Cape Peninsula University of Technology

BIOSURFACTANT PRODUCING BIOFILMS FOR THE ENHANCEMENT OF NITRIFICATION AND SUBSEQUENT AEROBIC DENITRIFICATION

by

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Thesis submitted in fulfilment of the requirements for the degree

Master of Engineering: Chemical Engineering

at the

Cape Peninsula University of Technology

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Cape Town

November 2018

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DECLARATION

I, **Yolanda Phelisa Mpentshu**, declare that the contents of this thesis represent my own unaided work, except where specifically acknowledged in text and that the thesis has not previously been submitted for academic examination towards any other qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology and the National Research Foundation of South Africa.

30/11/2018

Signed **Date**

ABSTRACT

Wastewater treatment methods have always gravitated towards the use of biological methods for the treatment of domestic grey water. This has been proven to offer a series of advantages such as the reduction of pollution attributed to the use of synthetic chemicals; therefore, this decreases the requirement of further costly post primary treatment methods. Although such biological methods have been used for decades, their efficiency and sustainability has always been challenged by inhibitory toxicants which renders the systems redundant when these toxins are prevalent in high concentrations, culminating in the deactivation of biomass which facilitates the treatment. In most instances, this biomass is anaerobic sludge. Hence, the proposal to utilize biofilms which are ubiquitous and self-sustaining in nature. The use of engineered biofilms in wastewater treatment and their behaviour has been studied extensively, with current research studies focusing on reducing plant footprint, energy intensity and minimal usage of supplementary synthetic chemicals. An example of such processes include traditional nitrification and denitrification systems, which are currently developed as simultaneous nitrification and aerobic denitrification systems, i.e. in a single stage system, from the historical two stage systems. However, there is limited literature on biofilm robustness against a potpourri of toxicants commonly found in wastewater; particularly for total nitrogen removal systems such as simultaneous nitrification and denitrification (SND).

This study was undertaken (aim) to assess the ability of biosurfactant producing biofilms in the removal of total nitrogen in the presence of toxicants, i.e. heavy metals and phenol, as biosurfactants have been proven to facilitate better mass transfer for pollutant mitigation. Unlike in conventional studies, the assessment of biosurfactant producers in total nitrogen removal was assessed in both planktonic and biofilm state. Since biofilms are known to have increased tolerance to toxic environmental conditions, they were developed thus engineered using microorganisms isolated from various sources, mainly waste material including wastewater as suggested in literature reviewed, to harness microorganisms' possessing specified traits that can be developed when organisms are growing under strenuous environments whereby they are tolerant to toxic compounds. The assessment of these engineered biofilms involved the development from individual microorganisms to form biofilms in 1L batch reactors where the isolated microorganisms were grown in basal media containing immobilisation surfaces.

The assessment of the total nitrogen efficiency was conducted using Erlenmeyer flasks (500mL) in a shaker incubator, with the biofilm TN removal efficiency being assessed in batch systems to ascertain simultaneous nitrification and denitrification rates even in the

presence of heavy metals (Cu^{2+}, Zn^{2+}) and C_6H_5OH. Ambient temperature and dissolved oxygen conditions were kept constant throughout the duration of biofilm development with microorganisms (initially n = 20) being isolated for the initiation of biosurfactant studies which included screening.

Results indicated that the engineered biofilms, constituted by biosurfactant producing organisms (n = 9), were consisiting of bacteria (97.19%), Protozoa (2.81%) and Archaea (0.1%) as identified using metagenomics methods. Some of the biosurfactant produced had the following functional group characteristics as determined by FTIR: $-CH_3$-CH_2, deformed N-H, $-CH_3$ amide bond, C-O, C=O, O-C-O of carboxylic acids, and C-O-C of polysaccharides. Other selected microorganisms (n = 5) tolerated maximum concentrations of the selected toxicants (Cu^{2+}, Zn^{2+} and C_6H_5OH) of 2400 mg/L, 1800 mg/L and 850 mg/L, respectively. Enzyme analysis of the total nitrogen removal experiments indicated a higher nitrogen removal rate to be the *Alcanigene* sp. at 180 mg/L/h.

Keywords: Ammonium, Biofilms, Denitrification, Heavy metals; Nitrification, Phenol, Wastewater

DEDICATION

*To my parents, my mother Mrs. Tamara Nokwakha Mpentshu (**Mthimkhulu**) and my father Mr. Mbuzeli Nelson Mpentshu (**S'dindi**).*

I thank you for your undying support throughout the ups and downs of this study.

ACKNOWLEDGEMENTS

I acknowledge:

- God for giving me the strength to endure and complete this study and thesis,
- My supervisor Prof. S.K.O. Ntwampe for the mentorship, support and guidance throughout the course of my studies,
- Ms. Ncumisa Mpongwana for her technical and personal advice,
- My siblings Kanyisa Mpentshu-Tinti, Lwandisa Mpentshu-Gongqa and Aviwe Mpentshu for their unwavering support,
- My dear friends and colleagues Vuyiseka Nkqenkqa, Zandile Jingxi, Melody Mukandi, Cynthia Dlangamandla for their support throughout the study. Your words of support are much appreciated,
- BioERG members and the Biotechnology staff Mr. N. Khatywa, Mr. M. Tobin and Ms. M. Mobo for allowing me to utilise their resources, and
- NRF–DAAD (Grant number 109767) and the CPUT URF (RK16) for financially supporting the research study

RESEARCH OUTPUTS

The following research outputs represent the contributions by the candidate to scientific knowledge and development during his doctoral candidacy (2016-2018):

The following DHET-accredited research articles and conference proceedings were published from the studies reported in this thesis:

1. **Y.P. Mpentshu**, S.K.O. Ntwampe, and N. Mpongwana. 2018. Biosurfactant Producing Metallo-Phenolic Tolerant Microbial Consortia for Nitrification. 10th Int'l Conference on Advances in Science, Engineering, Technology & Healthcare (ASETH-18) Nov. 19-20, 2018 Cape Town (South Africa). Pp 221-224, ISBN - 978-81-938365-2-1, https://doi.org/10.17758/EARES4.EAP1118254

The following DHET- accredited research articles were published from other studies related to this thesis:

2. S. Mbulawa, S.K.O. Ntwampe, M. Basitere, **Y. Mpentshu**, C. Dlangamandla and B.S. Chidi. 2018. Bio-delipidation of Dissolved Air Flotation Pre-treated Poultry Slaughterhouse Wastewater. 10th Int'l Conference on Advances in Science, Engineering, Technology & Healthcare (ASETH-18) Nov. 19-20, 2018 Cape Town (South Africa). Pp 33-38, ISBN - 978-81-938365-2-1, https://doi.org/10.17758/EARES4.EAP1118213
3. S. Mbulawa, S.K.O. Ntwampe, M. Basitere, **Y. Mpentshu**, C. Dlangamandla and B.S. Chidi. 2018. Microbial Screening for Bio-delipidation System of Pre-treated Poultry Slaughterhouse Wastewater: Impact of Solvents, Metal Ions and Detergents on Lipase Activity. 10th Int'l Conference on Advances in Science, Engineering, Technology & Healthcare (ASETH-18) Nov. 19-20, 2018 Cape Town (South Africa). Pp 210-215, ISBN - 978-81-938365-2-1, https://doi.org/10.17758/EARES4.EAP1118252
4. N. Mpongwana, S. K. O. Ntwampe, L. Mekuto, E. A. Akinpelu, S. Dyantyi, **Y. Mpentshu**. 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*. DOI: 10.2166/wst.2016.070
5. C. Dlangamandla, S. A. Dyantyi, **Y. P. Mpentshu**, S. K. O. Ntwampe, M. Basitere. 2016. Optimisation of bioflocculant production by a biofilm forming microorganism from poultry slaughterhouse wastewater for use in poultry wastewater treatment. *Water Science and Technology*. 73(8), pp.1963-1968. DOI: 10.2166/wst.2016.047

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Description
BRE	Biological removal efficiency
EPS	Extracellular polymeric substance
FTIR	Fourier-transform infrared spectroscopy
HPLC	High Performance Liquid Chromatography
NH_4-N	Ammonium nitrogen
NO_3-N	Nitrate nitrogen
NO_2-N	Nitrite nitrogen
TN	Total nitrogen
WWTP	Wastewater treatment plant

GLOSSARY

GLOSSARY/BASIC TERMS AND CONCEPTS

Biodegradable: the substance's capability to decompose via microorganisms or biological methods (Courtens et al., 2014),

Biosurfactants: are biodegradable, low toxicity extracellular cellular compounds produced by microorganisms with an ability to reduce surface tension (Mulligan, 2005),

Denitrification: Microbial conversion of NO_2-N and the residual NH_4-N to nitrogen gas and some NO_3-N (Courtens et al., 2014),

Eutrophication: is an excessive richness of the nutrients in rivers or other bodies of water, which causes a dense growth of microbial and plant life, driven by human related activities (Smith, 2003),

Extracellular polymeric substances: high molecular weight natural polymers secreted by microorganisms cellular membranes and biomass for protection from external environmental factors and maintenance of structure (Yang et al., 2011),

Heavy metal(s): a high density and high atomic weight metal commonly found in trace quantities in nature (Nakamura et al., 2017),

Nitrification: Microbial oxidation of total nitrogen to NO_2-N (Courtens et al., 2014),

Simultaneous nitrification and aerobic denitrification (SND): total nitrogen removal in biological systems that occurs in a single stage configured reactor system whereby nitrification and denitrification happen simultaneously(Chen et al., 2018),

Toxicity: the degree of harm a particular substance is capable of inflicting on biological life (Sinha et al., 2012), and

Wastewater: the resulting water after human consumption and/or usage culminating in industrial and domestic run off carrying a consortium of dissolved pollutants including suspended solids (Fu et al., 2009).

LAYOUT OF THESIS

The aim of this study was to use a biosurfactant producing biofilm to completely eradicate total nitrogen via nitrification and subsequent denitrification under phenolic conditions in the presence of heavy metals.

The thesis is divided into the following chapters:

Chapter 1: Introduction. The chapter provides a background on potable water shortages and wastewater treatment methods' applicability in domestic water treatment systems. It also contains a brief explanation of the important methods currently used in wastewater treatment highlighting justifications as to the importance of the research undertaken.

Chapter 2: This chapter discusses theory and currently used methods for total nitrogen removal, biofilm engineering methods and conditions, and the current methods for biosurfactant production and characterisation. It further discusses the role of heavy metals and other pollutants including their inhibitory effects in wastewater treatment.

Chapter 3: This chapter reports on the experimental procedure undertaken for this study and outlines methodology and analytical techniques followed for specific measurements, including stock solution preparation and equipment used to ensure the fulfilment of the stated objectives. Consulted methodologies reported elsewhere and used for thesis are properly referenced.

Chapter 4: This chapter articulates the results obtained from the different phases of the study and the interpretation of the results whereby comparative analysis was undertaken to elucidate previously obtained results and these are reported in this thesis with detailed exposition being rendered for analysed and interpreted experimental data.

Chapter 5: This chapter highlights the overall conclusions and elucidates the important results achieved and lists recommendations for further research as a continuation from the current study.

Chapter 6: References

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Background

Freshwater source contamination primarily contributes to water shortages globally. Therefore, treatment and recycling of wastewater is vital. The sustainability for fresh water sources in semi-arid regions such as South Africa is of utmost importance. By effectively, treating the wastewater, even when such treated wastewater is not recycled, can protect both aquatic and terrestrial life, if it is to be disposed off into raw and/or fresh water sources. Sources of contaminants in industrial wastewater vary, thus its quality characteristics are different for wastewater from different industries (Bogardi et al., 2012). Environmentally benign wastewater treatment systems are mostly biological as they use suitable microorganisms, which form conglomerates of biofilms, and /or granules in cases whereby sldge is used.

Biofilms are a consortium of different microorganisms attached to a surface of a carrier. They have a protective layer, which also facilitates their adhesion onto the carrier for survival under different conditions. As part of their beneficial properties, biofilms have the ability to self-clean and survive using available nutritional sources (Wingender and Flemming, 2011). They have the ability to withstand harsh environmental conditions through processes that include quorum sensing that enables microorganisms to communicate and strengthen their physiological properties, thus resistance to toxic contaminants in the wastewater they are exposed to (Shrout and Nerenberg, 2012). These properties render them useful for water treatment purposes (Berne, Kysela and Brun, 2010).

The removal of ammonium-nitrogen in wastewater is a process that requires nitrifying and denitrifying microorganisms. Nitrifying bacteria are the type of microorganisms that have the ability to carry-out the process of ammonium-nitrogen assimilation under varying conditions. Using biofilms under limited dissolved oxygen levels can combat the competition and limitations encountered in heterotrophic bacteria. The use membrane aerated bioreactors (MABR) can also assist not only in the removal of ammonia-nitrogen but also organic carbon (Sator *et al.*, 2004). Nitrification that occurs naturally, can also occur as a result of microorganisms being attached to material or surfaces. It has been found that nitrification rates are highest in dissolved oxygen rich or under aerobic conditions. In the aquatic environment, the maximum concentration for total nitrogen (TN) is 1g-N/L as higher

concentrations were shown to be toxic to fish of commercial value. Nitrifying microorganisms, due to their sensitivity have to be grown at specific conditions with controlled acidity levels and dissolved oxygen levels, as they are susceptible to detrimental acidic conditions thus lower pHs (Crab *et al.*, 2007). With organisms extracellular polymeric substances to protect against such acidic conditions. Other extracellular polymeric constituents released by microorganisms include biosurfactant, which are different from the extracellular substances facilitating biofilm attachment.

These biosurfactants are produced by a variety of organisms to increase the solubility of pollutants in bioremediation processes and can reduce the surface tension of liquefied matrices thus improve the mass transfer of pollutants in wastewater. However, the production of biosurfactants on an industrial scale is relatively expensive; therefore, the use of microorganisms that have the ability to produce these compounds in-situ for bioremediation purposes has become a cost effective strategy of exploiting their functionality and environmental benignity. The necessity of these compounds arose from their added advantages such as biodegradability, relatively low toxicity which render these compounds environmentally friendly and relatively inexpensive,and be used with other microorganisms (Thavasi et al., 2007; Ilori et al., 2007).

1.2 Research problem

Large quantities of wastewater containing different ammonium based compounds are produced through different industrial activities annually. This in turn, results in the contamination of water bodies when such water is released without treatment causing eutrophication, which requires intense treatment strategies. The common contaminants found in domestic wastewater are detergents and plant nutrients from soil run-off including human waste, which is constituted by urine that has urea, another source of ammonium nitrogen contamination, including faecal matter. Commonly used strategies to reduce total ammonium-nitrogen content from wastewater treatment plants (WWTP) are nitrification and denitrification; both these methods use microorganisms that are capable to oxidise ammonium-nitrogen to different nitrogenous by-products, which are further reduced to nitrogen gas. Traditionally, the two methods are operated in two different stages with current methods indicating that they can occur in a single stage system.

However, the ability of nitri-denitrifying microorganisms is challenged by the bioavailability of the nitrogenous compounds and the prevalence of toxic pollutantsin the wastewater to b

treated. In this study, microorganisms grown under controlled concentrations of C_6H_5OH and ammonium based compounds as total nitrogen were used in simultaneous nitrification and aerobic denitrification (SND). These microorganisms were further used in a biofilm state, since biofilms are known to have a higher toxicity tolerance thus degradation efficiency of pollutants which in a sessile state than in a planktonic state due to the protective mechanism provided when they are in a sessile state. Due to the complexity and varying degrees of toxicity, it is vital to introduce a pollutant for the testing of the microbial/biofilm strength particularly for SND system. Phenolic compounds including heavy metals constitute part of wastewater, as they can be found in most wastewater from petroleum to pharmaceutical industries. This necessitated the need to find and explore ways for the SND in the presence of these pollutants, as they are toxic to both humans and animals if wastewater is discharged without pollutant eradication and perhaps their biodegradation.

1.3 Hypothesis

The growth of these microorganisms as they are found in the environment is easily harnessed for biosurfactants and ultimately the removal of TN in wastewater in the presence of C_6H_5OH and selected heavy metals. However, challenges due to the presence of dual contamination are foreseen as these microbes are yet to undergo such harsh environmental conditions. The microbial consortium in the form of a biofilm can thus be able to degrade and remove total nitrogen using SND mechanism. However, owing to the presence of contaminants ie C_6H_5OH and heavy metals, slight inhibition is predicted.

1.4 Research Questions

The following research questions were developed for the research study:

- Are nitrifyers able to produce biosurfactants?
- What conditions best allow for the formation of nitrifying and aerobic denitrifying biofilms?
- Can the engineered biofilms withstand the effects of C_6H_5OH and heavy metals while sustaining biosurfactant production?
- What is the effect of biosurfactant production in simultaneous nitrification and subsequent denitrification?
- Are the biofilms sufficiently robust to allow nitrification and subsequent denitrification in under metallo-phenolic conditions?

1.5 Research Aims and Objectives

The aim of this study was to use biosurfactant producing biofilms to completely eradicate total nitrogen via nitrification and subsequent aerobic denitrification under metallo-phenolic conditions.

The research was divided into 3 Phases:

Phase 1: Aim 1: Isolation and Identification of biosurfactant producing microorganisms for nitrification and denitrification.

Objective 1: To isolate and identify a microbe or a consortium with high nitrogen removal rates, and

Objective 2: To evaluate whether the isolate (s)/consortia can produce biosurfactants using emulsification index assessments

Phase 2: Aim 2: To assess the nitrification of the isolates under metallo-phenolic conditions.

Objective 1: To assess the microorganisms' tolerance to C_6H_5OH and heavy metals,

Objective 2: Identify the isolates which retained the highest nitrification efficiency under metallo-phenolic conditions,

Objective 3: Sort and reclassify the isolate categories; producers and non-biosurfactant producers by assessing their ability to remove nitrogenous compounds under metallo-phenolic conditions, and

Objective 4: Assess and quantify the activity responsible for nitrifying and denitrifying enzymes for the highest performing isolates.

Phase 3: Aim 3: To assess the rate of total nitrogen removal by biofilms engineered to constitute the total nitrogen removal consortia.

Objective 1: To reassess the rate of nitrification and aerobic denitrification under phenolic conditions using the biofilms, and

Objective 2: To validate the robustness of biofilms in nitrification and denitrification under selected heavy metal conditions.

1.6 Significance of study

This study will benefit the wastewater treatment industry to ensure safe treatment of wastewater that could be detrimental to the health of human, aquatic and terrestrial life, using

engineered biofilms. Further research studies may be derived from the success of this study, as new strategies are required to develop robust large scale WWTP.

1.7 Delineation of the study

The following were not researched in this study:

- The large scale application of total nitrogen removing biofilms as all assessments were conducted at a laboratory scale,
- The mechanisms and enzymology associated with biosurfactant production,
- The quantification of overall oxygen transfers in used (Erlenmeyer flasks) and miniaturised bioreactors, and
- Field application of the produced biosurfactants and engineered biofilms.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Water scarcity challenges in South Africa

In the early 2000s, in South Africa an introduction of the water usage registration requirement was legislated against water wastage and to regulate use for commercial purposes. The regulations in place seek to quantify the water utilised by the numerous industries as studies have revealed that agro-processing is one to the highest consumers of surface waters contributing to water shortage challenges. The agricultural sector contributes to the pollution and runoff into rivers and other water bodies, contaminating such water with dissolved nitrogenous compounds due to fertilizer application for crop growth enhancement (Mendez-Barrientoz et al., 2016).

2.2 Ammonium-nitrogen and its by-products toxicity

The level of ammonium-nitrogen toxicity has been found to be associated with pH as reported by Russo et al. (1981) who noticed that the toxicity of ammonium-nitrogen is relatively high under alkaline conditions. To detoxify this contaminant in most industrial wastewater, conventional methods using microorganisms in planktonic state are often used; however, these microorganisms are relatively weaker in such a state compared to those attached to surfaces, i.e. biofilms, which are formed by a consortium of microorganisms (Liu et al., 2016). Another advantage in the removal of TN for treatment systems using biofilms, is that simultaneous nitrification and denitrification (SND) can be easily achieved with the biodegradable COD also being significantly reduced. By using biofilms, bioreactor performance allows for hydraulic retention time (HRT) adjustments, and DO requirement variations in order to manage the bioremediation process (Hocaoglu *et al.*, 2011). Additionally, although the roughness of the attachment surface carrier, biofilm formation is dependent on the strength of electrostatic forces and the rate of movement of essential nutrients within the biofilm which restrict the rate of mass flow of important nutrients. Overall biosurfactant producing biofilms can reduce such effects (Aslam, 2008).

The non-removal of total ammonium-nitrogen in wastewater including its by-products, can results in the overdosage of nutrients into receiving water bodies which can lead to algae formation, the major cause of eutrophication. To solve such a challenge, constructed

wetlands in certain instances have been found to be efficient which is a process facilitating ammonium-nitrogen and NO_x loading into the atmosphere contributing to the prevalence of pseudohalogens in the stratosphere, increasing global warming. Furthermore, the use of wetlands requires minimal rainfall as runoff from these constructed wetlands could lead to recontamination of surface water bodies. Therefore, this renders this method a seasonal solution with minimal environmental sustainability (Gregory et al., 2012).

The minimum allowed concentrations of the ionised form of ammonium-nitrogen, is 0.012mg/L in aquatic environments with higher concentrations being found to contribute to environmental toxicity of aquatic life. The presence of and type of ammonium-nitrogen including its by-products present in water is largely determined by the water temperature and pH whereby at both low pH and temperature, an increase in the unionized form of ammonium-nitrogen was found to increase (Gregory et al., 2012).

Generally, the removal of total nitrogen (TN) using biofilms, can cause operational challenges including clogging and sustained flux can cause periodic pressurization within a bioreactor resulting in biofilm detachment thus fouling. However, for processes such as nitrification, increased efficiency has been reported where minute ammonium-nitrogen concentrations were to be removed using engineered biofilms. The use of fixed-film reactors has been found to be proficient with efficiency reports showing 99.69% removal of NO_3-Ns using methanol to sustain the biofilm (Wang et al., 2009). In river water used by villagers in South Africa, ammonium nitrogen has been found to affect foetus growth resulting in stillbirth due to the ingestion of contaminated river water, due to the negligence of some industries operating in these regions in particular however water is discharged without proper treatment into water (river) bodies (Sun and Nemati, 2012).

2.3 Total nitrogen (TN) removal

The presence of ammonium-nitrogen and its by-products herein referred to as total nitrogen (TN) in various matrices renders this compound ubiquitous as it is found in both aquatic and terrestrial environments due to its production by anthropogenic activities and in excrement of wildlife (Körner *et al.*, 2001). In high concentrations in the aquatic environment, it is toxic to aquatic organisms; although, it is a metabolic by-product of fish excrement. Additionally TN concentrations are as a result of contributions from external sources including fertilizer run-off into water bodies and from spillages including inadequately treated wastewater from treatment plants to remedy TN contamination, suitable bioreactors can be used with some

being fixed-film (biofilm) reactors. Bioreactor removal efficiency of TN is susceptible to internal and external operational including environmental factors.

For example, an increase in temperature to 40˚C summer temperature in regions of South Africa favours an increase in the TN oxidation rates whereas a decrease to 6˚C and at extremely high temperatures above 50˚C, results in the reduced TN removal process. Similarly, saline conditions attributed to heavy metals and co-contaminants can also be an inhibiting factor in the removal of TN as observed in marine environments, leading to observed cumulative presence of ammonium nitrogen in receiving water bodies which negatively affects aquatic life (Sudarno et al., 2011). In ammonium nitrogen oxidation processes, i.e. nitrification, fluctuation in the distribution of overall dissolved oxygen in the biofilm matrix can negatively affect the process and this could be a result of competition for nutrients amongst the microbial species resulting in the low TN removal (Han et al., 2013).

The susequent denitrification is alsoinfluenced by a number of factors, i.e. dissolved oxygen (DO) whereby a high concentration of DO is detrimental to the anoxic state of denitrification. This results in an increase in the oxidation of NO_2-N to NO_3-N culminating in the disruption of the denitrification process in systems whereby simultaneous nitrification and denitrification systems are employed (He et al., 2009). Therefore, the establishment of the correlation of numerous factors around the removal of total ammonium nitrogen is required, which is the initial step in the removal of TN in wastewater treatment plants. The TN removal process occurs in two steps and is initiated by AOB which are typically abundant in aquatic environments. In the first step, the microorganisms capable of carrying out the nitrification process which is the microbial oxidation of ammonium/ammonia to NO_3-N, ensues; however, environmental and sudden changes in pH and the availability of DO have interrelated effects on the growth and mechanism of action of nitrifying organisms; thereby, affecting the overall efficiency of the process (Kim et al., 2006).

Similarly, the presence of toxicants such as phenol (C_6H_5OH) and cyanide can have similar detrimental effects on nitrification and denitrification (Papadimitriou et al., 2009). Some studies reported 99.8% ammonium nitrogen removal in the presence of phenol. Therefore, by reducing inhibitory effects of C_6H_5OH which is largely dependent on the concentration and overall process conditions, in order to have minimal impact on the removal of ammonium nitrogen (nitrification step) is desirable. However, when the microorganisms are no longer able to neither tolerate nor degrade phenol, this may affect the level and rate of nitrification culminating process redundancy. This then can create a phenomenon for high C_6H_5OH

biodegradation proportional to the level of nitrification without the toxicant (phenol) while the ammonium nitrogen accumulating within the process (Kim *et al.*, 2006).

The toxicity of accumulated ammonium nitrogen in aquatic systems and has been found to temper with the metabolic activities in fish species thereby causing problems such as physical fatigue in naturally active fish (Sinha et al., 2012). The use of biological treatment for TN removal offers a vast list of advantages, one of which is the absence of by-product residue that may require the wastewater to be subjected to further treatment which would require additional energy and costs (Mook et al., 2012). Most microorganisms capable of TN removal can tolerate high saline conditions; albeit below 10/L (Wang et al., 2016).

Biological wastewater treatment has been advanced with the use of membrane bioreactors where efficiency has been an ongoing challenge. This has been addressed through the utilization of membrane technology for the removal of TN whereby the use of MBR systems offers the option of simultaneous nitrification and denitrification including suspended solid removal thereby reducing the operational time required in conventional TN removal processes (Fu et al., 2009). Although MBR technology provides for required minimal operation footprint, low number plant personnel, high throughput rates for treated wastewater implies less overall costs; however, these membrane reactors have been shown to have a number of limitations such as the inefficiency in the transfer or the distribution of overall DO to maintain nitrification consortia with sparging being counterproductive for the aerobic denitrification process as it requires low concentrations of DO (Hocaoglu et al., 2011).

The microorganisms responsible for carrying out TN can be sourced in various matrices namely; soils where *Archea* and other lithotrophic nitrifyers are found including other industrial wastewater. Quantification of nitrification including denitrification rates by these organisms can be executed using reaction kinetics methods which quantifies the nitrification and denitrification performed over specified time intervals (Xiao et al., 2017; Tao et al., 2017). Environmental conditions such as lower temperatures have also been shown to have an impact on nitrification and denitrification rates; conditions such as batch experiments under low temperatures regions a temperature range of 3-20˚C allows for maximum denitrification rates (Welander and Mattioson, 2003). Overall, fluctuations in temperature can have varying effects in the overall ammonium nitrogen removal. At low temperatures of 10˚C, significant ammonium nitrogen removal can still be achieved in comparison to temperature above 30˚C where minimal ammonium nitrogen removal can be observed, indicating that temperature as an influential factor can affect TN removal (Yao et al., 2013).

2.3.1 Nitrification

In solving the challenges of toxic ammonium nitrogen in wastewater, municipalities' wastewater treatment plants use the nitrification process, which occurs as result of the action of ammonium nitrogen oxidizing bacteria that act in a two-step process that converts ammonia into NO_2-N and subsequently NO_2-N into NO_3-N (Ruiz et al., 2006). However, this process does not result in the production of nitrogen gas. The process required to convert NO_3-N subsequently to nitrogen gas is denitrification (Bothe et al., 2000). Accumulation of NO_2-N during nitrification can be a result of low dissolved oxygen concentrations as reported by Chen et al. (2006). Generally, in natural environments, *Nitrosomas* sp. are the types of microorganisms involved in the oxidation of ammonia into NO_2-N whereas the *Nitrobacter* sp. are responsible for the oxidation of NO_2-N to NO_3-N and at low pH these microorganisms operate aerobically (Chen et al., 2013).

$$NH_4{}^+ + 1.5O_2 \rightarrow 2H^+ + H_2\,O + NO_2 \quad (2.1)$$

$$NO_2 + 1.5O_2 \rightarrow NO \quad (2.2)$$

Nitrification is followed by denitrification, an anoxic process in two separate processing units and when compared to the simultaneous nitrification and denitrification (SND), DO conditions do differ for these two types of nitrogen removal processes (Wang *et al.*, 2009). For SND, NO_3-N or NO_2-N is further reduced ultimately to nitrogen gas in a process of aerobic denitrification, which is facilitated by organisms such as *Pseudomonas* sp*.* or *Thiobacillus denitrificans.* For the complete removal of TN, different enzymes are required for the major processes (Lu et al., 2014).

The primary enzymes involved in the nitrification process include hydroxylamine monooxygenase which catalyses the oxidation of ammonium into NH_2OH whereas the enzyme hydroxylamine oxyreductase catalyses the oxidation of NH_2OH into NO_2 (Ge et al., 2015). Nitrification is divided into steps, i.e. nitritation and nitratation where one is susceptible to temperature changes as temperatures above 30°C reduce the rate of nitrification (Ni *et al.*, 2008). Additionally, pH also plays a significant role in the initial and rate limiting step of nitrification; a trait which is also observed for SND whereby alkaline conditions above pH 7 consequently results in decreased rates whereas at neutral pH TN removal was determined to be highest (He et al., 2009). Similarly, an MBR is commonly used for TN removal due to its

versatility, with added advantages including efficiency improvement in the presence of contaminants (He et al., 2009; Sarioglu et al., 2009).

2.3.2 Denitrification

The initiation of the denitrification process depends on the prevalence of both NO_3-N reductase enzyme producing microorganisms which catalyses the reduction of NO_3-N including NO_3-N reductase producers which converts NO_3-N to NO_2-N then nitric oxide. Denitrification, the second step in the TN removal process, with the products being a weak acid and nitrogen ultimately in the form of gas. In SND this process generally occurs under aerobic conditions and at 3-30˚C. However, temperatures responsible for high denitrification rates are around 15˚C in biofilm systems. This is evidenced by the versatility offered by consortia biofilms, with interspecies nutrient transfer allowing for the manipulation of conditions in biofilm systems for effective denitrification (Welander and Mattiasson, 2003). Particular when the biofilms are exposed to biosurfactants, would assist in transfer and biodegradation of pollutants.

Nitrous oxide reductase producers initiate the last step of the denitrification process where N_2O is reduced to environmentally benign N_2 gas. The microorganisms responsible for these processes are typically abundant in soil environments (Levy-Booth *et al.*, 2014). The process of denitrification which ultimately leads N_2 gas production which is then reintroduced into the nitrogen cycle (Levy-Booth *et al.*, 2014). As an individual process, denitrification in microbial process as pH sensitive where pH needs to be kept above 7 to avoid the environmental impact of the toxic nitrous oxide which has been found to have global warming effect thus impacts on the ozone layer (Welander and Mattiasson, 2003).

2.3.3 Simultaneous nitrification and denitrification

Simultaneous nitrification and denitrification (SND) refers to the removal of total ammonium-nitrogen in WWTP, which occurs through the mechanism of aerobic nitrifyers facilitated by heterotrophic denitrifying bacteria. This two-stage process occurs optimally at low temperatures and requires minimal dissolved oxygen concentrations allowing for one stage design of the TN removal system, resulting in the minimization of operational costs.

However, the SND encounter a number of operational challenges that include the presence of high concentration of toxicants in the wastewater to be treated.

SND in wastewater results in the prevention of NO_3-N combination accumulation through the development of minimal dissolved oxygen zones that occur in the nitrification stage in microbial systems. In biofilm microbial treatment systems, high NO_x (a combination of NO_2-N and NO_3-N) concentrations are reduced because of the layer of dissolved oxygen, which exists at the core of biofilm structures (Yilmaz *et al.*, 2007). Nitrification in biofilms is generally affected by mass transfer which typically transports nutrients within the biofilm structure to encourage microbial growth; however, in the absence of adequate mass transfer in biofilms the result is a decrease in microbial efficiency; thereby; rates of TN transfer subsequently decrease. This problem can then be counteracted by the presence of biosurfactants, which can be increased by mass transfer in biofilm structures allowing for maximum microbial efficiency (Zhao *et al.*, 2017).

2.4 Biofilm engineering

Biofilms consist of a variety of microorganisms adhered to material and or surfaces with the assistance of extracellularly produced polymeric substance (EPS) that result from individual planktonic microorganisms. For survival, these microorganisms exchange physiological properties, thus are protected from environmental stress by the layer of EPS. Biofilms can largely be found in moisture containing settings (Yang et al., 2011; Shi and Zhu, 2009). The use of periphytic biofilms in wastewater treatment is a developing trend commonly used to treat biological waste. Naturally, biofilms occur under varying environmental conditions, even in the absence of natural light. Due to the nature of the environment in which biofilms are growing; however, susceptibility to toxicants could decrease TN removal even when exposed to irradiation. These biofilms are highly effective at an optimum temperature of 25˚C as higher and lower temperatures have been determined to have a negative impact on the growth of biofilm in terms reported overall thickness (Zhao et al., 2017).

Overall, the process of forming biofilms herein reported as biofilm engineering involves the purposeful adhesion of individual microorganisms on immobilization surface and biofilm carriers. This is feasible due to the proliferation of microorganisms or their sporangia in nutrient rich conditions in the presence of carriers, which culminates in the formation of biofilm on carriers. The process of quorum sensing is then initiated through cell to cell interaction and promoting symbiosis between different types of microorganisms. Although biofilm formation is affected by a number of factors, i.e. the nature of the immobilization

surface; where uneven surface tend to allow rapid biofilm growth, acidity and temperature including microbial proliferation and or proliferation can also affect the long term efficiency thus performance biofilm formation (Simões et al., 2010).

The microorganisms in a biofilm communicate and exchange genes including other materials that assist in the biofilms' survival. In such biofilms, individual microorganisms rearrange the structure within a biofilm with the organization depending on nutrient and dissolved availability oxygen including pollutant tolerance. Obligate aerobes would normally be found on the outer surface of the biofilm thus are exposed to atmospheric or a high concentration of dissolved oxygen and pollutants in the liquid matrix; albeit, being protected from the harsh environmental conditions by a layer of EPS with the EPS layer being responsible for maintaining the biofilms shape (De Kreuk *et al.*, 2005; Nogueira *et al.*, 2002). In a laboratory environment, it has been found that using biofilms, reduced experiment time by up to 4 days (Nogueira *et al.*, 2002). Biofilms are also robust in that several metabolic processes within the biofilm can be performed using the same biofilm with simultaneous and sequential biological processes, This can be due to a variety of microorganism in the biofilm which are building blocks of the consortia capable of different metabolic processes under specific environmental conditions, within which the biofilm performance can be altered due to nutrient availability. The use of biofilms in TN studies has increased for the development of efficient wastewater treatment technologies, to incorporate specific biofilm combinations to achieve specific outcomes (Wang *et al.*, 2016).

However, excessive biofilm formation in wastewater systems on a large scale causes problems such as filter clogging and contamination of the treated water by dead biomass, challenges which can be persistent. However, these traits although viewed negatively, can be used to the advantage of waste treatment systems; as biofilms can even perform best under harsh conditions with the process supportive be in the biodegradation of recalcitrant pollutants for longer periods. This is because biofilms are physically and biochemically stronger than the conventional planktonic microorganisms. Therefore, biofilm engineering, i.e. inducement for development of biofilms as collections of different microorganisms that are attached to a given surface either naturally and/or microbial debris for integration in bioreactors is encouraged (Van Houdt and Michiels, 2005).

Biofilms in comparison to planktonic microorganisms can sustain themselves due to the symbiotic relationship formed by microorganisms forming the biofilms. However, these relationships can be counter productive for some of the species within the biofilm as extracellular secretions form one microorganism could be toxic to other microorganisms.

Assessing the strength of biofilms, can be achieved by testing the biofilms against heavy metal contamination using the minimal biofilm-eradication concentration (MBEC) method which quantifies microbial resistance to the toxicity of a variety of heavy metals (Harrison *et al.*, 2005). Biofilms in the form of bacterial consortia can be induced whereby only desired microorganisms may be included in the consortium to achieve the desired effect (Kesaano and Sims, 2014).

2.5 Production and characterization of biosurfactants

Biosurfactants are microbial compounds that are produced by microorganisms under suitable conditions. These compounds are able to lower the surface tension with properties to emulsify hydrophobic compounds in aqueous matrices (Bodour et al., 2003). The production of these compounds can aid in both the solubilization thus increase the bioavailability of contaminants. Unlike chemically synthesized surfactants, biosurfactant have relatively low toxicity (Bodour and Miller-Maier, 1998). Biosurfactants characteristics vary, depending on the type of microorganisms they are produced from and emulsification is used to determine their usability (Youssef et al., 2004). The formation of a stable emulsion using different hydrocarbons is then indicative of the efficacy of the biosurfactants. Some of these biological compounds due to their extensive properties have been found to have antimicrobial properties. Das et al (2008) found that some types of biosurfactants with antimicrobial activity are produced by *Bacillus subtilis*, an organism commonly involved in the degradation of phenol, a recalcitrant contaminant.

Biosurfactants, due to their benignity attributed by their biodegradability, renders these compounds suitable for use in a variety of industries to solve different environmental challenges. These challenges include among many, the bioremediation of soil contaminated with hydrocarbons (Hemlata et al., 2015). Due to their non-toxicity, these biosurfactants can also be used in the food industry as bio-emulsifiers; however, clinical trials are required to ensure safety particularly for human consumption (Liu et al., 2015). Due to their versatility, biosurfactants can withstand extremes of pH, i.e. 2 - 12 and their stability has been shown in a study in which the compounds were produced by three isolates of *Bacillus* sp. grown on molasses and cheese waste as a primary source of nutrients (Joshi et al., 2008). This broadens the acidity/alkalinity spectrum and allows for their production including extraction under low pH conditions where most processes are operated to incur minimal cost (Hemlata et al., 2015).

2.5.1 Quantification methods for biosurfactants

Methods for quantification of biosurfactants vary depending on the availability of analytical (characterization) analyses and the fermentation conditions in which a suitable isolate utilized for the production of these molecules; which influences the selection of downstream processes. General methods used for the confirmation of biosurfactant production include an oil displacement test which flatten the oil droplet symbolizing a positive test for the production of the biosurfactant (Silva et al., 2014). However, this is not the only determining factor or method as a conclusion cannot be made from such a single method. A combination of other positive results using different methods are required for the confirmation of biosurfactant production provided that this includes the determination of the emulsification index which uses the formation of an emulsion as a positive indicator whereby the height of the emulsion as an indication of the solubilisation of oil over a 24 h period is determined; furthermore, a haemolytic test which can be used with blood agar plates for validation with positive results showing clear zones around the bacterial colony solution after the biosurfactant has been added. The characterisation of the biosurfactant produced can include FTIR where the recovered, dried extractant is mixed with KBr to determine different components and functional groups of the biosurfactant (Swapna et al., 2016).

2.5.2 Biosurfactant mechanisms in pollutant treatment systems

Since components in wastewater can include a variety of pollutants such as hydrocarbons which require emulsification, biosurfactant facilitated emulsification is then required due to the perceived toxicity, i.e. on the basis of hydrocarbon pollutant of the process (Daverey and Pakshirajan, 2016). Higher efficiency in the facilitation of removal of PAH contaminated soil whereby biosurfactants (rhamnolipids) from *Pseudomonas aeruginosa* and *Bacillus subtilis,* respectively, were used to study overall biodegradation efficiency of PAH in soil with results indicating a 63% efficiency was observed where synthetic surfactants Tween 80 and Surfactin showed 23% efficiency (Lai et al., 2009).

Since biosurfactants are typically water soluble as they possess a hydrophilic-end that complexes with water molecules and the hydrophilic-end which interacts with the hydrocarbon; they can be used in processes requiring a multitude of environmental remediation interventions. For example, with the functional groups in some biosurfactant being negatively charged metallic ions can attach to functional groups in the biosurfactants.

Biosurfactants can therefore be used in heavy metal detoxification in soils as they offer an added advantage as there is minimal risk of further environmental contamination that requires further treatment as their residue can be assimilated by natural processes (Juwarkar et al., 2007). Furthermore, biosurfactants are efficient in a pH range of 3-7 with efficiency and or functionality decreasing with increasing acidity. This was observed when Saponin, i.e. a biosurfactant was used for the removal of heavy metal, Pb^{2+} and Zn^{2+} in contaminated soils (Hong et al., 2002).

Additionally, high performance liquid chromatography can also be used to characterize biosurfactant constituents in a liquid sample unlike with FTIR where dried samples are used. The characterization of biosurfactants in a liquid form, forms the basis for molecular weight classification with high molecular weight biosurfactants being identified as being polymeric type whereas low molecular weight biosurfactants are classified as phospholipids (Nitschke and Costa, 2007). Other chromatography methods include thin layer chromatography, which has been used as a method for characterization of biosurfactants from agro-waste with its reduction being facilitated by organisms isolated from the agro-waste using a range of colors to detect positive production of biosurfactant. This method, however, does not specify the components of the biosurfactants produced (Luna et al., 2013). Acid precipitation is another method used for the extraction of biosurfactants from crude fermentation broth. Its principle is based on the formation of micelles (Liu et al., 2015).

In metal recovery biosurfactant, usage through metal precipitation, for high recovery rate, has been reported at 91% under alkaline conditions (Mulligan, 2005). Furthermore, biosurfactants have been reported to be metal chelators, with FTIR being employed to assess the chelation effect. For example, a chelation effect was reported for a *Bacillus subtilis* produced biosurfactant against chromium (Cr^{2+}) contamination over 72h whereby the method of metal precipitation was used (Swapna et al., 2016). In these studies, FTIR was the preferred method used for determining chelation ability.

2.5.3 Biosurfactant production conditions and effects in wastewater treatment

Biosurfactant production heavily depends on the operational conditions of the bioreactors used whereby the high concentration of dissolved oxygen concentration can alter production efficiency of the biosurfactants producing isolate. Similarly, sparging can result in high foam formation necessitating the use of antifoams, which can disrupt the metabolic mechanism for certain microbial isolates. Furthermore, the supplement of foam suppressors can result in the

falsification of the quantity of the biosurfactant being produced (Fahim et al., 2013). Biosurfactant production culminates in a soapy feel of fermentation broth due to the lowering of the surface tension and this characteristic is responsible for the formation of stable emulsions. For high biosurfactant production, broth agitation might be required although this can also result in foam formation (Ushikubo and Cunha, 2014).

2.6 Phenol (C_6H_5OH) as a metabolic inhibitor

C_6H_5OH is a compound that is formed as a by-product in various industrial processes in the chemical industry. It is relatively toxic to humans including aquatic life and is typically found in the same industrial wastewater that contains ammonium-based contaminants. Similarly, to the treatment of other pollutants in industrial wastewater, the treatment of industrial wastewater containing phenol, suitable treatment processes for the simultaneous removal or biodegradation of these compounds (phenol/ammonium nitrogen) is therefore required, in particular if such wastewater is to be reused, i.e. effective treatment processes for the restoration of the quality parameters of the water to its original is required (Khatami et al., 1998; Kottuparambil et al., 2014). As with any other pollutant, the higher the concentration of the toxicant, the lower the biodegradation efficiency, as the employed microorganisms proliferation and metabolic activity will be reduced due to the inhibitory effect of C_6H_5OH in the wastewater; although biodegradation of C_6H_5OH concentration up to 2500 mg/L has been reported in nitrifying experiments (Amor et al., 2005).

For C_6H_5OH biodegradation agitation was found to positively contribute to the overall biodegradation of C_6H_5OH whereby 700 mg/L of C_6H_5OH was biodegraded completely at an agitation speed of 250 rpm with microbial growth and biodegradation efficiently being observed to increase with agitation speed when the pH was 6.5 (Shourian et al., 2017). Industries that contribute to the presence of C_6H_5OH include the steel industry; which produce this pollutant as a by-product of steel production, culminating in the inhibition of nitrification indirectly whereby its biodegradation in aerobic processes, has been determined to be influenced by an overall DO concentration and heavy metal presence (Kim et al., 2006). There is an abundance of treatment methods for C_6H_5OH including other phenolic compounds commonly found in wastewater treatment systems due to their prevalence and concentration increasing with globalization due industrial activity.

Generally, C_6H_5OH in nitrification processes is an inhibiting factor at concentrations above 300 mg/L. However, in systems where total nitrogen removal is performed using microbial

consortia, moving-bed reactors containing biofilm were determined to be effective; albeit, microbial activity was severely decreased, thus increasing the overall toxicity which intensified the continuous exposure of the contaminant whereby there is simultaneous removal of COD and total nitrogen, microbial activity can be reduced especially in the presence of high concentrations of C_6H_5OH loading. C_6H_5OH shock loading are necessary to strengthen microbial consortia destined for C_6H_5OH biodegradation (Alves et al., 2017).

2.7 Heavy metal contamination in wastewater

The presence of heavy metals in nitrification systems has been found to be toxic, with toxicity associated with nickel, a commonly used metal in industrial applications (Ge et al., 2015). Overall, heavy metal contamination of biological systems is profound as they are difficult to remove even in biological systems in which bioaccumulation is the dominant mechanism of removal than assimilation. However, technological combination of biological and conventional methods such as acid extraction where Cu^{2+},Zn^{2+} and Cd^{2+} can be removed by pH reduction, to sustain biofilm efficiency, might provide an alternative to mitigate against metal toxicity. Furthermore, the presence of sulphate reducing bacteria in biofilms is also an added advantage in the removal of heavy metals; additionally,it was evident because 98% heavy metal removal was achieved using a biofilm consortia an indicated by Edwards and Kjellerup (2013).

The presence of heavy metal contaminants in wastewater is a result of industrial processes for which the wastewater is illegally dumped into municipal wastewater treatment plants (WWTP). The type of metal pollutants in wastewater determines how it reacts, including its cumulative effect on the microorganisms used in the wastewater treatment plants. Heavy metals commonly found in WWTP include Pb^{2+}, which is from the manufacturing industries of glass and printing material whereas Ag^{3+} is a by-product of the production of electronics. The microorganisms responsible for nitrification have been shown to be susceptible to high concentrations of these heavy metals (Çeçen et al., 2010). Therefore, heavy metal may result in the inhibition of the TN removal, a phenomenon which was observed in a study whereby Cu^{2+} and Zn^{2+} were used as potential inhibition substances with the results revealing significant inhibition by these metals (Nakamura et al., 2017).

2.8 Hydrocarbon, oil and grease contamination in wastewater

Numerous hydrocarbons including other organic based contaminants are also commonly found in wastewater, as they constitute a part of the manufacturing industrial processes i.e. as lubricants and/or sealers. There are processes in place for the physical of agglomerated hydrocarbons biodegradation of these contaminants that have been developed to handle these types of contaminants. These however, have been affected by fluctuations in temperature particularly for large scale operations where a temperature decrease results in a decrease in solubility of the contaminants which results in their non-eradication and reduced bioavailability (Margesin et al., 2013). However, such challenges can be addressed using biosurfactant and/or biosystems with emulsification capabilities in order to reduce organic pollutant hydrocarbon agglomeration formation thus reduced biodegradability (Deepa et al., 2015). It is for these reasons that a suitable process for TN removal in the presence of toxicants such as phenol and heavy metal perhaps including hydrocarbons must be designed.

2.9 Summary

Wastewater treatment is necessary for the recycling and reuse of potable (process) water to allow for optimal usage of the precious resource for the growing population. This is necessary because most of the available water is either contaminated or saline. Most methods for removal of contaminants in wastewater combine chemical precipitation methods and biological methods; this however is degradatory to the environment due to the residual synthetic chemical compounds found in the treated wastewater; hence, the use of a green chemistry approach through the introduction of sole biological methods especially for TN removal. The use of biological methods ensures safer methods of wastewater treatment for reuse especially through the use of biofilms in TN removal as they are naturally robust; however, due to the presence of EPS, mass transfer limitations can be inhibited; hence, the use of organisms with a capability of synthesizing biological surfactants which have been proven to improve mass transfer with minimal or no impact on microbial efficiency in the biofilms. The robustness of the biofilms to perform a defined function can be further assessed through the addition of further contaminants to stimulate actual industrial processes in particular for WWTP systems in which the presence of C_6H_5OH and heavy metals is prevalent. Biofilms through manipulation, i.e. biofilm engineering by manipulating various conditions in which they grow, can be harnessed to achieve the desired end-result of

the designed process with the combination of the process conditions needing further investigation, for enhanced process efficiency.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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3.1 Microbial isolation and characterisation

The microorganisms used in this study were sourced from different locations with the intention of discovering their hidden properties of evolved microbes found in these locations. The organisms used were sourced using the swab method where a sterile swab was introduced to a potential microorganism source and liquid media was used for the initial growth of the microorganisms. Pure cultures were obtained using the streak plate method where single colonies were attained through the use of saline serial dilutions. The use of single colonies in the initial stages of the study was to use the microbes in planktonic form to compare the overall efficiency in both biofilm and planktonic state. The isolates used in the study were identified using metagenomics, to ascertain the constituents (individual organisms) forming the engineered biofilms.

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix with the primers presented in Table 3.1. The PCR products were run on a gel with gel extraction being conducted using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on an ABI 3500xl Genetic Analyzer (Applied Biosystems, US) for each reaction thus sample CLC Bio Main Workbench v7.6 was used to analyse the files generated by the ABI 3500XL Genetic Analyzer and the microbial denitrification results were obtained by a BLAST search (NCBI) https://blast.ncbi.nlm.nih.gov.

Figure 3.1: Primers used for amplification

16S-27F 16S rDNA sequence 5’ AGAGTTTGATCMTGGCTCAG 3’
16S-1492R 16S rDNA sequence 5’ CGGTTACCTTGTTACGACTT 3’

3.2 Biofilm development in reactors (batch)

The inoculation of microorganisms into biofilm reactors was done using 36 hrs old cultures cultivated in a rotary shaker (37˚C)at a speed of 121 rpm, using a in sterile basal medium (pH 7) to allow for maximum microbial growth. A spectrometer (Jenway 7305 Spectrophotometer, Bibby Scientific Ltd, UK) was used to quantify growth at a wavelength of 660nm (OD_{660nm}) whereby turbidity was used to determine the isolates' growth rate using absorbance. The glass reactors (1.5 L) for engineered biolfims containing 1.2L of basal media were assembled and connected to a water bath to stabilize overall temperature at 37˚C with which was found to yield maximum microbial growth according to previous studies (Zhao et al., 2017). Aeration to allow for sufficient dissolved oxygen transfer was done through the use of diffusers connected to a medium sized air pump whereby each reactor had 3 diffusers submerged in the liquid media adjacent to the biofilm carriers for even distribution of air.

3.3 Biosurfactant production from isolates

Biosurfactants are surface-active compounds commonly used to improve hydrocarbon and toxicant dispersion thus bioavailability under different environmental conditions. In this study, biosurfactants were the preferred surface tension reducing agents biofilm reactors in order to overcome mass transfer challenges of pollutants including dissolved oxygen with the biofilms. The biosurfactants were produced in sterile glass Erlenmeyer flasks (250mL) containing mineral salt media at a pH of 7 in a shaker incubator (121 rotations per minute) for 5 days at 34˚C.

3.4 Biosurfactant screening tests

3.4.1 Emulsification tests

Samples were collected from the biosurfactant production medium and centrifuged at 4000g for 20min using a centrifuge. Cell free supernatants (10mL) were transferred to clean conical tubes (15mL)whereby a volume (1mL) of hydrocarbon (diesel, kerosene and vegetable oil) was aliquoted into each tube. Subsequent to intense vortexing for thorough emulsification, emulsified samples were left for 24 h at ambient temperature to allow for emulsification stability quantified via emulsification index where a stable emulsion would be a positive result (Singh et al., 2004).

3.4.2 Oil spreading/displacement method

This method is described in Deepa et al. (2015) uses the principle of oil displacement where a petri dish was used to allow the hydrocarbon droplet to be in place in the presence of biosurfactant aliquots. Aliquots (100µL) of the hydrocarbon were then added to the water containing the biosurafactants, the dispersing of the oil resulting from the addition of the supernatant was recorded as a positive result. For increased visibility, a non-reactive dye was introduced in the form of crystal violet, commonly used in microorganism identification.

3.5 Extraction and purification of biosurfactant produced

The purification of the produced biosurfactant was initiated through foam fractionation to separate the foam from the media. The foam fractionation was conducted using a two port flask (250mL) connected to an air pump (Resun air pump, AC-9906, Resun®, China) with silicone tubing. The extraction of the biosurfactant was conducted by further supplementing the foam formed with liquid extracts and cold acetone including ethyl acetate, which were found to be a suitable combination for efficient extraction of biosurfactant. The vaporisation of the excess acetone from samples was done in a desiccator (5.8 L Duran desiccator DN12491, Duran® group, Germany), resulting in a cake, which was analysed using the FTIR (Spectrum Two FT-IRTM spectrometer, PerkinElmer Inc., USA) for the identification of functional groups in the extracted biosurfactants.

Figure 3.2: Fractionation contraption for biosurfactant recovery

3.6 Phenol (C_6H_5OH) and heavy metal tolerance tests

For the assessment of microbial resistance to phenol, microorganisms were plated on solid media (nutrient agar) containing various concentrations of C_6H_5OH and heavy metals up to 850mg/L and heavy metals up to 2400mg/L respectively. and incubated at 37˚C for a minimum of 24 h with a growth being observed. The growth of microorganisms was visually observed for comparative growth analysis, with an increase in the concentration of C_6H_5OH and heavy metals (Zn^{2+} and Cu^{2+}) being used due to their prevalence in most wastewater treatment systems. This method however did not include agar diffusion as per the standard for toxicant tolerance tests because growth was done for qualitative purposes to establish the estimated maximum growth achievable since biofilms were to be utilized for the actual tolerance experiments and wastewater treatment efficiency.

3.7 Total nitrogen removal experiments

For the planktonic microorganisms, Erlenmeyer flasks (250 mL) covered in foil and cotton wool, were used. The isolated microorganisms were initially grown in solid media (nutrient agar 31g/L) at 37˚C as monoseptic cultures under aerobic conditions. Overnight cultures (24 hrs) were aseptically transferred into 50 mL basal media (pH 7), enriched with yeast extract .Enrichment was done to aid microbial growth. The liquid cultures were incubated at 37˚C for 48 h in the rotary (121 rpm) shaker incubator (Labwit ZWYR-240 shaking incubator, Labwit® Scientific, Australia). The experiments for the biofilm reactors were conducted at 37˚C determined to be optimal for planktonic batch-based cultures. Aeration i.e. DO was supplied through sparging via air pumps (Resun air pump, AC-9906, Resun®, China) with air diffusers (Mott element 6500, Mott Corporation, United States of America) attached for even air distribution.

3.8 Analysis of constituents constituting total nitrogen

Residual NH_4-N, NO_2-N and NO_3-N concentrations were quantified as per manufacturers' instruction using Merck ammonium-nitrogen (NH_4-N) (00683), nitrite-nitrogen (NO_2-N) (110057) and nitrate-nitrogen (NO_3-N) (14773) test kits. A Merck spectroquant® NOVA 60 was used to quantify the concentration of the analytes. The NH_4-N test kit works on the Berthelot reaction method between ammonia, chlorine and phenolic compounds to form indophenol dyes. The nitrate test kit makes use of concentrated sulphuric acid in the presence of a benzoic acid derivative. Nitrites were determined according to the method of Rider and Mellon

in which a reaction occurs between nitrite ions and 4-aminobenzenesulfonic acid and 1-aminoaphthalem, resulting in a reddish-pink colour which can be read at 520nm. The pH for each sample was measured using a Crison Basic 20 pH meter subjected to a routine daily calibration. The quantification of the population of microorganisms was conducted using a Jenway 7315 UV/visible spectrophotometer (Camlab, UK) at a wavelength of 600 nm.

3.9 Ammonium-nitrogen conversion essay experiments

The selected microorganisms were assessed for enzyme activity associated with NH_4-N, NO_2-N and NO_3-N degradation. This was achieved by growing the microorganisms in basal media containing 46 mg/L concentration of ammonium nitrogen and 15 mg/L of model toxicants (C_6H_5OH, Cu^{2+} and $Zn^{2+)}$ in a shaker incubator for 120 h to allow for maximum possible growth. The incubated cultures harvested and subsequently centrifuged at 4000g for 15 min, were used to form a cell pellet. The pellet was then washed (n = 3) with sterile deionised water to rinse off the toxicants initially spiked into the growth media.

The pellets were then resuspended in 20 mM phosphate buffer (pH 7) which was found to be conducive for enzyme stability (Hermanová et al., 2015). Cell lysis was done using a sonicator at 5 sec pulse intervals for 30 min to ensure complete cell lysis. The extraction of the enzymes was then conducted using ice cold acetone added to the cell lysis extracts whereby two phases here observed in the tubes. Finally, the samples were centrifuged to settle the residual acetone allowing for the enzyme solution to be extracted and resuspended into the phosphate buffer (pH 7). The procedure was conducted in ice to maintain the enzyme structure and to prevent the deactivation of the extracted enzymes. Enzyme activity was then assessed using NH_4-N as a substrate (46 mg/L). An experiment (5 min) was conducted whereby sampling was done per minute. The samples were then analysed using Merck test kits and a Nova Spectroquant to quantify residual nitrogen species in the samples.

3.10 Microbial identification

3.10.1 Planktonic microorganisms identification

The microorganisms were incubated in fresh solid media (Nutrient agar) and an external analysing institution was used for the identification of the microorganisms, which were selected as the highest in the total nitrogen removal experiments. The 16S with primer selection was conducted for the identification of the microorganisms. The microorganism identification was categorised into two, where the two highest nitrifyers and denitirifyers

including biosurfactant producers were identified using the 16S rDNA, prior to utilising in biofilm engineering.

3.10.2 Biofilm identification

The microorganisms in this study were inoculated into basal media in 1L reactors whereby adequate supply of both heat in the form of a heated water jacket using a water bath was used to ensure consistency in temperature conditions. The reactors were constantly supplied with nutrients in the form of yeast extract infused basal media for maximum microbial growth on a 3-week basis. In ensuring the microorganisms were firmly attached for increased biofilm formation efficiency, course sponge-like cubes were inserted after 48 h incubation of the original inoculum. Dissolved oxygen supply was ensured through the use of air pumps (Resun air pump, AC-9906, Resun®, China) where were connected using silicone tubing was connected to air diffusers (Mott element 6500, Mott Corporation, United States of America).

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Isolation and Identification of biosurfactant producing microorganism for nitrification and denitrification

4.1.1 Introduction

Biosurfactants are microbially produced compounds which can be commonly used in various industrial applications, due to their properties; namely, biodegradability, low toxicity, environmental benignity, which can provide an improvement in the interaction of pollutants and microorganisms for biodegradation and partial emulsification processes including other desired bioremediation processes. These compounds require a range of relatively cost effective raw materials for their production (Makkar and Cameotra, 2002; Lang, 2002).

Biosurfactants reduce the surface tension in aqueous environments and have been tested using different confirmation methods previously, these methods have been widely accepted as valid for identification and characterisation of biosurfactants (Joshi et al., 2008). The biosurfactant confirmatory tests include hydrocarbon emulsification (Deepa et al., 2015), starch hydrolysis and oil displacement tests.

4.1.2 Objectives

The objectives for this part of the study were to:

- Identify the biosurfactant producing microorganisms from the isolated isolates, and
- To use screening tests for the confirmation of biosurfactant production.

4.1.3 Emulsification index

Table 4.1 and 4.2 illustrates morphological characteristics and emulsification results for the isolated isolates (n = 9) which are from different environmental (contaminated) sources using three oil types whereby cell free supernatants were used for emulsification index analysis. Isolates A, E, H, F and G showed positive emulsification results observed after 24 h. Najafi et al. (2011) found that only after 24 h was suitable for the assessment of an emulsion to

determine its positive or negative status. For vegetable oil, all the isolates showed a positive emulsification index; however, for kerosene, isolate A had a negative emulsification index which is a microorganism isolated from an environment likely to contain fats/oil. Some isolates showed a positive emulsification for kerosene and vegetable oil but negative for diesel. Microorganisms from different sources showed a similar phenomena of emulsification under controlled conditions. The emulsification indices were found to be at an average of 45%.

Table 4.1: Morphological features of the microorganisms

Isolate	**Features**
A	Cream colonies with raised elevation growing in clustered colonies.
B	Cream-white colonies with flat elevation and cloud like colonies.
C	Cream flat colonies with irregular shaped growth patterns.
D	Yellowish translucent colonies with raised elevation with clustered growth patterns.
E	Dark brown colonies with flat elevation growing in smear like colonies growing releasing greenish pigment onto agar.
F	Brownish colonies with raised elevation with greyish pigment on agar.
G	Cream-white colonies with flat elevation clustered colonies.
H	White colonies, flat elevation
I	Cream colonies raised elevation

Table 4.2: Emulsification results for the isolated isolates

Isolate	Kerosene	E_{24} (%)	Vegetable oil	E_{24} (%)	Diesel	E_{24} (%)
A	+	30	+	40	+	20
B	-	34	+	60	+	50
C	+	28	+	50	-	0
D	+	40	+	62	-	0
E	+	36	+	34	+	30
F	+	30	+	36	+	26
G	+	34	+	40	+	34
H	+	56	+	50	+	40
I	+	34	+	30	-	0

(+) = Positive reaction/(-) = negative reaction

4.1.4 Oil displacement test

The principle of an oil displacement test is to assess the ability of the produced biosurfactants to dissociate hydrocarbon based materials. If the liquid in the medium (containing the produced biosurfactant) does not cause the hydrocarbon to destabilise, the isolate is recorded as a negative outcome for oil displacement. The extract from the production of biosurfactants was used to perform the experiment whereby water was used to fill up the petri dish with 20µL of oil based compound and 10 µL of the extracted biosurfactant being used to displace the hydrocarbon/oil compound. The ability to displace the kerosene oil was assessed using crystal violet as a dye. Results obtained indicated positives for a majority of the extracted biosurfactants; however, due to varied emulsification potential, the displacement showed different efficiency levels for all the selected isolates as indicated in Table 4.3. The principle as outlined in Deepa et al. (2004), indicated that the produced biosurfactant if it can disperse the oil based compound a confirmatory displacement outcome will be noted; a method widely used as a screening method universally for biosurfactant production.

Table 4.3: Displacement results using different hydrocarbons

Isolate	Kerosene	Vegetable oil	Diesel
A	+	+	+
B	-	+	+
C	+	+	-
D	+	+	-
E	+	+	+
F	+	+	+
G	+	+	+
H	+	+	+
I	+	+	-

(+) = Positive reaction/(-) = negative reaction

4.1.5 FTIR spectrum for the extracted biosurfactant

Biosurfactants as surface tension reducers, can be applied in several industries including the wastewater, food and chemical producing industries, due to their low toxicity. The production of biosurfactants was conducted using the isolated microorganisms incubated in mineral salt media, which was found to be the commonly used media for the growth of biosurfactant producing microorganisms. A volume (100 mL) of the media was incubated in a shaker in 250 mL Erlenmeyer flasks and incubated for 5 days. Foam fractionation was performed to separate the foam from the media. The sample was purified and extracted through the use of acetone and ethyl acetate to obtain a pure extract of the biosurfactant with functional groups being determined using FTIR, a process which can identify the uniqueness of the biosurfactants.

Table 4.4: The functional groups of the extracted biosurfactants for individual isolates

Functional group (Coates, 2000)	**Current study (cm^{-1})**	**Comparisons**	**References**
Normal polymeric OH stretch	3359	$-CH_3$-CH_2 aliphatic chains	Srirama et al., 2011
Caorboxyilic acid /conjugate ketone	1697	Deformed N-H aliphatic chains	Sivapathasekaran et al., 2010
Oletenic alkene	1643	$-CH_3$ amide bond	Bharali et al., 2009
Aromatic nitro compounds	1370	N-H aliphatic chains	Srirama et al., 2011
Aromatic phosphates	1236	C-O and C=O aliphatic chains	Sivapathasekaran et al., 2010
Tertiary alcohol	1423	N-H aliphatic chains	Srirama et al., 2011; Saravanan et al., 2012
Organic siloxane	1044	O-C-O carboxylic acids/ C-O-C polysaccharides	Huang et al., 2015 Bharali et al., 2009
Aliphatic iodo compounds	535	Methylene proteins	Morikawa et al., 1993
Carboxylic acid	1590	Amide bond	Huang et al., 2014

Biosurfactants are produced from microorganisms and according to Swapna et al. (2015), the FTIR method is one of the reliable methods for their structural identification; however, due to the nature of the results obtained through this method, results only indicate the functional groups, which require further identification methods to determine the exact type of biosurfactant produced. Analysis was done using FTIR with no Kbr being required as pure liquid samples were initially used unlike Unàs et al. (2018) whereby biosurfactant identification was done for dried composite samples using Kbr. According to Bordes et al. (2009), the functional groups in the amide groups are commonly found in biosurfactants and serve as a link between the hydrophobic and hydrophobic ends of a surfactant structure. Since the FTIR only shows the functional groups commonly found in biomaterials, the results of this study appeared to have similar peaks with the presence of aliphatic functional groups being observed, as they have a peak at 1637 cm^{-1} which has been found to represent the C=O functional group. The presence of alkyl groups was observed in a study by Saravanan et al. (2012), indicating peaks in a range of 1467 and 1379 cm^{-1} which are indicative of peaks as listed in Table 4.4, with the majority of the peaks commonly known to indicate the

presence of biosurfactants. The samples studied indicating a diversity of biosurfactant being produced by the numerous isolates studied, some with aromatic functional groups associated with tertiary alcohols which are mainly additives in the pharmacetical industries (Srirama et al., 2011). The phospates functional groups are however, an indication of the rhamnolipis type biosurfactant which are used in detergents, with charateristic phosphate functional peaks at 1236-1320 cm^{-1} according to Coates (2000).

4.1.6 Summary

A number of microorganisms were isolated and utilised for the production of biosurfactants whereby isolates (n = 8) were emulsifiers. FTIR identification of the functional groups in the produced biosurfactant depicted peptides commonly found in synthetic surfactants, which was indicative of the ability of the produced biosurfactants to emulsify different hydrocarbons.

4.2 Assessing the nitrification and denitrification potential of microorganisms under metallo-phenolic conditions

4.2.1 Introduction

The removal of total nitrogen in wastewater systems occurs in many different ways including inducement both by chemical conversion and biological processes (biocatalysis). However, the use of microorganisms to remove total nitrogen in wastewater involves both nitrification and denitrification processes. Additionally, nitrification occurs in multiple steps, which are nitritation, a nitrification rate-limiting step for biological conversion of ammonium nitrogen and subsequently, conversion of by-products to a final product via nitration (Bothe et al., 2000). Additionally, denitrification encompasses the conversion of the NO_3-N ultimately to nitrogen gas, an inert environmentally benign gas. The nitrification process has been found to be inhibited by relatively low temperatures (5˚C) as this inactivates the process of organisms facilitating total nitrogen assmialtion as reported by Zhang et al. (2014). In this study, part of the microorganisms was subjected to varied concentrations of ammonium-nitrogen under selected conditions with the incorporation of possible inhibitors such as phenol (C_6H_5OH) and heavy metals (Cu^{2+} and Zn^{2+}) under similar temperature conditions as the optimum temperature range of these microorganisms which was established at 30-40˚C. The selection of the heavy metals was conducted based on the literature reviewed as to which

metals are being loaded into WWTP; albeit with beneficial traits in low concentration as they are used as micro-nutrients; however, at high concentrations, especially in wastewater, they have detrimental consequences to the wastewater treatment process in particular ammonium-nitrogen removal efficiency (Pani et al., 2017).

4.2.2 Objectives

The objectives for this part of the study were to:

- To assess the microorganisms' tolerance to C_6H_5OH and heavy metals,
- Identify the microorganisms with the highest nitrification efficiency under metallo-phenolic metal conditions,
- Classify the microorganism into biosurfactant producers and non biosurfactant producers including their ability to remove ammonium nitrogen, and
- Assess and quantify enzyme activity for the highest performing microbes.

4.2.3 Tolerance tests for phenol and heavy metals

Prior to the tolerance experiments, the isolates were identified using visual identification with reference to the basic microbiological isolate and identification techniques whereby pure cultures were inoculated onto solid media for visual identification techniques. Table 4.5 shows the tolerance tests for C_6H_5OH and heavy metals isolates.

Table 4.5: Tolerance tests for C_6H_5OH and heavy metals in agar plates

Isolate	**Cu^{2+} (2400 mg/L)***	**Zn^{2+} (1800 mg/L)***	**C_6H_5OH (850 mg/L)***
A	+	+	+
D	+	+	+
E	+	+	+
G	+	+	+
I	+	+	-/+

(+) = Positive reaction/(-) = negative reaction *= maxumum tolerance

4.2.4 Tolerance tests for C_6H_5OH and heavy metals

The microorganisms in this part study were subjected to toxicity tests to determine the maximum tolerance concentration, which negatively affects their growth, using the spiking method. Toxicant tolerance was visually evaluated as an indicator whereby streaks of microorganisms onto contaminated solid media reduced proliferation rate with toxicant increases.The maximal highest heavy metal concentration was 1800mg Zn^{2+}/L and 2400mg Cu^{2+}/L. The presence of high concentrations of heavy metals in wastewater treatment systems is known to be inhibitory. These toxicants have been found to have advantageous properties at low concentrations with Fe^{2+}, being found to increase microbial growth and increase the rates of NO_2-N conversion, which increases the rate of nitrification thus shortening the wastewaters retention time in treatment systems for TN removal. Table 4.5 also shows the concentrations used for C_6H_5OH and selected heavy metals (Zn^{2+}, Cu^{2+}) which are typically found in wastewater as a result of wastewater from the manufacturing industry being dumped into domestic WWTP culminating in further reduction of microbial communities efficiency in these plants (Singh and Kalamdhad, 2013). Most of the isolates selected and listed in Table 4.2 have been found to tolerate thus proliferate under conditions that are highly toxic with toxicant concentrations being up 5-fold of what has been reported in previous studies. For C_6H_5OH microbial tolerance a maximum of 500mg/L was previously reported in nitrification experiments whereby varying concentrations were used for the assessment of C_6H_5OH toxicity (Feng et al., 2015); however, the isolates in Table 4.2/4.3, in particular, isolates A, D, E, G, and I, have been determined to have a C_6H_5OH tolerance of a maximum of 850 mg/L for these isolates. Visual observations of the media showed that there was a decrease in the overall microbial growth with an increase in C_6H_5OH concentration which indicated some isolates might be susceptible to C_6H_5OH toxicity. Isolate I was the only isolate which proved to be highly inhibited by the C_6H_5OH at 850 mg/L among those resistant to C_6H_5OH; whereby, minute growth was observed, with all others, i.e. B, C, F, H, showing no growth. For heavy metals, susceptibility analysis and comparative analysis was observed for coppe, whichr had somewhat an inhibitory effect on isolates which were also susceptible to C_6H_5OH with a maximum concentration of 2400mg/L only inhibiting the least number of isolates deemed suitable for biolfilm engineering in TN removal systems.

4.2.5 Toxicant free total nitrogen removal efficiency for metallo-phenolic tolerant isolates

The initial concentration of NH_4-N used in this part of the study was derived from a study by Zhang et al. (2014) whereby the initial NH_4-N concentration was 2 mg/L; albeit, other studies had an initial concentration of NH_4-N of 40-50mg/L (Chen et al., 2012) with complete removal being achieved in 16 h with the removal of other toxicants, i.e C_6H_5OH and heavy metals, being determined to be half or less than the initial concentration of the primary toxicant, i.e. ammonium nitrogen, for biosurfactant producers and non-producers.

Other parameters were kept constand or near neutral at standard pH of 7.5 owing to previous studies conducted indicating that in nitrification systems, the processes continually taking place in this case simultaneous nitrification and partial denitrification can possibly perform better in buffered wastewater whereby observations for nitrification, indicated that the resultant effects on the nitrification process culminates in an acidifying effect, which can affect denitrification, responsible for the alkalinity balance for NO_3-N disappearance in SND sytems (Liu et al., 2017, Yao et al., 2013).

This part of the study investigated the potential nitrifying and subsequent aerobic denitrification ability of the selected isolates under metallo-phenolic free conditions. Initially, several isolates (n = 9) were selected for their nitrogen removal ability assessed in various experiments; however, due to the difference in environments from which the isolates were obtained, exposure to a different environment and varied concentration of ammonium nitrogen, was a predetermining factor of selection for further studies. Consequently, due to the exposure of microorganisms to ammonium nitrogen, the activation of enzymes in individual isolates was observed as NO_2-N and NO_3-N accumulation was observed for all the isolates after a 6-day incubation in ammonium nitorgen media in a shaker incubator at 121 rpm and 37˚C. Due to the large number of isolates isolated, the highest ammonium nitrogen oxidisers were selected according to the highest concentration of NO_3-N formed, which is an indication of a complete nitrification according to Chen et al. (2014). Since, the isolated isolates were initially categorized according to their ability to produce biosurfactants, with a reference to results obtained from the screening tests for biosurfactant production, the highest nitrogen remover was a non-biosurfactant producing *Providencia* sp. (H); albeit, *Alcanigene* sp. (G) was another highest ammonium nitrogen remover with a biosurfactant producing potential according to the screening methods used. The efficiency of the microorganisms however was determined by assessing the production of the intermediate

compounds namely, NO_3-N and NO_2-N. The results indicated that a similar trend in the degradation behaviour of the selected toxicants.

4.2.6 Impact of C_6H_5OH concentration on nitrogen removal for individual isolates

Phenol (C_6H_5OH) is known for its toxic properties and has been found to inhibit total nitrogen removal processes whereby a drastic decrease in the overall efficiency in planktonic microorganisms used for TN removal, was observed. The isolated microorganisms indicated an accumulation of NO_3-N (Table 4.6), with the highest accumulators of NO_3-N being F and G over a 6 day incubation period. The highest residual concentration of NO_3-N was observed in G cultures with a concentration of 281 mg/L similar to a study conducted by Mojiri et al. (2017) whereby the overall removal of ammoniun nitrogen was 89% in the presence of C_6H_5OH; culmunating in a NO_3-N accumulation. Although, as observed in this study, isolates G and F were observed for overall ammonium nitrogen concentration decreases, with results indicating the highest accumulation of both NO_2-N and NO_3-N, the presence of C_6H_5OH seemed to have alevated by-product accumulation, without observed reduction to innocous N_2 gas, similar to a study conducted by Perez-Gonzalez et al. (2012).

Table 4.6: NO_3-N concentration for the isolates after a 6 day incubation

Isolate	**NO_3-N concentration (NH_4-N cultures) mg/L**	**NO_3-N concentration (NH_4-N + C_6H_5OH cultures) mg/L**
A	2.80	11.50
C	2.30	3.00
D	2.85	19.00
F	8.35	264.50
G	12.10	281.50
H	1.95	3.25

Table 4.6 shows the accumulation of the NO_3-N over a period of 6 days, whereby G and F cultures were the highest accumulators of NO_3-N which is an indication of the completion of the process of nitrification, as indicated by Yu et al. (2011) whereby the depletion of dissolved oxygen was also found to indicate that the performance of reactors used for total

nitrogen removal was ineffective as most isolates didn't proliferate in C_6H_5OH contaminated cultures. The current study used sealed reactors to restrict dissolved oxygen supply to the Erlenmeyer flasks used for the nitrogen removal experiments as the experiment proceeded, facilitating the SND mechanism but also for safety purposes as C_6H_5OH is a toxicant to humans as well; hence, the observed increase in the NO_3-N accumulation for the G and F particularly for the G (*Alcanigene* sp.) and H (*Providencia* sp.). Since there was no interval sampling, volatilisation was restricted, with the obtained results being detemined to be reproducible. Generally, the NO_2-N concentration was lower than that of NO_3-N concentration – see Table 4.7. Suprisingly, increases in ammonium nitrogen was observed for cultures G and F towards the end of the 6 day incubation (Table 5.8).

Table 4.7: NO_2-N concentration for the microbial isolates after 6 day incubation

Isolate	**NO_2-N concentration (NH_4-N cultures) mg/L**	**NO_2-N concentration (NH_4-N + C_6H_5OH cultures) mg/L**
H	0.75	0.91
C	0.67	0.97
A	0.64	0.94
D	0.59	1.015
G	1.09	0.09
F	1.07	0.14

Table 4.8: NH_4-N concentration for the microbial isolates after 6 day incubation

Isolate	**NH_4-N concentration (NH_4-N cultures) mg/L**	**NH_4-N concentration (NH_4-N + C_6H_5OH cultures) mg/L**
H	71.4	60.1
C	51.6	70.65
s1	66.7	73.35
A	60.6	77.1
D	75.0	65.5
G	55.65	148.5
F	65.4	167.5

4.2.7 Biosurfactant producers and non-producers for nitrogen removal

The microorganisms were further organised into planktonic consortiums according to their ability to produce biosurfactants as indicated by the results of the screening methods. The mixed cultures were then supplement with 45mg/L of NH_4-N, 15mg/L phenol, 15mg/L Zn^{2+} and 15mg/L Cu^{2+}, as the selected heavy metals of this study, using a 2 hr sampling interval for cultures incubated at 37˚C incubation in a rotatory shaker. The biological conversion of ammonium-nitrogen however, did not correspond to the accumulation of NO_2-N.

Generally, there is limited published literature on the efficiency of biosurfactant producers in comparison to non producers on the influence of total nitrogen removal, particularly in the presence of toxicants such as heavy metals and C_6H_5OH. Additionally, there is also a general limit of published literature on the impact of metallo-phenolic conditions on the total nitrogen removal efficiency of non-biosurfactant producing organisms. Results obtained indicated that the producers and non-producers were both to some extend inhibited by the toxicants used, i.e. heavy metals and phenol. Ammonium-nitrogen reduction indicated by the constant increase in the by-products of ammonium-nitrogen oxidation in the bioreactor. Residual NO_2-N concentration in individual bioreactors indicated similar trends for both consortia, i.e. non- and biosurfactant producers, while low residual NO_3-N concentration indicated possible nitrification, indicated by the initial increase and subsequent decrease in NO_3-N concentration as observed for both biosurfactant producers and non-producers.

Biosurfactants in nature are known to reduce the surface tension in of wastewater. They have been known to have a vast variety of application including in wastewater treatment whereby they have been found to improve the efficiency of toxicant removal in biological systems, with some biosurfactant being produced by the bioremediating microorganisms themselves. A similar trend of an initial increase in NH_4-N attributed to cell lysis was observed for both sets of experiments; however, the accumulation of NO_3-N and NO_2-N differed for each set of microbial consortia. The NO_3-N was indicated as the highest residual concentration of the nitrogen constituents quantified to non-producer cultures; whereas, the residual NO_3-N concentration for biosurfactant producers was low. The overall total nitrogen trend however, showed the steady initial increase of ammonium nitrogen and subsequent to a gradual decrease, i.e. particularly of NH_4-N concentration, which was presumably influenced by the start of denitrification, which is primarily based on the conversion of NO_2-N to nitrogen gas (Chen et al., 2015). For nitrification efficiency, the highest conversion rates observed was for biosurfactant producers. The residual concentration of up to 0.815 mg/L, was observed.

4.2.8 Inhibition of total nitrogen removal by selected heavy metals

A comparative analysis involving non- and biosurfactant producing consortium was evaluated to ascertain performance sustainability in cultures grown for 24 h without toxicant loading subsequent to spiking with the known concentrations of toxicants, i.e. heavy metals. Results indicated that initially, the NH_4-N concentration was gradually increasing within the cultures, for both the biosurfactant producing consortium and non-producers. Phenomena attributed to NH_4-N in media components being degraded and perhaps cell lysis. (Wu et al., 2005). The increase in NH_4-N concentration was simultaneously observed with an increase in the presence of NO_2-N which is the primary by-product in the nitrification process indicating the presence of nitritation, with the gradual accumulation of NO_3-N, indicating the presence of the subsequent process of nitratation. Since heavy metals are ubiquitous in nature, their concentrations ranges from the naturally available, freely available in minute concentrations, high concentration contributed by anthropogenic activity. These compounds' toxicity depends on their overall concentration in the wastewater being treated, as they tamper with the metabolic efficiency of some microorganisms, inhibiting intermediate processes; thereby, leading to the inefficiency of biologically driven processes such as sequential nitrification and denitrification by pathway disruption (Wang et al*.,* 2017).

In planktonic microorganisms, the tolerance to environmental and synthetic toxicants is higher when the growth rate escalates, as observed in the tolerance (850 mg/L) of C_6H_5OH observed after a culture incubation period; albeit, proofing to significantly affect growth, with an increase in toxicant concentration. A study conducted by Juliastuti et al. (2003) led to the conclusion that, the toxicity of Cu^{2+} and its ability to result in inhibition of the physiological process in microorganisms was higher than that of Zn^{2+} whereby a comparison of overall inhibition capacity revealed a maximum of 50% inhibition of the nitrification process for Cu^{2+} at concentration of 0.08mg/L whereas Zn^{2+} was only 12%. The lowest concentrations which were observed to start the inhibition were 0.05 mg/L Cu^{2+} and 0.3 mg/L Zn^{2+}, respectively.

C_6H_5OH however as a toxicant, was found to have a toxicity of up to 50% at 3mg/L with complete inhibition recorded at 50mg/L C_6H_5OH in liquid cultures for TN removal. C_6H_5OH as a toxicant in this study was selected to determine the maximum microbial tolerance to the pooled consortia as it is a toxicant commonly found in wastewater treatment systems. C_6H_5OH is conventionally detected in minute quantities in wastewater treatment plants; therefore, microorganisms with the highest tolerance to C_6H_5OH inhibition had to form the basis of the engineered consortia. Juliastuti et al. (2003) reported in a study investigating the inhibition of nitrification by C_6H_5OH, observing a rapid increase in inhibition resulting in a drastic decrease of 78% nitrification rates of microorganisms primarily known to facilitate

nitrification. In this study however, a gradual decrease in the residual concentration of NH_4-N was observed, indication of the nitrification; albeit the process was stunted as similarly observed in a study by Tao et al. (2017).

Since the only quantifiable parameters were ammonium, NO_3-N and NO_2-N, the partial denitrification was denoted by the decrease in the concentration of NO_2-N. This process however, due to its occurrence in the same bioreactor (single stage) can be termed as the simultaneous and/or nitrification and aerobic (partial) denitrification. Although the processes occurred in a single stage system, and potentially being observed as being performed simultaneously at industrial scale, these are individual processes that occur in sequential reactors depending on environmental conditions in large scale operations.

This part of the study included the use of both non- and biosurfactant producers for the total nitrogen removal under metallo-phenolic conditions with some inhibition being observed, result similar to those reported in a study conducted by Zheng et al. (2011). Generally, inhibition by heavy metals was observed when a drastic decrease in total nitrogen removal efficiency was reduced from 80.3 to 24%, hypothesised to be associated with the deactivation of the ammonia monooxygenase and NO_2-N oxyreductase, enzymes which are responsible for the initiation of both nitrification and nitratation, respectively, which then required the assessment of the enzymatic activity of the consortia selected for biofilm engineering to ascertain whether the enzymes responsible for total nitrogen removal are actively produced by the consortia as well.

4.2.9 Single isolates: Total nitrogen removal under phenolic conditions

The microorganisms that were selected as the highest nitrogen removers according to the initial experiments, were conducted for individual microorganisms at different ammonium-nitrogen and C_6H_5OH concentration to assess the impact of the increase in concentration of ammonium nitrogen, to determine overall removal efficacy for the phenolic monoseptic cultures. The results for the *Providencia* sp. (F) indicated that the overall increase in NO_3-N in the presence of phenol. The increase in C_6H_5OH concentration however, was such that this particular isolate (F), had residual NO_3-N reduction from 7mg NO_3-N/L to 5.75mg/L within 24 h indicating the minimised impact of the C_6H_5OH toxicity on the nitratation process. These results could be interpreted as an indication of the initiation of the partial denitrification process as results indicated a decrease in the residual NO_3-N indicating the possibility of denitrification (Jahangir *et al.*, 2012).

C_6H_5OH impact was studied for selected isolates, whereby 24 hr cultures were used (Liu et al., 2011), with a few of the initial isolates (n = 2) chosen for further experimentation. A ratio of 46:15 (ammonium-nitrogen:C_6H_5OH) of the highest total nitrogen removers, *Providencia* sp. (H) and *Alcanigene* sp. (G) were determined to be efficient in total nitrogen removal.

The characteristics of the *Alcanigene* sp. (isolated from crude oil waste), identified as a biosurfactant producer, with observable reduction in the concentration of ammonium nitrogen when ammonium nitrogen was introduced as a toxicant together with C_6H_5OH at a concentration of 46mg/L and 15mg/L, respectively, resulted in a trend which was observed to increase residual NO_2-N whereby initially the concentration was 0.20mg NO_2-N/L, with further increases to 0.42mg/L being observed. An increase in the C_6H_5OH loading culminated in insignificant inhibition, contrary to the results obtained by Amor et al. (2005). A reasonable assertion attributed to conditions as observed by Alves et al. (2017) whereby significant inhibitions occur at only 300mg/L C_6H_5OH concentrations means some isolates have a higher tolerance threshold making them suitable to be incoporated in consortia for bioremdiation of metallo-phenolics contaminated wastewater. However, sudden shock loading and toxicity of C_6H_5OH have been found to be increased by other environmental factors such as temperature; hence, significant inhibition can be observed in such instances (Alves et al., 2017; Amor et al., 2005).

For the *Providencia* sp. (H), there was an increase in the concentration of ammonium nitrogen from experiments initiation stage through to 3 hrs. A similar trend was observed for residual NO_2-N whereby a decrease in concentration was observed at 4 hr of bioreactor operation, stabilised when C_6H_5OH was supplemented, albeit an increase in the ammonium nitrogen concentration increased substantially from its initial value, resulting in a gradual increase in the concentration of both residual NO_2-N and ammonium-nitrogen. Furthermore, residual NO_3-N showed an increase prior to the increase loading of the phenol as a toxicant. Figure 4.1 illustrates differentiation in (A) Nitrogen removal for the *Alcanigenes* sp. (G) at 46 mg/L NH_4-N and 15 mg/L C_6H_5OH concentration and (B) *Providencia* sp. (G) 46 mg/L NH_4-N and 15 mg/L C_6H_5OH concentration. Isolate cultures of *Alcanigenes* sp. (G) at 46mg/L and 30 mg/L C_6H_5OH and NH_4-N concentration and (D) *Providencia* sp. 30 mg/L C_6H_5OH to 46 mg/L NH_4-N, showed higher accumulation of NO_2-N and NO_3-N. Figure 4.2 also indicates residual concentrations of (A) NO_3-N (B) NO_2-N and (c) NH_4-N for biosurfactant producer and non-producer consortia.

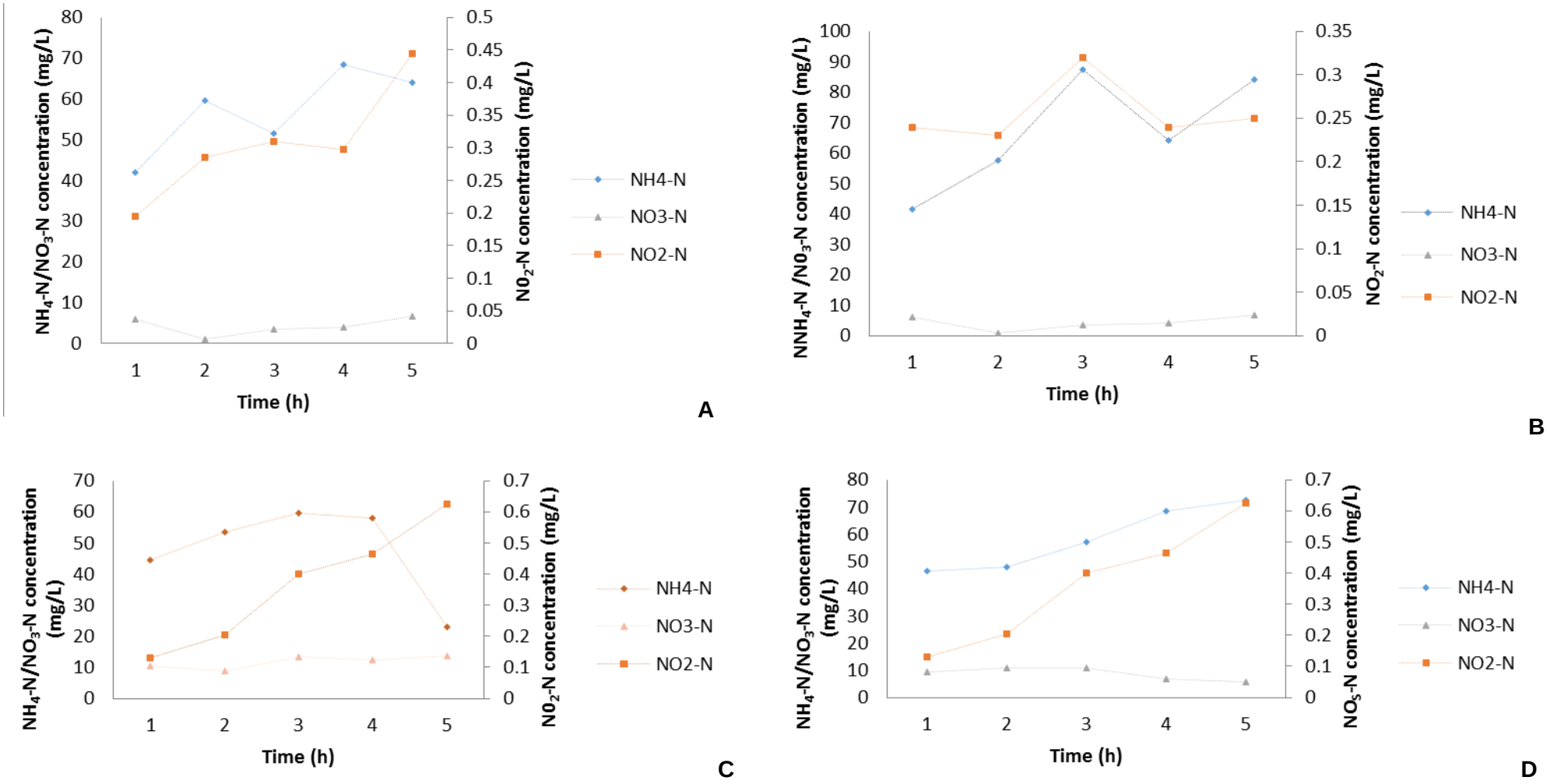

Figure 4.1: (A) Nitrogen removal for the *Alcanigenes* sp. at 46 mg/L NH_4-N and 15 mg/L C_6H_5OH concentration (B) *Providencia* sp. 46 mg/L NH_4-N to 15 mg/L C_6H_5OH concentration (C) *Alcanigenes* sp.at 46mg/L and 30 mg/L $C_6H_5OH$and NH_4-N concentration and (D) *Providencia* sp. 30 mg/L C_6H_5OH to 46 mg/L NH_4-N

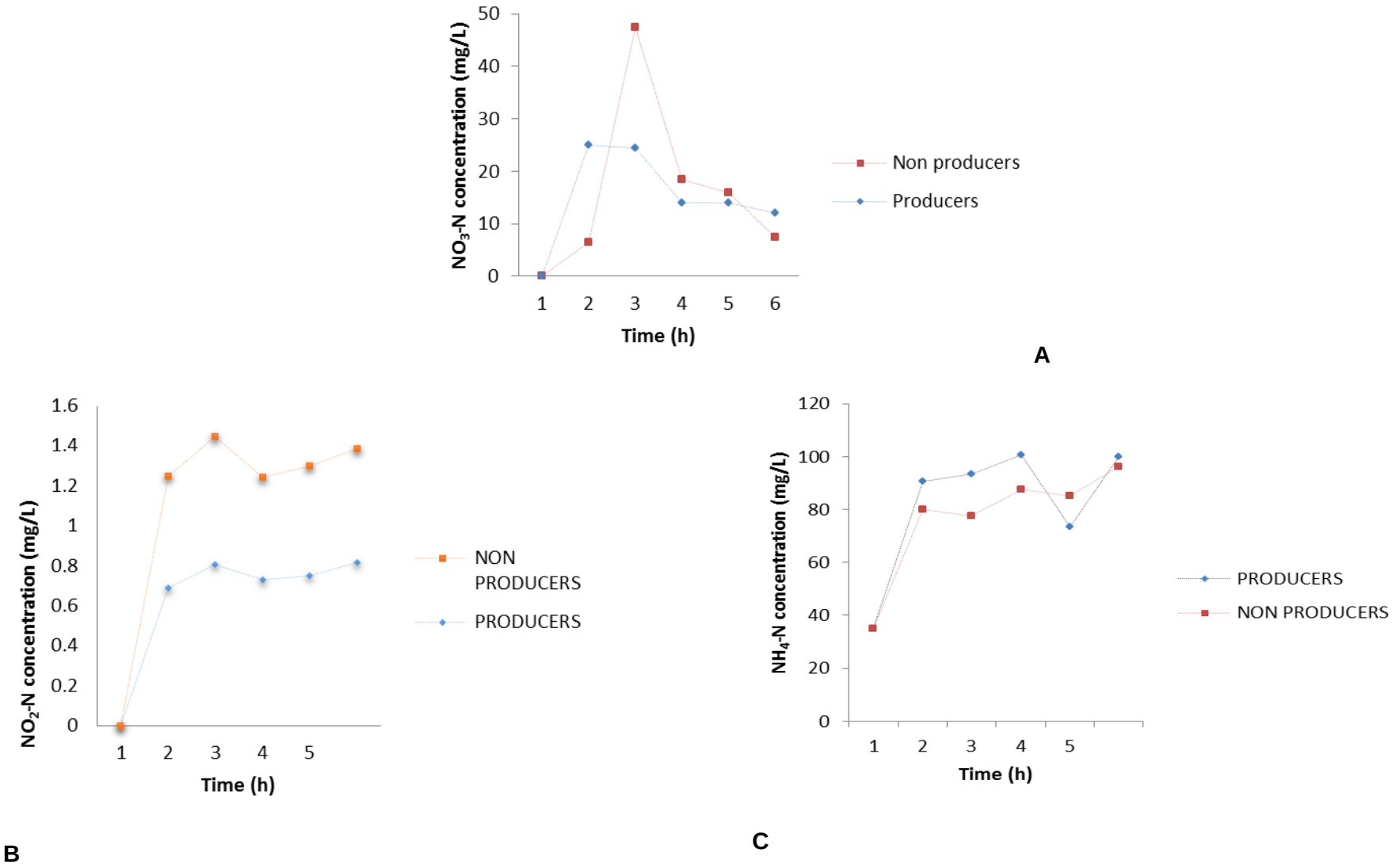

Figure 0.1: Residual concentrations of (A) NO_3-N (B) NO_s-N and (C) NH_4-N for biosurfactant producer and non-producer consortia

4.2.10 Enzyme activity for consortia used in total nitrogen removal systems

The selected microorganisms for the highest total nitrogen removal were used to perform enzyme activity experiments whereby the microorganisms were grown in a shaker incubator in the presence of 15mg/L of C_6H_5OH mg/L and ammonium nitrogen including 20mg/L of Zn^{2+} and Cu^{2+}. The extraction of crude enzymes was done using acetone in ice with the results indicating that microorganisms exposure to toxicants for 5 days, their enzyme functionality was not tremendously affected. The primary enzymes assessed was ammonia monooygenase with an activity which was determined to be suitable for initiating and sustaining nitrification as it is commonly found in *Nitrosomonas* cultures. However, further assessment of the nitrification with the quantification of NO_2-N and susbsequently NO_3-N to ascertain total nitrogen removal efficacy is required. With ammonium as a substrate, the enzymatic activity showed a decrease in the initial substrate concentration which indicated the presence and activity of ammonium monooxygenase.

The enzymes responsible for the total nitrogen removal rate in planktonic microorganisms was evaluated for *Providencia* sp*.* and *Alcanigene* sp. isolates whereby 5 day cultures were lysed using a sonicator and enzyme extraction was performed with ice cold acetone in 20mmoL phosphate buffer (pH 7). The semi-purified enzymes were spiked using 46 mg/L of ammonium nitrogen, i.e. used as substrate. The substrate reduction profiles were observed over a 5 hr period to ascertain substrate conversion with the final concentration being 39.4mg/L. This indicated the presence of ammonia monooxygenase which was identified as the enzyme responsible for the oxidation of ammonium to yield NO_2-N.

The initial accumulation and subsequent conversion of both NO_3-N and NO_2-N was also observed; an indication that all the enzymes required for nitrogen removal were present in the crude enzyme extract solution. The enzyme activity represented as a decrease in NH_4-N concentration, as observed for ammomium monooxygenase assays, was found to be 108mg/L/h for NH_4-N for the *Providencia* sp., indicating a correlation to the oxidation of ammonium resulting in an NH_4-N decrease, with an accumulation in NO_2-N. Similarly, for the *Alcanigene* sp., activity was indicated by a decrease in NH_4-N concentration correlating with an increase in NO_2-N concentration. For the *Alcanigene* sp. Cultures, NO_3-N decreased with time as it was the initial concentration of 3.8mg/L which was reduced to 3.3 mg/L at the termination of the experiment which was an indication of partial SND.

Table 4.9: Enzyme activity for *Alacanigene* sp.

Time (minutes)	**NH_4-N (mg/L)**	**NO_3-N (mg/L)**	**NO_2-N (mg/L)**	**BRE % Current study**	**BRE % comparative study 1**	**BRE % comparative study 2**
1	35.5	3.8	0.58	With C_6H_5OH and 2 heavy metals(Cu^{2+} and Zn^{2+}) as toxicants	Joo et al. (2006) No indicated toxicants	Joo et al. (2005) No indicated toxicants
2	30.5	4.0	0.56			
3	28.0	2.0	0.58			
4	23.5	2.9	0.57	**180mg/L/h**	**30mg/L/h**	**26.1mg/L/h**
5	20.8	3.3	0.59	42.2% after partial denitrification	65% after denitrification	90% after denitrification

Similarly, the residual concentrations of NO_3-N and NO_2-N were simultaneously quantified to indicate the activity of the other nitrification enzymes, i.e. NO_3-N reductase (NR) and NO_2-N reductase (NiR). This resulted in the determination of activity for these enzymes for both *Providencia* sp. and *Alacanigene* sp., with minute decreases from 0.59mg/L of NO_2-N concentration to 0.58mg/L at after 5 min for *Alcanigene* sp.; whereas *Providencia* sp. had a similar trend of NO_2-N increase from 0.39mh/L to 0.42mg/L over the similar 5 min period. This is an indication of the efficiency of the NO_2-N reductase enzyme (Kumar et al., 2010).

Denitrification in biological systems is indicted by a decrease in the concentration of NO_3-N; hence, the enzymes in the crude extracts responsible was evaluated through the quantification of the NO_3-N concentration. The results as indicated in Table 4.9 obtained for the nitrogen removal rates indicating 180mg/L/h removal after the enzyme essaying was conducted for the *Alcanigene* sp. were anticipated to be due to the biosurfactant producing ability of the isolate (Bharali et al., 2011) whereas a recording of 30 mg/L was reported for the same species by Joo et al. (2006). Despite the presence of C_6H_5OH in the nutrient media as a model toxicant, the microrganisms were able to achieve a 42.2% reduction in TN, over a period of 5 min indicating the potential of nitrogen removal as the microorganisms

tolerance has been recorded against C_6H_5OH (Bastos et al., 2001). The mechanism therefore for the specified isolate was determined to be associated with the simultaneous nitrogen removal ability and toxicant (C_6H_5OH) tolerance. Removal rates of 26.1mg/L/h was reported, observing a 90% removal efficiency in a Joo et al. (2005) study; whereby there was no specified toxicants.

For the *Providencia* sp. the removal rates shown in Table 4.10 for the NH_4-N in the current study were found to be 108mg/L/h after a 5 min experiment whereas a rate of 18.7mg/L/h, was observed in similar microbial species as reported by Zhao et al. (2010). Additionally, the removal percentage in the Zhao et al. (2010) study, was lower than that observed in the current study.

Table 4.10: Enzyme activity of *Providencia* sp

Time	**NH_4-N (mg/L)**	**NO_3-N (mg/L)**	**NO_2-N (mg/L)**	**BRE % NH_4-N Current study**	**BRE % Comparative 1**	**BRE % Comparative 2**
1	46	0	0	With C_6H_5OH and 2 heavy metals(Cu^{2+} and Zn^{2+}) as toxicants	(Ye et al., 2016) No specified toxicants	(Zhao et al., 2010) Zn^{2+} as one of the toxicants
2	45.8	2.7	0.39			
3	42.3	1.4	0.33			
4	39.4	2.3	0.32	108mg/L/h	No specified removal rates	18.7mg/L/h
5	37.1	2.6	0.42	19.3%	72% removal	69% removal

4.2.11 Summary

From the isolates determined to be biosurafctant producers, not all had a higher tolerance for heavy metals and phenol, especially at hihgher concentrations, in particular, Cu^{2+} (2400 mg/L), Zn^{2+} (1800 mg/L) and C6H5OH (850 mg/L). Isolates F and G cultures had a higher residual NO_3-N concentration when phenol was the toxicant, indicating a stunted overall total nitrogen conversion, with a higher residual NH_4-N concentration towards the end of a 6 day experimentation period, suggesting cell lysis.

4.3 To assess the rate of total nitrogen removal by engineered biofilms

4.3.1 Introduction

Biofilms are microorganisms collected into a structure and are attached to a surface. They are commonly found in various habitats including wastewater and can form part of fouling in water pipes whereby they are observed as a contaminant. Furthermore, in natural habitats the formation of biofilms is an indication of diverse microorganisms types. This is an advantage for biofilms as it results in increased overall tolerance to environmental toxicants. This is due to the presence of EPS, which protects the biofilm from toxin attack. Biofilms in terms of degradation efficiency have been found to have an increased potential efficiency thus due to the combined effect of the diverse microorganisms commonly found in natural biofilms. This study evaluated the total nitrogen removal efficiency with the following objectives.

4.3.2 Objectives

The objectives for this part of the study were:

- To assess the rate of nitrification under phenolic conditions,
- To validate the robustness of biofilms in nitrification and denitrification under selected heavy metal conditions, and
- To use metagenomics for the identification of biofilm microorganisms.

4.3.3 Biofilm performance in nitrogen removal

A 3-month old biofilm engineered using isolates determined to be resistant to heavy metals and phenol, was evaluated based on overall nitrogen removal. The experiment was conducted in a 1L batch reactor under controlled temperatures of 37°C under constant supply of air (dissolved oxygen) at a lowly rate to avoid volatilization of the toxicants used. The initial conditions were 40mg/L of NH_4-N, 30mg/L phenol, 20mg/L of Cu^{2+} and Zn^{2+} which were the selected heavy metals for this part of the study. Results indicated a gradual increase in pollutant concentration within the system at the initiation of the experiment, with the accumulation of NO_2-N which is a theoretical indication of the initialisation of the nitrification process where the initial ammonium nitrogen is converted to NO_2-N in a process of nitritation which is the initial and rate limiting step of the nitrification process. Additionally, acidity conditions were maintained at 7.4 as the optimum pH as it was previously found that complete nitrification can be achieved at a range of a 6.45 – 8.95 according to a study conducted by Ruiz et al. (2008).

Similarly, Xiao et al. (2017) observed conditions of higher efficiency with near neatral pH when soil nitrification was studied. This study however, indicated NH_4-N accumulation as observed at with the highest concentration at 52.5mg NH_4-N/L which was an indication of either cell death due to C_6H_5OH and or ammonium intoxication including shock which resulted in the release of nitrogenous compounds into wastewater being treated. Another study conducted by Ma et al. (2017), focussed on the ability of biofilms to remove total nitrogen in a SBBR system, attaining results which indicated the coexistence of nitrifyers and denitrifyers, a phenomena which was found to be prevelant through the use of minute aeration. The denitrification as observed through the results indicated the presence of an anoxic zone; however, there was only limited denitrification occurring. Additionally, in biofilm systems, Miao et al. (2016) found that inhibition due to the presence of toxicants was minimal to negligible; however, a decrease in the microbial diversity due to the toxicity of the added compounds might ensue, with reduced performance of the biofilms over the long term, especially for systems operated in a continuous mode.

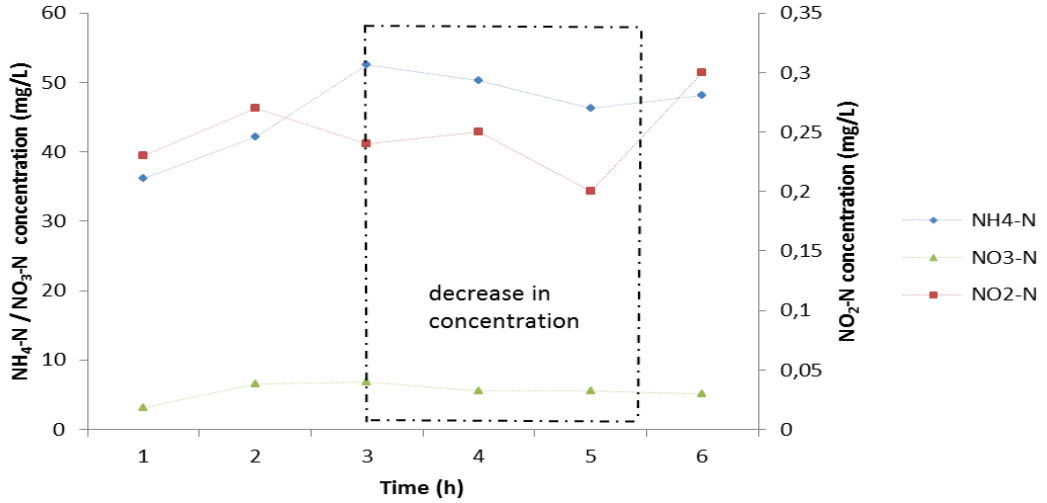

Figure 4.3: Graphical representation of the nitrogen removal results for biofilm in the presence of C_6H_5OH and heavy metals

4.3.4 Simultaneous nitrification and partial denitrification

Traditionally, simultaneous nitrification and denitrification occurs in complete reactions where the requirement of anoxic catalyses for the completion of the denitrification process is advocated for. These processes however, can be incomplete owing to different operational variations such as inadequate supply of nutrients, inhibition by toxicants and aeration discrepancies (Tsuneda et al., 2006). Lochmatter et al. (2013) found that the inhibition in complete SND was the COD concentration when sludge was used for total nitrogen removal in the presence of phosphorus. Due to the observed insignificant impact of the added C_6H_5OH for the planktonic micoorganisms, a suggestion for the incomplete denitrification as depicted in the minimal decrease in NO_3-N concentration for both the planktonic consortia (biosurfactant producers and non-producers), i.e. where no significant increase in nitrification was observed, was attributed to the heavy metals introduced into the media as they have been found to negatively impact the nitrogen removal process (Papadimitriou et al., 2009; Ge et al., 2015). The biofilm total nitrogen experiments however, showed an improvement in the decrease of NO_3-N concentrations as biofilms have been proven to have increased overall efficiency in comparison to planktonic microorganisms; albeit not to an extend intially envisaged.

Other reasons for the partial denitrification could be a result of microbial growth as it was found to cause pH fluctuations resulting in alkaline conditions which have been found to decrease the total nitrogen efficiency and in adequate temperature whereby exceeding the optimum temperature of 30-37°C could be detrimental to the growth and efficiency of the engineered biofilm (He et al., 2009; Ni et al., 2008).

4.3.5 Biofilm composition: Microbial identification using metagenomics

Since the microorganisms as per the assumption were nutrient specific, however a form of universal media was used to cater for the majority of the microorganisms. This allowed the vital nutrients commonly required by microorganism such as carbon source and nitrogen source be sufficiently availableto to the organisms (Bedi et al., 2017). The identification method of the microorganisms of this study used the 16S bacterial identification which indicated that the majority of the microorganisms were bacteria with 97.19% followed by protozoa 2.81% and an insignificant percentage of unknown organisms and Archaea. Similarly, the biofilm inoculum indicated that 45.78% of the bacteria were unknown. This is also depicted in the family identification profile with the highest percentage of 84.78%. Similarly, the blast output results indicated the highest percentage of 45.06% of uncultured bacteria with a significant 39.00% constituted of uncultured *Cholorflexi*. The family classification showed a majority of 84.78% of unknown/unculturable microoganisms with a 6.39% *Bacillaceae* with 2.81 *Spathidiidae,* and 1.12% *Alcanigenaceae* – see Figure 4.4.

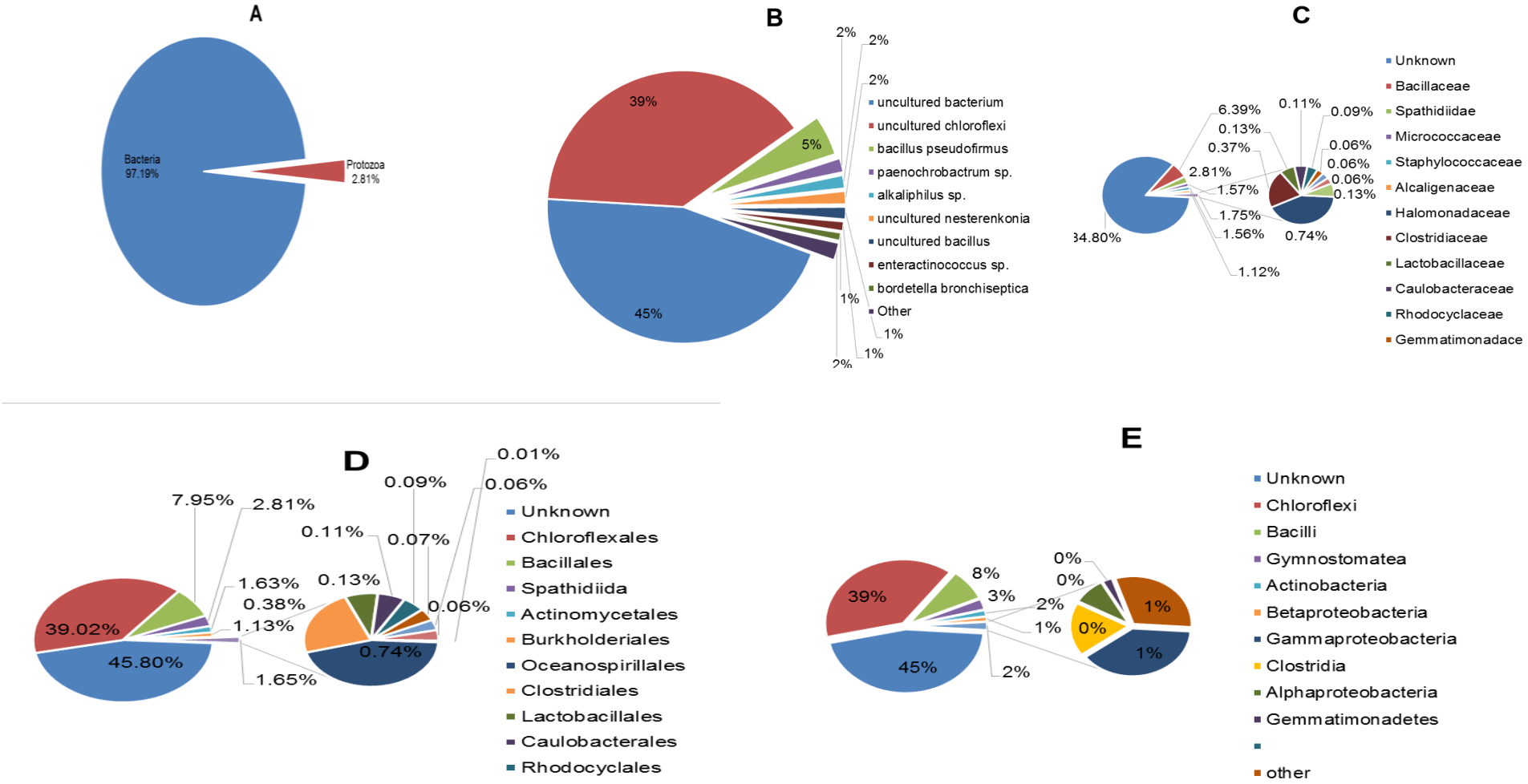

Figure 4.4: Biofilm metagenomic identification profiles (A) Taxonomy, (B) Species, (C) Family, (D) Order and (E) Phylum

CHAPTER 5

CONCLUSIONS

AND

RECOMMENDATIONS

CHAPTER 5

4 CONCLUSIONS AND RECOMMENDATIONS

5.1 Overview

The total nitrogen removal was the initial aim of the current study; albeit under metallo-phenolic conditions. The results revealed that the isolated microorganisms had the ability to reduce concentrations of nitrogenous compounds artificially introduced in synthetically prepared wastewater. However, due to the differentiation in microorganism types, only partial denitrification was achieved in both planktonic and biofilm microorganisms as indicated in the results whereby a decrease in the NO_3-N concentrations was minimal indicating a newly found phenomenon termed simultaneous nitrification and *partial* denitrification, since a single unit reactor was used for total nitrogen removal.

Providencia sp. (KX394623.1) and *Alcanigene* sp. (KY495219.1) were identified using the 16S rDNA identified using a selected from a list of microorganisms that were isolated due to the observed high efficiency in TN removal. *Alcanigene* sp. was found to be a producer of biosurfactants when screening for biosurfactant production was conducted; however, *Providencia* sp. was evidently a non-producer; however, there was insignificant differences in the efficiency of the two microorganism types when total nitrogen removal was assesed. Microbial identification using the metagenomics study revealed that the highest percentage of microorganisms in the engineered biofilms were bacterial isolates (97.19%) followed by protozoa (2.81%) with the least abundant microorganisms' type found to be Archaea (0.1%). The latter two classifications were hypothetically assumed to have introduced in the system unintentionally. Additionally, nitrification and denitrification efficiency was found to be lower in planktonic cultures than in the biofilm studies particularly in the presence of similar concentrations of NH_4-N and heavy metals; however, C_6H_5OH in biofilms was increased initially to assess the robustness of the biofims with inhibition being found to be negligible. Results indicated that only 8 of the isolated microorganisms were able to produce biosurfactants. The FTIR samples indicated resemblance to synthetic surfactants as observed using the functional group identification method with C=O and alkyl groups being the most common. The activity of enzymes responsible for nitrification and denitrification indicated a degradation rate of for *Providencia* sp. was 108mg/L/h whereas the rate for *Alacanigene* sp. was 180mg/L/h; determined through enzyme kinetics with BRE (%) of 19.3% and 42.2%, respectivley. It was then concluded that biofilms performed better in total nitrogen removal studies under metallo-phenolic conditions. Nitrogen removal was minimally

inhibited by an increase in concentration of phenol; however, long term operation of the engineered biofilms is still in doubt. Addionally, biofilms (engineered) showed their robustness in the presence of increased concentrations of heavy metals, than phenol, when both toxicants were supplied continuosly. Finally, the biosurfactants although commonly used in reducing toxicity, played no significant role in the total nitrogen removal efficiency.

5.2 Recommendations

Due to the challenges encountered such as biofilm development, a recommendation for future studies would be the use of raw samples initially, as petri dish grown of inoculum showed slow growth. Furthermore, the material for biofilm growth which was used had temperature fluctuation challenges which slowed microbial growth with the glass reactor culminating in the system being affected by the overall room temperature.

Consistency in the monitoring and optimization of aeration must be considered, as oversparging the system could result in dissolved oxygen stripping total nitrogen constituents including phenol resulting in decreased bioreactor thus bioremediation efficiency.

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7. APPENDICES

Appendix A: Media preparation

Basal medium (g/L)

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

1 ml traces elemental per litter

Trace elemental solution

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

Appendix B: Analytical procedures

Pre-treatment of sample

Samples were collected

Centrifuged 5ml at 4000rpm for 4 min to separate cells and supernatant

Pipette 1ml of sample into 10ml volumetric flask and make up to the mark with distilled water

NH_4-N analysis

Pipette 5ml of NH_4-1 reagent into test tube

Add 1 level blue micro spoon of NH_4-2 reagent

Add 0.10ml of pre-treated sample

Shake vigorously until reagent mix thoroughly

Incubate at room temperature for 15 min and analyse using spectroquant

NO_3-N Test

5ml of NO_3-2 pipetted into test tube 15ml

1x level blue microspoon of NO_3-1 added to the 5ml

Vigorous shaking for 1min until NO_3-1 dissolves to completion

1.5ml of pretreated sample added followed by brief vortexing

10 min room temperature incubation to allow reaction to take place

Immediate analysis using spectroquant

NO_2-N test

5ml of pretreated pipetted sample into test tube

Addition of 1 level microspoon of NO_2-1

10 min room temperature incubation to allow reaction to take place

Immediate analysis using spectroquant

Appendix c

Figure 7.1:**The batch reactor setup used for the nitrogen removal experiments**

Biological removal efficiency equation

$$BRE\% = \frac{Initial\ concentration - final\ concentration}{Initial\ concentration} * 100$$

Emulsification index equation

$$E_{24}\,\% = \frac{height\ of\ emulsion}{height\ of\ solution} * 100$$