0, 34 °C, 140 mg CN^-/L and 205 mg SCN^-/L, respectively. This study was the first report on the co-metabolism of CN^- and SCN^- under alkaline conditions, especially by the aforementioned organisms. As a result of the effectiveness of these organisms, they were supplemented in a continuous cyanide and thiocyanate degradation system, to aid in the successful destruction of these chemical compounds.

Early microbial ecology studies revealed that the vast majority of microbial biodiversity in ecological niches has been missed due to the utilization of cultivation-based techniques. Hence, SCN^--degrading organisms (TDO's) were isolated using a gravimetric technique in liquid media since CN^--degrading organisms (CDO's) were observed to be unable to degrade SCN^- (Chapter 6). 16S rRNA amplicon gene sequencing revealed the microbial diversity within these microbial communities which were mainly dominated by *Myroides odoratimimus* and *Proteus* sp. at 37.82% and 30.5% for CDOs, and 35.26% and 17.58% for TDOs, respectively. Contrarily, cultivation-based identification techniques only detected a small fraction of organisms that were detected by the 16S rRNA amplicon gene sequencing procedure. The organisms detected by the cultivation-based technique were dominated by *Bacillus* sp. for the CDO's, while the TDO's were dominated by *Bacillus* sp., *Klebsiella oxytoca*, *Providencia* sp. and *Pseudomonas* sp. The co-culturing of these organisms proved to be advantageous as they were able to co-metabolise CN^- and SCN^-, resulting in degradation efficiencies exceeding 99%. Additionally, the increased biodegradative capacity of the co-culture was mainly ascribed to the biochemical changes in key metabolic enzymes, which were assessed using the VITEK 2 Compact system.

Furthermore, ammonium (NH_4^+) and nitrates (NO_3^-) utilization studies were undertaken in cynogenic and non-cynogenic conditions. The nitrification and denitrification capacity of the CDO's and TDO's singularly and in co-cultures was effective irrespective of the tested CN^- and SCN^- load (Chapter 7). This proved the heterotrophic nitrification and aerobic denitrification capacity of these organisms and the positive interaction between the microbial communities. Hence, these organisms should be used in the secondary processes downstream of the biodegradation processes since these organisms are not susceptible to CN^- and SCN^-.

The data that was generated throughout this study was used for the construction of an effective microbial community of high-strength for the continuous biodegradation of SCN^- and CN^- under varying

physicochemical conditions (Chapter 8). The biodegradation of SCN^- and CN^- was successful throughout the duration of the experimental run under varying hydraulic retention times, SCN^- and CN^- concentrations, except when the organisms were temporarily shocked by the introduction of CN^- and increase in pH (from 7 to 9.9). The system later regained the high biodegradation efficiency which was coupled with the observed nitrification and aerobic denitrification capacities. This confirmed the effectiveness and robustness of the microbial consortium.

This study provided fundamental research which is necessary for the successful operation pilot studies. The scientific advancements that were achieved from this study are:

- The application of mixed microbial culture which was isolated using an uncommon microbial isolation technique (i.e. gravimetric method), proved to be robust and effective in the biodegradation of CN^- and SCN^- in batch and continuous systems, under varying physicochemical conditions.
- Culture-independent techniques for the characterization of the microbial communities involved in the biodegradation of the mentioned contaminants have proven to be effective as compared to culture-dependent techniques.
- The utilization of the biodegradation by-products by the employed mixed microbial cultures capable of CN^- and SCN^- biodegradation was a demonstration of the capacity of the isolates to conduct nitrification and denitrification in CN^-/SCN^--laden conditions. Furthermore, it was evident that the nitrification to denitrification ratio was not 1:1 as is normally reported.

Therefore, the application of such organisms on real CN^-/SCN^--laden wastewaters would prove to be robust, effective and sustainable for extended periods as was observed in this study.

9.2 Recommendations for future work

This study presented successful biodegradation of SCN^- and CN^-, which are the major contaminants of cyanidation wastewaters. However, cyanidation wastewaters are complex and contain CN_{WAD} and CN_{SAD} species which were not covered on this work. Therefore, further research should focus on the degradation of these metal complexes. Furthermore, the undertaken study should be expanded to incorporate the enzymatic degradation studies. This would be done to understand the underlying biochemical pathways associated with the degradation of these contaminants using pure and mixed microbial communities. Understanding the biochemical pathways in mixed cultures is somewhat challenging and this would need a much more sophisticated approach, such as metabolomics.

The sulphates (SO_4^{2-}) produced from the biodegradation of SCN^- were not utilised by the employed organisms as a source of sulphur. This was evident in the continuous reactor system where the SO_4^{2-}

accumulated through the experimental run. Hence, further studies should focus on the removal of SO_4^{2-} such that the resultant wastewater meets discharge standards. This could be accomplished by the supplementation of magnetite zeolite particles in the biological treatment systems. The magnetite zeolite nanoparticles would act as adsorbents for the SO_4^{2-} while serving as a microbial attachment surface to facilitate biofilm formation. However, this approach would necessitate the establishment of regeneration and reusability procedures, which must be investigated. Furthermore, the toxicity of such adsorbents on the microbial species would necessitate for further investigations.

Additionally, this study detected microbial organisms which have been previously determined to be pathogens, causing a variety of diseases. Therefore, a virulence study needs to be undertaken to assess the pathogenicity of these microbial communities.

The observed heterotrophic nitrification and aerobic denitrification capabilities of these organisms is still poorly understood. There are currently no reports on the mechanisms of heterotrophic nitrification and aerobic denitrification. The mechanisms of this process would aid in a better understanding of the stoichiometry associated with this process and also determine the nitrification and denitrification ratios. For improved process performance, it is recommended that that the reactor configuration be improved to incorporate a pre-denitrification stage for optimised TN removal using the consortium that is responsible for SCN^- and CN^- biodegradation.

SUPPLEMENTARY FILES

Mekuto, L., Muchatibaya, G., Ntwampe, S.K.O. & Mudumbi, J.B.N. Sulphate production: An indirect technique for microbial growth, activity and thiocyanate biodegradation efficiency. Submitted to *Indian Journal of Microbiology* [Manuscript ID: INJM-D-17-00312]

This manuscript has been inserted on this section as submitted to the aforementioned journal.

Supplementary files

10.1 Supplementary file

ABSTRACT

Biodegradation of thiocyanate results in the production of ammonium and sulphates and since the thiocyanate degrading organisms are unable to utilise the produced sulphates as a source of sulphur, the sulphates can be utilised as an indirect technique to assess microbial growth, activity and thiocyanate biodegradation efficiency. In this study, the production of sulphates from thiocyanate biodegradation had a good correlation in comparison to the traditional methods of assessing microbial growth and activity i.e. direct cell counts (DCC), heterotrophic counts (CFU) and use of fluorescein production from fluorescein diacetate (FDA). The concentration of the produced sulphates demonstrated a similar logarithmic trend with the FDA, DCC and CFU techniques, thus confirming that the production of sulphates from thiocyanate biodegradation systems can be used as an indirect technique for microbial growth, activity and thiocyanate biodegradation efficiency.

Keywords: Biodegradation, Sulphates, Thiocyanate, Fluorescein diacetate, Heterotrophic cell counts, Direct cell counts.

10.1.1 Introduction

Thiocyanate containing wastes are produced from a variety of industries, which include amongst others, the gasification, mineral processing and coal to coke industries (Baxter and Cummings, 2006). Biological treatment techniques are preferred for the treatment of thiocyanate containing wastewaters, and in this technique, microorganisms are utilised to accelerate the biodegradation of thiocyanate according to Eq. 2.14. Aerobic biodegradation systems are preferred as compared to anaerobic systems (Akcil, 2003, Akcil and Mudder, 2003), due to the high degradation rates achieved in such systems. Thiocyanate is metabolised via two interconnected pathways and these have been elucidated elsewhere (Gould et al., 2012).

Ammonium production is normally used as an indirect measure for thiocyanate biodegradation. However, recent studies have demonstrated that the microbial species which are responsible for the biodegradation of thiocyanate (Mekuto et al., 2015), are able to utilise the formed ammonium as a source of nitrogen and therefore, the use of ammonium as an indirect measure of thiocyanate biodegradation may not truly represent the performance of the microorganisms in the biodegradation process, since these organisms are able to utilise the formed ammonium. On the contrary, these organisms are unable to utilise the produced sulphates. The inability to utilise sulphates by these organisms can be used as an indirect technique for thiocyanate biodegradation and microbial growth. Therefore, this study was aimed at evaluating the feasibility of using sulphate production as an indirect

measure for microbial growth, activity and thiocyanate biodegradation, in comparison to traditional techniques which are used for microbial growth and activity.

10.1.2 Materials and methods

10.1.2.1 Microbial culture and growth conditions

Thiocyanate degrading organisms (TDO) were isolated from thiocyanate containing wastewater. The TDOs were analysed using the 16S rRNA amplicon gene sequencing approach to reveal the bacterial species that were present within the organisms and this data is available in a public repository handle (http://hdl.handle.net/11189/5110).

The culture was grown in minimal media as described in Mekuto et al. (2016). The media was supplemented with thiocyanate, to achieve a concentration of 150 mg SCN^-/L. The flasks were incubated in an orbital shaker at 30 °C and 180 rpm. Samples were collected at various time intervals and centrifuged at 10 000 *g* for 5 min prior to the analysis of thiocyanate and sulphates. Thiocyanate biodegradation and sulphate production was evaluated using the ferric iron method (Katayama et al., 1992) and turbidimetrically using barium salts (Kolmert et al., 2000) respectively. Ammonium nitrogen was determined according to the method developed by Patton and Crouch (1977). The effect of the initial thiocyanate concentration on sulphate production, microbial growth and activity was evaluated in batch cultures for a period of 98 h.

10.1.2.2 Assessing microbial activity using fluorescein diacetate (FDA)

The microbial activity of the microbial cultures within the biodegradation processes was assessed using the FDA method as described in Adam and Duncan (2001) . Briefly, this method works on the hydrolysis of FDA intracellularly by active microorganisms, releasing a fluorescent fluorescein which can be detected spectrophotometrically (Chrzanowski et al., 1984).

10.1.2.3 Heterotrophic plate counting

The number of viable cells were estimated using the heterotrophic plate count procedure. The plate count procedure was achieved with autoclaved nutrient agar (Meat extract 1.0 g/L, peptone 5.0 g/L, yeast extract 2.0 g/L, NaCl 8.0 g/L, agar 15 g/L). Samples were collected particular intervals and an aliquot (200 µL) of the culture was spread plated after appropriate dilutions. This was followed by incubation at 30 °C for 48 h. The visible microbial colonies were counted as colony forming units (CFU).

10.1.2.4 Direct cell counting (DCC)

DCC was achieved by the utilisation of a Thoma counting chamber (Hawksley, UK). 5 µl of the planktonic microorganisms sampled from the thiocyanate degradation experiments were evenly distributed in the chamber and counted directly in an Olympus CX21 phase contrast light microscope

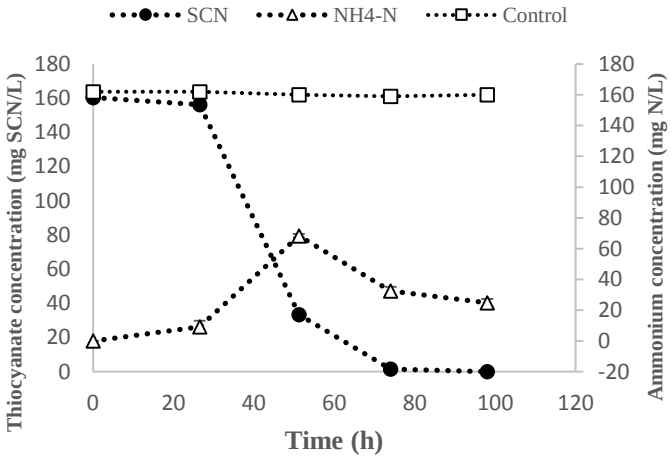
(New York, U.S.A.). The mobile cells were the only cells that were counted as these were considered to be active and/or viable while the non-mobile cells were ignored as they were considered to be non-viable or dead.

10.1.3 Results and discussion

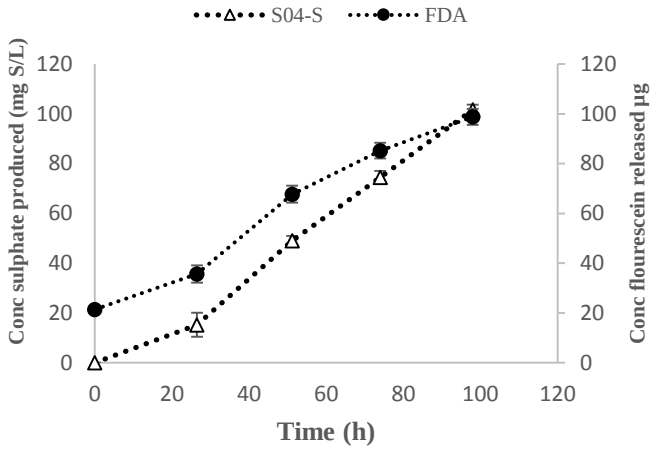
A microbial consortia was used for the biodegradation of thiocyanate under alkaline conditions, as defined by Mekuto et al (2016). Thiocyanate was completely degraded after 98 h (see Fig 1a), producing ammonium and sulphate (see Fig 1b) as products of biodegradation. The concentration of ammonium increased to 68.4 mg NH_4^+-N/L after 52 h and thereafter, the concentration decreased due to metabolism by the TDO. This phenomenon was also observed by Razanamahandry et al. (2016) when cyanide degrading bacteria (CDB) were able to metabolise the produced ammonium from the biodegradation of free cyanide. In addition, Mpongwana et al. (2016) observed ammonium metabolism when free cyanide was degraded by species belonging to *Yersinia*, *Enterobacter* and *Serratia*, thus demonstrating the ability of most cyanide and thiocyanate degrading organisms in metabolising the produced ammonium from the biodegradation process. The biodegradation of thiocyanate was accompanied by the production of sulphates (see Fig 1b). The sulphate concentration during the biodegradation process assumed a logarithmic trend which correlated very well with the amount of fluorescein produced from FDA hydrolysis, a technique used to measure microbial activity. Similarly, the sulphate concentration assumed a similar trend when compared to the CFU and DCC techniques (see Fig 1c).

The effect of initial thiocyanate concentration on sulphate production, microbial growth and activity was evaluated in batch cultures over a 98 h period. The concentration of sulphates increased up to 317 mg SO_4^{2-}-S/L when the initial concentration of thiocyanate was at 600 mg SCN^-/L and thereafter, the concentration decreased with an increase in the initial thiocyanate concentration, suggesting substrate inhibition. The sulphate production data correlated with the FDA profile, which showed a similar trend to that of sulphate concentration. Similarly, the DCC and CFU methods assumed a similar trend to that observed for sulphate production, reaffirming the feasibility of using the produced sulphates as an indirect technique for thiocyanate biodegradation. This is the first report for indirect evaluation of microbial performance for thiocyanate biodegradation.

A



B



C

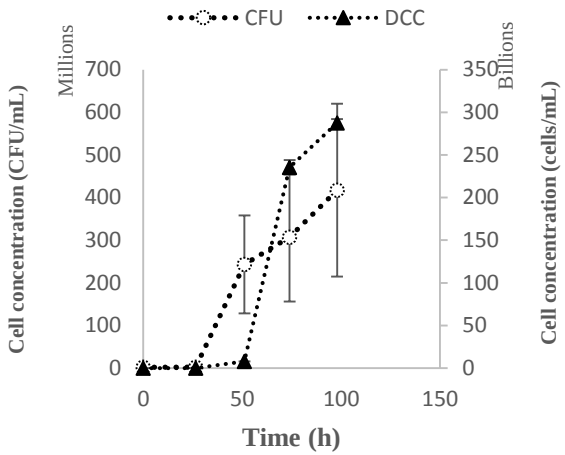
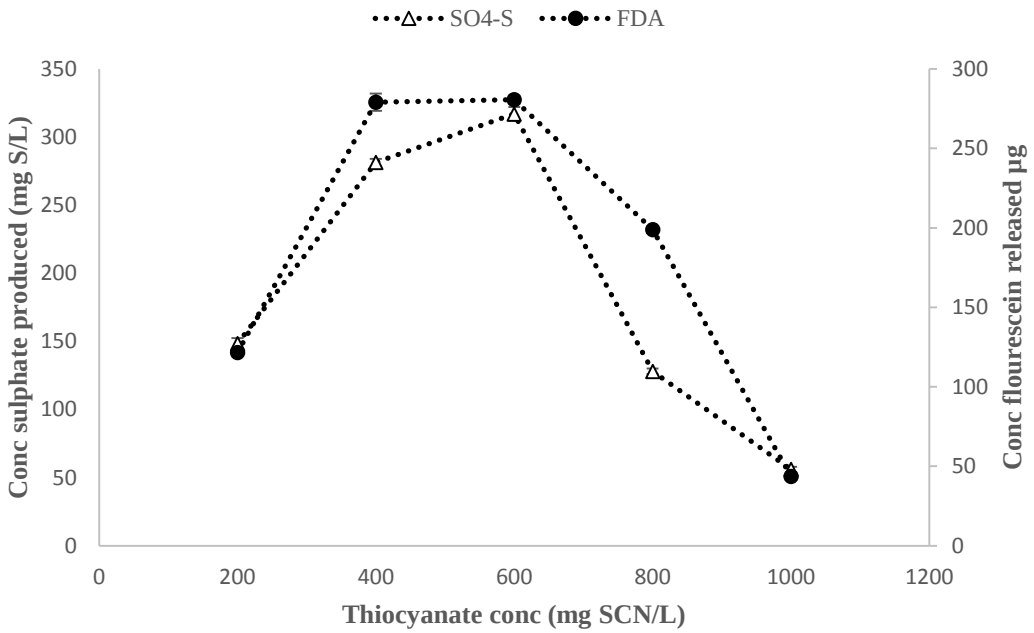


Fig. 10.1: Graphical profiles representing (a) thiocyanate biodegradation, (b) sulphate concentration produced and microbial activity, and (c) direct cell counts and heterotrophic plate counts. Error bars represent deviations.

A



B

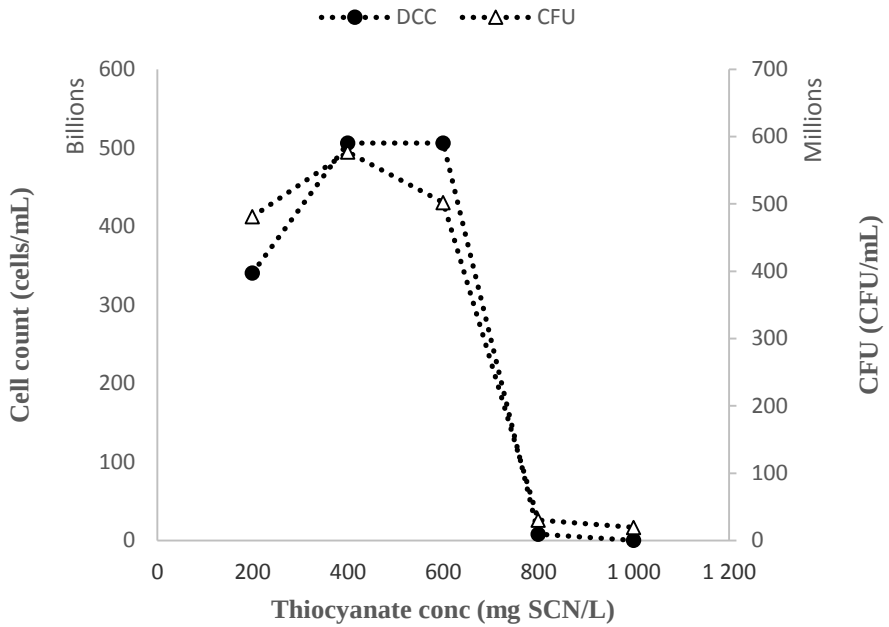


Fig. 10.2: The effect of initial thiocyanate concentration on (a) sulphate production and microbial activity, and (b) direct cell counts and heterotrophic plate counts. Error bars represent deviations.

10.1.4 Conclusion

Sulphate production can be utilized as an indirect method for assessing thiocyanate biodegradation efficiency, microbial growth and activity. This technique had a good correlation when compared with the traditional techniques which are used for the direct determination of microbial growth and activity. This is the first study to report on sulphate production as an indirect technique for microbial growth, activity and thiocyanate biodegradation efficiency.

CHAPTER 11

BIBLIOGRAPHY

Bibliography

ADAM, G. & DUNCAN, H. 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry,* 33**,** 943-951.

ADAMS, M. & LLOYD, V. 2008. Cyanide recovery by tailings washing and pond stripping. *Minerals Engineering,* 21**,** 501-508.

ADAMS, M. D. 2013. Impact of recycling cyanide and its reaction products on upstream unit operations. *Minerals Engineering,* 53**,** 241-255.

ADJEI, M. D. & OHTA, Y. 1999. Isolation and characterization of a cyanide-utilizing *Burkholderia cepacia* strain. *World Journal of Microbiology and Biotechnology,* 15**,** 699-704.

AHN, J.-H., LEE, S. & HWANG, S. 2005. Growth kinetic parameter estimation of *Klebsiella sp*. utilizing thiocyanate. *Process Biochemistry,* 40**,** 1363-1366.

AHN, J. H., KIM, J., LIM, J. & HWANG, S. 2004. Biokinetic evaluation and modeling of continuous thiocyanate biodegradation by *Klebsiella sp. Biotechnology progress,* 20**,** 1069-1075.

AKCIL, A. 2002. First application of cyanidation process in Turkish gold mining and its environmental impacts. *Minerals Engineering,* 15**,** 695-699.

AKCIL, A. 2003. Destruction of cyanide in gold mill effluents: biological versus chemical treatments. *Biotechnology Advances,* 21**,** 501-511.

AKCIL, A. 2010. A New Global Approach of Cyanide Management: International Cyanide Management Code for the Manufacture, Transport, and Use of Cyanide in the Production of Gold. *Mineral Processing and Extractive Metallurgy Review,* 31**,** 135-149.

AKCIL, A., KARAHAN, A. G., CIFTCI, H. & SAGDIC, O. 2003. Biological treatment of cyanide by natural isolated bacteria (*Pseudomonas* sp.). *Minerals Engineering,* 16**,** 643-649.

AKCIL, A. & MUDDER, T. 2003. Microbial destruction of cyanide wastes in gold mining: process review. *Biotechnology Letters,* 25**,** 445-450.

AMANN, R. I., LUDWIG, W. & SCHLEIFER, K. H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological reviews,* 59**,** 143-169.

AMOR, K. B., VAUGHAN, E. E. & DE VOS, W. M. 2007. Advanced molecular tools for the identification of lactic acid bacteria. *The Journal of nutrition,* 137**,** 741S-747S.

AWOLUSI, O. O., ENITAN, A. M., KUMARI, S. & BUX, F. 2015a. Nitrification Efficiency and Community Structure of Municipal Activated Sewage Sludge. *World Academy of Science, Engineering and Technology, International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering,* 9**,** 970-977.

AWOLUSI, O. O., KUMARI, S. K. S. & BUX, F. 2015b. Ecophysiology of nitrifying communities in membrane bioreactors. *International Journal of Environmental Science and Technology,* 12**,** 747-762.

BARCLAY, M., TETT, V. A. & KNOWLES, C. J. 1998. Metabolism and enzymology of cyanide/metallocyanide biodegradation by Fusarium solani under neutral and acidic conditions. *Enzyme and Microbial Technology,* 23**,** 321-330.

BARRY, J., BROWN, A., ENSOR, V., LAKHANI, U., PETTS, D., WARREN, C. & WINSTANLEY, T. 2003. Comparative evaluation of the VITEK 2 Advanced Expert System (AES) in five UK hospitals. *Journal of Antimicrobial Chemotherapy,* 51**,** 1191-1202.

BAXTER, J. & CUMMINGS, P. S. 2006. The current and future applications of microorganism in the bioremediation of cyanide contamination. *Antonie van Leeuwenhoek,* 90**,** 1-17.

BEZSUDNOVA, E. Y., SOROKIN, D. Y., TIKHONOVA, T. V. & POPOV, V. O. 2007. Thiocyanate hydrolase, the primary enzyme initiating thiocyanate degradation in the novel obligately chemolithoautotrophic halophilic sulfur-oxidizing bacterium Thiohalophilus thiocyanoxidans. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1774**,** 1563-1570.

BOTZ, M. & MUDDER, T. 2000. Modelling of natural cyanide attenuation in tailings impoundments. *Mineral and Metallurgical Processing,* 17**,** 228-233.

BOTZ, M. & MUDDER, T. Treatment of solutions and slurries for cyanide removal. *In:* MIULAR, A., HALBE, D., BARRATT, D.(EDS.), ed. Mineral Processing Plant Design, Practice and Control, 2002a Vancouver, Canada. Society for Mining, Metallurgy and Exploration, Inc., 1866-1885.

BOTZ, M., MUDDER, T. & AKCIL, A. 2005a. Cyanide treatment: physical, chemical and biological processes. *In:* ADAMS, M. (ed.) *Advances in Gold Ore Processing.* Amsterdam: Elsevier Ltd.

BOTZ, M., MUDDER, T. & AKCIL, A. 2005b. Cyanide treatment: physical, chemical and biological processes. *In:* ADAMS, M. (ed.) *Advances in Gold Ore Processing.* Amsterdam: Elsevier.

BOTZ, M., MUDDER, T. & AKCIL, A. 2015. Cyanide treatment: Physical, chemical and biological. *In:* ADAMS, M. (ed.) *Advances in Gold Ore Processing.* Amsterdam: Elsevier Ltd.

BOTZ, M. M. & MUDDER, T. I. Treatment of solutions and slurries for cyanide removal. *In:* MULAR, A. L., HALBE, D. N. & BARRATT, D. J., eds. Mineral Processing Plant Design, Practice and Control, 2002b Vancouver, Canada. Society for Mining, Metallurgy and Exploration, Inc., 1866-1885.

BOUCABEILLE, C., BORIES, A. & OLLIVIER, P. 1994. Degradation of thiocyanate by a bacterial coculture. *Biotechnology Letters,* 16**,** 425-430.

CABUK, A., UNAL, A. T. & KOLANKAYA, N. 2006. Biodegradation of Cyanide by a White Rot Fungus, *Trametes versicolor*. *Biotechnology Letters,* 28**,** 1313-1317.

CAMPOS, M. G., PEREIRA, P. & ROSEIRO, J. C. 2006. Packed-bed reactor for the integrated biodegradation of cyanide and formamide by immobilised Fusarium oxysporum CCMI 876 and Methylobacterium sp. RXM CCMI 908. *Enzyme and Microbial Technology,* 38**,** 848-854.

CARLING, G. T., DIAZ, X., PONCE, M., PEREZ, L., NASIMBA, L., PAZMINO, E., RUDD, A., MERUGU, S., FERNANDEZ, D. P., GALE, B. K. & JOHNSON, W. P. 2013. Particulate and Dissolved Trace Element Concentrations in Three Southern Ecuador Rivers Impacted by Artisanal Gold Mining. *Water, Air, & Soil Pollution,* 224**,** 1-16.

CHAPATWALA, D. K., BABU, V. G. R., VIJAYA, K. O., KUMAR, P. K. & WOLFRAM, H. J. 1998. Biodegradation of cyanides, cyanates and thiocyanates to ammonia and carbon dioxide by immobilized cells of *Pseudomonas putida*. *Journal of Industrial Microbiology and Biotechnology,* 20**,** 28-33.

CHAUDHARI, A. U. & KODAM, K. M. 2010. Biodegradation of thiocyanate using co-culture of *Klebsiella pneumoniae* and *Ralstonia* sp. *Applied Microbiology and Biotechnology,* 85**,** 1167-1174.

CHEN, C. Y., CHEN, S. C., FINGAS, M. & KAO, C. M. 2010. Biodegradation of propionitrile by *Klebsiella oxytoca* immobilized in alginate and cellulose triacetate gel. *Journal of Hazardous Materials,* 177**,** 856-863.

CHEN, C. Y., KAO, C. M. & CHEN, S. C. 2008. Application of Klebsiella oxytoca immobilized cells on the treatment of cyanide wastewater. *Chemosphere,* 71**,** 133-139.

CHEN, F., XIA, Q. & JU, L.-K. 2003. Aerobic denitrification of *Pseudomonas aeruginos*a monitored by online NAD(P)H fluorescence. *Applied and Environmental Microbiology,* 69**,** 6715-6722.

CHEN, P., LI, J., LI, Q. X., WANG, Y., LI, S., REN, T. & WANG, L. 2012. Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium *Rhodococcus sp*. CPZ24. *Bioresource Technology,* 116**,** 266-270.

CHEN, Q. & NI, J. 2011. Heterotrophic nitrification–aerobic denitrification by novel isolated bacteria. *Journal of Industrial Microbiology & Biotechnology,* 38**,** 1305-1310.

CHEN, Q. & NI, J. 2012. Ammonium removal by *Agrobacterium sp*. LAD9 capable of heterotrophic nitrification–aerobic denitrification. *Journal of Bioscience and Bioengineering,* 113**,** 619-623.

CHRZANOWSKI, T. H., CROTTY, R. D., HUBBARD, J. G. & WELCH, R. P. 1984. Applicability of the fluorescein diacetate method of detecting active bacteria in freshwater. *Microbial Ecology,* 10**,** 179-185.

COKER, C., POORE, C. A., LI, X. & MOBLEY, H. L. T. 2000. Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes and Infection,* 2**,** 1497-1505.

COWAN, D., MEYER, Q., STAFFORD, W., MUYANGA, S., CAMERON, R. & WITTWER, P. 2005. Metagenomic gene discovery: past, present and future. *Trends in Biotechnology,* 23**,** 321-329.

CUMMINS, A. B. 1973. *SME mining engineering handbook*, Society of Mining Engineers, American Institute of Mining, Metallurgical, and Petroleum Engineers.

DASH, R. R., GAUR, A. & BALOMAJUMDER, C. 2009. Cyanide in industrial wastewaters and its removal: A review on biotreatment. *Journal of Hazardous Materials,* 163**,** 1-11.

DORR, J. V. N. & BOSQUI, F. L. 1950. *Cyanidation and concentration of gold and silver ores*, McGraw-Hill.

DURSUN, A. Y., ÇALıK, A. & AKSU, Z. 1999. Degradation of ferrous(II) cyanide complex ions by Pseudomonas fluorescens. *Process Biochemistry,* 34**,** 901-908.

EBBS, S. 2004. Biological degradation of cyanide compounds. *Current Opinion in Biotechnology,* 15**,** 231-236.

EBEL, M., EVANGELOU, M. W. H. & SCHAEFFER, A. 2007. Cyanide phytoremediation by water hyacinths (Eichhornia crassipes). *Chemosphere,* 66**,** 816-823.

EMERSON, D., AGULTO, L., LIU, H. & LIU, L. 2008. Identifying and characterizing bacteria in an era of genomics and proteomics. *Bioscience,* 58**,** 925-936.

ESTAY, H., ORTIZ, M. & ROMERO, J. 2013. A novel process based on gas filled membrane absorption to recover cyanide in gold mining. *Hydrometallurgy,* 134–135**,** 166-176.

EZZI, M. I. & LYNCH, J. M. 2005. Biodegradation of cyanide by *Trichoderma spp*. and *Fusarium spp*. *Enzyme and Microbial Technology,* 36**,** 849-854.

FARROW, J., ASH, C., WALLBANKS, S. & COLLINS, M. 1992. Phylogenetic analysis of the genera *Planococcus*, *Marinococcus* and *Sporosarcina* and their relationships to members of the genus Bacillus. *FEMS microbiology letters,* 93**,** 167-172.

FARROW, J. A. E., WALLBANKS, S. & COLLINS, M. D. 1994. Phylogenetic Interrelationships of Round-Spore-Forming Bacilli Containing Cell Walls Based on Lysine and the Non-Spore-Forming Genera *Caryophanon*, *Exiguobacterium*, *Kurthia*, and *Planococcus*. *International Journal of Systematic and Evolutionary Microbiology,* 44**,** 74-82.

GAGNON, I., ZAGURY, G. & DESCHÊNES, L. Natural attenuation potential of cyanide in groundwater near a SPL landfill. Proceedings of the 8th International Symposium on Environmental Issues and Management of Waste in Energy and Mineral Production SWEMP, 2004. 451-456.

GIBBONS, T. 2005. International cyanide management code. *In:* ADAMS, M. D. (ed.) *Advances in Gold ore.* Amsterdam: Elsevier.

GOULD, D. W., KING, M., MOHAPATRA, B. R., CAMERON, R. A., KAPOOR, A. & KOREN, D. W. 2012. A critical review on destruction of thiocyanate in mining effluents. *Minerals Engineering,* 34**,** 38-47.

GRIGOR'EVA, N. V., KONDRAT'EVA, T. F., KRASIL'NIKOVA, E. N. & KARAVAIKO, G. I. 2006. Mechanism of cyanide and thiocyanate decomposition by an association of *Pseudomonas putida* and *Pseudomonas stutzeri* strains. *Microbiology,* 75**,** 266-273.

GRIGOR'EVA, N. V., SMIRNOVA, Y. V. & DULOV, L. E. 2009. Thiocyanate decomposition under aerobic and oxygen-free conditions by the aboriginal bacterial community isolated from the waste water of a metallurgical works. *Microbiology,* 78**,** 402-406.

GRIGOR'EVA, N. V., SMIRNOVA, Y. V., TEREKHOVA, S. V. & KARAVAIKO, G. I. 2008. Isolation of an aboriginal bacterial community capable of utilizing cyanide, thiocyanate, and ammonia from metallurgical plant wastewater. *Applied Biochemistry and Microbiology,* 44**,** 502-506.

GUO, Q., SHI, Z.-J., YANG, C.-C., HUANG, M., XU, J.-L., XU, Y.-Q., NI, W.-M. & JIN, R.-C. 2017. Individual and combined inhibition of phenol and thiocyanate on microbial activity of partial nitritation. *Environmental Science and Pollution Research***,** 1-11.

GUPTA, A. B. 1997. *Thiosphaera pantotropha*: a sulphur bacterium capable of simultaneous heterotrophic nitrification and aerobic denitrification. *Enzyme and Microbial Technology,* 21**,** 589-595.

GUPTA, N., BALOMAJUMDER, C. & AGARWAL, V. K. 2010. Enzymatic mechanism and biochemistry for cyanide degradation: A review. *Journal of Hazardous Materials,* 176**,** 1-13.

GURBUZ, F., CIFTCI, H. & AKCIL, A. 2009. Biodegradation of cyanide containing effluents by Scenedesmus obliquus. *Journal of Hazardous Materials,* 162**,** 74-79.
HABASHI, F. 1966. The theory of cyanidation. *Transactions of the mineralogical society of AIME,* 235**,** 236-239.
HAN, Y., JIN, X., WANG, Y., LIU, Y. & CHEN, X. 2014. Inhibitory effect of cyanide on nitrification process and its eliminating method in a suspended activated sludge process. *Environmental Science and Pollution Research,* 21**,** 2706-2713.
HANDELSMAN, J. 2004. Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and molecular biology reviews,* 68**,** 669-685.
HE, T., LI, Z., SUN, Q., XU, Y. & YE, Q. 2016. Heterotrophic nitrification and aerobic denitrification by *Pseudomonas tolaasii* Y-11 without nitrite accumulation during nitrogen conversion. *Bioresource Technology,* 200**,** 493-499.
HILSON, G. & MONHEMIUS, A. J. 2006. Alternatives to cyanide in the gold mining industry: what prospects for the future? *Journal of Cleaner Production,* 14**,** 1158-1167.
HOUPIKIAN, P. & RAOULT, D. 2002. Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. *Emerging infectious diseases,* 8**,** 122-131.
HU, C., YOU, L., LIU, H. & QU, J. 2015. Effective treatment of cadmium–cyanide complex by a reagent with combined function of oxidation and coagulation. *Chemical Engineering Journal,* 262**,** 96-100.
HUDDY, R. J., VAN ZYL, A. W., VAN HILLE, R. P. & HARRISON, S. T. L. 2015. Characterisation of the complex microbial community associated with the ASTER™ thiocyanate biodegradation system. *Minerals Engineering,* 76**,** 65-71.
HUERTAS, M. J., SÁEZ, L. P., ROLDÁN, M. D., LUQUE-ALMAGRO, V. M., MARTÍNEZ-LUQUE, M., BLASCO, R., CASTILLO, F., MORENO-VIVIÁN, C. & GARCÍA-GARCÍA, I. 2010. Alkaline cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Influence of pH. *Journal of Hazardous Materials,* 179**,** 72-78.
HUNG, C. H. & PAVLOSTATHIS, S. G. 1999. Kinetics and modeling of autotrophic thiocyanate biodegradation. *Biotechnology and bioengineering,* 62**,** 1-11.
ISHII, S., SONG, Y., RATHNAYAKE, L., TUMENDELGER, A., SATOH, H., TOYODA, S., YOSHIDA, N. & OKABE, S. 2014. Identification of key nitrous oxide production pathways in aerobic partial nitrifying granules. *Environmental microbiology,* 16**,** 3168-3180.
JEONG, Y.-S. & CHUNG, J. S. 2006. Biodegradation of thiocyanate in biofilm reactor using fluidized-carriers. *Process Biochemistry,* 41**,** 701-707.
JI, B., YANG, K., WANG, H., ZHOU, J. & ZHANG, H. 2015. Aerobic denitrification by Pseudomonas stutzeri C3 incapable of heterotrophic nitrification. *Bioprocess and Biosystems Engineering,* 38**,** 407-409.
JOHNSON, C. A. 2015. The fate of cyanide in leach wastes at gold mines: An environmental perspective. *Applied Geochemistry,* 57**,** 194-205.
JONES, M., BICKAR, D., WILSON, M., BRUNORI, M., COLOSIMO, A. & SARTI, P. 1984. A re-examination of the reactions of cyanide with cytochrome c oxidase. *Biochem. J,* 220**,** 57-66.
JOO, H.-S., HIRAI, M. & SHODA, M. 2005. Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. *Journal of Bioscience and Bioengineering,* 100**,** 184-191.
KAEWKANNETRA, P., IMAI, T., GARCIA-GARCIA, F. J. & CHIU, T. Y. 2009. Cyanide removal from cassava mill wastewater using *Azotobactor vinelandii* TISTR 1094 with mixed microorganisms in activated sludge treatment system. *Journal of Hazardous Materials,* 172**,** 224-228.
KANG, D.-H., HONG, L. Y., PAUL SCHWAB, A. & BANKS, M. K. 2007. Removal of Prussian blue from contaminated soil in the rhizosphere of cyanogenic plants. *Chemosphere,* 69**,** 1492-1498.
KANTOR, R. S., ZYL, A. W., HILLE, R. P., THOMAS, B. C., HARRISON, S. T. & BANFIELD, J. F. 2015. Bioreactor microbial ecosystems for thiocyanate and cyanide degradation unravelled with genome-resolved metagenomics. *Environmental microbiology,* 17**,** 4929-4941.

KAO, C. M., LIU, J. K., LOU, H. R., LIN, C. S. & CHEN, S. C. 2003. Biotransformation of cyanide to methane and ammonia by *Klebsiella oxytoca*. *Chemosphere,* 50**,** 1055-1061.

KAPOOR, V., ELK, M., LI, X. & SANTO DOMINGO, J. W. 2016. Inhibitory effect of cyanide on wastewater nitrification determined using SOUR and RNA-based gene-specific assays. *Letters in applied microbiology,* 63**,** 155-161.

KARAMBA, K. I., AHMAD, S. A., ZULKHARNAIN, A., SYED, M. A., KHALIL, K. A., SHAMAAN, N. A., DAHALAN, F. A. & SHUKOR, M. Y. 2016. Optimisation of biodegradation conditions for cyanide removal by *Serratia marcescens* strain AQ07 using one-factor-at-a-time technique and response surface methodology. *Rendiconti Lincei***,** 1-13.

KARAVAIKO, G. I., KONDRAT'EVA, T. F., SAVARI, E. E., GRIGOR'EVA, N. V. & AVAKYAN, Z. A. 2000. Microbial degradation of cyanide and thiocyanate. *Microbiology,* 69**,** 167-173.

KATAYAMA, Y., HIRAISHI, A. & KURAISHI, H. 1995. Paracoccus thiocyanatus sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of Thiobacillus versutus to the genus Paracoccus as *Paracoccus versutus* comb. nov. with emendation of the genus. *Microbiology,* 141**,** 1469-1477.

KATAYAMA, Y., NARAHARA, Y., INOUE, Y., AMANO, F., KANAGAWA, T. & KURAISHI, H. 1992. A thiocyanate hydrolase of *Thiobacillus thioparus*: A novel enzyme catalyzing the formation of carbonyl sulfide from thiocyanate. *Journal of Biological Chemistry,* 267**,** 9170-9175.

KIM, Y. M., CHO, H. U., LEE, D. S., PARK, C., PARK, D. & PARK, J. M. 2011a. Response of nitrifying bacterial communities to the increased thiocyanate concentration in pre-denitrification process. *Bioresource Technology,* 102**,** 913-922.

KIM, Y. M., CHO, H. U., LEE, D. S., PARK, D. & PARK, J. M. 2011b. Comparative study of free cyanide inhibition on nitrification and denitrification in batch and continuous flow systems. *Desalination,* 279**,** 439-444.

KIM, Y. M., LEE, D. S., PARK, C., PARK, D. & PARK, J. M. 2011c. Effects of free cyanide on microbial communities and biological carbon and nitrogen removal performance in the industrial activated sludge process. *Water Research,* 45**,** 1267-1279.

KIM, Y. M., PARK, D., LEE, D. S. & PARK, J. M. 2008. Inhibitory effects of toxic compounds on nitrification process for cokes wastewater treatment. *Journal of Hazardous Materials,* 152**,** 915-921.

KITIS, M., KARAKAYA, E., YIGIT, N. O., CIVELEKOGLU, G. & AKCIL, A. 2005. Heterogeneous catalytic degradation of cyanide using copper-impregnated pumice and hydrogen peroxide. *Water Research,* 39**,** 1652-1662.

KNOWLES, R. 1982. Denitrification. *Microbiological reviews,* 46**,** 43.

KOLMERT, Å., WIKSTRÖM, P. & HALLBERG, K. B. 2000. A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. *Journal of Microbiological Methods,* 41**,** 179-184.

KOREN, D. W., GOULD, W. D. & BÉDARD, P. 2000. Biological removal of ammonia and nitrate from simulated mine and mill effluents. *Hydrometallurgy,* 56**,** 127-144.

KORNAROS, M. & LYBERATOS, G. 1998. Kinetic modelling of *Pseudomonas denitrificans* growth and denitrification under aerobic, anoxic and transient operating conditions. *Water Research,* 32**,** 1912-1922.

KORTE, F., SPITELLER, M. & COULSTON, F. 2000. The Cyanide Leaching Gold Recovery Process Is a Nonsustainable Technology with Unacceptable Impacts on Ecosystems and Humans: The Disaster in Romania. *Ecotoxicology and Environmental Safety,* 46**,** 241-245.

KSIĄŻCZYK, M., KUCZKOWSKI, M., DUDEK, B., KORZEKWA, K., TOBIASZ, A., KORZENIOWSKA-KOWAL, A., PALUCH, E., WIELICZKO, A. & BUGLA-PŁOSKOŃSKA, G. 2016. Application of Routine Diagnostic Procedure, VITEK 2 Compact, MALDI-TOF MS, and PCR Assays in Identification Procedure of Bacterial Strain with Ambiguous Phenotype. *Current Microbiology,* 72**,** 570-582.

KUYUCAK, N. & AKCIL, A. 2013. Cyanide and removal options from effluents in gold mining and metallurgical processes. *Minerals Engineering,* 50–51**,** 13-29.

KWON, H., WOO, S. & PARK, J. 2002a. Thiocyanate degradation by *Acremonium strictum* and inhibition by secondary toxicants. *Biotechnology Letters,* 24**,** 1347-1351.
KWON, H. K., WOO, S. H. & PARK, J. M. 2002b. Degradation of tetracyanonickelate (II) by Cryptococcus humicolus MCN2. *FEMS microbiology letters,* 214**,** 211-216.
LA BROOY, S. R., LINGE, H. G. & WALKER, G. S. 1994. Review of gold extraction from ores. *Minerals Engineering,* 7**,** 1213-1241.
LAMBERT, J. L., RAMASAMY, J. & PAUKSTELIS, J. V. 1975. Stable reagents for the colorimetric determination of cyanide by modified Koenig reactions. *Analytical Chemistry,* 47**,** 916-918.
LARSEN, M., TRAPP, S. & PIRANDELLO, A. 2004. Removal of cyanide by woody plants. *Chemosphere,* 54**,** 325-333.
LIU, Y., WANG, Y., LI, Y., AN, H. & LV, Y. 2015. Nitrogen removal characteristics of heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* C16. *Chinese Journal of Chemical Engineering,* 23**,** 827-834.
LOGSDON, M. J., HAGELSTEIN, K. & MUDDER, T. 1999. *The management of cyanide in gold extraction*, International Council on Metals and the Environment Ottawa.
LOPES, R. F. & JOHNSON, R. J. A technical review of heap leaching. *In:* LOOTENS, D. J., GREENSLADE, W. M. & BARKER, J. M., eds. Environmental Management for the 1990s, August 1988 Denver, Colorado. Society for Mining, Metallurgy, and Exploration, Inc.
LUQUE-ALMAGRO, V., BLASCO, R., HUERTAS, M., MARTÍNEZ-LUQUE, M., MORENO-VIVIÁN, C., CASTILLO, F. & ROLDÁN, M. 2005a. Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344. *Biochemical Society Transactions,* 33**,** 168-169.
LUQUE-ALMAGRO, V. M., HUERTAS, M.-J., MARTÍNEZ-LUQUE, M., MORENO-VIVIÁN, C., ROLDÁN, M. D., GARCÍA-GIL, L. J., CASTILLO, F. & BLASCO, R. 2005b. Bacterial degradation of cyanide and its metal complexes under alkaline conditions. *Applied and environmental microbiology,* 71**,** 940-947.
MACHINGURA, M. & EBBS, S. D. 2010. Increased beta-cyanoalanine synthase and asparaginase activity in nitrogen-deprived wheat exposed to cyanide. *J Plant Nutr Soil Sci,* 173**,** 808-810.
MACHINGURA, M., SIDIBE, A., WOOD, A. J. & EBBS, S. D. 2013. The β-cyanoalanine pathway is involved in the response to water deficit in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry,* 63**,** 159-169.
MANIYAM, N. M., SJAHRIR, F., IBRAHIM, A. & CASS, A. G. 2013. Biodegradation of cyanide by *Rhodococcus* UKMP-5M. *Biologia,* 68**,** 177-185.
MARAKI, S., SARCHIANAKI, E. & BARBAGADAKIS, S. 2012. *Myroides odoratimimus* soft tissue infection in an immunocompetent child following a pig bite: case report and literature review. *The Brazilian Journal of Infectious Diseases,* 16**,** 390-392.
MASSEY, V. & EDMONDSON, D. 1970. On the mechanism of inactivation of xanthine oxidase by cyanide. *Journal of Biological Chemistry,* 245**,** 6595-6598.
MECHICHI, T., STACKEBRANDT, E. & FUCHS, G. 2003. *Alicycliphilus denitrificans* gen. nov., sp. nov., a cyclohexanol-degrading, nitrate-reducing β-proteobacterium. *International Journal of Systematic and Evolutionary Microbiology,* 53**,** 147-152.
MEKUTO, L. 2014. *BIODEGRADATION OF CYANIDE AND SUBSEQUENT NITRIFICATION-AEROBIC DENITRIFICATION IN CYANIDE CONTAINING WASTEWATER.* Masters Thesis, Cape Peninsula University of Technology.
MEKUTO, L., NTWAMPE, S. K. O. & JACKSON, V. A. 2013. Biodegradation of free cyanide using *Bacillus safensis*, *Lichenformis* and *Tequilensis* strains: A bioprocess supported solely with whey. *Journal of Bioremediation and Biodegradation,* S:18.
MEKUTO, L., NTWAMPE, S. K. O. & JACKSON, V. A. 2015. Biodegradation of free cyanide and subsequent utilisation of biodegradation by-products by *Bacillus* consortia: optimisation using response surface methodology. *Environmental Science and Pollution Research,* 22**,** 10434-10443.
MEKUTO, L., NTWAMPE, S. K. O., KENA, M., GOLELA, M. T. & AMODU, O. S. 2016. Free cyanide and thiocyanate biodegradation by *Pseudomonas aeruginosa* STK 03 capable of heterotrophic nitrification under alkaline conditions. *3 Biotech,* 6**,** 1-7.

MEYERS, P. R., GOKOOL, P., RAWLINGS, D. E. & WOODS, D. R. 1991. An efficient cyanide-degrading *Bacillus pumilus* strain. *Journal of general microbiology,* 137**,** 1397-1400.

MOSHER, J. B. & FIGUEROA, L. 1996. Biological oxidation of cyanide: A viable treatment option for the minerals processing industry? *Minerals Engineering,* 9**,** 573-581.

MPONGWANA, N., NTWAMPE, S. K. O., MEKUTO, L., AKINPELU, E. A., DYANTYI, S. & MPENTSHU, Y. 2016. Isolation of high-salinity-tolerant bacterial strains, *Enterobacter sp*., *Serratia sp*., *Yersinia sp*., for nitrification and aerobic denitrification under cyanogenic conditions. *Water Science and Technology,* 73**,** 2168-2175.

MUDDER, T. & BOTZ, M. 2004. Cyanide and society: a critical review. *European Journal of Mineral Processing and Environmental Protection,* 4**,** 62-74.

NEUFELD, R., GREENFIELD, J. & RIEDER, B. 1986. Temperature, cyanide and phenolic nitrification inhibition. *Water Research,* 20**,** 633-642.

NSIMBA, E. B. 2009. *Cyanide abd cyanide complexes in the Gold-mine polluted land in the East and Central Rand Goldfields, South Africa.* Master of Science, University of Witwatersrand.

OUDJEHANI, K., ZAGURY, G. & DESCHÊNES, L. 2002. Natural attenuation potential of cyanide via microbial activity in mine tailings. *Applied Microbiology and Biotechnology,* 58**,** 409-415.

PAPEN, H., VON BERG, R., HINKEL, I., THOENE, B. & RENNENBERG, H. 1989. Heterotrophic nitrification by *Alcaligenes faecalis*: NO_2^-, NO_3^-, N_2O, and NO production in exponentially growing cultures. *Applied and Environmental Microbiology,* 55**,** 2068-2072.

PATIL, Y. & PAKNIKAR, K. 2000. Biodetoxification of silver–cyanide from electroplating industry wastewater. *Letters in applied microbiology,* 30**,** 33-37.

PATIL, Y. B. & PAKNIKAR, K. M. 1999. Removal and recovery of metal cyanides using a combination of biosorption and biodegradation processes. *Biotechnology Letters,* 21**,** 913-919.

PATTON, C. J. & CROUCH, S. R. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. *Analytical Chemistry,* 49**,** 464-469.

PETERSEN, J. & DIXON, D. G. 2007. Modeling and optimization of heap bioleach processes. *Biomining.* Springer.

RAZANAMAHANDRY, L. C., ANDRIANISA, H. A., KAROUI, H., KOUAKOU, K. M. & YACOUBA, H. 2016. Biodegradation of free cyanide by bacterial species isolated from cyanide-contaminated artisanal gold mining catchment area in Burkina Faso. *Chemosphere,* 157**,** 71-78.

RIDER, B. & MELLON, M. 1946. Colorimetric determination of nitrites. *Industrial & Engineering Chemistry Analytical Edition,* 18**,** 96-99.

ROBERTSON, L. & KUENEN, J. G. 1990. Combined heterotrophic nitrification and aerobic denitrification in Thiosphaera pantotropha and other bacteria. *Antonie van Leeuwenhoek,* 57**,** 139-152.

ROBERTSON, L. A. & KUENEN, J. G. 1984. Aerobic denitrification — old wine in new bottles? *Antonie van Leeuwenhoek,* 50**,** 525-544.

ROBERTSON, L. A., VAN NIEL, E. W., TORREMANS, R. A. & KUENEN, J. G. 1988. Simultaneous nitrification and denitrification in aerobic chemostat cultures of Thiosphaera pantotropha. *Applied and environmental microbiology,* 54**,** 2812-2818.

RUIZ, G., JEISON, D. & CHAMY, R. 2003. Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. *Water Research,* 37**,** 1371-1377.

RYU, B.-G., KIM, W., NAM, K., KIM, S., LEE, B., PARK, M. S. & YANG, J.-W. 2015. A comprehensive study on algal–bacterial communities shift during thiocyanate degradation in a microalga-mediated process. *Bioresource Technology,* 191**,** 496-504.

SAMIOTAKIS, M. & EBBS, S. D. 2004. Possible evidence for transport of an iron cyanide complex by plants. *Environmental Pollution,* 127**,** 169-173.

SANTOS, B. A. Q., NTWAMPE, S. K. O. & DOUGHARI, J. H. 2013. Continuous biotechnological treatment of cyanide contaminated waters by using a cyanide resistant species of *Aspergillus awamori*. *Environmental Biotechnology–New Approaches and Prospective Applications, M. Petre, Ed. Croatia: InTech***,** 123-146.

SATOKARI, R. M., VAUGHAN, E. E., AKKERMANS, A. D., SAARELA, M. & DE VOS, W. M. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* 67**,** 504-513.

SCHLOSS, P. D. & HANDELSMAN, J. 2003. Biotechnological prospects from metagenomics. *Current Opinion in Biotechnology,* 14**,** 303-310.

SCHRÖTTNER, P., RUDOLPH, W. W., EING, B. R., BERTRAM, S. & GUNZER, F. 2014. Comparison of VITEK2, MALDI-TOF MS, and 16S rDNA sequencing for identification of *Myroides odoratus* and *Myroides odoratimimus*. *Diagnostic Microbiology and Infectious Disease,* 79**,** 155-159.

SHARMA, N. K. & PHILIP, L. 2014. Effect of cyanide on phenolics and aromatic hydrocarbons biodegradation under anaerobic and anoxic conditions. *Chemical Engineering Journal,* 256**,** 255-267.

SILVA-AVALOS, J., RICHMOND, M. G., NAGAPPAN, O. & KUNZ, D. A. 1990. Degradation of the metal-cyano complex tetracyanonickelate (II) by cyanide-utilizing bacterial isolates. *Applied and environmental microbiology,* 56**,** 3664-3670.

SINGH, R., PAUL, D. & JAIN, R. K. 2006. Biofilms: implications in bioremediation. *Trends in Microbiology,* 14**,** 389-397.

SOROKIN, D. Y., ABBAS, B., VAN ZESSEN, E. & MUYZER, G. 2014. Isolation and characterization of an obligately chemolithoautotrophic *Halothiobacillus* strain capable of growth on thiocyanate as an energy source. *FEMS microbiology letters,* 354**,** 69-74.

SOROKIN, D. Y., KOVALEVA, O. L., TOUROVA, T. P. & MUYZER, G. 2010. *Thiohalobacter thiocyanaticus* gen. nov., sp. nov., a moderately halophilic, sulfur-oxidizing gammaproteobacterium from hypersaline lakes, that utilizes thiocyanate. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 444-450.

SOROKIN, D. Y., TOUROVA, T., APOS, P., Y., ANTIPOV, A. N., MUYZER, G. & KUENEN, J. G. 2004. Anaerobic growth of the haloalkaliphilic denitrifying sulfur-oxidizing bacterium *Thialkalivibrio thiocyanodenitrificans* sp. nov. with thiocyanate. *Microbiology,* 150**,** 2435-2442.

SOROKIN, D. Y., TOUROVA, T. P., LYSENKO, A. M. & KUENEN, J. G. 2001. Microbial thiocyanate utilization under highly alkaline conditions. *Applied and Environmental Microbiology,* 67**,** 528-538.

STOTT, M. B., FRANZMANN, P. D., ZAPPIA, L. R., WATLING, H. R., QUAN, L. P., CLARK, B. J., HOUCHIN, M. R., MILLER, P. C. & WILLIAMS, T. L. 2001. Thiocyanate removal from saline CIP process water by a rotating biological contactor, with reuse of the water for bioleaching. *Hydrometallurgy,* 62**,** 93-105.

STROUS, M., VAN GERVEN, E., ZHENG, P., KUENEN, J. G. & JETTEN, M. S. M. 1997. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. *Water Research,* 31**,** 1955-1962.

SUN, Y., FENG, L., LI, A., ZHANG, X., YANG, J. & MA, F. 2017. Ammonium assimilation: An important accessory during aerobic denitrification of *Pseudomonas stutzeri* T13. *Bioresource Technology,* 234**,** 264-272.

SUN, Y., LI, A., ZHANG, X. & MA, F. 2015. Regulation of dissolved oxygen from accumulated nitrite during the heterotrophic nitrification and aerobic denitrification of *Pseudomonas stutzeri* T13. *Applied Microbiology and Biotechnology,* 99**,** 3243-3248.

TAKAYA, N., CATALAN-SAKAIRI, M. A. B., SAKAGUCHI, Y., KATO, I., ZHOU, Z. & SHOUN, H. 2003. Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Applied and environmental microbiology,* 69**,** 3152-3157.

TAYLOR, S. M., HE, Y., ZHAO, B. & HUANG, J. 2009. Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL. *Journal of Environmental Sciences,* 21**,** 1336-1341.

TRAPP, S., LARSEN, M., PIRANDELLO, A. & DANQUAH-BOAKYE, J. 2003. Feasibility of cyanide elimination using plants. *ejmp & ep (European Journal of Mineral Processing and Environmental Protection),* 3**,** 128-137.

VAN ASWEGEN, P. C., VAN NIEKERK, J. & OLIVIER, W. 2007. The BIOX™ process for the treatment of refractory gold concentrates. *Biomining.* Springer.

VAN BUUREN, C., MAKHOTLA, N. & OLIVIER, J. W. 2011. The ASTER process: technology development through to piloting, demonstration and commercialization. *ALTA 2011 Nickel–Cobalt–Copper, Uranium and Gold Conference.* Perth.

VAN BUUREN, K. J. H., NICHOLLS, P. & VAN GELDER, B. F. 1972. Biochemical and biophysical studies on cytochrome aa3. VI. Reaction of cyanide with oxidized and reduced enzyme. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 256**,** 258-276.

VAN HILLE, R. P., DAWSON, E., EDWARD, C. & HARRISON, S. T. L. 2015. Effect of thiocyanate on BIOX® organisms: Inhibition and adaptation. *Minerals Engineering,* 75**,** 110-115.

VAN NIEL, E. W. J., BRABER, K. J., ROBERTSON, L. A. & KUENEN, J. G. 1992. Heterotrophic nitrification and aerobic denitrification in *Alcaligenes faecalis* strain TUD. *Antonie van Leeuwenhoek,* 62**,** 231-237.

VAN ZYL, A. W., HARRISON, S. T. & VAN HILLE, R. P. Biodegradation of thiocyanate by a mixed microbial population. *In:* RUDE, F. W., ed. Mine Water–Managing the Challenges (IMWA 2011), 2011 Aachen, Germany. 119-123.

VAN ZYL, A. W., HUDDY, R., HARRISON, S. T. L. & VAN HILLE, R. P. 2015. Evaluation of the ASTER^{TM} process in the presence of suspended solids. *Minerals Engineering,* 76**,** 72-80.

VILLEMUR, R., JUTEAU, P., BOUGIE, V., MÉNARD, J. & DÉZIEL, E. 2015. Development of four-stage moving bed biofilm reactor train with a pre-denitrification configuration for the removal of thiocyanate and cyanate. *Bioresource Technology,* 181**,** 254-262.

VU, H., MU, A. & MOREAU, J. 2013. Biodegradation of thiocyanate by a novel strain of *Burkholderia phytofirmans* from soil contaminated by gold mine tailings. *Letters in applied microbiology,* 57**,** 368-372.

WATLING, H. R. 2006. The bioleaching of sulphide minerals with emphasis on copper sulphides — A review. *Hydrometallurgy,* 84**,** 81-108.

WEISS, N. L. 1985. SME mineral processing handbook.

WHITLOCK, J. L. 1990. Biological detoxification of precious metal processing wastewaters. *Geomicrobiology Journal,* 8**,** 241-249.

WILD, S. R., RUDD, T. & NELLER, A. 1994. Fate and effects of cyanide during wastewater treatment processes. *Science of The Total Environment,* 156**,** 93-107.

WOOD, P. A., KELLY, P. D., MCDONALD, R. I., JORDAN, L. S., MORGAN, D. T., KHAN, S., MURRELL, C. J. & BORODINA, E. 1998. A novel pink-pigmented facultative methylotroph, *Methylobacterium thiocyanatum* sp. nov., capable of growth on thiocyanate or cyanate as sole nitrogen sources. *Archives of Microbiology,* 169**,** 148-158.

YAO, S., NI, J., CHEN, Q. & BORTHWICK, A. G. L. 2013. Enrichment and characterization of a bacteria consortium capable of heterotrophic nitrification and aerobic denitrification at low temperature. *Bioresource Technology,* 127**,** 151-157.

YU, L., YUAN, Y., CHEN, S., ZHUANG, L. & ZHOU, S. 2015. Direct uptake of electrode electrons for autotrophic denitrification by *Thiobacillus denitrificans*. *Electrochemistry Communications,* 60**,** 126-130.

YU, X.-Z., LU, P.-C. & YU, Z. 2012. On the role of β-cyanoalanine synthase (CAS) in metabolism of free cyanide and ferri-cyanide by rice seedlings. *Ecotoxicology,* 21**,** 548-556.

YU, X., TRAPP, S. & ZHOU, P. 2005a. Phytotoxicity of Cyanide to Weeping Willow Trees. *Environmental Science and Pollution Research,* 12**,** 109-113.

YU, X., TRAPP, S., ZHOU, P. & HU, H. 2005b. The effect of temperature on the rate of cyanide metabolism of two woody plants. *Chemosphere,* 59**,** 1099-1104.

YU, X., TRAPP, S., ZHOU, P., WANG, C. & ZHOU, X. 2004. Metabolism of cyanide by Chinese vegetation. *Chemosphere,* 56**,** 121-126.

YUMOTO, I., HISHINUMA-NARISAWA, M., HIROTA, K., SHINGYO, T., TAKEBE, F., NODASAKA, Y., MATSUYAMA, H. & HARA, I. 2004. *Exiguobacterium oxidotolerans* sp. nov., a novel alkaliphile exhibiting high catalase activity. *International Journal of Systematic and Evolutionary Microbiology,* 54**,** 2013-2017.

ZHANG, J., WU, P., HAO, P. & YU, Z. 2011. Heterotrophic nitrification and aerobic denitrification by the bacterium *Pseudomonas stutzeri* YZN-001. *Bioresource Technology,* 102**,** 9866-9869.

ZHANG, Q.-L., LIU, Y., AI, G.-M., MIAO, L.-L., ZHENG, H.-Y. & LIU, Z.-P. 2012. The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. *Bioresource Technology,* 108**,** 35-44.

ZUMFT, W. G. 1997. Cell biology and molecular basis of denitrification. *Microbiology and molecular biology reviews,* 61**,** 533-616.

Appendices

12.1 Appendix A: Supplementary data from Chapter 5

Table A1: Consensus sequences of the isolated organisms.

Exiguobacterium acetylicum

TCCTTRCGGTTACCTCACCGGCTTCGGGTGTTGCAAACTCTCGTGGTGTGACGGGCGGT
G
TGTACAAGACCCGGGAACGTATTCACCGCAGTATGCTGACCTGCGATTACTAGCGATT
CC
GACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGGGAACGGCTTTATGGGATT
GG
CTCCACCTCGCGGTCTCGCTGCCCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAA
C
TCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
T
CTCCCTAGAGTGCCCAACTAAATGCTGGCAACTAAGGATAGGGGTTGCGCTCGTTGCG
GG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCAT
TG
TCCCCGAAGGGAAAACTTGATCTCTCAAGCGGTCAATGGGATGTCAAGAGTTGGTAAG
GT
TCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAAT
T
CCTTTGAGTTTCAGCCTTGCGGCCGTACTCYCCCAGGCGGAGTGCTTAATGCGTTAGCT
T
CAGCACTGAGGGGCGGAAACCCCCCAACACCTAGCACTCATCGTTTACGGCGTGGACT
AC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACC
A
AAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGT
G
GAATTCCACTCTTCTCTTCTGTACTCAAGCCTTCCAGTTTCCAATGGCCCTCCCCGGTTG
AGCSGGGGGCTTTCACATCAGACTTAAAAGGCCGCCTGCGCGCGCTTTACGCCCAATA
AT
TCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTG
GC
TTTCTCGTAAGGTACCGTCAAGGTACGAGCATTACCTCTCGTACGTGTTCTTCCCTTAC
A
ACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCATCAGACTTT
C
GTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT
C
CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTCGCCTTGGTGGGCCGTTA
C
CTCACCAACTAGCTAATGCACCGCAAGGCCATCTCAAGGTGACGCCGAAGCGCCTTTC
AT
CAGCGGACCATGCGGTCCGTTGAACTATCCGGTATTAGCTCCGATTTCTCGGAGTTATC
C
CAATCCTTGAGGCAGGTTCCTTACGTGTTACTCACCCGTCCGCCGCTCATTCCRCTGCC
T

TCCCTCCGAAGAGTTCCGTCAGTTCCTGCGCTCGA

Bacillus marisflavi

GAGCGGATCGATGGGAGCTTGCTCCCTGAGATCAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATA
ACACCTACCCCCGCATGGGGGAAGGTTGAAAGGTGGCTTCGGCTATCACTTACAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCC
GTTCGAATAGGGCGGCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT
CATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCG
GTGAAATGCGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGG
GACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTA
AGATGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGGGCTGCAAGACCG
CGAGGTTTAGCCAATCCCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCC
TACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG
AGGTAACCTTT

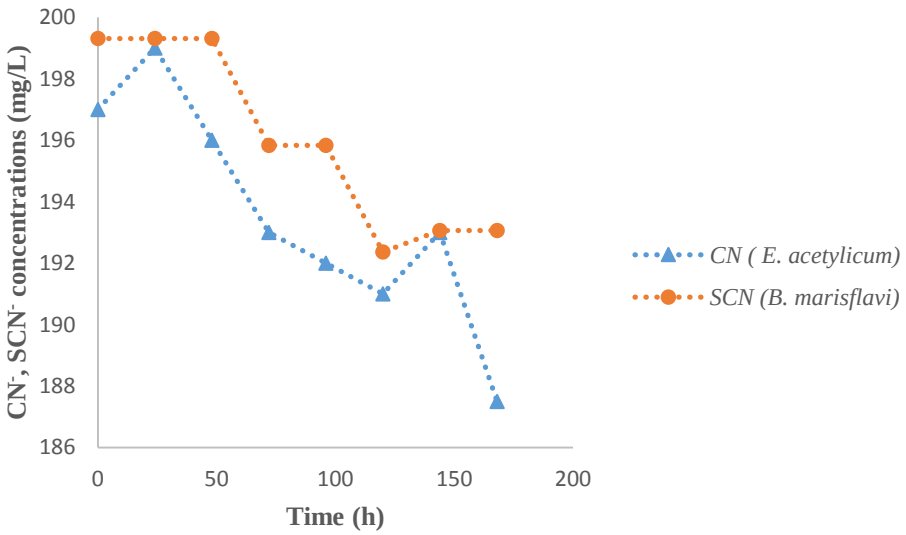


Fig. A1: Free cyanide and thiocyanate degradation profile by *Exiguobcterium acetylicum* and *Bacillus marisflavi*.

12.2 Appendix B: Supplementary data from Chapter 6

Table B1: The composition of the CDO as identified by 16S rDNA amplicon gene sequencing**.**

Organism/HIT	Cluster size	%	Accession	e-value	Fastq header
Myroides odoratimimus	3147	37.82	gi|163932218|gb|EU331413.1|	1.2932e-114	M01232:1:000000000-AP50K:1:1104:14758:19235
Proteus vulgaris	2538	30.50	gi|923095386|gb|KP969052.1|	2.72887e-79	M01232:1:000000000-AP50K:1:1103:23348:5397
Uncultured bacterium	558	6.71	gi|648092936|gb|KJ604130.1|	3.69942e-100	M01232:1:000000000-AP50K:1:1104:13883:7954
Myroides sp.	400	4.81	gi|736012191|gb|CP010327.1|	5.99806e-93	M01232:1:000000000-AP50K:1:1108:8233:2199
Uncultured proteus	211	2.54	gi|506969934|gb|KC896751.1|	1.62701e-93	M01232:1:000000000-AP50K:1:2101:19182:18633
Stenotrophomonas maltophilia	187	2.25	gi|194346582|gb|CP001111.1|	1.46331e-98	M01232:1:000000000-AP50K:1:1119:11670:13420
Uncultured providencia	128	1.54	gi|926458287|dbj|LC079061.1|	3.43811e-41	M01232:1:000000000-AP50K:1:1104:5503:20191
Acidovorax sp.	72	0.87	gi|120604516|gb|CP000539.1|	1.3288e-33	M01232:1:000000000-AP50K:1:1101:2376:13124
Delftia sp.	56	0.67	gi|333741867|gb|CP002735.1|	3.87294e-18	M01232:1:000000000-AP50K:1:1108:16411:13013
Delftia acidovorans	41	0.49	gi|160361034|gb|CP000884.1|	3.47335e-57	M01232:1:000000000-AP50K:1:1103:9363:7746
Pseudomonas syringae	30	0.36	gi|63253978|gb|CP000075.1|	2.99499e-63	M01232:1:000000000-AP50K:1:1105:18596:23856
Citrobacter koseri	29	0.35	gi|673531252|emb|LK931336.1|	8.60737e-12	M01232:1:000000000-AP50K:1:1104:4495:18351
Alicycliphilus denitrificans	23	0.28	gi|329308025|gb|CP002657.1|	3.05065e-16	M01232:1:000000000-AP50K:1:1105:8530:19327
Ralstonia solanacearum	22	0.26	gi|916490054|gb|CP011997.1|	1.00598e-75	M01232:1:000000000-AP50K:1:1108:24493:12339
Uncultured thiobacillus	21	0.25	gi|698322799|gb|KM595276.1|	5.50735e-148	M01232:1:000000000-AP50K:1:1105:12282:9420
Pseudomonas aeruginosa	20	0.24	gi|660504631|gb|CP008749.1|	1.19213e-144	M01232:1:000000000-AP50K:1:1103:9815:13566
Sideroxydans lithotrophicus	20	0.24	gi|291582584|gb|CP001965.1|	4.90792e-53	M01232:1:000000000-AP50K:1:1108:11320:23576
Oceanimonas sp.	20	0.24	gi|444439651|ref|NR_074966.1|	7.35192e-27	M01232:1:000000000-AP50K:1:1119:3722:11659
Serratia marcescens	19	0.23	gi|560171871|emb|HG326223.1|	1.85544e-49	M01232:1:000000000-AP50K:1:2101:18939:17690
Comamonas testosteroni	18	0.22	gi|672605233|gb|CP006704.1|	3.59636e-90	M01232:1:000000000-AP50K:1:1105:10705:11355

Ralstonia pickettii	16	0.19	gi|546340292|gb|CP006668.1|	1.82435e-08	M01232:1:000000000-AP50K:1:2101:18026:5128
Providencia sp.	16	0.19	gi|815932210|gb|KR232641.1|	2.72887e-79	M01232:1:000000000-AP50K:1:1103:25689:12753
Cellulomonas flavigena	13	0.16	gi|296019684|gb|CP001964.1|	2.62834e-31	M01232:1:000000000-AP50K:1:1101:19393:21486
Pseudomonas putida	12	0.14	gi|158392725|dbj|AB333783.1|	2.2351e-49	M01232:1:000000000-AP50K:1:1104:20333:19760
Acidovorax ebreus	11	0.13	gi|221728669|gb|CP001392.1|	4.52077e-89	M01232:1:000000000-AP50K:1:2101:15985:18363
Pseudomonas chlororaphis	9	0.11	gi|787852299|gb|CP011110.1|	2.86247e-61	M01232:1:000000000-AP50K:1:1101:11528:15699
Achromobacter xylosoxidans	9	0.11	gi|408362959|gb|JX448550.1|	7.23576e-132	M01232:1:000000000-AP50K:1:1101:11140:19395
Klebsiella oxytoca	8	0.10	gi|828959694|gb|CP011636.1|	6.02932e-138	M01232:1:000000000-AP50K:1:1119:15407:13718
Pseudoxanthomonas suwonensis	7	0.08	gi|317464132|gb|CP002446.1|	5.35019e-44	M01232:1:000000000-AP50K:1:1103:12838:17520
Proteus sp.	7	0.08	gi|914702447|gb|KP823034.1|	1.22143e-144	M01232:1:000000000-AP50K:1:1105:16106:13146
Myroides profundi	7	0.08	gi|753770668|gb|CP010817.1|	2.72036e-42	M01232:1:000000000-AP50K:1:1119:24298:13050
Herbaspirillum seropedicae	7	0.08	gi|852454696|gb|CP011930.1|	9.9185e-86	M01232:1:000000000-AP50K:1:1108:17746:12414
Alcaligenes sp.	7	0.08	gi|409103805|dbj|AB754812.1|	3.58594e-99	M01232:1:000000000-AP50K:1:1103:11208:1154
Bacillus cereus	6	0.07	gi|755995789|gb|CP009605.1|	1.1257e-139	M01232:1:000000000-AP50K:1:1105:14482:24175
Pseudomonas plecoglossicida	5	0.06	gi|752308899|gb|CP010359.1|	1.67789e-78	M01232:1:000000000-AP50K:1:1108:15191:16124
Propionibacterium acnes	4	0.05	gi|657118275|gb|CP006032.1| e	2.54479e-136	M01232:1:000000000-AP50K:1:1103:8162:17028
Azotobacter chroococcum	4	0.05	gi|747125374|gb|CP010415.1|	2.93158e-36	M01232:1:000000000-AP50K:1:2101:13802:22643
Alcaligenes faecalis	4	0.05	gi|913125411|gb|KP859538.1|	1.81552e-77	M01232:1:000000000-AP50K:1:1119:9072:15900
Cupriavidus necator	4	0.05	gi|338167938|gb|CP002878.1|	1.74718e-68	M01232:1:000000000-AP50K:1:1105:6376:19499
Proteus mirabilis	3	0.04	gi|806981489|gb|KP401767.1|	5.52136e-34	M01232:1:000000000-AP50K:1:1108:19368:22094

Pseudomonas protegens	3	0.04	gi|68342549|gb|CP000076.1|	1.24973e-39	M01232:1:000000000-AP50K:1:1101:20194:24301
Enterobacter cloacae	3	0.04	gi|846395709|gb|KT157602.1|	1.00666e-19	M01232:1:000000000-AP50K:1:1105:11563:20149
Xanthomonas sacchari	3	0.04	gi|743685209|gb|CP010409.1|	6.08919e-73	M01232:1:000000000-AP50K:1:1101:20357:11233
Pseudomonas stutzeri	3	0.04	gi|390981275|gb|CP003677.1|	1.51862e-128	M01232:1:000000000-AP50K:1:1103:11149:2076
Xanthomonas translucens	3	0.04	gi|828451590|gb|CP008714.1|	3.81639e-40	M01232:1:000000000-AP50K:1:1108:10215:10753
Thiobacillus denitrificans	3	0.04	gi|74055513|gb|CP000116.1|	2.20356e-77	M01232:1:000000000-AP50K:1:1119:12892:5872
Xanthomonas arboricola	3	0.04	gi|814589807|gb|KP314293.1|	1.64046e-22	M01232:1:000000000-AP50K:1:1119:21701:4832
Escherichia coli	3	0.04	gi|926539378|gb|CP012632.1|	2.10591e-97	M01232:1:000000000-AP50K:1:1108:9185:19967
Herbaspirillum frisingense	3	0.04	gi|896689380|gb|KP713806.1|	2.19511e-137	M01232:1:000000000-AP50K:1:1108:19475:16710
Uncultured dokdonella	3	0.04	gi|107785044|gb|DQ533520.1|	5.97996e-81	M01232:1:000000000-AP50K:1:1101:23990:19816
Pigmentiphaga sp.	3	0.04	gi|338319330|gb|JN000323.1|	5.06188e-133	M01232:1:000000000-AP50K:1:1108:16603:23108
Pseudomonas alkylphenolia	3	0.04	gi|675318909|gb|CP009048.1|	8.16048e-47	M01232:1:000000000-AP50K:1:1101:13291:10977
Thiobacillus sp.	2	0.02	gi|239836913|gb|FJ982929.1|	6.91686e-38	M01232:1:000000000-AP50K:1:1103:22517:10320
Cupriavidus metallidurans	2	0.02	gi|93352797|gb|CP000352.1|	5.55178e-24	M01232:1:000000000-AP50K:1:1108:22378:4923
Uncultured stenotrophomonas	2	0.02	gi|410699391|gb|JX575903.1|	1.22403e-124	M01232:1:000000000-AP50K:1:1119:23419:5859
Pseudomonas entomophila	2	0.02	gi|95101722|emb|CT573326.1|	2.95291e-96	M01232:1:000000000-AP50K:1:1103:16389:8246
Bordetella bronchiseptica	2	0.02	gi|408767172|emb|HE965806.1|	1.00913e-55	M01232:1:000000000-AP50K:1:1103:26322:7711
Pseudomonas cremoricolorata	2	0.02	gi|691224436|gb|CP009455.1|	1.05031e-35	M01232:1:000000000-AP50K:1:2101:21852:15501
Xanthobacter sp.	2	0.02	gi|399905791|gb|JX178938.1|	3.57787e-135	M01232:1:000000000-AP50K:1:1101:22732:9041
Stenotrophomonas rhizophila	2	0.02	gi|627787876|gb|CP007597.1|	7.77009e-87	M01232:1:000000000-AP50K:1:1103:13343:23439
Acidovorax avenae	2	0.02	gi|323371659|gb|CP002521.1|	2.01012e-62	M01232:1:000000000-AP50K:1:1108:10203:16891
Pseudomonas sp.	2	0.02	gi|568237447|gb|CP007012.1|	2.40657e-72	M01232:1:000000000-AP50K:1:1104:8727:20913
Dechloromonas aromatica	2	0.02	gi|71845263|gb|CP000089.1|	1.16024e-25	M01232:1:000000000-AP50K:1:1101:11771:4495

Pseudomonas fluorescens	2	0.02	gi|800910178|gb|CP011117.1|	2.11681e-92	M01232:1:000000000-AP50K:1:1101:27585:14039
Burkholderia glumae	2	0.02	gi|755901546|gb|CP002581.1|	1.80799e-13	M01232:1:000000000-AP50K:1:1103:16164:15424
Anaplasma marginale	2	0.02	gi|5852413|gb|AF112479.1|	5.57527e-138	M01232:1:000000000-AP50K:1:1119:25107:13616
Uncultured glaciimonas	1	0.01	gi|814556810|gb|KP794262.1|	5.44244e-108	M01232:1:000000000-AP50K:1:1101:17921:23187
Gemmatirosa kalamazoonesis	1	0.01	gi|575461033|gb|CP007129.1|	1.58003e-18	M01232:1:000000000-AP50K:1:1108:19813:13307
Streptomyces avermitilis	1	0.01	gi|148878541|dbj|BA000030.3|	4.88333e-44	M01232:1:000000000-AP50K:1:1108:6176:5007
Dyella jiangningensis	1	0.01	gi|612502010|gb|CP007444.1|	6.607e-18	M01232:1:000000000-AP50K:1:1119:12136:3897
Ramlibacter tataouinensis	1	0.01	gi|334728683|gb|CP000245.1|	1.78717e-28	M01232:1:000000000-AP50K:1:1108:14678:6930
Xanthomonas campestris	1	0.01	gi|807234606|gb|CP011256.1|	1.26649e-74	M01232:1:000000000-AP50K:1:1103:21055:2238
Burkholderia multivorans	1	0.01	gi|773019683|gb|CP009830.1|	8.70161e-37	M01232:1:000000000-AP50K:1:1105:6870:12764
Pseudomonas resinovorans	1	0.01	gi|512374267|dbj|AP013068.1|	1.39069e-78	M01232:1:000000000-AP50K:1:1108:4168:6698
Enterobacter asburiae	1	0.01	gi|918042828|gb|CP010360.2|	3.88117e-94	M01232:1:000000000-AP50K:1:1104:12559:22562
Kurthia sp.	1	0.01	gi|512134489|gb|KF023517.1|	3.51958e-85	M01232:1:000000000-AP50K:1:2101:21640:20880
Pseudomonas fulva	1	0.01	gi|333113473|gb|CP002727.1|	6.09224e-78	M01232:1:000000000-AP50K:1:1105:13315:17367
Bradyrhizobium japonicum	1	0.01	gi|736032532|gb|CP010313.1|	3.56572e-45	M01232:1:000000000-AP50K:1:1101:7505:23933
Modestobacter marinus	1	0.01	gi|388483940|emb|FO203431.1|	2.57433e-17	M01232:1:000000000-AP50K:1:1119:11022:20109
Uncultured aminobacter	1	0.01	gi|507579830|gb|KC923256.1|	3.8135e-62	M01232:1:000000000-AP50K:1:1103:20934:5907
Laribacter hongkongensis	1	0.01	gi|226713858|gb|CP001154.1|	1.13729e-20	M01232:1:000000000-AP50K:1:1105:18399:14089
Pseudomonas mendocina	1	0.01	gi|145573243|gb|CP000680.1|	2.70299e-41	M01232:1:000000000-AP50K:1:1108:5781:14023
Saccharothrix espanaensis	1	0.01	gi|407879691|emb|HE804045.1|	4.50833e-05	M01232:1:000000000-AP50K:1:1105:17029:25180
Blastococcus saxobsidens	1	0.01	gi|378781357|emb|FO117623.1|	2.3524e-77	M01232:1:000000000-AP50K:1:1108:20155:17758
Arsenophonus endosymbiont	1	0.01	gi|820660972|emb|LN829878.1|	1.32302e-09	M01232:1:000000000-AP50K:1:1119:20738:5938
Nocardioides sp.	1	0.01	gi|119534933|gb|CP000509.1|	3.01954e-11	M01232:1:000000000-AP50K:1:1105:19224:4938

Methylovorus sp.	1	0.01	gi|312439093|gb|CP002252.1|	2.78443e-36	M01232:1:000000000-AP50K:1:1105:24289:17306
Clostridium sp.	1	0.01	gi|923889233|gb|KM083029.1|	3.44947e-30	M01232:1:000000000-AP50K:1:1119:12173:7246
Dechlorosoma suillum	1	0.01	gi|359353254|gb|CP003153.1|	1.47032e-108	M01232:1:000000000-AP50K:1:1105:4375:9732
Burkholderia cepacia	1	0.01	gi|685655815|gb|CP007785.1|	9.83606e-06	M01232:1:000000000-AP50K:1:1119:16950:13814
Pseudomonadaceae bacterium	1	0.01	gi|915642179|gb|CP012365.1|	4.27957e-144	M01232:1:000000000-AP50K:1:1119:14734:22296
Pseudomonas trivialis	1	0.01	gi|902687210|gb|CP011507.1|	3.75952e-100	M01232:1:000000000-AP50K:1:1101:15256:17020
Xanthomonas citri	1	0.01	gi|780460238|gb|CP009039.1|	6.18065e-118	M01232:1:000000000-AP50K:1:1108:12086:4164
Uncultured propionibacteriaceae	1	0.01	gi|545345141|gb|KF508373.1|	5.93388e-133	M01232:1:000000000-AP50K:1:1105:6659:18480
Caulobacter segnis	1	0.01	gi|295429362|gb|CP002008.1|	1.68138e-36	M01232:1:000000000-AP50K:1:1101:11506:18029
Pandoraea thiooxydans	1	0.01	gi|827097060|gb|CP011568.1|	2.40553e-11	M01232:1:000000000-AP50K:1:2101:25977:8720
Tenacibaculum sp.	1	0.01	gi|923091939|gb|KT276410.1|	1.37719e-17	M01232:1:000000000-AP50K:1:2101:18458:11220
Parvibaculum lavamentivorans	1	0.01	gi|154154406|gb|CP000774.1|	2.29925e-29	M01232:1:000000000-AP50K:1:1101:14565:4126
Uncultured ruminobacillus	1	0.01	gi|388890795|gb|JQ724340.1|	8.80841e-131	M01232:1:000000000-AP50K:1:1105:8600:23285
Craurococcus sp.	1	0.01	gi|578897545|gb|KF309173.1|	5.93388e-133	M01232:1:000000000-AP50K:1:1105:6458:11514
Corallococcus coralloides	1	0.01	gi|380727201|gb|CP003389.1|	8.91475e-07	M01232:1:000000000-AP50K:1:1104:14625:10752
Acidovorax citrulli	1	0.01	gi|120587178|gb|CP000512.1|	4.23107e-44	M01232:1:000000000-AP50K:1:1105:23289:17689
Herbaspirillum sp.	1	0.01	gi|834963733|gb|KR296697.1|	5.27165e-88	M01232:1:000000000-AP50K:1:1104:5122:11962
Devosia sp.	1	0.01	gi|901895891|gb|CP011300.1|	1.39026e-34	M01232:1:000000000-AP50K:1:1119:9483:6762
Bacillus gaemokensis	1	0.01	gi|926657055|dbj|LC076295.1|	1.22202e-40	M01232:1:000000000-AP50K:1:1104:15201:19946
Acidiphilium multivorum	1	0.01	gi|325049009|dbj|AP012035.1|	6.12555e-23	M01232:1:000000000-AP50K:1:1101:15315:23043
Streptosporangium roseum	1	0.01	gi|270504784|gb|CP001814.1|	5.4811e-08	M01232:1:000000000-AP50K:1:2101:15995:20274
Ricinus communis	1	0.01	gi|255603892|ref|XM_002538083.1|	2.11948e-81	M01232:1:000000000-AP50K:1:1101:25137:19348
Arthrobacter sp.	1	0.01	gi|222546856|gb|FJ610336.1|	6.81216e-25	M01232:1:000000000-AP50K:1:1105:17040:4664

Uncultured acidovorax	1	0.01	gi|922671874|gb|KP967502.1|	6.91686e-38	M01232:1:000000000-AP50K:1:1105:9255:21314
Uncultured ochrobactrum	1	0.01	gi|926657571|dbj|LC001074.1|	1.67328e-81	M01232:1:000000000-AP50K:1:1119:10734:15760
Mesorhizobium ciceri	1	0.01	gi|317165637|gb|CP002447.1|	1.08237e-30	M01232:1:000000000-AP50K:1:1101:6481:9079
Azoarcus aromaticum	1	0.01	gi|56311475|emb|CR555306.1|	2.75857e-111	M01232:1:000000000-AP50K:1:1104:17293:11038
Pseudomonas mosselii	1	0.01	gi|684194542|gb|CP009365.1|	1.04619e-50	M01232:1:000000000-AP50K:1:1108:10764:6978
Mycobacterium rhodesiae	1	0.01	gi|359817839|gb|CP003169.1|	6.88467e-43	M01232:1:000000000-AP50K:1:1119:26019:17592
Janthinobacterium Agaricidamnosum	1	0.01	gi|571265423|emb|HG322949.1|	1.91386e-23	M01232:1:000000000-AP50K:1:1104:6020:12652
Variovorax paradoxus	1	0.01	gi|239799596|gb|CP001635.1|	9.36593e-11	M01232:1:000000000-AP50K:1:1108:22494:17203
Micrococcus luteus	1	0.01	gi|758182997|gb|CP007437.1|	8.50581e-72	M01232:1:000000000-AP50K:1:1101:7524:5746
Desulfobacterium Autotrophicum	1	0.01	gi|223689840|gb|CP001087.1|	6.61231e-08	M01232:1:000000000-AP50K:1:1108:10710:15747

Table B2: Composition of the TDO as identified by 16S rDNA amplicon gene sequencing.

Organism/HIT	Cluster size	%	Accession	e-value	Fastq header
Myroides odoratimimus	4847	35.26	gi|922317158|gb|KR349266.1|	3.77534e-46	M01232:1:000000000-AP50K:1:1103:13905:16356
Proteus sp.	2417	17.58	gi|189409506|gb|EU710747.1|	2.00902e-137	M01232:1:000000000-AP50K:1:1108:12924:3193
Myroides sp.	668	4.86	gi|914702437|gb|KP823024.1|	1.37719e-17	M01232:1:000000000-AP50K:1:1103:24983:10053
Stenotrophomonas maltophilia	534	3.88	gi|194346582|gb|CP001111.1|	5.57527e-138	M01232:1:000000000-AP50K:1:1119:19648:16035
Proteus mirabilis	533	3.88	gi|333353439|gb|JF772095.1|	9.68362e-111	M01232:1:000000000-AP50K:1:1119:24016:17977
Uncultured Enterobacteriaceae	530	3.86	gi|294613661|gb|GU905819.1|	2.62951e-106	M01232:1:000000000-AP50K:1:2101:25386:13705
Uncultured *Proteus*	469	3.41	gi|506969934|gb|KC896751.1|	1.21527e-99	M01232:1:000000000-AP50K:1:1104:7992:2893
Proteus vulgaris	229	1.67	gi|340025986|gb|JN092605.1|	1.24072e-129	M01232:1:000000000-AP50K:1:1108:28974:16380
Delftia sp.	180	1.31	gi|333741867|gb|CP002735.1|	2.35216e-48	M01232:1:000000000-AP50K:1:2101:23435:5502
Uncultured *Thiobacillus*	173	1.26	gi|926657308|dbj|LC000812.1|	3.38393e-125	M01232:1:000000000-AP50K:1:1108:9976:11963
Uncultured *Providencia*	149	1.08	gi|926458287|dbj|LC079061.1|	4.01632e-46	M01232:1:000000000-AP50K:1:1119:28844:12035
Delftia acidovorans	101	0.73	gi|160361034|gb|CP000884.1|	5.87347e-107	M01232:1:000000000-AP50K:1:1108:20088:23867
Myroides profundi	67	0.49	gi|753770668|gb|CP010817.1|	9.73837e-31	M01232:1:000000000-AP50K:1:1105:18480:8337
Proteus penneri	55	0.40	gi|919500502|gb|KT427910.1|	2.37446e-81	M01232:1:000000000-AP50K:1:1104:11655:2456
Providencia vermicola	54	0.39	gi|340026009|gb|JN092796.1|	1.56114e-133	M01232:1:000000000-AP50K:1:1103:8136:16885
Klebsiella pneumoniae	51	0.37	gi|926677775|gb|CP012300.1|	7.18337e-21	M01232:1:000000000-AP50K:1:1105:23300:6043
Pseudomonas syringae	51	0.37	gi|63253978|gb|CP000075.1|	4.73653e-61	M01232:1:000000000-AP50K:1:1104:7000:18283
Acidovorax sp.	46	0.33	gi|407894523|gb|CP003872.1|	3.9268e-14	M01232:1:000000000-AP50K:1:1105:7372:15086
Alcaligenes sp.	38	0.28	gi|485951523|gb|KC534482.1|	1.27439e-114	M01232:1:000000000-AP50K:1:1104:13743:9344
Serratia marcescens	33	0.24	gi|560171871|emb|HG326223.1|	1.16284e-144	M01232:1:000000000-AP50K:1:1103:6353:17408
Uncultured *Dokdonella*	31	0.23	gi|107785044|gb|DQ533520.1|	4.25281e-139	M01232:1:000000000-AP50K:1:1108:25742:19359
Providencia sp.	30	0.22	gi|815932210|gb|KR232641.1|	6.01749e-148	M01232:1:000000000-AP50K:1:1108:21355:17715
Cupriavidus necator	29	0.21	gi|338167938|gb|CP002878.1|	7.25413e-67	M01232:1:000000000-AP50K:1:1104:18041:23936
Pseudomonas aeruginosa	29	0.21	gi|915391195|dbj|AP014839.2|	6.17238e-33	M01232:1:000000000-AP50K:1:1119:13684:24016
Pseudomonas chlororaphis	26	0.19	gi|829490642|gb|CP011020.1|	3.07976e-16	M01232:1:000000000-AP50K:1:1119:25016:6318
Alicycliphilus denitrificans	26	0.19	gi|329312633|gb|CP002658.1|	6.51491e-81	M01232:1:000000000-AP50K:1:1119:16258:20862
Thiobacillus sp.	21	0.15	gi|239836913|gb|FJ982929.1|	7.38599e-54	M01232:1:000000000-AP50K:1:1101:12930:7768

Achromobacter xylosoxidans	21	0.15	gi|777196577|emb|LN831029.1|	4.88815e-84	M01232:1:000000000-AP50K:1:1105:21838:15968
Microbacteriaceae bacterium	19	0.14	gi|601039660|gb|KJ023347.1|	1.24862e-149	M01232:1:000000000-AP50K:1:1105:23690:13978
Ralstonia solanacearum	19	0.14	gi|469772332|gb|CP004012.1|	1.12762e-65	M01232:1:000000000-AP50K:1:1119:10340:2237
Variovorax paradoxus	19	0.14	gi|239799596|gb|CP001635.1|	1.00844e-80	M01232:1:000000000-AP50K:1:1119:19727:9107
Cellulomonas flavigena	17	0.12	gi|296019684|gb|CP001964.1|	2.55423e-31	M01232:1:000000000-AP50K:1:1105:13783:6005
Uncultured *Stenotrophomonas*	16	0.12	gi|821346181|dbj|LC053309.1|	2.52978e-20	M01232:1:000000000-AP50K:1:1104:24756:18070
Providencia stuartii	14	0.10	gi|684200017|gb|CP008920.1|	4.41388e-56	M01232:1:000000000-AP50K:1:1103:6346:13968
Uncultured *Pseudomonas*	13	0.09	gi|923142338|emb|LN875100.1|	6.06021e-143	M01232:1:000000000-AP50K:1:1104:6710:18297
Acidovorax ebreus	12	0.09	gi|221728669|gb|CP001392.1|	1.58887e-13	M01232:1:000000000-AP50K:1:1103:25280:18609
Comamonas testosteroni	12	0.09	gi|672605233|gb|CP006704.1|	2.66179e-29	M01232:1:000000000-AP50K:1:1103:26782:15303
Herbaspirillum seropedicae	10	0.07	gi|852454696|gb|CP011930.1|	1.32919e-44	M01232:1:000000000-AP50K:1:1104:21074:20151
Sideroxydans lithotrophicus	10	0.07	gi|291582584|gb|CP001965.1|	6.09744e-68	M01232:1:000000000-AP50K:1:1108:14355:17157
Pseudomonas putida	9	0.07	gi|764072275|gb|CP010979.1|	4.70475e-89	M01232:1:000000000-AP50K:1:1104:7851:7801
Citrobacter koseri	9	0.07	gi|673531252|emb|LK931336.1|	8.34654e-12	M01232:1:000000000-AP50K:1:1105:8269:13992
Orientia tsutsugamushi	9	0.07	gi|33320065|gb|AF478127.1|	3.37748e-125	M01232:1:000000000-AP50K:1:1103:7270:11422
Pseudomonas stutzeri	8	0.06	gi|390981275|gb|CP003677.1|	2.59749e-131	M01232:1:000000000-AP50K:1:1105:21094:18564
Uncultured acidovorax	8	0.06	gi|926655361|dbj|LC001616.1|	3.40326e-125	M01232:1:000000000-AP50K:1:1101:18683:14419
Uncultured *Hydrogenophilaceae*	8	0.06	gi|821254099|gb|KP292554.1|	2.57936e-48	M01232:1:000000000-AP50K:1:1101:9948:3857
Bacillus sp.	7	0.05	gi|925176648|gb|KR006321.1|	6.49292e-132	M01232:1:000000000-AP50K:1:1104:12716:23643
Herbaspirillum frisingense	7	0.05	gi|378404942|gb|JN869241.1|	8.52369e-131	M01232:1:000000000-AP50K:1:1104:12346:23855
Uncultured *Klebsiella*	7	0.05	gi|926657574|dbj|LC001077.1|	1.37719e-17	M01232:1:000000000-AP50K:1:1119:12742:4053
Xanthomonas sacchari	5	0.04	gi|743685209|gb|CP010409.1|	2.22908e-82	M01232:1:000000000-AP50K:1:1104:9161:3819
Parastrongyloides trichosuri	5	0.04	gi|687022128|emb|LM523277.1|	2.07111e-102	M01232:1:000000000-AP50K:1:1108:17853:10783
Klebsiella oxytoca	4	0.03	gi|828959694|gb|CP011636.1|	3.41767e-130	M01232:1:000000000-AP50K:1:1104:21217:13341
Aestuariibaculum scopimerae	4	0.03	gi|926663105|ref|NR_132699.1|	4.83672e-17	M01232:1:000000000-AP50K:1:1108:8816:18593
Uncultured vibrionaceae	4	0.03	gi|588293873|gb|KF941760.1|	2.03886e-21	M01232:1:000000000-AP50K:1:1119:12113:16557
Burkholderia multivorans	4	0.03	gi|773019683|gb|CP009830.1|	9.03205e-37	M01232:1:000000000-AP50K:1:1103:14186:4658
Uncultured *Alcaligenes*	4	0.03	gi|364515557|gb|JN860163.1|	3.72554e-125	M01232:1:000000000-AP50K:1:1104:14089:9091
Laribacter hongkongensis	4	0.03	gi|226713858|gb|CP001154.1|	8.90157e-21	M01232:1:000000000-AP50K:1:1119:16383:3337
Clavibacter michiganensis	4	0.03	gi|472820487|emb|HE614873.1|	7.86571e-47	M01232:1:000000000-AP50K:1:1104:5972:8945
Pseudomonas sp.	4	0.03	gi|808352541|gb|KP289281.1|	9.36474e-136	M01232:1:000000000-AP50K:1:1105:18131:13296

Pseudomonas fluorescens	4	0.03	gi|666084728|gb|CP008896.1|	2.60607e-49	M01232:1:000000000-AP50K:1:1103:5811:7452
Agrobacterium rhizogenes	4	0.03	gi|300669338|dbj|AB289616.1|	3.33337e-145	M01232:1:000000000-AP50K:1:1103:10729:19778
Pseudomonas plecoglossicida	3	0.02	gi|752308899|gb|CP010359.1|	1.53648e-68	M01232:1:000000000-AP50K:1:1104:20145:24300
Streptomyces sp.	3	0.02	gi|822591927|gb|CP011492.1|	0.00174135	M01232:1:000000000-AP50K:1:1119:18029:23483
Spongitalea numazuensis	3	0.02	gi|926663071|ref|NR_132665.1|	4.83672e-17	M01232:1:000000000-AP50K:1:1103:10377:6756
Escherichia coli	3	0.02	gi|926539378|gb|CP012632.1|	1.03027e-95	M01232:1:000000000-AP50K:1:1105:23506:18275
Ralstonia pickettii	3	0.02	gi|187724002|gb|CP001068.1|	9.98039e-91	M01232:1:000000000-AP50K:1:1103:24365:18270
Uncultured *Phaselicystis*	3	0.02	gi|304422147|gb|HQ018497.1|	4.25525e-144	M01232:1:000000000-AP50K:1:1108:11011:14887
Xanthobacter sp.	3	0.02	gi|399905791|gb|JX178938.1|	4.52218e-139	M01232:1:000000000-AP50K:1:1104:10445:20234
Stenotrophomonas rhizophila	3	0.02	gi|627787876|gb|CP007597.1|	2.30476e-16	M01232:1:000000000-AP50K:1:1108:16612:24154
Uncultured *Proteobacterium*	3	0.02	gi|110754486|gb|DQ829173.1|	4.81929e-124	M01232:1:000000000-AP50K:1:1104:9072:2651
Salinicola zeshunii	3	0.02	gi|926663123|ref|NR_132717.1|	1.91635e-27	M01232:1:000000000-AP50K:1:1104:21540:24452
Pseudomonas alkylphenolia	3	0.02	gi|675318909|gb|CP009048.1|	1.04277e-55	M01232:1:000000000-AP50K:1:1119:18207:15716
Aminobacter sp.	3	0.02	gi|808352622|gb|KP792998.1|	2.92452e-63	M01232:1:000000000-AP50K:1:1101:14457:21845
Rhodopseudomonas palustris	2	0.01	gi|192282182|gb|CP001096.1|	3.24307e-31	M01232:1:000000000-AP50K:1:1101:10107:11214
Ramlibacter tataouinensis	2	0.01	gi|334728683|gb|CP000245.1|	6.456e-33	M01232:1:000000000-AP50K:1:1101:14558:7299
Pseudoxanthomonas suwonensis	2	0.01	gi|807383831|gb|CP011144.1|	3.79229e-08	M01232:1:000000000-AP50K:1:1105:7165:13578
Sulfuritalea hydrogenivorans	2	0.01	gi|572099409|dbj|AP012547.1|	1.29547e-34	M01232:1:000000000-AP50K:1:1119:15635:22920
Enterobacter asburiae	2	0.01	gi|918042828|gb|CP010360.2|	1.54314e-39	M01232:1:000000000-AP50K:1:1101:11586:14538
Tistrella mobilis	2	0.01	gi|388531416|gb|CP003239.1|	6.19294e-06	M01232:1:000000000-AP50K:1:1119:19058:23555
Pseudoxanthomonas Jiangsuensis	2	0.01	gi|926663118|ref|NR_132712.1|	2.72036e-42	M01232:1:000000000-AP50K:1:1108:18564:16578
Bacillus cereus	2	0.01	gi|925175099|gb|KP998178.1|	6.6322e-117	M01232:1:000000000-AP50K:1:1104:14071:8360
Pigmentiphaga sp.	2	0.01	gi|338319330|gb|JN000323.1|	4.68187e-82	M01232:1:000000000-AP50K:1:1101:17204:19117
Pseudomonas entomophila	2	0.01	gi|95101722|emb|CT573326.1|	6.66362e-77	M01232:1:000000000-AP50K:1:1108:13845:5976
Aeromonas salmonicida	2	0.01	gi|142849896|gb|CP000644.1|	8.28939e-16	M01232:1:000000000-AP50K:1:1119:25813:20025
Serratia sp.	2	0.01	gi|676307183|gb|CP003424.1|	4.6514e-56	M01232:1:000000000-AP50K:1:1105:21737:8086
Marinobacter similis	1	0.01	gi|582024311|gb|CP007151.1|	2.29529e-17	M01232:1:000000000-AP50K:1:1103:13306:7456
Uncultured *Spirochaeta*	1	0.01	gi|294821864|gb|HM049865.1|	1.29462e-79	M01232:1:000000000-AP50K:1:1108:15441:22338
Actinoplanes missouriensis	1	0.01	gi|381368402|dbj|AP012319.1|	2.85154e-61	M01232:1:000000000-AP50K:1:1108:7406:8793
Uncultured *Actinobacterium*	1	0.01	gi|146429963|gb|EF220779.1|	5.52247e-138	M01232:1:000000000-AP50K:1:1108:15175:19488
Brevundimonas sp.	1	0.01	gi|143024276|gb|EF486314.1|	5.65974e-138	M01232:1:000000000-AP50K:1:1105:19075:3508

Brevundimonas subvibrioides	1	0.01	gi|302191744|gb|CP002102.1|	2.9103e-56	M01232:1:000000000-AP50K:1:1119:20428:17355
Sphingomonas asaccharolytica	1	0.01	gi|780749594|gb|KP191993.1|	7.18337e-21	M01232:1:000000000-AP50K:1:1101:7781:20498
Salinicoccus iranensis	1	0.01	gi|343198876|ref|NR_043937.1|	0.00068987	M01232:1:000000000-AP50K:1:1104:17030:4255
Azoarcus sp.	1	0.01	gi|119668705|emb|AM406670.1|	1.4117e-06	M01232:1:000000000-AP50K:1:1119:8538:22070
Cronobacter muytjensii	1	0.01	gi|924333052|gb|CP012268.1|	6.91686e-38	M01232:1:000000000-AP50K:1:1108:20035:10625
Acidiphilium sp.	1	0.01	gi|908660394|emb|LN866594.1|	2.31342e-59	M01232:1:000000000-AP50K:1:1104:13914:10793
Delftia tsuruhatensis	1	0.01	gi|149774731|gb|EF469602.1|	8.57296e-121	M01232:1:000000000-AP50K:1:1104:11900:8800
Xanthomonas campestris	1	0.01	gi|902821146|gb|CP012145.1|	9.16951e-51	M01232:1:000000000-AP50K:1:1101:3105:16542
Kineococcus radiotolerans	1	0.01	gi|196121877|gb|CP000750.2|	4.17197e-88	M01232:1:000000000-AP50K:1:2101:21228:23567
Pusillimonas sp.	1	0.01	gi|687199282|gb|KM054873.1|	4.61262e-83	M01232:1:000000000-AP50K:1:1101:5549:4857
Halomonas campaniensis	1	0.01	gi|641739997|gb|CP007757.1|	1.72049e-88	M01232:1:000000000-AP50K:1:1104:14866:17080
Pseudomonas fulva	1	0.01	gi|333113473|gb|CP002727.1|	2.62623e-51	M01232:1:000000000-AP50K:1:1103:11079:11827
Bradyrhizobium japonicum	1	0.01	gi|736032532|gb|CP010313.1|	9.4916e-05	M01232:1:000000000-AP50K:1:1119:2017:13951
Leptothrix cholodnii	1	0.01	gi|170774137|gb|CP001013.1|	1.99438e-17	M01232:1:000000000-AP50K:1:1101:12071:11566
Cupriavidus metallidurans	1	0.01	gi|93352797|gb|CP000352.1|	1.77221e-38	M01232:1:000000000-AP50K:1:1108:13285:6448
Pseudomonas mendocina	1	0.01	gi|145573243|gb|CP000680.1|	7.95873e-62	M01232:1:000000000-AP50K:1:1105:18267:8397
Xanthomonas oryzae	1	0.01	gi|856757157|gb|CP011961.1|	6.73954e-63	M01232:1:000000000-AP50K:1:1103:15563:18137
Nocardioides sp.	1	0.01	gi|119534933|gb|CP000509.1|	1.15819e-35	M01232:1:000000000-AP50K:1:1101:12688:21346
Mycobacterium chelonae	1	0.01	gi|807052974|gb|CP010946.1|	1.62957e-28	M01232:1:000000000-AP50K:1:1101:15655:1760
Clostridium sp.	1	0.01	gi|723265869|gb|KM454168.1|	9.47417e-141	M01232:1:000000000-AP50K:1:1104:12563:11135
Burkholderia gladioli	1	0.01	gi|772900580|gb|CP009322.1|	1.10349e-15	M01232:1:000000000-AP50K:1:1119:23014:19597
Thiobacillus denitrificans	1	0.01	gi|74055513|gb|CP000116.1|	1.18486e-30	M01232:1:000000000-AP50K:1:1104:15545:11426
Alcaligenes faecalis	1	0.01	gi|913125411|gb|KP859538.1|	1.17868e-72	M01232:1:000000000-AP50K:1:1119:16844:9553
Uncultured *Vampirovibrio*	1	0.01	gi|765567848|gb|KP108877.1|	1.72989e-55	M01232:1:000000000-AP50K:1:1105:22928:21695
Rubrivivax gelatinosus	1	0.01	gi|381376528|dbj|AP012320.1|	1.09005e-25	M01232:1:000000000-AP50K:1:1119:12241:9263
Citrobacter freundii	1	0.01	gi|828983113|gb|CP011657.1|	2.9948e-31	M01232:1:000000000-AP50K:1:1108:18565:17737
Geobacillus sp.	1	0.01	gi|909995672|gb|CP008903.1|	6.13154e-22	M01232:1:000000000-AP50K:1:1103:21864:18986
Herbaspirillum hiltneri	1	0.01	gi|917675518|gb|CP011409.1|	1.82059e-23	M01232:1:000000000-AP50K:1:1101:21629:10741
Caulobacter segnis	1	0.01	gi|295429362|gb|CP002008.1|	4.918e-39	M01232:1:000000000-AP50K:1:1105:9409:21765
Pimelobacter simplex	1	0.01	gi|723622094|gb|CP009896.1|	1.10078e-10	M01232:1:000000000-AP50K:1:1108:6955:12298
Xanthomonas arboricola	1	0.01	gi|910827970|gb|CP012251.1|	3.01283e-32	M01232:1:000000000-AP50K:1:1105:22121:13711

Pseudomonas cremoricolorata	1	0.01	gi|691224436|gb|CP009455.1|	9.24404e-36	M01232:1:000000000-AP50K:1:1105:17844:3878
Bifidobacterium longum	1	0.01	gi|320456974|dbj|AP010889.1|	1.93619e-87	M01232:1:000000000-AP50K:1:1108:25682:17588
Caulobacter crescentus	1	0.01	gi|220962111|gb|CP001340.1|	2.94987e-61	M01232:1:000000000-AP50K:1:1104:9818:9158
Acidithiobacillus caldus	1	0.01	gi|640840007|gb|CP005986.1|	1.68975e-13	M01232:1:000000000-AP50K:1:1101:13004:15267
Pseudomonas simiae	1	0.01	gi|646231607|gb|CP007637.1|	1.28861e-89	M01232:1:000000000-AP50K:1:1105:7226:9188
Uncultured *Curvibacter*	1	0.01	gi|741986181|gb|KM603398.1|	4.83672e-17	M01232:1:000000000-AP50K:1:1101:8990:7351
Polyangium brachysporum	1	0.01	gi|826168461|gb|CP011371.1|	2.87179e-56	M01232:1:000000000-AP50K:1:1108:24108:17213
Burkholderia ambifaria	1	0.01	gi|171994659|gb|CP001026.1|	2.33425e-12	M01232:1:000000000-AP50K:1:1108:20370:3233
Aeromonas veronii	1	0.01	gi|328802836|gb|CP002607.1|	1.54168e-23	M01232:1:000000000-AP50K:1:1103:20874:20124
Bordetella bronchiseptica	1	0.01	gi|408445111|emb|HE965807.1|	8.7383e-17	M01232:1:000000000-AP50K:1:1101:16030:10070
Bacillus gaemokensis	1	0.01	gi|926657055|dbj|LC076295.1|	2.39633e-26	M01232:1:000000000-AP50K:1:1105:19352:19047
Sphingomonas sanxanigenens	1	0.01	gi|569540043|gb|CP006644.1|	2.48935e-27	M01232:1:000000000-AP50K:1:1103:25091:12200
Luteibacter jiangsuensis	1	0.01	gi|926663115|ref|NR_132709.1|	1.69768e-16	M01232:1:000000000-AP50K:1:1105:18796:18324
Comamonas sp.	1	0.01	gi|478895874|gb|KC771559.1|	2.87084e-115	M01232:1:000000000-AP50K:1:1103:4605:7699
Comamonas acidovorans	1	0.01	gi|3608182|dbj|AB009273.1|	3.26534e-75	M01232:1:000000000-AP50K:1:1103:7360:21176
Polaribacter sp.	1	0.01	gi|440546819|gb|JX272927.1|	1.91635e-27	M01232:1:000000000-AP50K:1:1101:26431:14022
Lysinibacillus sp.	1	0.01	gi|902947085|gb|KP728971.1|	1.53549e-71	M01232:1:000000000-AP50K:1:1105:11731:15534
Uncultured *Bacteroidetes/chlorobi*	1	0.01	gi|84663643|gb|DQ211467.2|	3.5341e-105	M01232:1:000000000-AP50K:1:1108:9790:1227
Xanthomonas translucens	1	0.01	gi|828451590|gb|CP008714.1|	1.12576e-26	M01232:1:000000000-AP50K:1:1101:14729:7486
Acidovorax avenae	1	0.01	gi|323371659|gb|CP002521.1|	1.27289e-47	M01232:1:000000000-AP50K:1:1105:15660:23895
Arthrobacter sp.	1	0.01	gi|767256459|gb|CP011005.1|	1.21266e-05	M01232:1:000000000-AP50K:1:1119:24624:19176
Pseudomonas denitrificans	1	0.01	gi|472247168|gb|CP004143.1|	1.23612e-09	M01232:1:000000000-AP50K:1:1101:9871:8087
Bosea sp.	1	0.01	gi|171191219|gb|EU373419.1|	1.97705e-147	M01232:1:000000000-AP50K:1:1119:7758:6111
Sphingomonas taxi	1	0.01	gi|695169020|gb|CP009571.1|	1.33667e-13	M01232:1:000000000-AP50K:1:1104:10083:12187
Methylobacterium populi	1	0.01	gi|179342784|gb|CP001029.1|	8.63565e-07	M01232:1:000000000-AP50K:1:1101:10878:22159
Uncultured ochrobactrum	1	0.01	gi|926657571|dbj|LC001074.1|	1.53081e-28	M01232:1:000000000-AP50K:1:1108:19790:25004
Intrasporangium calvum	1	0.01	gi|315587265|gb|CP002343.1|	3.74369e-40	M01232:1:000000000-AP50K:1:2101:25050:4735
Bradyrhizobium sp.	1	0.01	gi|146189981|emb|CU234118.1|	2.6384e-07	M01232:1:000000000-AP50K:1:1103:12172:8824
Xanthomonadaceae bacterium	1	0.01	gi|321156606|emb|FR774560.1|	7.31561e-87	M01232:1:000000000-AP50K:1:1119:23379:4142
Bacillus pumilus	1	0.01	gi|924878695|gb|KT624198.1|	2.45626e-37	M01232:1:000000000-AP50K:1:1103:20824:18248
Thiomonas intermedia	1	0.01	gi|295794626|gb|CP002021.1|	1.95876e-18	M01232:1:000000000-AP50K:1:1101:22371:21250

Labilithrix luteola	1	0.01	gi|913610804|gb|CP012333.1| e	1.83698e-06	M01232:1:000000000-AP50K:1:1101:20154:9066
Pseudogulbenkiania sp.	1	0.01	gi|345641016|dbj|AP012224.1|	3.29437e-31	M01232:1:000000000-AP50K:1:1104:5848:18614
Caulobacter sp.	1	0.01	gi|167346403|gb|CP000927.1|	1.86858e-42	M01232:1:000000000-AP50K:1:1103:16557:4994
Methylobacterium oryzae	1	0.01	gi|689271676|gb|CP003811.1|	1.76166e-19	M01232:1:000000000-AP50K:1:1108:10675:5885
Mycobacterium rhodesiae	1	0.01	gi|359817839|gb|CP003169.1|	1.43798e-39	M01232:1:000000000-AP50K:1:1104:8915:20740
Uncultured *Sphingobium*	1	0.01	gi|383617482|gb|JQ288598.1|	9.39564e-131	M01232:1:000000000-AP50K:1:1119:15936:12333
Xanthomonas axonopodis	1	0.01	gi|346647687|gb|CP002914.1|	2.86078e-56	M01232:1:000000000-AP50K:1:1105:16003:14237
Vitreoscilla stercoraria	1	0.01	gi|219846304|ref|NR_025894.1|	5.94979e-28	M01232:1:000000000-AP50K:1:1105:17422:1221

Table B3: Biochemical reactions on the GP-1 colorimetric card of the Vitek® 2 Compact System

Biochemical test	Acronym	CDO	TDO	CDO + TDO
D-Amygdalin	AMY	-	-	-
Ala-Phe-Pro arylamidase	APPA	+	-	-
Leucine arylamidase	LeuA	+	+	+
Alanine arylamidase	AlaA	+	+	+
D-Ribose	dRIB	+	+	+
Novobiocin resistance	NOVO	+	+	+
D-Raffinose	dRAF	+	+	+
Optochin resistance	OPTO	+	+	+
Phosphatidylinositol phospholipase C	PIPLC	-	-	-
Cyclodextrin	CDEX	-	-	-
L-Proline arylamidase	ProA	-	-	-
Tyrosine arylamidase	TyrA	-	-	-
L-Lactate alkalinisation	ILATk	+	+	+
Growth in 6.5% NaCl	N.C.6.5	-	-	-
O/129 resistance (comp. vibrio)	O129R	+	+	+
D-Xylose	dXYL	-	+	-
L-Aspartate arylamidase	AspA	+	-	-
β-Glucuronidase	BGURr	-	-	-
D-Sorbitol	dSOR	-	+	-
Lactose	LAC	-	+	-
D-Mannitol	dMAN	+	+	+
Salicin	SAL	+	+	+
Arginine dihydrolase 1	ADH1	+	-	+
β-Galactopyranosidase	BGAR	-	-	-
α-Galactosidase	AGAL	+	+	+
Urease	URE	+	+	+
N-Acetyl-D-Glucosamine	NAG	+	+	+
D-Mannose	dMNE	+	+	+
Saccharose/ Sucrose	SAC	+	+	+
β-Galactosidase	BGAL	+	+	+
α-Mannosidase	AMAN	-	-	-
L-Pyrrolidonyl-arylamidase	PyrA	-	-	-
Polymixin B resistance	POLYB	+	-	+
D-Maltose	dMAL	-	+	-
Methyl-β-D-Glucopyranoside	MBdG	+	+	+
D-Trehalose	dTRE	+	+	+
α-Glucosidase	AGLU	+	-	-
Phosphatase	PHOS	+	-	+
β-Glucuronidase	BGUR	-	-	-
D-Galactose	dGAL	+	+	+
Bacitracin resistance	BACI	+	+	+
Pullulan	PUL	-	-	-
Arginine dihydrolase 2	ADH2s	-	+	-

Table B4: Biochemical reactions on the GN-1 colorimetric card of the Vitek® 2 Compact System.

Biochemical test	Acronym	CDO	TDO	CDO + TDO
Ala-Phe-Pro-Arylamidase	APPA	+	-	-
Hydrogen sulphide production	H2S	+	-	+
β-glucosidase	BGLU	+	+	+
L-Proline arylamidase	ProA	-	+	+
Saccharose/Sucrose	SAC	+	+	+
L-lactate alkalinization	ILATk	+	+	+
Glycine arylamidase	GlyA	-	(+)	-
O/129 resistance (comp. vibrio)	O129R	+	+	+
Adonitol	ADO	+	+	+
β-N-Acetyl-glucosaminidase	BNAG	+	-	+
D-Maltose	dMAN	+	+	+
Lipase	LIP	-	+	-
D-Tagatose	dTAG	-	+	+
α-Glucosidase	AGLU	+	(+)	-
Ornithine decarboxylase	ODC	+	-	-
Glu-Gly-Arg-Arylamidase	GGAA	-	-	-
L-Pyrrolydonyl-arylamidase	PyrA	-	+	-
Glutamyl arylamidase pNA	AGLTp	-	-	-
D-Mannitol	dMAN	+	+	+
Palatinose	PLE	+	+	+
D-Trehalose	dTRE	+	+	+
Succinate alkalinisation	SUCT	+	+	+
Lysine decarboxylase	LDC	+	+	+
L-malate assimilation	IMLTa	-	+	-
L-Arabitol	IARL	-	-	-
D-Glucose	dGLU	+	+	+
D-Mannose	dMNE	+	+	+
Tyrosine arylamidase	TyrA	-	+	-
Citrate (Sodium)	CIT	+	+	+
β-N-Acetyl galactosaminidase	NAGA	-	-	-
L-Histidine assimilation	IHISa	-	-	-
Ellman	ELLM	+	-	+
D-Cellobiose	dCEL	+	+	+
γ-Glutamyl-transferase	GGT	+	+	+
β-Xylosidase	BXYL	+	+	-
Urease	URE	+	+	+
Malonate	MNT	+	+	+
α-galactosidase	AGAL	+	+	+
Coumarate	CMT	+	+	+
L-Lactate assimilation	ILATa	-	+	-
β-galactosidase	BGAL	+	+	+
Fermentation/Glucose	OFF	+	+	+
β-Alanine arylamidase	BAIap	-	-	-
D-Sorbitol	dSOR	+	+	+
5-Keto-D-Gluconate	5KG	-	+	-
Phosphatase	PHOS	+	+	+
β-Glucoronidase	BGUR	-	-	-

Table B5: Biochemical reactions on the YST-1 colorimetric card of the Vitek® 2 Compact System.

Biochemical test	Acronym	CDO	TDO	CDO + TDO
L-Lysine-arylamidase	LysA	-	-	-
Tyrosine arylamidase	TyrA	+	+	(+)
D-Glucose assimilation	dGLUa	+	+	+
D-Raffinose assimilation	dRAFa	+	+	+
L-Rhamnose assimilation	IRHAa	+	+	+
D-Turanose assimilation	dTURa	-	-	-
L-Glutamate assimilation	IGLTa	+	+	+
L-Proline assimilation	IPROa	+	+	+
L-Malate assimilation	IMLTa	+	+	+
β-N-Acetyl glucosaminidase	BNAG	+	-	+
Lactose assimilation	LACa	+	+	+
PNP-N-Acetyl-BD-galactosaminidase 1	NAGA1	-	-	-
Xylitol assimilation	XLTa	-	-	-
D-Trehalose assimilation	dTREa	+	+	+
D-Xylose assimilation	dXYLa	+	+	+
2-Keto-D-Gluconate assimilation	2KGa	+	+	+
Leucine arylamidase	LeuA	+	+	+
Arbutin assimilation	ARBa	+	+	+
Methyl-α-D-Glucopyranoside assimilation	MAdGa	-	+	-
D-Mannose assimilation	dMNEa	+	+	+
D-Sorbitol assimilation	dSORa	+	+	+
Nitrate assimilation	NO3a	+	-	+
DL-Lactate assimilation	LATa	+	+	+
N-Acetyl-Glucosamine assimilation	NAGa	-	(-)	-
Arginine GP	ARG	+	+	+
Amygdalin assimilation	AMYa	+	+	-
D-Cellobiose assimilation	dCELa	+	+	+
D-Melibiose assimilation	dMELa	+	+	+
Saccharose/ Sucrose assimilation	SACa	+	+	+
L-Arabinose assimilation	lARAa	+	+	+
Acetate assimilation	ACEa	+	+	+
D-Gluconate	dGNTa	-	+	+
Erythritol assimilation	ERYa	+	-	-
D-Galactose assimilation	dGALa	+	+	+
Gamma-Glutamyl-transferase	GGT	+	+	+
D-Melezitose assimilation	dMLZa	+	+	+
Urease	URE	+	+	+
D-Galacturonate assimilation	dGATa	+	+	+
Citrate (Sodium) assimilation	CITa	+	+	+
Glycerol assimilation	GLYLa	+	+	+
Gentiobiose assimilation	GENa	+	+	+
D-Maltose assimilation	dMALa	+	+	+
L-Sorbose assimilation	ISBEa	+	+	+
α-Glucosidase	AGLU	+	+	+
Esculin hydrolysis	ESC	+	+	+
Glucoronate assimilation	GRTas	+	+	(-)

Table B6: Biochemical reactions on the BCL-1 colorimetric card of the Vitek® 2 Compact System

Biochemical test	Acronym	CDO	TDO	CDO + TDO
β-Xylosidase	BXYL	+	+	+
β-Galactosidase	BGAL	+	+	+
Ala-Phe-Pro arylamidase	APPA	+	(+)	+
Ellman	ELLM	+	-	+
D-Mannose	dMNE	+	+	+
β-Mannosidase	BMAN	-	-	-
Inulin	INU	-	-	-
Oleandomycin resistance	OLD	+	+	+
L-Lysine arylamidase	LysA	-	+	-
L-Pyrrolydonyl-arylamidase	PyrA	+	(+)	(-)
Cyclodextrin	CDEX	-	-	-
Methyl-D-Xyloside	MdX	-	-	-
D-Melezitose	dMLZ	+	+	+
Phosphoryl choline	PHC	+	-	(-)
D-Glucose	dGLU	+	+	+
Esculin hydrolysis	ESC	+	+	+
L-Aspartate arylamidase	AspA	+	-	+
α-Galactosidase	AGAL	+	+	+
D-Galactose	dGAL	+	+	+
α-Mannosidase	AMAN	-	-	-
N-Acetyl-D-Glucosamine	NAG	+	+	+
Pyruvate	PVATE	+	+	-
D-Ribose	dRIB	+	+	+
Tetrazolium red	TTZ	+	+	+
Leucine-arylamidase	LeuA	+	+	+
Alanine arylamidase	AlaA	+	+	+
Glycogen	GLYG	-	-	-
Maltotriose	MTE	+	+	+
Palatinose	PLE	+	+	+
α-Glucosidase	AGLU	-	-	-
Putrescine assimilation	PSCNa	+	-	-
Polymixin B resistance	POLYB_R	+	+	+
Phenylalanine arylamidase	PheA	+	+	+
Tyrosine arylamidase	TyrA	+	(+)	+
myo-Inositol	INO	-	(+)	-
Glycine arylamidase	GlyA	-	-	-
L-Rhimnose	IRHA	+	+	+
D-Tagatose	dTAG	+	+	+
Growth in 6.5% NaCl	Nacl 6,5%	+	+	+
L-Proline arylamidase	ProA	+	-	+
β-N-Acetyl-Glucosaminidase	BNAG	+	-	+
Methyl-A-D-Glucopyranoside acidification	MdG	-	+	-
D-Mannitol	dMAN	+	+	+
β-Glucosidase	BGLU	+	+	+
D-Trehalose	dTRE	+	+	+
Kanamycin resistance	KAN	+	+	+

12.3 Appendix C: Supplementary data from Chapter 8

Table C1: Composition of the inoculum as identified by 16S amplicon gene sequencing.

Organism/HIT	Cluster size	%	Accession	e-value	FastQ header
Uncultured *bacterium*	758	15,33	gi|187964183|gb|EU536078.1|	8.1793e-112	M01232:3:000000000-AP509:1:1109:21984:4617
Thiobacillus sp.	568	11,49	gi|21490240|gb|AC104439.2|	2.88002e-126	M01232:3:000000000-AP509:1:1118:4725:11028
Serratia sp.	297	6,01	gi|767910003|ref|XM_011509813.1|	5.48532e-88	M01232:3:000000000-AP509:1:1106:3997:11146
Lactobacillus curvatus	193	3,9	gi|855112495|dbj|LC063167.1|	8.10758e-117	M01232:3:000000000-AP509:1:1118:7160:2734
Oxalobacteraceae *bacterium*	190	3,84	gi|695102737|gb|KM187519.1|	1.02854e-130	M01232:3:000000000-AP509:1:1116:28380:10331
Anaplasma marginale	133	2,69	gi|161406800|gb|EU281852.1|	3.20889e-49	M01232:3:000000000-AP509:1:1101:18401:20866
Propionibacterium acnes	100	2,02	gi|657118275|gb|CP006032.1|	7.58931e-11	M01232:3:000000000-AP509:1:1119:15754:20671
Proteus mirabilis	95	1,92	gi|529186758|gb|CP004022.1|	2.25365e-49	M01232:3:000000000-AP509:1:1103:16709:11429
Sphingomonas aerolata	86	1,74	gi|397747027|gb|JX122158.1|	2.77993e-151	M01232:3:000000000-AP509:1:1115:18627:6882
Mycobacterium abscessus	66	1,33	gi|731175139|dbj|AP014547.1|	4.08443e-60	M01232:3:000000000-AP509:1:2106:21844:17301
Uncultured *bacillus*	63	1,27	gi|756420863|gb|KM819153.1|	4.71809e-139	M01232:3:000000000-AP509:1:1118:17463:9315
Methylobacterium oryzae	61	1,23	gi|689271676|gb|CP003811.1|	1.33752e-12	M01232:3:000000000-AP509:1:1106:19002:24894
Uncultured *hymenobacter*	47	0,95	gi|156118672|gb|EU071507.1|	2.40736e-76	M01232:3:000000000-AP509:1:2107:20629:17286
Xanthomonas campestris	42	0,85	gi|167731156|emb|AM920689.1|	5.36666e-66	M01232:3:000000000-AP509:1:1108:15029:2551
Uncultured *microorganism*	26	0,53	gi|379354914|gb|JN907367.1|	3.7191e-125	M01232:3:000000000-AP509:1:1116:7523:5682
Uncultured *janthinobacterium*	22	0,44	gi|726973671|gb|KM391598.1|	1.60488e-28	M01232:3:000000000-AP509:1:1116:21920:1984
Micrococcus yunnanensis	22	0,44	gi|456371513|gb|KC494328.1|	2.91601e-116	M01232:3:000000000-AP509:1:1108:22919:11737
Micrococcus lylae	21	0,42	gi|343527293|gb|JN377810.1|	2.83956e-136	M01232:3:000000000-AP509:1:1109:14306:17738
Moraxella lacunata	21	0,42	gi|310974961|ref|NR_036825.1|	2.78998e-146	M01232:3:000000000-AP509:1:1116:19063:9317
Methylobacterium adhaesivum	21	0,42	gi|397787467|dbj|AB698696.1|	1.32359e-129	M01232:3:000000000-AP509:1:2116:15933:13071
Kytococcus sedentarius	17	0,34	gi|256687298|gb|CP001686.1|	8.97158e-38	M01232:3:000000000-AP509:1:2108:15078:11974
Bradyrhizobium japonicum	17	0,34	gi|736032532|gb|CP010313.1|	0.00104159	M01232:3:000000000-AP509:1:2116:15754:21899
Micrococcus luteus	15	0,3	gi|924426520|gb|KT340107.1|	1.33762e-124	M01232:3:000000000-AP509:1:2107:19638:12271
Enterobacter sp.	13	0,26	gi|755163242|gb|KM975677.1|	6.35686e-108	M01232:3:000000000-AP509:1:2109:14701:13264
Methylobacterium extorquens	11	0,22	gi|240006747|gb|CP001510.1|	9.42148e-41	M01232:3:000000000-AP509:1:1103:13642:6800

Uncultured *planctomycetales*	10	0,2	gi|165968489|gb|EU370841.1|	3.79887e-110	M01232:3:000000000-AP509:1:1101:23480:19322
Uncultured *actinomycetales*	8	0,16	gi|297346871|gb|HM080417.1|	1.77952e-81	M01232:3:000000000-AP509:1:2118:10227:20793
Streptomyces sp.	7	0,14	gi|83367161|gb|DQ304115.1|	7.95108e-132	M01232:3:000000000-AP509:1:1101:13739:9144
Uncultured *alpha*	7	0,14	gi|238953204|emb|FM252843.1|	4.84511e-119	M01232:3:000000000-AP509:1:2102:19296:7153
Jeotgalicoccus sp.	7	0,14	gi|339478260|gb|HQ433462.4|	2.25421e-117	M01232:3:000000000-AP509:1:2106:18957:17265
Methylobacterium sp.	7	0,14	gi|924860953|gb|KP844649.1|	1.33994e-124	M01232:3:000000000-AP509:1:1107:7222:21064
Uncultured *burkholderiales*	6	0,12	gi|375153407|gb|JN634253.1|	1.52545e-108	M01232:3:000000000-AP509:1:1102:9987:24190
Massilia sp.	6	0,12	gi|909841042|gb|CP012201.1|	2.51092e-42	M01232:3:000000000-AP509:1:1112:22601:22277
Staphylococcus simulans	6	0,12	gi|525458232|gb|KC849411.1|	1.02867e-125	M01232:3:000000000-AP509:1:2119:26373:5990
Pseudomonas sp.	5	0,1	gi|646237114|gb|CP007638.1|	1.71217e-128	M01232:3:000000000-AP509:1:1118:2888:16976
Nocardia farcinica	4	0,08	gi|239809049|gb|GQ121036.1|	1.75803e-113	M01232:3:000000000-AP509:1:2113:20587:10742
Stenotrophomonas rhizophila	4	0,08	gi|627787876|gb|CP007597.1|	2.61526e-12	M01232:3:000000000-AP509:1:1115:7673:9906
Pseudomonas putida	3	0,06	gi|764072275|gb|CP010979.1|	1.98641e-23	M01232:3:000000000-AP509:1:2112:23293:10209
Triticum aestivum	3	0,06	gi|669026884|emb|HG670306.1|	2.15231e-11	M01232:3:000000000-AP509:1:2101:21951:18288
Uncultured *proteobacterium*	3	0,06	gi|380837539|gb|JN866455.1|	4.92267e-109	M01232:3:000000000-AP509:1:2108:23764:7159
Uncultured *sphingomonas*	3	0,06	gi|209421835|gb|FJ192215.1|	1.01952e-135	M01232:3:000000000-AP509:1:1103:7999:19905
Raoultella ornithinolytica	3	0,06	gi|480474683|gb|CP004142.1|	1.11967e-70	M01232:3:000000000-AP509:1:1114:25420:16733
Moraxella catarrhalis	2	0,04	gi|696614304|gb|CP007669.1|	3.7455e-120	M01232:3:000000000-AP509:1:1115:11810:19048
Klebsiella oxytoca	2	0,04	gi|828945832|gb|CP011618.1|	1.0052e-145	M01232:3:000000000-AP509:1:1118:19660:13418
Sphingomonas sp.	2	0,04	gi|891166527|dbj|AB974275.1|	6.14656e-133	M01232:3:000000000-AP509:1:1113:22893:15103
Serratia marcescens	2	0,04	gi|560171871|emb|HG326223.1|	4.6514e-56	M01232:3:000000000-AP509:1:2114:20248:4308
Acinetobacter baumannii	2	0,04	gi|924308394|gb|KR067422.1|	8.91185e-101	M01232:3:000000000-AP509:1:1104:28274:15034
Microbacterium sp.pcob-2	2	0,04	gi|1536917|emb|Y07842.1|	1.30256e-144	M01232:3:000000000-AP509:1:1103:11596:13596
Pseudomonas mendocina	2	0,04	gi|328915200|gb|CP002620.1|	5.00336e-51	M01232:3:000000000-AP509:1:2107:25212:15815
Nocardioides sp.	2	0,04	gi|582014856|dbj|AB851233.1|	1.04139e-120	M01232:3:000000000-AP509:1:2110:10268:12871
Microbacterium lacticum	2	0,04	gi|636559479|ref|NR_115539.1|	3.69928e-130	M01232:3:000000000-AP509:1:2119:21110:19045
Massilia aurea	2	0,04	gi|674640772|emb|LM994753.1|	1.83108e-83	M01232:3:000000000-AP509:1:1114:2093:16065
Geodermatophilus obscurus	2	0,04	gi|284061874|gb|CP001867.1|	4.10421e-41	M01232:3:000000000-AP509:1:2119:20178:19098
Delftia sp.	2	0,04	gi|333741867|gb|CP002735.1|	5.96473e-153	M01232:3:000000000-AP509:1:1117:25756:6583

Lactobacillus graminis	2	0,04	gi|636558859|ref|NR_114916.1|	6.20179e-123	M01232:3:000000000-AP509:1:1103:10997:15857
Acinetobacter johnsonii	2	0,04	gi|924818590|gb|KR002423.1|	2.52978e-20	M01232:3:000000000-AP509:1:2104:21737:23769
Uncultured *oxalobacteraceae*	2	0,04	gi|726973613|gb|KM391540.1|	3.12496e-85	M01232:3:000000000-AP509:1:1105:3974:6136
Uncultured *arthrobacter*	2	0,04	gi|675911485|gb|KJ624618.1|	3.0049e-96	M01232:3:000000000-AP509:1:2116:12997:15128
Uncultured *massilia*	1	0,02	gi|381283158|gb|JQ290991.1|	1.33762e-124	M01232:3:000000000-AP509:1:1114:25421:19461
Klebsiella variicola	1	0,02	gi|749145882|gb|CP010523.1|	2.23832e-122	M01232:3:000000000-AP509:1:1115:7017:14487
Klebsiella pneumoniae	1	0,02	gi|742679781|gb|CP006738.1|	1.01233e-140	M01232:3:000000000-AP509:1:1119:6267:22428
Uncultured *actinobacterium*	1	0,02	gi|238953099|emb|FM252738.1|	6.10323e-138	M01232:3:000000000-AP509:1:2115:24401:23467
Comamonadaceae bacterium	1	0,02	gi|635597072|dbj|AP014569.1|	6.51302e-05	M01232:3:000000000-AP509:1:1116:13824:18895
Amycolatopsis sp.	1	0,02	gi|524853206|gb|KF218953.1|	1.80536e-93	M01232:3:000000000-AP509:1:2114:26258:14128
Pantoea vagans	1	0,02	gi|918042713|gb|KT375349.1|	1.28426e-154	M01232:3:000000000-AP509:1:1110:19752:12931
Wisteria floribunda	1	0,02	gi|743433694|gb|KM103376.1|	5.78447e-22	M01232:3:000000000-AP509:1:1105:11074:21401
Streptomyces xiamenensis	1	0,02	gi|914410687|gb|CP009922.2|	5.55178e-24	M01232:3:000000000-AP509:1:1102:15304:8772
Uncultured *cyanobacterium*	1	0,02	gi|583826816|emb|HG917244.1|	1.35904e-114	M01232:3:000000000-AP509:1:2104:28317:15758
Lactobacillus sakei	1	0,02	gi|925176646|gb|KR006319.1|	1.64046e-22	M01232:3:000000000-AP509:1:2119:27571:17207
Micrococcus sp.	1	0,02	gi|772519990|gb|KP455684.1|	6.35686e-108	M01232:3:000000000-AP509:1:1102:17878:2632
Marinobacter hydrocarbonoclasticus	1	0,02	gi|381337457|emb|FO203363.1|	2.65169e-111	M01232:3:000000000-AP509:1:1111:16247:13402
Sphingomonas aurantiaca	1	0,02	gi|19702229|emb|AJ429237.1|	2.2422e-122	M01232:3:000000000-AP509:1:1115:18700:24678
Uncultured *microbacterium*	1	0,02	gi|220682307|gb|FJ542914.1|	2.22254e-127	M01232:3:000000000-AP509:1:1114:26379:15885
Corynebacterium sp.	1	0,02	gi|7110403|gb|AF227825.1|	3.43829e-75	M01232:3:000000000-AP509:1:1115:6367:4211
Bacillus licheniformis	1	0,02	gi|693585404|gb|KM099482.1|	1.33994e-124	M01232:3:000000000-AP509:1:1109:5184:8785
Citricoccus zhacaiensis	1	0,02	gi|914408088|gb|KR047787.1|	4.84511e-119	M01232:3:000000000-AP509:1:1104:20473:14049
Stenotrophomonas maltophilia	1	0,02	gi|922795094|gb|CP011305.1|	1.10394e-80	M01232:3:000000000-AP509:1:2101:24892:9431
Sphingobium sp.	1	0,02	gi|345135066|dbj|AP012222.1|	1.29111e-59	M01232:3:000000000-AP509:1:1105:16935:20207
Uncultured rubrivivax	1	0,02	gi|926657283|dbj|LC000787.1|	5.41698e-28	M01232:3:000000000-AP509:1:1106:20732:13199
Uncultured *nocardioides*	1	0,02	gi|242346274|gb|GQ183231.1|	1.06373e-105	M01232:3:000000000-AP509:1:2113:22448:6696
Acinetobacter sp.	1	0,02	gi|676899856|gb|KJ920200.1|	2.30656e-102	M01232:3:000000000-AP509:1:2109:16395:7657
Staphylococcus hominis	1	0,02	gi|387289151|gb|JQ795928.1|	2.33133e-92	M01232:3:000000000-AP509:1:2103:24785:16004
Burkholderia ambifaria	1	0,02	gi|171994659|gb|CP001026.1|	1.8736e-68	M01232:3:000000000-AP509:1:2115:10543:16741

Uncultured *actinomycete*	1	0,02	gi|674275465|gb|KJ834239.1|	2.29857e-102	M01232:3:000000000-AP509:1:2115:12939:7932
Microbacterium paraoxydans	1	0,02	gi|390633708|gb|JX130390.1|	7.82442e-43	M01232:3:000000000-AP509:1:2116:16399:11030
Nocardia sp.	1	0,02	gi|919401576|gb|KT372140.1|	4.92267e-109	M01232:3:000000000-AP509:1:1108:11235:17508
Alicycliphilus denitrificans	1	0,02	gi|329308025|gb|CP002657.1|	1.26923e-109	M01232:3:000000000-AP509:1:1104:8017:13148
Navicula arenaria	1	0,02	gi|768321723|gb|KJ961668.1|	2.85477e-131	M01232:3:000000000-AP509:1:2111:21697:9816
Staphylococcus epidermidis	1	0,02	gi|445065299|gb|KC213934.1|	4.61961e-149	M01232:3:000000000-AP509:1:1111:17986:5625
Uncultured *micrococcaceae*	1	0,02	gi|389547004|gb|JQ401932.1|	4.84511e-119	M01232:3:000000000-AP509:1:2105:26939:16197
Chelatococcus sp.	1	0,02	gi|919432499|gb|CP012398.1|	5.12723e-79	M01232:3:000000000-AP509:1:1113:8287:20627
Rhodococcus aetherivorans	1	0,02	gi|816214082|gb|CP011341.1|	2.61526e-12	M01232:3:000000000-AP509:1:2102:16762:3942
Uncultured *kineosporiaceae*	1	0,02	gi|389546973|gb|JQ401901.1|	2.90047e-121	M01232:3:000000000-AP509:1:2105:18357:10574
Klebsiella sp.	1	0,02	gi|861556752|gb|KR189681.1|	5.95439e-153	M01232:3:000000000-AP509:1:1118:14359:15601
Uncultured *klebsiella*	1	0,02	gi|257074314|gb|GQ418033.1|	2.27021e-112	M01232:3:000000000-AP509:1:1118:14031:23764
Ramularia mali	1	0,02	gi|610723276|gb|KJ504778.1|	1.66192e-146	M01232:3:000000000-AP509:1:2103:20885:11351