Bioaugmentation with *Bacillus* spp. for the bioremediation of synthetic wastewater using a fluidised-bed bioreactor

Submitted in fulfilment of the degree of Master of Applied Science: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

Yrielle Roets

August 2019

Supervisor	:	Prof Faizal Bux
Co-Supervisor	:	Dr Raj Lalloo
Co-Supervisor	:	Dr Sheena Kumar

Declaration

Bioaugmentation with *Bacillus* spp. for the bioremediation of synthetic wastewater using a fluidised-bed bioreactor

Yrielle Roets

I, Yrielle Roets and Professor Faizal Bux do hereby declare that in respect of the dissertation and the content herein is entirely my own work. It has never before been submitted for any diploma, degree or examination to any other University, Technikon or Tertiary Educational Institute.

Yrielle Roets **Date**

Reference Declaration in Respect of a Master's Dissertation

I Yrielle Roets and Professor Faizal Bux do hereby declare that in respect of the following dissertation:

Bioaugmentation with *Bacillus* spp. for the bioremediation of synthetic wastewater using a fluidised-bed bioreactor

As far as we know and can ascertain: No other similar dissertation exists: All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

Signature of Student **Date**

Signature of Supervisor **Date**

Signature of Co-Supervisor **Date**

Signature of Co-Supervisor **Date**

Approval

I hereby approve the final submission of the following dissertation.

Prof. F. Bux

Supervisor

Doctoral Degree in Technology: Biotechnology

Durban University of Technology (South Africa)

Dr R. Lalloo

Co-Supervisor

Doctor of Philosophy: Process engineering

University of Stellenbosch

Dr. S. Kumari

Co-Supervisor

Doctor of Philosophy: Bioscience

Mangalore University (India)

This _25___day of ___March_____ 2020, at the Durban University of Technology

Dedication

I dedicate this thesis to my parents, my mum who eagerly awaited this day but sadly passed before it could be completed. Your Love, kindness and support have been the rock on which I have leaned on and has comforted me on this journey. This was meant for you before and it is still in your honour. Lastly to my Papa, who had no limits to making us happy and was the perfect example of a father, you set the bar, gone too soon. Love always.

Acknowledgements

I would like to take this opportunity to show my gratitude to those who have assisted me in a myriad of ways:

I would first like to express my gratitude to my co-supervisor Dr Rajesh Lalloo. This thesis was a journey not only academically. This master's study would not have happened if it weren't for his guidance and mentorship not only during the course of this masters but in my growth professionally and personally.

Within the University I have also had the support of my co-supervisor Dr Sheena Kumari and supervisor Prof Faizal Bux. Thank you for your support and allowing this to happen.

I would like to acknowledge and thank my CSIR family. Firstly, Dr Ghaneshree Moodley, who was a pillar throughout this process always encouraging me to soldier on. Secondly, Dr Nodumo Zulu who encouraged me to apply for this masters study and started the journey with me. Lastly the bioprocess development group Santosh, Monique, Prisha, Wynand, Sthembiso and Tsepo for their support.

Lastly to my family:

My Sisters and Brother, Gracey, Sumaya, Tayren and William, we have been through so much heartache. We have grown in love and we are closer than ever before. You are always telling me to do what is possible to succeed and always being there such that I never feel like I am away from home. Tayren, my sister and best friend, I am always grateful for your love and support which has pulled me through the toughest of times. I have always looked up to you, from when we were babies you took care of me, you have been my rock, always protecting me, Love always.

To my new family, my husband, Livelethu who has been steering, pushing and making sure things happen in the background. Always patient and listening to my troubles and stories with interest, showing me how much they matter. Finally, to Levi, our son, who I hope I inspire, with this work.

Abstract

South Africa's freshwater resources, including rivers, man-made lakes and groundwater are under severe threat due to an ever-expanding population and economy, which is depleting these resources. The increase in population has a direct correlation with the increase in wastewater generated. The remaining fresh water resources need to be preserved therefore recycling of wastewater, to replenish our water supplies and preserve the environment, is a solution to the problem. For a developing country, it is important to use treatment methods that are cost effective and do not exert a negative impact on the environment, such as biological wastewater treatment options. One of the systems commonly used in biological wastewater treatment is the fluidized-bed bioreactor (FBBR) due to its advantages such as higher biomass concentration and a higher mass transfer thus resulting in a higher rate of biodegradation. This study focused on evaluating the efficacy of augmenting with *Bacillus* spp. to enhance the bioremediation of wastewater using a FBBR. *Bacillus* spp. used in this study were isolated from a municipal wastewater treatment plant (10 isolates) and the remaining three isolates were selected from the CSIR *Bacillus* database. The isolates (13 in total) were screened for 1) their ability grow in wastewater, 2) ability to reduce high concentrations of COD, ammonium, nitrates and phosphates in flask studies containing synthetic wastewater (SWW) and 3) ability to produce common enzymes such as amylase, cellulase, lipase and protease.

Isolates showed varying bioremediation potential for different compounds analysed. Isolate B006 showed the highest phosphate removal rate (3.290 $mg.L^{-1}.h^{-1}$) where as D005 showed the highest growth rate (0.955 h^{-1}), COD reduction rate (55 $mg.L^{-1}.h^{-1}$) and cellulase activity (5.485 mm) among all the isolates. Isolate D014 presented the highest ammonium removal rate (12.43 $mg.L^{-1}.h^{-1}$), amylase (5.00 mm) and protease (10.00 mm) activity whilst B001 displayed the highest nitrate removal rate (9.4 $mg.L^{-1}.h^{-1}$). The results for the individual

assays were assessed and weighted in a matrix and the isolates that scored above 50% were selected for consortium studies. Four *Bacillus* spp. that scored above 50% in the scoring matrix were then evaluated for their ability to co-exist as a consortium. The consortium studies were then compared with results obtained for individual isolates.

The selected *Bacillus* isolates were identified and assessed for their safety to the environment and to the end user. Identification was conducted using 16s rDNA sequencing and results showed that B006 identified as *B. cereus*, D005 as *B. cereus* and D014 as *B. subtilis*. Isolates, B006 and D005 were further assessed for enterotoxin production and the presence of anthrax virulent plasmids pX01 and pX02. After conducting the biosafety assays, the isolates were rendered safe for use. The isolates were then cryopreserved as spores in 25% glycerol and stored at -80 °C. The impact of the cryopreservation method and the storage conditions on the viability of the isolates was assessed after six months of storage and it was established that the isolates were still viable and that the method was adequate.

The bioremediation potential of the consortium was further evaluated using a 17 L Pilot scale fluidised-bed bioreactor. The reactors were fed at three different flow rates of 1.5 $L.h^{-1}$, 2 $L.h^{-1}$ and 3 $L.h^{-1}$ over steady state conditions (~3months). The results showed that the FBBR augmented with the selected *Bacillus* isolates, resulted in improved nutrient (COD, ammonium and phosphates) removal efficiencies compared to the non-bioaugmented control. The highest ammonium removal (62.8%) was observed at a flow rate of 1.5 $L.h^{-1}$ (11.30 h retention time), whereby there was an overall 29.8% improvement in ammonia removal in comparison to the non-augmented control. Similarly, an overall improvement in phosphate (14.73%) was observed at a flow rate of 2 $L.h^{-1}$ (8.48 h retention time) with 50% removal efficiency. The highest COD removal was observed at a flow rate of 1.5 $L.h^{-1}$ (11.30

h retention time) whereby 74.5% COD was reduced with a 32.6% improvement when compared to the non-bioaugmented control. Our work has demonstrated the potential application of *Bacillus* as bioaugmentation agents to enhance wastewater treatment efficiency as a potential solution to water challenges in developing countries. This technology could also be utilized for addressing the challenges of a wider range of different effluents.

Preface

Research Outputs:

- 2 Conference posters:

South African Society of Microbiology (SASM) 2013 – 8th Biennial conference held in November 2015 in Bela, Limpopo

Poster title: Bioaugmentation using indigenous *Bacillus* spp. for bioremediation of wastewater using a fluidised-bed bioreactor.

Authors: Roets, Y., Kumari S., Bux, F and Lalloo, R.

Water Institute of Southern Africa (WISA) 2015 – 4th Biennial and 1st African YWP conference of Held in November at the CSIR ICC, Pretoria.

Poster title: Using *Bacillus* spp. as bioaugmentation agents in the treatment of synthetic wastewater using a fluidised bed bioreactor**.**

Authors: Roets, Y**.,** Kumari S., Bux, F and Lalloo, R

TABLE OF CONTENTS

List of tables

List of figures

LIST OF ABBREVIATIONS

ASP	-	Activated sludge process
BNR	-	Biological nutrient removal
BOD	-	Biological oxygen demand
CFU	-	Colony forming units
CMC	-	Carboxy methyl cellulose
COD	-	Chemical oxygen demand
CV	-	Coefficient of variance
DNA	-	Deoxyribonucleic acid
EBPR	-	Enhanced biological phosphate removal
EPS	-	Exopolysaccharide
HRT	-	Hydraulic retention time
MBBR	-	Moving bed biofilm rector
NA	-	Nutrient agar
OD	-	Optical density
PAO	-	Polyphosphate accumulating organisms
PCA	-	Plate count agar
PCR	-	Polymerase chain reaction
PHA	-	Polyhydroxy butyrate
PVC	-	Polyvinyl chloride
RBC	-	Rotating biological contactor
rRNA	-	Ribosomal ribonucleic acid
SBR	-	Sequencing batch reactor
SE	-	Sporulation efficiency
SM	-	Sporulation media
SWW	-	Synthetic wastewater
TCA	-	Trichloroacetic acid
TMC	-	Total microscopic counts
TOC	-	Total organic carbon
TSB	-	Tryptone soy broth
UV	-	Ultraviolet
VFA	-	Volatile fatty acids
VMBR	-	Vertical moving biofilm reactor
VOC	-	Volatile organic compounds

Chapter 1: General introduction

As the global population increases, the demand for water also increases resulting in exhaustion of natural freshwater resources. In addition to population increases, other factors such as climate change, economic growth and standards of living increase the requirements for water (Sulieman, A et al., 2006a). Hence, the available water ecosystems are the most endangered (Dudgeon, 2005).

Fresh water resources are at risk from depletion and contamination by pollutants (Edokpayi et al., 2017). To mitigate against these risks water recycling is being used to ease water shortages whilst also preserving the environment. Wastewater treatment systems are designed and managed to protect human and environmental health by alleviating pollution. Wastewater treatment plants are highly mechanized, expensive to build and maintain, therefore, significant time and planning is needed for successful treatment of pollutants (Muga and Mihelcic, 2008). Economically viable, efficient and effective methods of treating wastewater is of utmost importance, especially in less economically developed parts of the world. For these reasons, wastewater treatment and recycling has become the most researched technology of our century.

Wastewater contains a wide range of pollutants that are responsible for environmental contamination; the most common and concerning are compounds that emanate from carbon (C), nitrogen (N) and phosphorus (P). These compounds include ammonium, nitrates, nitrites, phosphates and oxidizable organic material that contributes to high chemical oxygen demand (COD). An influx of these nutrients causes excessive nutrient enrichment of natural water systems, which is termed eutrophication and can cause deterioration in the quality of the receiving water body (Hilton et al., 2006; Smith et al., 2016). In developing countries, challenges such as cost of infrastructure, lack of skills in operating the infrastructure and

excessive load on effluent treatment facilities, influence the quality of treated wastewater entering the environment (Mara, 2013). This results in polluted effluent being released into natural water systems and the environment (Edokpayi et al., 2017). The improvement of wastewater treatment by more efficient methods for removal of COD, ammonium, nitrates and phosphates is a critical challenge to mitigate eutrophication, reduce pollution and preserve the sustainability of water resources (Chávez-Crooker and Obreque-Contreras, 2010). There is a constant search for robust new methods of treating wastewater at a low cost whilst using less energy and minimizing the use of numerous process stages. Conventionally, wastewater is treated using a combination of physical, chemical or biological methods which are illustrated in Figure 1.1. This study focuses on the biological aspect of wastewater treatment, also known as bioremediation and is an option employed to remove oxygen-demanding organic pollutants using microorganisms, which offers the possibility to render various contaminants harmless (Vidali, 2001). For bioremediation to be successful, it is important to have the right microorganisms in the system (Boopathy, 2000). Bioremediation techniques are typically more economical, utilise natural attenuation and have also been publicly accepted more than other technologies (Vidali, 2001).

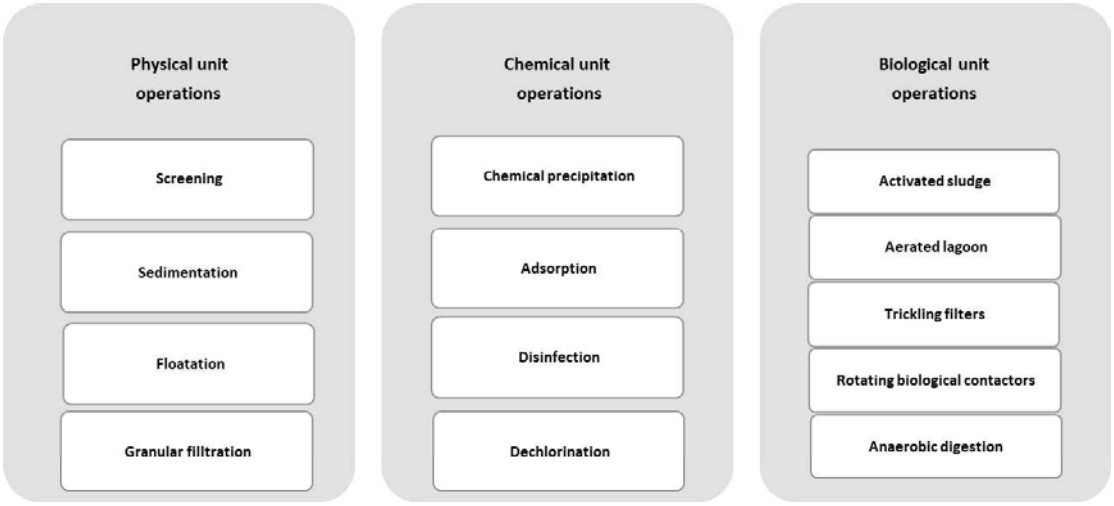

Figure 1.1: Conventional wastewater treatment methods (adapted from Muga and Mihelcic, 2008)

The ultimate goal for researchers is to establish a process that can treat wastewater effectively and efficiently (Muga and Mihelcic, 2008). Some of the ways that have been used to improve bioremediation efficiency are; 1) modifying the treatment system or bioreactors, 2) altering the flow rate and 3) adding microorganisms, i.e. bioaugmentation. Bioreactors that have been used for wastewater treatment have evolved over time from the conventional trickling towers and activated sludge to membrane bioreactors, rotating biological contactors (RBC) and to more modern technologies such as fixed film submerged filters and fluidised bed bioreactors (FBBR). Some researchers have modified their system by using more than one bioreactor such as Chan et al. (2009) who enhanced wastewater treatment by coupling aerobic and anaerobic reactors. Since microorganisms are responsible for removing pollutants during biological treatment, bioaugmentation has offered an alternative method to improve the waste removal efficiencies (Jianlong et al., 2002; Tyagi et al., 2011). Bioaugmentation has been studied to enhance wastewater treatment efficiency of wastewater treatment plants (Stephenson and Stephenson 1992; Martín-Hernández et al., 2012; Wakako et al., 2013; Raper et al., 2018; Nguyen et al., 2019).

The present study focuses on enhancing wastewater treatment efficiency of FBBR by bio-augmenting with specially selected *Bacillus* isolates. FBBR technology allows a microbial biofilm to attach to bio-material whilst the wastewater is continuously fed through an inlet at a specific flow rate (Abdulgader et al., 2007; Papirio, 2012; Butler and Boltz, 2013; Nelson et al., 2017). The microorganisms added into the system form a biofilm along with other naturally occurring microorganisms. The characteristics of the microbial strain or consortia of microorganisms used in bioaugmentation are a key criterion. *Bacillus* spp. offer an attractive option as bioaugmentation agents, mainly, due to their ability to survive harsh environments and utilise the nutrients in the wastewater (Nemutanzhela et al., 2014). *Bacillus* spp. have been used as biological agents for enhancement of water quality and to

remove typical waste metabolites (ammonium, nitrite, nitrate and phosphate) in aquaculture (Lalloo et al. 2007) and ammonia nitrogen from wastewater (Joong et al. 2005).

In addition, *Bacillus* isolates are suitable as bioremediation agents because of fast growth rates, tolerate a wide range of physiological conditions, sporulating microorganisms, cost effective to produce as they grow on multiple carbon sources and can be processed into products with exceptional shelf-life (Lalloo et al., 2007). For bioaugmentation to be successful it is essential to isolate microorganisms that are indigenous to the contaminated site as the nonindigenous microbes in most cases compete with the indigenous populations (Vidali, 2001).

The aim of this study was to enhance bioremediation of wastewater using FBBR technology bioaugmented with indigenous *Bacillus* spp. In order to achieve the aim, the following objectives were addressed:

- Isolation, screening and characterisation of indigenous isolate, derived from a wastewater treatment plant, as putative biological agents for the treatment of wastewater (Chapter 3)
- Identification, safety, preservation and storage of selected *Bacillus* isolates (Chapter 4)
- Evaluation of the ability of *Bacillus* isolates to remove pollutants in wastewater using a laboratory scale FBBR (Chapter 5)

The *Bacillus* spp. were isolated from wastewater treatment systems. The isolated *Bacillus* spp. were then screened for their ability to breakdown wastewater pollutants such as ammonium, nitrates, phosphates and COD. Their prolific growth in synthetic effluent systems and the synthesis of enzymes of interest were also assessed. Once suitable isolates were selected, evaluation for the ability to co-exist was determined. The isolates

that had the highest percentage in the population study were selected and evaluated for their bioremediation potential. The isolates that possessed an inherently strong bioremediation capability were assessed for their efficacy in consortia. The results obtained from the consortium trial were compared with the results obtained from the selected individual isolates. The findings of this aspect of the research are captured in Chapter 3. Once the isolates were selected it was imperative that the isolates were identified. The identities of the promising candidates were elucidated in order to mitigate any potential risk to end users or the environment. Some *Bacillus* spp. are toxic and can cause disease in humans such as those belonging to the *B. cereus* group. For these reasons it was imperative that a safety assessment be conducted to ensure that the end user is protected. After the isolates were rendered safe for use, the spore suspension of each isolate was preserved in 25% glycerol and stored in cryovials at -80 °C. The viability of the isolates after 6 months of storage was also assessed by cultivating the cryopreserved cultures in media as tracking growth every hour. This part of the research is captured in Chapter 4.

The efficacy of the organisms as bioremediation agents was determined by conducting *in-vitro* lab scale demonstration studies. Two lab scale FBBR's were designed and fabricated in order to conduct these studies. One of the FBBR's was used as the test reactor that was bioaugmented with the selected *Bacillus* isolates. The second FBBR was used as a control with no addition of *Bacillus* isolates. These FBBR's were packed with bio-adsorbents, after which synthetic wastewater was fed through the reactor at different flow rates in order to determine the benefits of biological enhancement for the treatment of wastewater. These findings are highlighted in Chapter 5. Due to the nature of this study, and its potential for wide applications, key findings, conclusion and recommendations of the technology have been identified in Chapter 6.

Chapter 2: Literature review

2.1 Water – a global shortage

Clean water is essential for drinking as well as for recreational purposes and only approximately 1% of the earth's water occurs as freshwater which is a limited resource (Bouwer, 2000; Sonune and Ghate, 2004).). Due to population growth, the available freshwater resources have been negatively impacted (Loucks and van Beek, 2005).

Water is essential for life to continue, therefore management of water quality and availability is essential, since it is predicted that the demand for water will outstrip its supply by 2025 (Kamara and Sally, 2003). Every year, contaminated water, coupled with the lack of sanitation, kills at least 1.6 million children. At the beginning of the "Water for Life" decade, 1.1 billion people did not have access to an improved source of drinking water (Unicef and WHO, 2004).

2.1.1 Water shortage in South Africa

South Africa is a third world country and the existing freshwater resources are under increasing stress from a growing population and an expanding economy. In addition, almost all of the country's fresh water resources have declined in quality due to increased pollution caused by industry, urbanization, forestation, mining, agriculture and power generation (Oberholster and Ashton, 2008). At this pace, it is expected that South Africa's freshwater resources will be fully depleted by the year 2030 (Department of water and sanitation, 2018).

Since South Africa's population is growing rapidly and the volume of waste entering the environment is increasing, a large portion of the sewage emanating from South African

urban and rural areas is not treated appropriately prior to discharge. This is attributed to incomplete sewer systems or sewage treatment plants being overloaded. In some instances, sewage is not even treated such as in rural settings, where pit latrines and septic tanks are used and either overflow or seep into the ground contaminating groundwater. This causes a decline in the quality of fresh water bodies, water quality problems arise when an increasing amount of untreated wastewater is released into surrounding water bodies (Tchobanoglous et al., 1991). The Department of water and sanitation green drop certification, (2009) stated that over 50% of wastewater treatment plants in South Africa are below international standards. The main reasons were attributed to lack of skills to operate the highly specialised treatment systems and also the inadequate treatment technologies employed. For a developing country, it is important to use methods that are cost effective and do not exert negative impacts on the environment (Oberholster and Ashton, 2008)

2.1.2 Wastewater reuse as a solution to water shortages

There is increasing interest over wastewater reuse in many parts of the world which is aimed at promoting sustainable, efficient and appropriate water use (El et al., 2019; Sulieman, A et al., 2006b). Feng and Chu, (2004) stated that wastewater reuse has a potential to improve water resources as systems such as groundwater, rivers and dams will remain preserved. The heightened interest in wastewater reuse is driven by two factors which are scarcity of freshwater resources and environmental concerns emanating from wastewater discharge into the environment. The aim of wastewater treatment plants and technologies is to provide sufficient high quality water that meets environmental standards (Anderson, 2003; Naddafi et al., 2009). Therefore, wastewater must not be viewed as waste to be disposed of but as a resource that must be reused as the onset of waste beneficiation has become a global sensation (Dzionek et al., 2016; Mccarty et al., 2011; Peter et al., 2016).

2.1.3 Sources of wastewater

Wastewater originates predominantly from water used in residences, commercial, agricultural and industrial establishments, together with groundwater, surface water and storm water (Shon et al., 2007; Parker, 2011). The most common type of wastewater is municipal wastewater which contains a broad spectrum of contaminants resulting from the mixing of water from different sources. Industrial waste is as varied as the industry that generates the waste and is commonly treated before discharge into sewers or treated separately through suitable treatment processes or released untreated into municipal sewer lines mixing with domestic wastewater (Sulieman, Yousif and Mustafa, 2006). Agricultural waste emanates from aquaculture, livestock and vegetative farms (Sulieman et al., 2006; Lawrie et al., 2010). Domestic wastewater includes typical wastes from the kitchen, bathroom, toilet and laundry (Eriksson et al., 2002). An estimated 28% of the water that is withdrawn is for municipal use, whilst the agricultural sector uses 62% and industry is estimated to use 10% (Donnenfeld et al., 2018). Figure 2.1 illustrates the different methods in which wastewater enters the environment contaminating fresh water bodies (Loucks and van Beek, 2005).

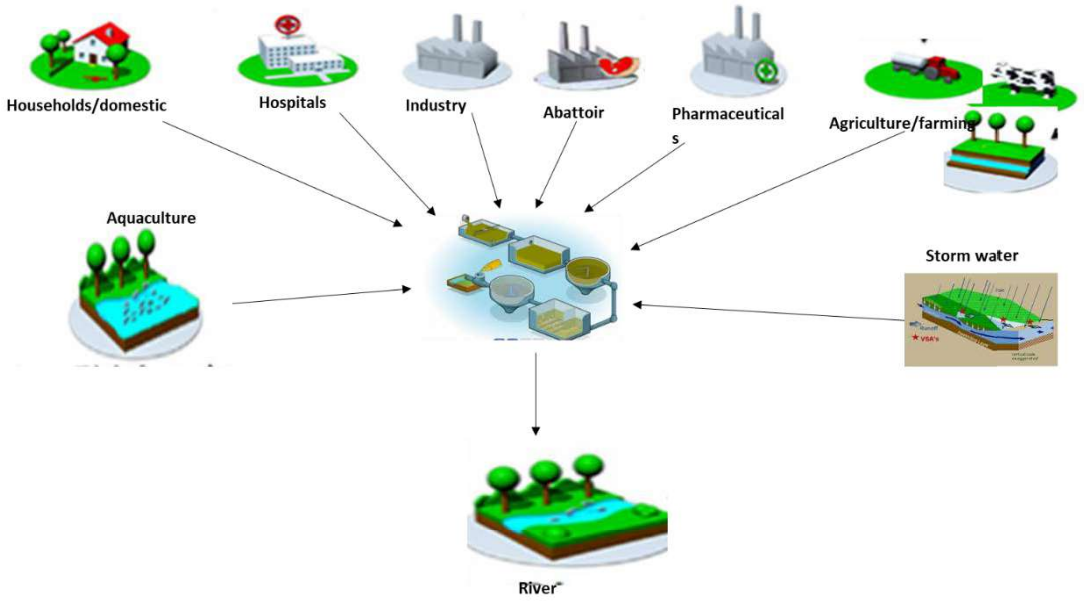

Figure 2.1: Major sources of wastewater from different sectors

2.1.4 Wastewater Characteristics

Knowledge of the composition of wastewater is important, since this allows an understanding of reactions and interactions that occur in it and assists when evaluating disposal and reuse methods (Eriksson et al., 2002). Wastewater quality may be defined by its physical, chemical, and biological characteristics. Industrial waste mostly contains toxic contaminants such as heavy metals, aromatic compounds and dyes because of their wide industrial use. Agriculture wastewater contains substantial amounts of uneaten feed, faeces, pesticides and fertilisers, which contribute to high concentrations of nutrients (Crab et al., 2007). Domestic wastewater contains a lot of food particles, excreta, urine and bath water containing soap (Loucks and van Beek, 2005).

Wastewater emanating from different sources has two major contaminating components classified as organic and inorganic compounds. Organic contaminants include dissolved or undissolved volatile organic compounds (VOC) which include phenols, chlorobenzene and hydrocarbons. Dissolved or undissolved non-volatile organic compounds are designated as biological oxygen demand (BOD) or COD (Shon et al., 2007). Total organic carbon (TOC) such as carbohydrates, fats and starches also contribute to the organic load in wastewater. Inorganic pollutants may include compounds of trace minerals, sulphides, chlorides, nitrogen and phosphorous. These components occur in all types of wastewater in different concentrations. Essential plant nutrients nitrogen and phosphorus, coupled with organic pollutants, are the principal components of concern in wastewater discharges. The discharge of these common pollutants can cause an adverse effect on the environment (Smith et al., 1998; Geissen et al., 2015).

2.2 Wastewater impact on the environment

The effects of wastewater pollution are not only nutrient based, some are caused by the microorganisms found in wastewater. Some of these microorganisms are disease causing bacteria and viruses. Diseases caused by viruses and bacteria originate mainly from the gastrointestinal tract of humans and animals (Naddeo et al., 2009). Typical microorganisms associated with water borne diseases and infections are *E. coli*, Faecal coliforms, Total coliforms, *Enterococcus fecalis*, *Staphylococcus aureus*., *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, Enterovirus and bacteriophages (Sharma et al., 2003; Salgot et al., 2006). Craun and Calderon, (2001) examined causes of reported waterborne outbreaks from 1971 to 1998 and noted that, in community water systems, 30% of 294 outbreaks were associated with wastewater polluted systems.

Common wastewater pollutants released into natural water sources can either stimulate the growth of aquatic biota or cause negative effects on the environment (Shannon et al., 2008). The most common pollutants or nutrients found in wastewater originate from nitrogen and phosphorus (Connell et al., 2009; Amin et al., 2014; Zhou et al., 2014). Nitrogen and phosphorus are essential for growth of all living organisms, however in excess they can be detrimental to the environment. If left untreated nitrogen and phosphorus strongly influence the growth of algae and vascular plants when released into freshwater streams (Smith et al., 1998). The influx of these nutrients and the effect thereafter is termed eutrophication and has a huge impact on water, such as an increase in algal biomass, shifts in phytoplankton composition that maybe toxic or inedible, reduced water clarity, undesirable taste and odour. The increase in algal biomass causes water filtration problems, health risks in water supply, elevated pH, dissolved oxygen depletion in the water and an increase in fish mortalities (Effler et al., 1996; Hilton et al., 2006; Elser et al., 2007; Smith et al., 2016). Figure 2.2 describes the process leading up to eutrophication and the effects.

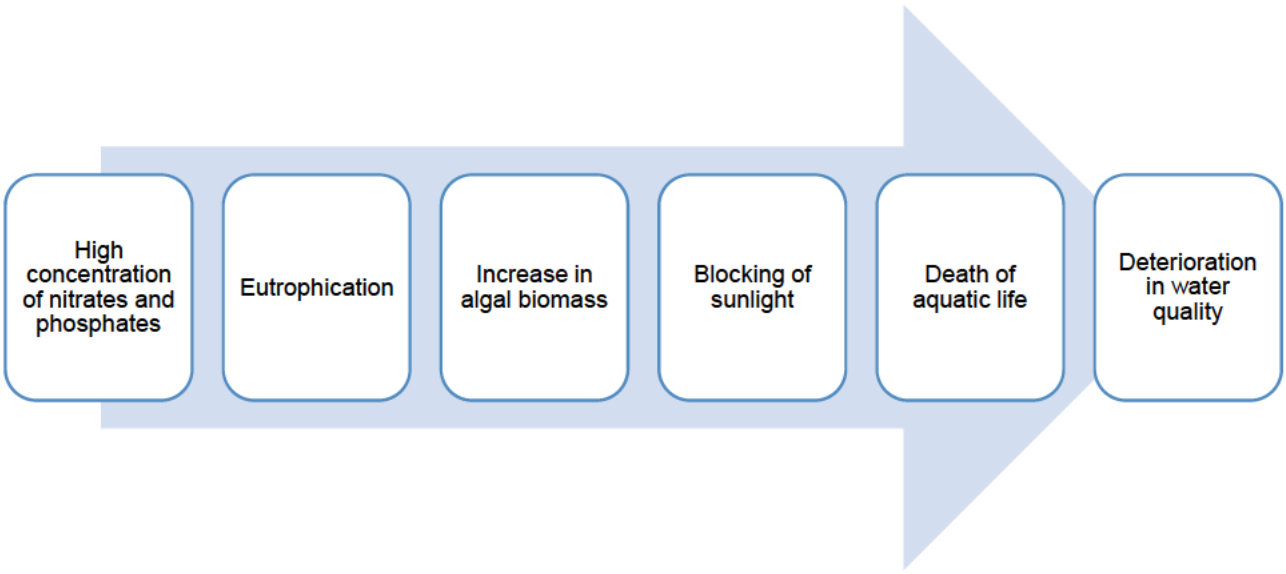

Figure 2.2: An illustration on the process of eutrophication and its effects on a water body.

2.2.1 Phosphorus in wastewater

Human activity has strong effects on the fluxes of phosphorus in the environment. Large quantities of phosphorus are mined and processed to create phosphorus-containing fertilizers, and are heavily applied worldwide even to soils that already contain ample reserves (Liu et al., 2008; Bouwman et al., 2009). Phosphorus readily combines with oxygen to form phosphate (PO_4). In wastewater, phosphorus exists as one of several possible phosphate compounds including orthophosphate (PO_4^{-3}), polyphosphate (P_2O_7) and organic phosphate (Rossle and Pretorius, 2001). Wastewater treatment plant discharges account for 5% of anthropogenic phosphorus loadings (Lombardo, 2006). Phosphorus and nitrogen control are critical to mitigate eutrophication.

2.2.2 Nitrogen in wastewater

Similarly, human activity has profoundly altered the global biogeochemical cycle of Nitrogen (Zhu et al., 2017). Different forms of nitrogen such as ammonium (NH_4), nitrites (NO_2), and nitrates (NO_3) are present in wastewater (Robertsaon and Groffman, 2007; Robertson and Groffman, 2015). While traveling through sewer pipes nitrogen contained in raw sewage as

urea is converted to ammonia through a process called hydrolysis. Ammonia that is formed (ammonium) is then converted to nitrite and nitrate through biological oxidation of ammonia. However, significant concentrations of nitrogen in wastewater may be difficult to treat and can end up in fresh water systems (Mulder et al., 1995; Gupta et al., 2015; Cruz et al., 2018). There is public health concerns regarding high concentration of nitrogen in drinking water which is the cause of methaemoglobinaemia (Patterson, 2003).

2.2.3 Organic pollution in wastewater

Organic pollution occurs when an excess of organic matter, such as manure or sewage, enter a water system. Indicators of organic pollution in water systems are BOD and COD. Together with phosphorus, nitrates in excess amounts can further accelerate eutrophication, causing dramatic increases in aquatic deterioration.

2.3 Methods of reducing wastewater pollutants

Wastewater treatment is a technology that was initiated to protect freshwater resources such as ground water, rivers and dams and ensure that these naturally occurring water bodies are not polluted (Rout, 2013). Wastewater treatment employs three methods of reducing pollution which are physical, chemical and biological (Tchobanoglous et al., 1991). In conventional wastewater treatment, these three methods are accomplished in preliminary, primary, secondary and tertiary treatment illustrated in Figure 2.3 (Sonune and Ghate, 2004). What does preliminary treatment involve? Primary treatment involves the partial removal of solids and organic matter by means of physical operations such as screening and sedimentation. Secondary treatment involves the biological removal of dissolved organic matter and some inorganics ions (Rout, 2013). Tertiary treatment is the disinfection process which can involve ultra violet (UV) treatment, chlorination and filtration to reduce

pathogen load (Sonune and Ghate, 2004; Shon et al., 2007; Rout, 2013). Some processes still use chemical treatment, however biological treatment is better as chemical treatment methods have several drawbacks. Biological treatment processes have been advocated in the last few decades because of significant ecological and economic advantages (Singh et al., 2006; Akpor and Muchie, 2010).

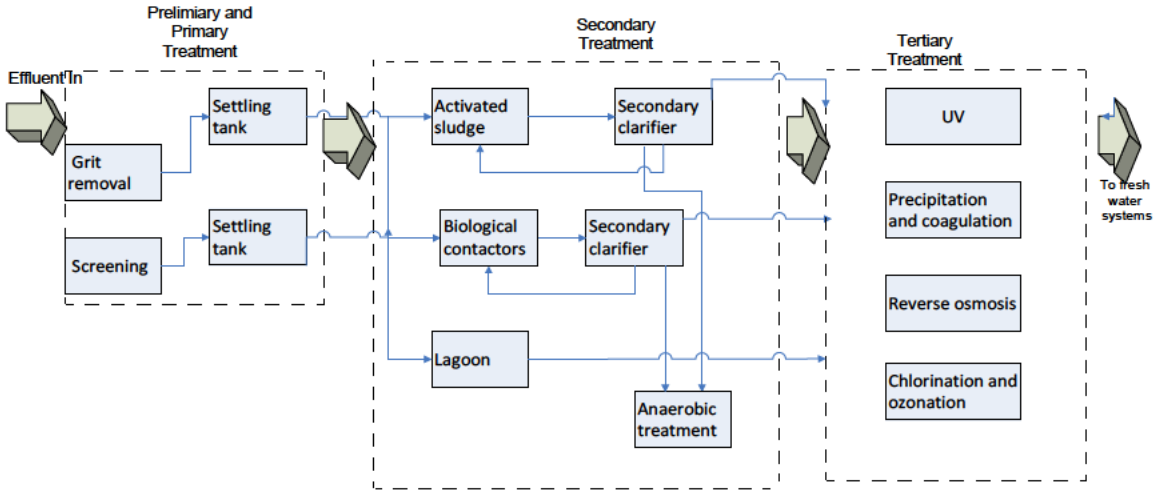

Figure 2.3: Diagrammatic representation of a typical Conventional biological wastewater treatment process

2.4 Biological wastewater treatment

Various strategies are being developed and further research is currently underway to improve means of sustaining the environment by protecting it from toxic pollutants. The development and improvement of the existent methods of biological treatment of wastewater may offer a solution to this problem (Shon et al., 2007; Nozhevnikova et al., 2012). Biological treatment (bioremediation) is the most effective, environmentally responsible in situ technology for the clean-up of environmental pollutants using naturally occurring microorganisms (Kumar et al., 2011; Vidali, 2001). In this process, microorganisms use the pollutants as nutrients to grow and multiply. Biological wastewater treatment can be either aerobic, which employs oxygen utilising microorganisms, or anaerobic which employs microorganisms that do not need oxygen (Mittal, 2011).

In aerobic treatment processes, oxygen is supplied to the microorganisms at a rate sufficient to allow the organisms to metabolize the organic matter (Boopathy, 2000; Singh et al., 2006; Shon et al., 2007).

All biological processes used for treating wastewater contain microorganisms, either attached or suspended. The fixed film process is whereby the microorganisms are fixed on a surface forming a biofilm. The suspended growth process produces biomass which is suspended in the wastewater or sludge (Tchobanoglous et al., 1991). These methods have been used for decades and are currently used in conventional wastewater treatment processes. Conventional wastewater treatment methods are constantly being modified to accommodate high concentrations of nutrients found in wastewater, higher influent volumes and to be cost effective. The wastewater influent characteristics can vary dramatically, such as when there is a sudden large industrial load, or another shock load added. The most common wastewater treatment processes employed all over the world are activated sludge processes (ASP), which makes use of suspending the microorganisms in sludge, and trickling filters which use an attached growth systems (Rajasulochana and Preethy, 2016a).

The activated-sludge process is an aerobic, continuous flow system containing a mass of activated microorganisms that are suspended and capable of treating organic matter (Bhargava, 2016). The ASP can handle large volumes of wastewater however the main disadvantage is that it needs a lot of land and at times electrical power (Wei et al., 2003). Trickling filters are the second most used system that consists of a bed of tightly packed permeable medium. The filter medium to which microorganisms attach may be made of stones or plastic medium (Kornaros and Lyberatos, 2006). Advantages of trickling filters include: low maintenance, cheap installation, and great tolerance of differences in hydraulic and organic loads (Lekang and Kleppe, 2000). Some of the disadvantages of trickling filters

is that they are complex to construct, operate and clogging by growing biomass if too much nutrients are added (Cox and Deshusses, 2001).

Over the past three decades, more technologies have emerged giving new possibilities of improving efficiency in wastewater treatment. Table 2.1 illustrates the different types of conventional treatment and emerging methods of treating wastewater using suspended or attached microorganisms. It has been demonstrated that fixed cultures are less affected by changes in environmental conditions than suspended cultures (Lazarova and Manem, 1995). The first moving biofilm reactor (MBBR) facility became operational in early 1990 (Zafarzadeh et al., 2010a).

When comparing biofilm reactors such as the rotating biological contactor (RBC), the moving bed biofilm reactor (MBBR), the vertical moving bed biofilm reactor (VMBR) and the fluidised bed bioreactor (FBBR), Rodgers and Zhan (2003) concluded that these biofilm reactors were good when treating wastewater with varying waste concentrations, when upgrading a treatment system, treating water from small sources and for removing nutrients.

Table 2.1: The different methods of treating wastewater conventionally and emerging technologies using fixed or suspended growth reactors.

Process	Conventional	Emerging technologies / alternate
Fixed film	• Trickling filters • Rotating Biological contactors (RBC)	• Fixed film submerged filters (FFSF) • Moving bed biofilm reactor (MBBR) • FBBR • Biofilm Airlift Suspension (BAS) • Vertically moving biofilm reactor (VMBR)
Suspended	• Activated sludge • Membrane bioreactor • Anaerobic digesters	• Plug flow • Membrane filtration • Nanotechnology • Automatic variable filtration technology

2.5 Biological removal of common wastewater pollutants

2.5.1 Biological phosphorus removal

Phosphorus is prevalent in nature and is used in many industries, in agriculture and domestically. Phosphorus is one of the contributing nutrients to eutrophication (Correll, 1996), therefore phosphorus needs to be treated before release into the environment. Biological phosphate removal can be achieved in two ways, stoichiometric coupling to microbial growth or enhanced storage in the biomass as polyphosphate which is known as luxury uptake. The latter is also known as enhanced biological phosphorus removal (EBPR) and promotes the removal of phosphates from wastewater without the need for chemical precipitants (Mino et al., 1998). During EBPR the microorganisms used to remove phosphorus are mainly polyphosphate accumulating organisms (PAO) (Akpor and Muchie,

2010). These organisms have the ability to store phosphate as intracellular polyphosphate leading to phosphate removal (Ekama et al., 1986; Jeon et al., 2003; Oehmen et al., 2007). In this process, the wastewater sludge is subjected to anaerobic and aerobic conditions. Some of the sludge from this process is recycled or returned to the anaerobic zone where it mixes with new influent wastewater (Blackall et al., 2002). In the anaerobic zone facultative heterotrophic bacteria breakdown organics into volatile fatty acids (VFA) which serve as a food source for PAO. The VFA and polyphosphate are then converted and stored as orthophosphate and polyhydroxybutyrate (PHB) within the cell (Anand et al., 2014). This process causes the total phosphate concentration in the anaerobic phase to increase. This is followed by the aerobic zone where in the presence of oxygen, the PHB is consumed generating energy for uptake of orthophosphate. The PAO's multiply rapidly and therefore uptake a large amount of phosphates (Baetens, 2001; Seviour et al., 2003). Some of the phosphates are used in cell production and some are stored as polyphosphate inside the cell which is known as luxury uptake. After this process the sludge then goes through a clarifier which separates the water from sludge. Some of the sludge is then recycled to re inoculate the influent wastewater as mentioned whilst the rest is used for energy or agricultural purposes (Loosdrecht and Brdjanovic, 1997; Blackall et al., 2002; Seviour et al., 2003).

2.5.2 Biological nitrogen removal

Nitrogen is one of the major nutrients contained in wastewater and it occurs in different constituents which are formed during biological breakdown. Biological degradation can be performed in different conditions and by different types of organisms during wastewater treatment (Nozhevnikova et al., 2012). The biological reduction of nitrogen in wastewater is performed in two major stages, the first stage is the conversion of ammonium to nitrate via nitrite in a process called nitrification. Nitrate is further converted to nitrogen gas in a process

called denitrification under anoxic conditions (Isaacs and Henze, 1995; Ma et al., 2016; Zanetti et al., 2012).

2.5.2.1 Nitrification

The nitrogenous substance in wastewater, particularly municipal wastewater is mostly found in the form of ammonium ion. Ammonium is a preferred source of nitrogen for many bacterial species (Wang et al., 2016). It is commonly accepted that ammonium is oxidised to nitrate in a process called nitrification. The first step is the conversion of ammonium to nitrite by ammonia oxidising bacteria (AOB), this reaction is illustrated below (Zafarzadeh et al., 2010a).

$$\mathbf{NH_4^+ + O_2 \rightarrow 2H^+ + 2H_2O + NO_2^-} \qquad \textbf{(Eq.2.1)}$$

Thereafter, a second group of phylogenetically different bacteria known as nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate as depicted below:

$$\mathbf{2NO_2^- + O_2 \rightarrow 2NO_3^-} \qquad \textbf{(Eq.2.2)}$$

Nitrite is formed as an intermediate product during ammonium oxidization and proceeds rapidly to nitrate so that it seldom accumulates in the environment (Zafarzadeh et al., 2010a). The three main factors that determine nitrification rate are; 1) load of organic matter, 2) ammonium concentration and 3) oxygen concentration (Ødegaard, 2006).

2.5.2.2 Denitrification

Specialised group of denitrification bacteria perform denitrification, these bacteria perform denitrification by converting nitrates to nitrogen gas as illustrated in Equation 2.3:

$$\mathbf{Food\ (BOD) + NO_3^- \rightarrow OH^- + N_2 + Energy} \qquad \textbf{(Eq.2.3)}$$

For maximum efficiency, nitrification and denitrification require a long retention time and more than one (aerobic and anoxic) treatment conditions. It is recommended that nitrifiers be allowed enough time to thrive in a system because they will wash out and nitrification will be lost (Ebeling, 2006). However some studies have demonstrated that the consolidation of nitrogen removal into a single unit is feasible, thereby reducing the initial capital as well as operating costs (Matějů et al., 1992; Munch and Lant, 1996; Pochana and Keller, 1999; Holman and Wareham, 2005).

2.5.2.3 Heterotrophic nitrogen removal

From Section 2.5.2.1 and 2.5.2.2 it can be deduced that conventional biological nitrogen removal processes include an aerobic stage whereby autotrophs covert ammonium to nitrates followed by an anoxic stage whereby heterotrophs convert nitrates to nitrogen gas. To date, researchers have proven that heterotrophic bacteria are capable of simultaneous nitrification-denitrification (Mével and Prieur, 2000; Yan et al., 2006; Lalloo et al., 2007;; Yang et al., 2017; Khanitchaidecha et al., 2018). These heterotrophic microorganisms have been isolated from soil and wastewater and were discovered to be *Paracoccus denitrificans*, *Alcaligenes faecalis*, *Pseudomonas stutzeri*, *Microvirgula aerodenitrificans* and *Bacillus* spp. (Joo et al., 2006; Kim et al., 2008; Yang et al., 2011; Ye et al., 2017). Su et al., (2015) stated that heterotrophs have the advantage of a high growth rate, ability to convert ammonium to nitrogen gas aerobically, less acclimation problems and less buffer quantity because alkalinity generated during denitrification can partly compensate for the alkalinity consumption in nitrification. The pathway that has been suggested for heterotrophic nitrification and aerobic denitrification is; ammonium → hydroxylamine → nitrite → nitrate (Taylor et al., 2009; Khanitchaidecha et al., 2018; Su et al., 2019;). Research conducted in this discipline has used a host of heterotrophic microorganisms, reactors and operational

parameters. Some of the examples of this research to illustrate the ability of heterotrophic nitrification and aerobic denitrification are described in Table 2.2.

Table 2.2: Research showing heterotrophic nitrification and de-nitrification

Heterotrophic species used	Reference
Bacillus spp.	Joong et al., 2005
Bacillus spp.	Yan et al., 2006
Bacillus spp.	Lalloo et al., 2007
Pseudomonas putida	Kim et al., 2008
Providencia rettgeri	Taylor et al., 2009
B. subtilis	Yang et al., 2011
Cupriavidus sp.	Sun et al., 2016
Bacillus sp.	Yang et al., 2017
Pseudomonas putida	Ye et al., 2017
B. licheniformis	Khanitchaidecha et al., 2018
Acinetobacter harbinensis	Zheng et al., 2018
Acinetobacter sp.	Su et al., 2019
Acinetobacter sp.	Yang et al., 2019

From Table 2.2 it is evident that *Bacillus* spp. are the most commonly researched heterotrophs capable of nitrogen removal. *Bacillus* spp. are advantageous over the others because they consist of aerobes and facultative anaerobes which live in a wide range of habitats, easily isolated, non-toxic and tolerant to temperature, pH and salt conditions (Khanitchaidecha et al., 2019).

2.5.3 Biological removal of organic materials

Traditionally organic matter has been measured as BOD and COD. Both BOD and COD are a measure of the relative oxygen depletion effect of a waste contaminant and both are an indicator of pollution (Jouanneau et al., 2014). The BOD test measures the oxygen demand of biodegradable pollutants whereas the COD is a measure of the oxygen demand of biodegradable pollutants plus the oxygen demand of the non-biodegradable oxidizable pollutants (Pisarevsky et al., 2005). COD in wastewater occurs as three common fractions, these fractions are biodegradable (readily degradable), non-biodegradable along with the biomass fraction as seen in Figure 2.4.

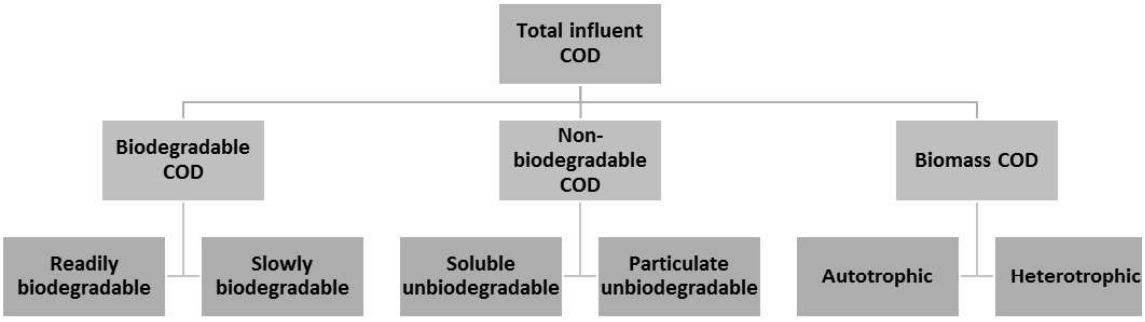

Figure 2.4: Fractions of COD found in wastewater (Pasztor et al., 2009)

2.5.4 Microorganisms responsible for bioremediation

The key to efficient biological wastewater treatment relies on knowing the organisms involved and how they respond to different operational conditions (Xia et al., 2008). The overall performance of any wastewater treatment process, whether in activated aerobic sludge stages or anaerobic digesters, is controlled by the internal microbial structure and composition (Whiteley and Lee, 2006). All biological-treatment processes take advantage of the ability of microorganisms to use diverse wastewater constituents to provide the energy for microbial metabolism and the building blocks for cell synthesis (Rajasulochana and

Preethy, 2016b). The two major pollutants in wastewater are nitrogen and phosphorus derivatives, therefore the microorganisms present in wastewater should have the ability to breakdown these major pollutants and use them as a food source whilst thriving in their presence (Akpor et al., 2014).

Another criterion that wastewater microorganisms should possess is the ability to feed on organic pollutants that contribute to high COD. Coupled to these it would be advantageous for the microorganisms to either form biofilms as flocs or attach to biomaterial in the treatment system. Table 2.3 shows the vast array of microorganisms that have shown the potential to remove wastewater nutrients.

Table 2.3: The different microorganisms responsible for the removal of pollutants found wastewater (Kim et al., 2008 and Zhu et al., 2008)

Mode of action	**Microorganisms**
Nitrifiers	*Nitrosomonas, Nitosococcus, Nitrosospira and Nitrosolobus (Nitrosommonas eutropha, N. Mobilis, N.oligotropha, N.communis, Nitrosolobus multiformis), Nitrobacter, Nitrospira, Nitrococcus and Nitrospira (Nitrospira briensis, Nitrospira sp.etc).*
Denitrifiers	*Thiobacillus denitrificans, Micrococcus denitrificans, Paracoccus denitrificans (Wgner et al., 2005). B. cereus and B. licheniformis*
Phosphorus reducers	*Microlunatus phosphovorus*, *Lampropedia* spp., *Acinetobacter calcoaceticus*, *Acinetobacter iwoffi*, *Aeromonas hydrophila*
COD reducers	*Aerobic bacteria, heterotrophic bacteria*
BOD reducers	*Bacillus spp., Psedomonas spp, entrobacter spp, Lactobaciluus. spp.*

2.5.5 *Bacillus* spp. as robust bioremediation agents

Bacillus spp. are saprophytic, gram-positive, rod shaped, facultative bacteria common in soil, water, dust and air (Nicholson, 2002). *Bacillus* spp. are attractive industrial microorganisms set to become the preferred hosts for the production of many new and improved products (Schallmey et al., 2004). Some *Bacillus* spp. are used for the synthesis of a very wide range of important medical, agricultural, pharmaceutical and other industrial products (Lyngwi and Joshi, 2014). Some *Bacillus* spp. can also break down various naturally-occurring pollutants, which are useful for environmental bioremediation purposes (Das and Chandran, 2010; Nemutanzhela et al., 2014).

Approximately half of the present commercial production of bulk enzymes are derived from strains of *Bacillus* spp. These include proteases, which emanate from species such as *B. alcalophilus, B. amyloliquefaciens, B. lentus* and *B. licheniformis*. Amylases are also produced by many Bacillus species such as *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* (Deb et al., 2013). Members of the *Bacillus* genus are also used in agriculture as bio-fertilisers, plant growth promoting factors and as insecticides (Radhakrishnan et al., 2017).

Bacillus spp. have been involved in bioremediation of wastewater by reducing ammonium, nitrate, phosphate and COD concentrations. Joong et al., (2005) established that *Bacillus* spp. had the ability to fix nitrogen and a *B. subtilis* strain was involved in nitrification, whilst *B. cereus* and *B. licheniformis* strains were involved in converting nitrates to nitrogen gas. Ajao et al., (2011) used *B. subtilis* and *P. aeruginosa* in bioremediation of textile industrial effluent. Yan et al., (2006) proved that a strain designated *Bacillus* sp. LY isolated from a membrane bioreactor, was responsible for COD and total nitrogen reduction by 71.7 % and 61.2 % respectively. Yang et al., (2017) treated synthetic wastewater by bioaugmenting a

SBR with a *Bacillus* isolates that were capable of simultaneous nitrification and denitrification and removal efficiencies of 98% and 95% for COD and ammonium respectively. These researchers have proven the ability of *Bacillus* spp. as bioremediation agents.

2.6 Bioprospecting

The effective operation of a biological treatment plant depends on having the right microorganisms in the wastewater treatment system (Boon et al., 2000). The introduction of microorganisms into a site for bioremediation purposes is termed bioaugmentation. For bioaugmentation to be successful, it is preferable to use indigenous microorganisms (Vogel, 1996). Indigenous microbes are more likely to survive and propagate when reintroduced into the site, as compared to transient or alien strains (Bento et al., 2005; Thompson et al., 2005). The search for pollutant-degrading microorganisms, understanding their degradation ability and developing methods for their application in the field has become an important tool when isolating for an ideal microorganism for bioremediation (Megharaj et al., 2011). Classical sampling, isolation, screening and selection rationale may include collection of background information, acquisition of isolates, purification of isolates and evaluation based on pre-determined criteria (Nemutanzhela et al., 2014).

2.6.1 Sampling and isolation

An ideal environment to sample for microorganisms that can reduce environmental pollutants would be wastewater, water and soil sediments. Methods used for isolating various *Bacillus* spp. were based mainly on resistance of their endospores to elevated temperatures. Földes et al., (2000) used a technique whereby cells were blended with a special enrichment medium which induced vegetative cells to sporulate followed by incubation to allow formation of mature spores in large quantities. Földes et al., 2000 does

not mention the incubation temperature but Lalloo et al., 2007 incubated at 30°C for 24 hours followed by 45°C for 10 minutes, thereafter the culture was mixed with ethanol and centrifuged. The resultant pellet was then incubated at 105°C for 5 minutes. Additionally, the resistance of *Bacilli* to the antibiotic polymyxin B assists in the selection of this group of bacteria whilst eliminating most gram-negative bacteria (Nemutanzhela et al., 2014). Cells can be further characterised by microscopic appearance, gram stain, catalase test and cell morphology (Földes et al., 2000; Nemutanzhela et al., 2014).

2.6.2 Screening and selection of potential candidates from the environment

The screening of potential microorganisms that can be used in treatment of wastewater should be based on the ability of the isolates to breakdown pollutants of interest commonly found in wastewater. Also an essential advantage would the proliferation of the isolates under the desired environment.

The most common wastewater quality variables of concern used for screening a good biological agent for wastewater remediation are ammonium, nitrate, phosphate and COD reduction (Akpor and Muchie, 2010). The microorganisms of choice should be able to reduce these pollutants and can synthesizedesired enzymes. The ability of *Bacillus* spp. to remediate wastewater pollutants has been proven by researchers as mentioned in Section 2.5.5 therefore making them ideal candidates. The isolated *Bacillus* spp. will be screened for their bioremediation ability and the isolates with the best removal rates and enzyme production will be selected.

2.6.3 Identification and safety of selected *Bacillus* isolates

In the 1980s, a new standard for identifying bacteria began to be developed. It was shown that phylogenetic relationships of bacteria and all life-forms, could be determined by comparing a stable part of the genetic code (Woese, 1987). Candidates for this genetic area

in bacteria included the genes that code for the 5S, the 16S and the 23S ribosomal ribonucleic acid (rRNA). The part of the deoxyribonucleic acid (DNA) now most commonly used for taxonomic purposes for bacteria is the 16S rDNA gene. 16S rDNA gene sequences allow bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing (Clarridge and Alerts, 2004).

Bacillus spp. are used in agriculture, bioremediation and medicine either as whole cells, spores or their derivatives (Schroeter et al., 2013). However, some *Bacillus* spp. are considered unsafe for use and therefore a biosafety assessment must be conducted. The strain with members that may be unsafe for biotechnological uses belong to the *Bacillus cereus* group (Zhu et al., 2016). *B. cereus* is the most important cause of food poisoning from this group due to its ability to produce enterotoxins and emetic toxin (Zhu et al., 2016). *Bacillus* anthracis, *Bacillus cereus*, and *Bacillus* thuringiensis are members of the *Bacillus cereus* group of bacteria, demonstrating widely different phenotypes and pathological effects (Nakamura 1998). *B. anthracis* causes the acute fatal disease anthrax and is a potential biological weapon due to its high toxicity. *B. thuringiensis* produces intracellular protein crystals toxic to a wide number of insect larvae and is the most commonly used biological pesticide worldwide. Although many strains of *B. cereus* are ubiquitous and excellent biological agents, some strains are opportunistic pathogens that commonly cause food poisoning (Helgason et al., 2000a).

2.7 Enhancing bioremediation efficiency

For economic and efficiency purposes, development of innovative systems that allow the concomitant removal of waste pollutants in a single reactor is essential in bioremediation (Shukla et al., 2010). A successful bioremediation strategy should be economically viable,

use less land space and be less labour intensive. For these reasons, it would be beneficial to enhance bioremediation efficiency in wastewater treatment systems. One of the options would be bioaugmentation. Using a single bacterial strain or a bacterial consortium, with an array of waste degrading abilities that provide selective advantages in a given environment would be beneficial (Díaz, 2004; Megharaj et al., 2011). Another advantage would be to achieve simultaneous nitrification and denitrification in one vessel which has an advantage over two separated processes such as less land use, energy and labour.

2.7.1 Bioaugmentation

Successful application of bioaugmentation techniques is dependent on the isolation of appropriate microbial strains, and their subsequent survival and activity once released into the target habitat (Thompson et al., 2005). The performance of a biological system is often enhanced through bioaugmentation of one or more species of microorganisms. Using a microbial consortium rather than a single culture for bioremediation is more advantageous as it provides the metabolic diversity and robustness needed for field applications (Rahman et al., 2002; Koul and Gauba, 2014). Alisi et al., (2009) discovered that a mixture of three bacteria was more effective for the removal of COD, nitrate, phosphate and odour compared to using a single isolate. Jiang et al., (2007) also alleged that bioaugmentation using a group of microorganisms may improve reliability compared to using a single strain. It is important for the introduced isolates to establish stable microbial communities within the indigenous bacterial consortium for treatment improvement (Chen et al., 2009). Nzila et al., (2016) stated that harnessing microorganisms present in wastewater treatment plants is one of the most important strategies to remove organic contaminants from wastewater by bioaugmentation.

2.7.2 Fluidised bed bioreactor (FBBR)

The FBBR represents a recent innovation in biofilm processes (Burghate and Ingole, 2013). Immobilization of microorganisms on the fluidised particles of the adsorbent medium results in a higher reactor biomass holdup which enables the process to be operated at significantly higher volumetric throughputs with the practical absence of biomass wash-out (Jena et al., 2005). FBBR's are packed with mobile supports in which particles covered with biofilm are fluidised by the recirculation of liquid. Cells are "immobilized" and move in the fluid while the biomaterial creates a large surface area for cells to attach (Burghate and Ingole, 2013). These characteristics of a FBBR enables an operation at high volumetric loading, a fact that makes them a choice for wastewater treatment (Ochieng et al., 2003). The FBBRs have a major advantage over conventional systems when using modern bio-material, because it supports a higher biomass concentration and a higher mass transfer thus resulting in a higher rate of biodegradation (Ochieng et al., 2003). When compared to other biofilm reactors the FBBR was found to be the best at maximizing pollutant removal compared to the MBBR and the vertical moving biofilm reactor (VMBR) (Rodgers and Zhan, 2003). More importantly, treatment is accomplished in less space and time, which can be translated into a cost effective wastewater treatment process. The efficiency and effectiveness of treating wastewater using the FBBR has been shown by Patel et al., (2006) who achieved high biological nitrogen and phosphorus removal efficiencies using a FBBR bioaugmented with *Enterobacter cloacae*.

2.7.3 Biomaterial

Conventional treatment plants suffer from problems related to their large space requirement, emissions into environments from large open reactors, low process efficiencies, large surplus sludge production and high energy consumption (Chan et al., 2009). The use of carrier materials often provides a physical support for biomass, along with a better access

to nutrients, moisture and aeration, which extend the survival rate of the microbes (Mishra et al., 2001). The elements used (modern biomaterial) in FBBR systems should have a large surface area to volume ratio, have a longer lifespan, enable efficient control of biofilm thickness and support nutrient removal to extremely low concentrations. Different types of bio-material exist in the market but the bio-material seen in Figure 2.5 have proven efficient for use in FBBR systems (Pfeiffer and Wills, 2011).

The biofilm carriers are made from high-density polyethylene or polypropylene, have a large surface area and low density (Figure 2.5). These technologies have a major advantage over the conventional biofilm reactors. The advantages are a large surface area for biomass attachment, no bulking problems, no sludge recycling and no clogging problems in the reactor and a large biomass can be maintained (Andreottola et al., 2000; Pfeiffer and Wills, 2011). Filling fraction of a bioreactor is recommended at less than 70% and bio-carriers are kept in suspension and in continuous movement within the bioreactor (Welander and Mattiasson, 2003).

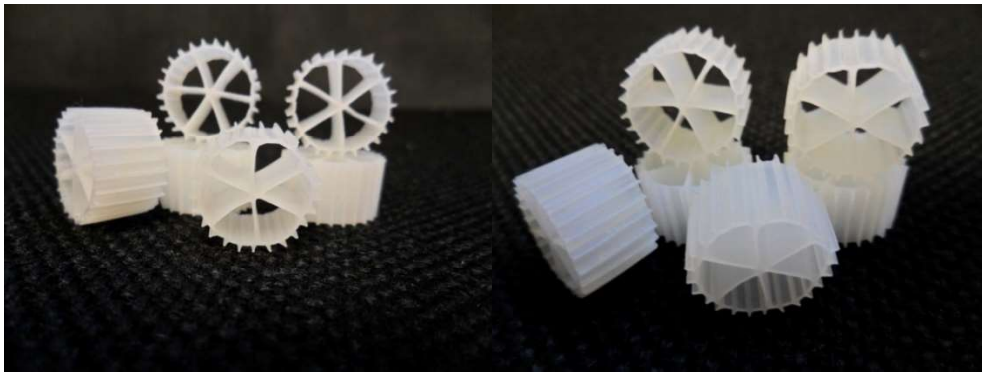

Figure 2.5: Modern biomaterial used in a FBBR**.**

2.8 Biofilm application in bioremediation

The ability for microorganisms to congregate in sessile biofilm structures allows for many advantages compared with their free-living planktonic counterparts. These advantages

include protection from the surrounding environment, ability to communicate and exchange genetic material and collectively manage waste nutrient load. Biofilms can consist of single or multiple species of microorganisms (Edwards and Kjellerup, 2013). Simultaneous nitrification and denitrification can be achieved under aerobic conditions in the bulk water phase (Khanitchaidecha et al., 2015; Su et al., 2015). Oxygen will only penetrate to a certain depth in the biofilm giving an outer aerobic environment, where nitrification occurs. The deeper layers will be anoxic with denitrifying bacteria utilising the nitrate produced by the nitrifiers in the outer layer. Several *Bacillus* spp. have biofilm forming ability and previous research has revealed many genes that express biofilm formation in *B. subtilus*. Critical to biofilm formation is the exopolysaccharide (EPS) operon, which is believed to be responsible for the biosynthesis of an exopolysaccharide that binds chains of cells together in bundles (Kearns et al., 2005; Morikawa, 2006). *B. cereus* has been shown to form biofilms on different surfaces including plastic and metal substances (Hsueh et al., 2006).

Chapter 3: Isolating, screening and characterizing *Bacillus* spp. for potential use in bioremediation of wastewater

3.1 Introduction

A successful bioaugmentation strategy to enhance wastewater treatment requires isolation, screening and characterization of suitable candidates (Van Der Gast et al., 2003; Thompson et al., 2005). It is necessary for the strains to be able to degrade the common chemical components in the waste, as well as to survive and be active at the target site, particularly in the presence of toxic co-contaminants present in various effluents (Jianlong et al., 2002; Van Der Gast et al., 2003; Tyagi et al., 2011).

Bacillus spp. offer an attractive option as bioaugmentation agents, mainly due to their ability to survive harsh environments as a result of their ability to form spores (Nemutanzhela et al., 2014). Research conducted by Lalloo et al., (2007) demonstrated the potential of adding *Bacillus* spp. to enhance water quality. Joong et al., (2005) discovered that some *Bacillus* isolates had the ability to remove ammonium ion from wastewater. Some researchers have used a consortium that included *Bacillus* spp. For example Antony and Philip, (2006) used a consortium that included different *Bacillus* spp. and *Lactobacillus* spp. to break down organic pollutants in aquaculture wastewater. One of the main beneficial traits using *Bacillus* spp. is the ability to produce a variety of important enzymes. Some of these enzymes are pertinent in wastewater treatment such as amylase, cellulase, lipase and protease (Karigar and Rao, 2011). These hydrolytic enzymes breakdown substrates to small molecules, which are then taken up as energy sources by other organisms.

Thompson et al., (2005) stated that in most cases, a bioaugmentation strategy is effective if the site for isolation is similar to the environment in which the strains will be applied. There

is increasing evidence from literature to support this and showing that the best way in which to overcome ecological barriers is to look for microorganisms isolated from the same ecological niche as the area in which they will be applied (El Fantroussi and Agathos, 2005). Individual strains or consortia of organisms should be able to multiply at sites of use. Furthermore characterization of selected strains could assist in selecting the most appropriate candidates without any negative impact on the environment (Van Der Gast et al., 2003). Chapter 3 forms part of a study, which was conducted, with the aim of isolating, purifying, screening and selecting organisms based on pre-defined criteria for bioremediation of wastewater, more specifically to augment sewage treatment systems.

3.2 Methodology

3.2.1 Sampling and isolation of spore forming *Bacillus* isolates

3.2.1.1 Sample collection

Most of the samples were collected from the primary and secondary stages of a wastewater treatment plant as illustrated in Figure 3.1. The treatment plant that was selected for sampling was a municipal treatment plant situated in the City of Tshwane (GPS: 25° 43' 16.72" S, 28° 03' 14.07" E) after permission was obtained from the municipality to collect samples. Liquid samples were collected using a long wooden stick with a cup attached at one end. Wastewater was collected (approximately 30 mL) and transferred into sterile labelled 50 mL McCartney bottles. Swabs were used to wipe of residues from solid surfaces, such as rocks from trickling filter towers, and placed back into housing vials. Sampling details such as locality details, date, time and sample type were also recorded during the time of sampling. All samples were stored in a cooler box during sampling and transportation; upon arrival they were stored at 4 °C in a laboratory fridge, until further use.

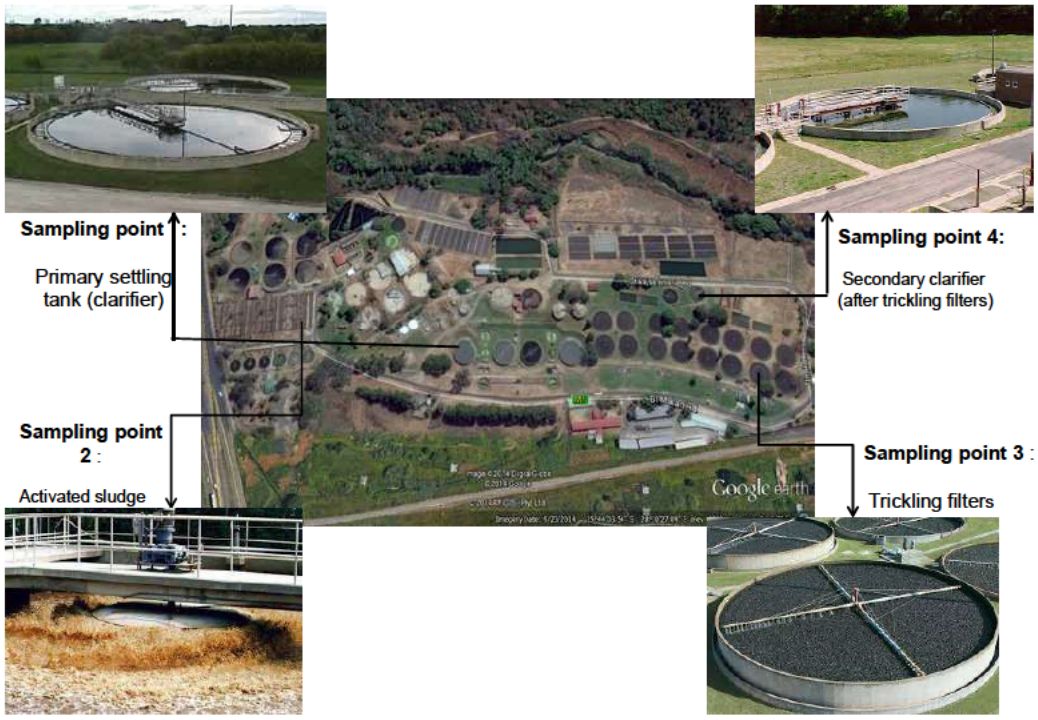

Figure 3:1: A Google earth image of one of the treatment plant that was sampled at the City of Tshwane and an illustration of the different points that where sampled.

3.2.1.2 Isolation of spore forming *Bacillus* spp. from samples

Sporulation media (SM) was prepared according to the recipe listed in Table 3.1 The components that were used to make SM were all molecular grade manufactured by Merck, Darmstadt, Germany. The prepared media (100 mL) was added into 1000 mL Erlenmeyer flasks. The flasks were then sterilised by autoclaving at 121 °C for 15 minutes, after which they were allowed to cool to room temperature before inoculation. The samples that were collected in Section 3.2.1.1 were then aseptically transferred into the flasks under a biological safety cabinet. Wastewater (liquid) samples were vortexed and 1 mL was transferred into sterile Erlenmeyer flasks containing SM. Swab samples were transferred aseptically by cutting of the cotton bud, into sterile Erlenmeyer flasks containing SM, using a sterile scissor. Soil and sludge samples were weighed (1 g) aseptically and added into sterile Erlenmeyer flasks containing SM. All flasks were then incubated at 32 °C on a rotary

platform shaker set at 180 rpm. Samples from the flasks were analysed daily and observed under the microscope. The cells and spores were counted microscopically using a Haemocytometer counting chamber (Thoma®, Hawksley and Sons, London) to determine sporulation efficiency using the formula below:

$$\textbf{\textit{Sporulation efficiency}} = \frac{\textit{no. of spores}}{\textit{no. of spores} + \textit{no. of cells}} \times \mathbf{100} \qquad \textbf{\textit{(Eq. 3.1)}}$$ (Lalloo et al., 2009)

Once a sporulation efficiency between 90-100% was determined, the flasks were removed from the incubator and spore forming *Bacillus* spp. were isolated

Table 3.1: Media constituents (molecular grade) and composition of sporulation medium (Lalloo et al., 2007)

Compound	Unit	Amount
Yeast extract	$g.L^{-1}$	1
$MgSO_4.7H_2O$	$g.L^{-1}$	0.7
$MnSO_4.4H_2O$	$g.L^{-1}$	0.7
$CaCl_2$	$g.L^{-1}$	0.14
Make up with distilled water and adjust pH to 7 using either HCL or NaOH		

A volume of 4 mL from each flask was transferred into 50 mL corning® centrifuge tubes. Ethanol (50% $v.v^{-1}$) was then added to each falcon tube and made up to a volume of 20 mL. The contents were mixed using a vortex and centrifuged at 10 000 x*g*, thereafter, the supernatant was decanted and the resultant pellet was incubated at 105 °C in a convection oven for 5 minutes. The dry pellet was then reconstituted into 20 mL sterile distilled water and serially diluted from 10^{-1} to 10^{-4}. Aliquots (0.1 mL) of each serial dilution were spread onto nutrient agar plates and incubated at 32 °C. The method used to isolate spore forming

Bacillus spp. from environmental samples was adapted from methods outlined by Lalloo et al., (2007)

Colonies formed on culture plates from the isolation step were purified using a three-stage passage method to achieve pure colonies, which involves using a four-way streak technique to isolate single distinct colonies. Colonies were purified by streaking onto fresh nutrient agar plates until only one colony type was visible on the agar plates (Figure 3.2).

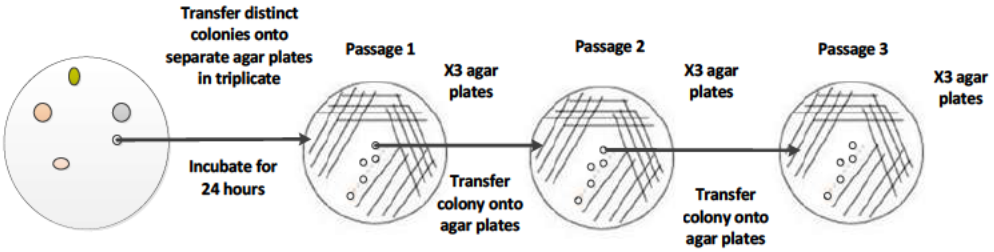

Figure 3:2: An illustration of the pruification of a mixed culture from environmental sample to a pure culture.

3.2.1.3 Verification of isolates as *Bacillus* spp.

General characteristics of *Bacillus* spp. are that these isolates are gram positive, spore forming, rod shaped and catalase positive (Amin et al., 2015). Based on these characteristics the isolated cultures were subjected to gram staining, catalase testing, cell morphology observations and the ability to form spores. Each morphologically distinct gram-positive isolate that produced the catalase enzyme was cryopreserved and data was captured in an in-house database.

3.2.1.3.1 Gram staining

Bacillus spp. are gram positive, to verify this, the isolated, purified cultures were subjected to a gram stain. A colony from each culture plate was smeared onto a microscope slide and

stained according to methods outlined by Claus, (1992) and Thaira et al., (2014). Once the cells were appropriately stained, slides were viewed microscopically using an Olympus microscope at 400 × and 1000 × magnification to substantiate the cultures as either gram positive or gram negative. The cultures that were observed as gram positive were retained and the gram-negative cultures were discarded.

3.2.1.3.2 Catalase test

Bacillus spp. are catalase positive, and this was tested using a qualitative assay. The catalase test was conducted using the hydrogen peroxide method outlined by Iwase et al., 2013, whereby, a pure colony was smeared onto a glass slide using an inoculation loop and a drop of hydrogen peroxide was dropped onto the smeared colony. In the presence of the catalase enzyme, bubbles are seen forming on the colony and in the absence there are no bubbles formed. Catalase is an abundant enzyme in nature decomposing hydrogen peroxide to water and molecular oxygen (Costa *et al*., 2001)

3.2.1.3.3 Cell morphology observation

Isolates that tested gram positive and were able to produce the catalase enzyme were observed microscopically to assess cell morphology of the isolates. *Bacillus* spp. are rod shaped and to verify this, a colony from each culture was smeared on a microscope slide and observed using an Olympus microscope at 1000 × magnification.

3.2.1.3.4 Sporulation test

All cultures that presented as gram positive, catalase producing rods were assessed for their ability to sporulate. In as much as only spore forming isolates were selected in Section 3.2.1.2, this test was the final verification test conducted to ascertain that only *Bacillus* spp. were isolated. The ability of isolated and purified cultures to sporulate was determined by

inoculating a colony from each culture into sterile 1000 mL Erlenmeyer flasks containing 100 mL SM (Table 3.1). Flasks were incubated at 32 °C on an orbital shaker set at 180 rpm and sampled daily to observe and assess the presence of spores along with measuring the sporulation efficiency (Equation 3.1). Isolates that tested positive in the verification tests were then assessed for their ability to utilise and reduce wastewater nutrients that cause pollution.

3.2.2 Screening and selection to determine bioremediation potential of *Bacillus* spp.

3.2.2.1 Inoculum preparation

Pure colonies obtained in Section 3.2.2.3 and three in-house *Bacillus* isolates were used as the source of inoculum for this experiment. A two-stage inoculum was conducted by taking a loopful of colonies from agar plates of each isolate and inoculating each isolate separately into 30 mL McCartney bottles containing sterile 9 mL Tryptone Soy Broth (TSB) (Merck, Darmstadt, Germany). The bottles were mixed for approximately two minutes using a vortex to ensure cell homogeneity. Thereafter, 1 mL of this mixture was aseptically transferred into 1000 mL Erlenmeyer flasks containing 200 mL sterile TSB (autoclaved at 121 °C for 15 minutes). The flasks were then incubated at 32 °C on a rotary platform shaker (New Brunswick Scientific, New Jersey, USA) at 180 rpm for 24 hours until mi- exponential growth phase was obtained. The growth of the flasks was monitored by determining turbidity at an OD of 660 nm. When the mid-exponential growth phase was reached, the concentration of cells was standardised to a final concentration of 1×10^{08} cells $.mL^{-1}$. The cells and spores were counted microscopically using a Haemocytometer counting chamber (Thoma®, Hawksley and Sons, London). The standardised cell concentration was used as an inoculum for the assays described in Section 3.2.2.2. The Sterility of the culture media

and the monoseptic status of cultures were both assessed by streak plating on nutrient agar plates and microscopic observations.

3.2.2.2 Cultivation of *Bacillus* spp. in synthetic wastewater

The ability of the selected isolates to grow in wastewater, remove waste ions, COD and produce pertinent enzymes was assessed by conducting cultivation studies using synthetic wastewater (SWW). The SWW was prepared by mixing all the components in Table 3.2 with distilled water and aliquoting 200 mL into 1000 mL Erlenmeyer flasks after which the flasks were sterilised by autoclaving at 121 °C for 15 minutes. The components that were used to make SWW were all molecular grade manufactured by Merck, Darmstadt, Germany.

Table 3.2: Composition of synthetic wastewater (all compounds used were molecular grade) as reported by (Bracklow et al., 2007; Nopens et al., 2001; Zafarzadeh et al., 2010b)

Compound	Concentration
$NaHCO_3$	48 mg. L^{-1}
KH_2PO_4	22 mg.L^{-1}
NH_4Cl	100 mg.L^{-1}
$CaCl_2$	36 mg.L^{-1}
$MgSO_4.7H_2O$	35 mg.L^{-1}
K_2HPO_4	22 mg.L^{-1}
YE	62.5 mg.L^{-1}
Glucose	62.5 mg.L^{-1}
Trace element Stock solution	1 mL.L^{-1}

Minerals and trace metals: $CaCl_2$, 5 mg.L^{-1}; $FeSO_4.7H_2O$ 10 mg.L^{-1} ; $CuCl_2.2H_2O$, 480 µg.L^{-1}; $Cr(NO_3)_3$ .9H2O, 680 µg.L^{-1}; $MnSO_4.H_2O$, 100 µg.L^{-1}; $NiSO_4.6H_2O$, 300 µg.L^{-1}; $CoCl_2.6H_2O$, 50 µg.L^{-1}; $ZnCl_2$, 180 µ g.L^{-1}; K_2MoO_4, 20 µg.L^{-1}; EDTA, 0.22 µg.L^{-1}.

Minerals and trace metals were adapted from methods outlined by Bracklow et al., (2007) and Nopens et al., (2001) and were prepared once in a stock solution which was used for the duration of the study.

A 2 mL culture containing each isolate in equal proportion, obtained in Section 3.2.2.1, was used to inoculate triplicate flasks. Samples (10 mL) were taken from the flasks before and after inoculation for analysis, to obtain an initial uniform concentration of waste ions in the SWW. The inoculated flasks were then incubated at 32 °C for 12 h on a rotary platform shaker (New Brunswick Scientific, New Jersey, USA) at a speed of 180 rpm. Thereafter, a volume of 10 mL samples were harvested hourly from each flask for analysis which is described in Section 3.2.2.3. A negative control was used and contained no culture addition or inoculum, however, no positive control was used in this study.

3.2.2.3 Analysis to determine wastewater bioremediation potential of *Bacillus* spp.

The samples obtained in Section 3.2.2.2 were analysed for turbidity, the reduction of waste ions and COD, along with the ability to synthesize enzymes such as amylase, cellulase, protease and lipase. Turbidity was assessed by dispensing 2 mL of sample into a cuvette and measuring optical density (OD 660 nm) using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter Inc, California USA) to determine the growth of each isolate in SWW. The growth profile was calculated using the following equation:

$$\mu = \frac{d \ln OD_{(660\,nm)}}{dt} \qquad \textit{(Eq. 3.2)} \text{ (Arana et al., 2013)}$$

The ability of the isolates to breakdown waste ions and COD was assessed by dispensing 6 mL of sample from Section 3.2.2.2 into Eppendorf tubes and centrifuging at 10 000 x*g* for five minutes. The resultant supernatant was used to assess the reduction of COD,

ammonium, nitrate and phosphate. The analysis was conducted according to the methods outlined on Merck test kit inserts, catalogue numbers 1:00683.0001, 1:14773.0001, 1:14848.0001 and 1:14895.0001 respectively and in Section 3.2.2.3.1 to Section 3.2.2.3.4. Rates of waste reduction were calculated by determining the slope (conforming to linearity r^2>0.9) of concentration ($mg.L^{-1}$) plotted against time (hours). Enzyme assays were conducted according to methods outlined in Section 3.2.2.3.5 to Section 3.2.2.3.8.

3.2.2.3.1 COD analysis (1:14895.0001)

The COD Merck test is based on the amount of oxygen originating from potassium dichromate that reacts with the oxidizable substances contained in a sample. The supernatant (2 mL) from each filtered sample from section 3.2.2.3 was slowly added into sample vials found in the kit. Each vial contained sulphuric solution of potassium dichromate, with silver sulphate as the catalyst. The vials were homogenized using a vortex, after which, they were placed in a thermoreactor for 120 min at 148 °C. After the incubation period, the vials were removed from the thermoreactor and allowed to cool to room temperature, thereafter COD was measured by placing the cooled vials in a Pharo 300 Spectroquant® (Merck Milipore, Massachusetts, USA). The resultant measurement appearing on the Spectroquant® screen was recorded and multiplied by the dilution factor for a final concentration.

3.2.2.3.2 Ammonium analysis (1:00683.0001)

In strongly alkaline solution ammonium, nitrogen is present almost entirely as ammonia, which reacts with hypochlorite ions to form monochloramine. This in turn reacts with a substituted phenol to form a blue indophenol derivative that is determined photometrically. To assess ammonium concentration in the samples, reagent one from the test kit was added into each test tube (5 mL), thereafter 0.10 mL of supernatant from each respective sample

was added into the test tube followed by a scoopful of reagent two. The test tubes were homogenised and allowed to stand for 15 mins for the reaction to occur. After 15 mins the contents in the tubes were transferred into a glass 10 mm cuvette and ammonium concentration was measured using a Pharo 300 Spectroquant® (Merck Milipore, Massachusetts, USA). The resultant measurement appearing on the Spectroquant® screen was recorded and multiplied by the dilution factor for a final concentration.

3.2.2.3.3 Nitrate analysis (1:14773.0001)

In concentrated sulphuric acid nitrate ions react with a benzoic acid derivative to form a red nitro compound that is determined photometrically. To test for nitrate concentration in our samples, a level spoon full (spoon was provided in the kit) of reagent one powder (NO_3-1) was added into clean dry test tubes. Reagent two (NO_3-2) (5 mL) was added into each test tube and vortexed until the reagent one had completely dissolved. After the mixture had completely homogenised, 1.5 mL of the filtered samples were aliquoted into respective test tubes and allowed to stand for 10 mins reaction time. After the reaction period had elapsed, samples were analysed for nitrate concentration by adding into a glass 10 mm cuvette, which was the placed in a Pharo 300 Spectroquant® (Merck Milipore, Massachusetts, USA) and nitrate concentration was determined. The resultant measurement appearing on the Spectroquant® screen was recorded and multiplied by the dilution factor for a final concentration

3.2.2.3.4 Phosphate test (1.4848.0001)

In sulphuric solution orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically. The supernatant obtained from the filtered samples (5 ml) was pipetted into a test tube. Thereafter, reagent one (PO_4-1) was added by releasing 5

drops into the test tubes of each sample, followed by one level spoonful of reagent 2 (PO_4-2). The contents were homogenised by vortexing and allowed a reaction time of 5 mins after which, a cuvette (10 mm) was filled and used to measure phosphate concentration using a Pharo 300 Spectroquant® (Merck Milipore, Massachusetts, USA). The resultant measurement appearing on the Spectroquant® screen was recorded and multiplied by the dilution factor for a final concentration

3.2.2.3.5 Amylase

Amylase activity was detected using starch agar basic medium which was prepared by adding 1 g soluble starch and 28 g nutrient agar into 1000 mL deionised water and sterilised for 15 minutes at 121 °C. After cooling to approximately 60 °C, the agar was aseptically poured into sterile plastic petri dishes and left to solidify. After solidifying a well was made in the agar using the back end of a sterile pipette tip after which 100 µL of culture of each test isolate, was added to the well in triplicate plates. The plates were then incubated at 32 °C for 24 hours. After the incubation period, the plates were flooded with Gram's iodine solution for 3 to 5 minutes (grams iodine was prepared by adding 2 g potassium iodide and 1 g iodine in 300 mL distilled water). Zones of clearing were measured using a Vernier calliper and an average of the three plates was recorded. This method was adapted from methods outlined by Alariya et al., (2013).

3.2.2.3.6 Cellulase

Screening for cellulase producing isolates was done using nutrient agar plates containing 1 g carboxymethyl cellulose (CMC) and 28 g nutrient agar into 1000 mL of distilled water. The agar was then sterilized, inoculated and incubated according to Section 3.2.2.3.1. After the incubation period, the plates were flooded with Gram's iodine solution for 3 to 5 minutes. Zones of clearing around each well were measured using a Vernier calliper and an average

of the three plates was recorded. This method was adapted from methods outlined by (Kasana et al., 2008).

3.2.2.3.7 Lipase

Lipase activity was measured using a modified version of the method published by Kouker and Jaeger, (1987). Growth medium containing nutrient agar was supplemented with gum Arabic (10 $g.L^{-1}$), Rhodamine B solution (10 $g.L^{-1}$) and olive oil (10 $mL.L^{-1}$). Media was sterilised in an autoclave at 121 °C for 15 minutes and cooled to approximately 60 °C. Thereafter, calcium chloride ($CaCl_2$) solution was added into the medium, mixed gently then poured into plastic petri-dishes and left to solidify. Single wells were made on triplicate agar plates and 100 µL of culture was added into each well for each of the test isolates. The plates were incubated at 32 °C for 48 hours. Presence of the lipase enzymes was observed by zones of clearing when viewing under UV light. Zones of clearing were an indicator that the lipase enzyme had degraded the olive oil in the media (Haba et al., 2000). Due to the sensitivity of the assay when viewing under UV light, zones are seen within seconds and vanish just as easily therefore results are noted as positive or negative.

3.2.2.3.8 Protease

To assess the production of proteases 28 g nutrient agar supplemented with casein (10 g) and milk powder (10 g) were mixed in 1000 mL of distilled water. The agar was sterilised, inoculated and incubated according to Section 3.2.2.4.1. After incubation, the plates were flooded with a 25% trichloroacetic acid (TCA) solution and incubated for 15 minutes at 45 °C. In the presence of the protease enzyme, zones of clearing were observed around the wells, these zones were measured using a Vernier calliper and an average of the three plates was recorded. This method was adapted from methods outlined by (Vijayaraghavan et al., 2013).

3.2.2.4 Selection of putative isolates based on performance against pre-defined bioremediation criteria in a scoring matrix

A scoring matrix is a decision making tool that is used to narrow a list of options based on several criteria. An in-house scoring matrix, developed at the CSIR, based on results obtained and statistics was used to rate each isolate whereby, each criteria was given a weighted score (Table 3.3). The isolates with the highest scores obtained a high percentage in that criteria. Performance of each isolate was assessed after analysing the data obtained from each respective trial. Isolates were selected based on their resultant growth rate in SWW, their ability to reduce waste ions, COD and the ability to synthesis pertinent enzymes. The cumulative number of positive responses or indicators was also a measurement criterion. The scoring matrix was configured using these indicators, such that potential isolates could be selected based on their performance (Table 3.3). Thereafter, the selected isolates were used to develop different consortium combinations. The isolates scoring above 50% were selected to be included into subsequent consortium studies.

Table 3.3: A list of the different responses that were used to configure the scoring matrix and the weights in percentage of each response

Responses	% Weight
Growth rate (μmax in SWW)	10
Ammonium reduction	15
Nitrate reduction	15
Phosphate reduction	15
COD reduction	15
Amylase	5
Cellulase	5

Protease	5
Lipase	5
Total positive responses	10

3.2.2.5 Evaluating the ability of selected isolates to coexist

The selected isolates were assessed for their ability to coexist. A 2 mL volume consisting of each isolate, obtained from an inoculum prepared in Section 3.2.2.1, was used to inoculate triplicate Erlenmeyer flasks containing SWW (prepared and sterilised as in Section 3.2.2.2). Prior to inoculation, the concentration of each isolate was standardised such that an equal concentration (1×10^{08} cells.mL^{-1}) of each isolate was achieved in the 2 mL sample that was used to inoculate. The flasks were incubated at 32 °C, 180 rpm and samples (2 mL) were taken every hour for 12 hours. Samples were analysed for OD at 660 nm to monitor the growth profile of the consortium. The sample that was taken at end of exponential growth phase was used to assess the ability of the isolates to coexist by conducting spread plates on plate count agar (PCA) Merck, Darmstadt, Germany). The agar plates were incubated for 24 hrs at 32 °C. Following incubation, colonies appearing on PCA plates were assessed by identifying them on agar plates by morphological assessment and determining the concentration of each isolate using colony forming units (CFU). All isolates that were selected were combined and assessed for their ability to dominate in SWW. Thereafter, the isolates that isolate that possessed the lowest dominance (below 10 %) was eliminated.

3.2.3 Determining the bioremediation potential of selected isolates in consortium

3.2.3.1 Inoculum preparation

Agar plates containing purified selected isolates (B006, D005 and D014) were used to inoculate and prepare a two-stage inoculum as in Section 3.2.2. Following incubation and growth at mid to end of exponential growth phase based on tracking OD 660nm

measurements, cell concentration was determined at the end point using total microscopic count (TMC), to enable standardization of the inoculum to a cell concentration of 1×10^{08} cells.mL^{-1} before use.

3.2.3.2 Cultivation of selected isolates in consortium

A 2 mL volume containing each isolate in equal concentrations was used to inoculate sterile Erlenmeyer flasks containing 200 mL SWW prepared and sterilised as in Section 3.2.2.2. The flasks were incubated at 32 °C at a shaker speed of 180 rpm on a rotary platform shaker (New Brunswick Scientific, New Jersey, USA). The ability of the consortium to grow in wastewater, remove waste ions, COD and produce pertinent enzymes was assessed.

3.2.3.3 Determination of bioremediation potential of consortium

Samples (10 mL) were taken from the flasks before and after inoculation and every hour subsequently for analysis, to obtain an initial OD 660 nm, concentration of waste ions and COD in the SWW. Analysis were conducted according to methods in Section 3.2.2.3.

3.2.3.4 Final selection of combination or single isolate for inclusion in a biological product

The consortium results were compared with the results obtained for single isolates. The data was assessed in a scoring matrix based on data obtained for growth rate in which contributed 10% to the total weighting matrix. The ability to reduce waste ions and COD contributed 15 % each, whilst the synthesis of enzymes each contributed 5% (Table 3.4).

Table 3.4: A list of the different responses that were used to configure the scoring matrix and the scores in percentage of each response

Responses	% Weight
Growth rate (μmax in SWW)	10
Ammonium reduction	15
Nitrate reduction	15
Phosphate reduction	15
COD reduction	15
Amylase	5
Cellulase	5
Protease	5
Lipase	5
Total positive responses	10

3.3 Results and Discussion

3.3.1 Sampling and isolation of spore forming *Bacillus* spp.

Overall, 10 samples were collected from a treatment plant in the City Tshwane. This included four swab samples, four liquid samples and two sludge samples from different areas of the treatment plant as shown in Table 3.5.

Table 3.5: Sample type, source and location of samples collected

Sample #	Type of sample	Source	Sampling location
1	Swab	Trickling filter	Treatment plant
2	Swab	Trickling filter	Treatment plant
3	Swab	Trickling filter	Treatment plant
4	Liquid	Trickling filter	Treatment plant
5	Liquid	Primary treatment	Treatment plant
6	Liquid	Grid chamber	Treatment plant
7	Liquid	After screening	Treatment plant
8	Sludge	Activated sludge	Treatment plant
9	Swab	Mucous on skin of Carp	Pond
10	Sludge	Mud from pond	Pond

The organisms (10) that were isolated from the samples listed in Table 3.5 were presumptively identified as members of the *Bacillus* genus as they exhibited characteristics listed in Table 3.7. The swabs obtained from trickling filters yielded the highest number of isolates as highlighted. The selected CSIR isolates showed good bioremediation potential in previous independent studies conducted at the CSIR and reported by Lalloo et al., (2007). *Bacillus* species were targeted in this study because of its ability to replicate rapidly, high growth rate, tolerance to a multitude of environmental conditions and their ability to form spores under stress conditions (Nemutanzhela et al., 2014). Bioremediation ability of *Bacillus* spp were also previously reported by various researchers Joong et al., (2005); Lalloo et al., (2008); Yang et al., (2011); Zhang et al., (2012a,) and Yang et al., (2017b).

Table 3.6: List of pure *Bacillus* spp. that were used in the selection study

Samples	Type of sample	Source
B001	Cryovial	CSIR database
B006	Cryovial	CSIR database
B008	Cryovial	CSIR database
D003	Swab	Trickling filter
D004	Swab	Trickling filter
D005	Swab	Trickling filter
D006	Liquid	Trickling filter
D012	Liquid	Primary Treatment
D014	Sludge	Primary treatment
D015	Liquid	Grid chamber
D016	Swab	After screening
D018	Sludge	Activated sludge
D019	Liquid	Primary treatment

All the 13 isolates were assessed for cell and colony morphology, their ability to produce catalase enzyme and spore formation to verify the isolates as belonging to the *Bacillus* genus. The isolates were all gram positive, catalase positive, spore-forming rods as depicted in Table 3.7. The morphologies were in line with morphologies of *Bacillus* spp. described in literature (Fujikawa, 1994; Lu et al., 2018). Coupled to this, Amin et al. (2015) stated that the ability to produce catalase enzyme, being gram positive and the ability to form spores are some of the basic characteristics of *Bacillus* spp.

Table 3.7: A description of the number of isolates obtained their designation, microscopic and colony morphology, catalase and sporulation test results.

Isolate	Microscopic Morphology	Colony Morphology	Catalase test	Spore formation
D003	Slender, long rods appear in pairs	Rough dry mat, raised centre, circular and entire	+	+
D004	Short and small rods	creamy orange shiny, circular, umbonate and entire	+	+
D005	Short rods and appear in pairs	white to creamy, raised, circular, undulate with a dry appearance	+	+
D006	Thick and medium sized rods	Cream coloured, irregular, flat and undulate.	+	+
D012	Short rods appearing in pairs	Extreme orange, circular, entire and raised colonies	+	+
D014	Slender and long rods appearing in pairs	White rough dry matted centre with smooth edges, flat and undulate.	+	+
D015	Thick and short rods	A crateriform centre, circular, rough and orange	+	+
D016	Thick and short rods	Shinny white, circular, umbonate and entire	+	+
D018	Short rods, forming long chains	Pink, small, shiny, circular, raised and entire	+	+

D019	Thick rods, forming long chains	Orange fading to cream colour, raised, circular and undulate	+	+
B001	Medium sized rods in chains.	Round with smooth undulate margins and flat	+	+
B006	Long thick rods in pairs.	Creamy, raised and with a dry appearance, circular and undulate	+	+
B008	Long fat rods, some rods curved.	Cream coloured with rhizoid, filiform appearance and flat	+	+

3.3.2 Screening and selection to determine bioremediation potential of isolates

3.3.2.1 Growth rate

All the 13 isolates were screened for their ability to grow in SWW. Figure 3.3 is an illustration of the growth rates exhibited by the isolates in SWW. Isolate D005 (0.955 h^{-1}) displayed the highest growth rate followed by D014 (0.838 h^{-1}) and B006 (0.636 h^{-1}) (Figure 3.3). The growth of an organism in simulated or synthetic media is an important evaluation as it could provide an indication of the growth potential of the organism in actual wastewater. The benefits of an isolates ability to grow and proliferate in wastewater is related to the ability to use the available nutrients, therefore, the faster the growth rate, the faster their ability to reduce wastewater nutrients. The merit of using indigenous microorganisms isolated from similar environments to the actual intended application is that these isolates are may be better adapted to the functional purpose (Rahman et al., 2002). Of the 13 isolates evaluated, three isolates (D012, D015 and D019) did not show any sign of growth within the assessment period of 12 h in SWW and were therefore excluded from further studies Therefore, these isolates were eliminated because of their long lag phase and when applied

to an actual environment would not be able to compete with the strains that showed a high growth rate. No growth was noted in the negative control flasks.

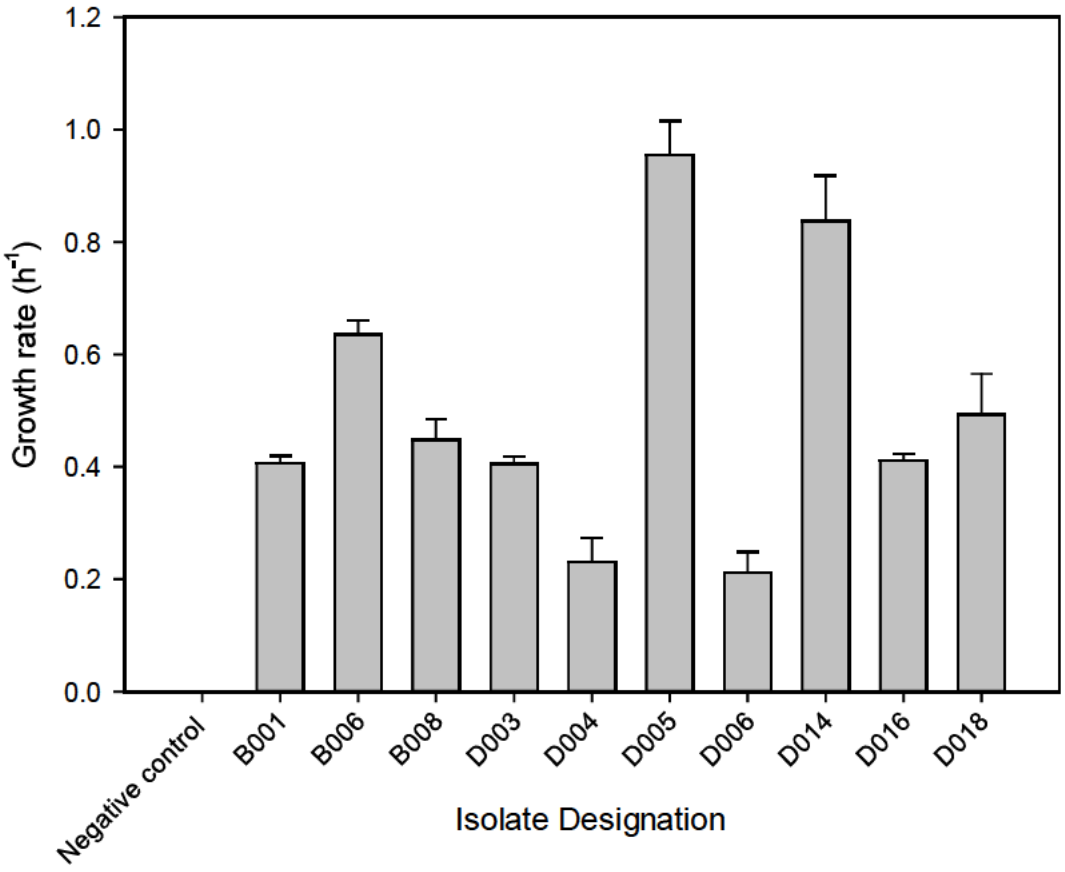

Figure 3:3: Growth rates obtained by all isolates cultivated in SWW

3.3.2.2 Bioremediation potential of isolates

3.3.2.2.1 COD removal

The ability of the isolates to reduce COD in SWW was determined. Figure 3.4 indicate the COD removal rate exihibited by different isolates. Isolates D005 showed the highest COD removal rate (55 $mg.L^{-1}.h^{-1}$), followed by D003 (48 $mg.L^{-1}.h^{-1}$) and D014 (45 $mg.L^{-1}.h^{-1}$). COD is the main carbon source for heterotrophs in wastewater which is used for biomass accumulation and growth. As mentioned, the isolates with a faster growth rate could use nutrients faster as observed in this study. The isolate with the higehst growth rate (D005)

also showed the highest COD removal rate. Previous results by Yang et al., (2011) using a SBR system, reported a COD reduction of up to 96.5% using a *B. subtilis* strain.

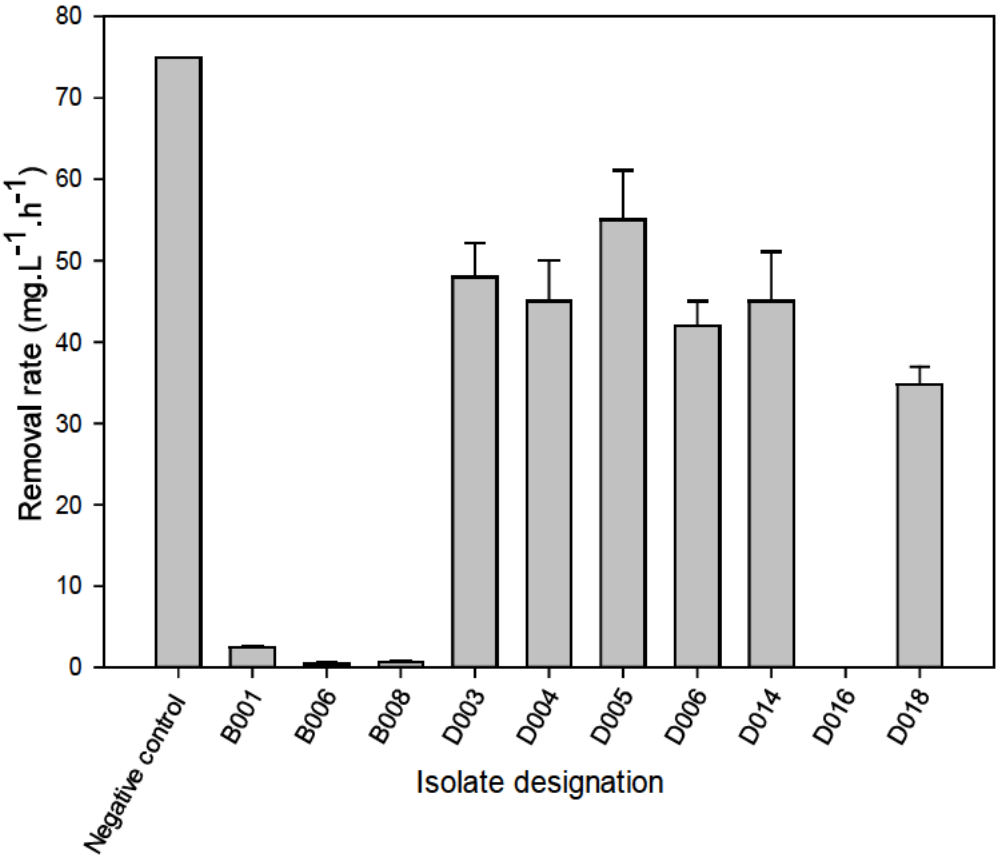

Figure 3:4: Data representing COD removal rates exhibited by each isolate

3.3.2.2.2 Nutrient removal

The removal of pertinent nutrients such as phosphates and nitrogen compounds in wastewater is very important (De-Bashan and Bashan, 2004). Figure 3.5 shows the ammonium, nitrate and phosphate removal rate by the isolates. Isolate D014 exhibited the highest ammonium removal rate (12.43 $mg.L^{-1}.h^{-1}$) when compared to the other isolates and was also able to remove nitrates (9.90 $mg.L^{-1}.h^{-1}$) and phosphates (3.13 $mg.L^{-1}.h^{-1}$) at notable rates. This isolate displayed a better nitrification ability than the other isolates tested, nonetheless. The isolate B006 presented the highest phosphate removal rate of 3.29 $mg.L^{-1}.h^{-1}$ and was also capable of removing ammonium (7.72 $mg.L^{-1}.h^{-1}$) and nitrate (3.77 $mg.L^{-1}.h^{-1}$). The results obtained from these two isolates (D014 and B006) indicated the ability of

Bacillus spp. to perform simultaneous nitrification-denitrification. This ability was previously demonstrated by Zhang et al., (2009) who reported a maximum ammonium and nitrate removal rate of 51.58 $mg.L^{-1}.d^{-1}$ and 5.81 $mg.L^{-1}.d^{-1}$, respectively. With regards to nitrate removal, isolate B001 removed 9.4 $mg.L^{-1}.h^{-1}$ of nitrates, which was also the highest reduction rate obtained indicating their denitrification potential. This isolate was also capable of ammonium and phosphate reduction, however was lower than the rates obtained by B006 and D014. Isolate D005 had the highest growth rate and COD removal rate. The COD results obtained by D005 correlate with the nutrient removal results, as heterotrophic bacteria also need organic carbon for denitrification, D005 also showed that it had a stronger denitrification ability compared to its nitrification ability. Even though D005 had a low ammonium and nitrate removal rate, when compared to the other isolates, the nitrate removal rate was still higher than the ammonium removal rate.

In wastewater systems, nitrite rarely accumulates to detectable levels. Nitrite is an intermediate compound formed during nitrification and denitrification. However, under suboptimal conditions nitrite can accumulate in the system (Philips et al., 2002). The nitrite concentration in this study fluctuated between 3 and 8 $mg.L^{-1}$ possibly due to the transient nature of this ion. Results from our study indicated that, the selected *Bacillus* isolates were able to reduce ammonium, nitrates and phosphates simultaneously from SWW (Figure 3.4).

.

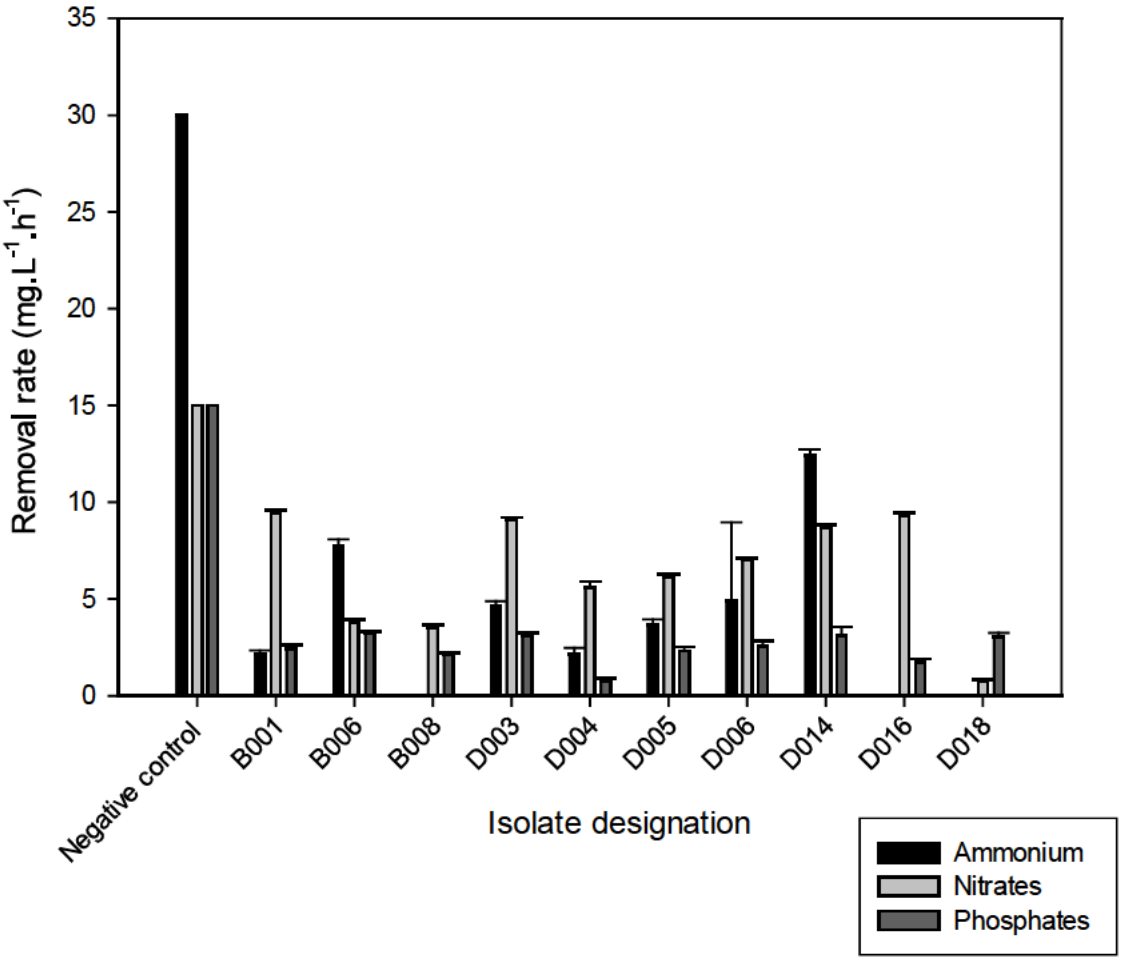

Figure 3:5: Ammonium, nitrate and phosphate removal rates of single isolates cultured in synthetic wastewater

3.3.2.2.3 Enzyme production

The isolates were also screened for their ability to produce enzymes such as amylase, cellulase, lipase and protease. Figure 3.6 shows the production of different enzymes by the isolates. The isolates B001, B006, D003, D004, D005, D014 and D016 were capable of producing all the enzymes (Figure 3.6) but with variable production rates. D014 produced the most protease and amylase whilst D005 produced the most cellulase when compared to the other isolates (Figure 3.6). Both isolates also had the highest growth rates and displayed high bioremediation potential compared to the other isolates. The ability to produce the key enzymes could be advantageous in the overall bioremediation process, as wastewater contains complex organic matter such as proteins, fats, cellulose and

carbohydrates which need to be broken down into simpler compounds that can be easily assimilated easily by the microorganisms (Burgess and Pletschke, 2008). Ellis, (2012) and Yao, (2014) characterised wastewater and reported that it composed of 31% protein, 16% carbohydrate, and 45% lipids. The cellulose content varies according to the type of wastewater as it consists of fibrous material from human diets and pulp from drains. Furthermore, *Bacillus* spp. are known to be good enzyme producers and have been widely used to produce enzymes for different applications (Schallmey et al., 2004; Fábio et al., 2013; Pant et al., 2015; Singh and Kumar, 2017).

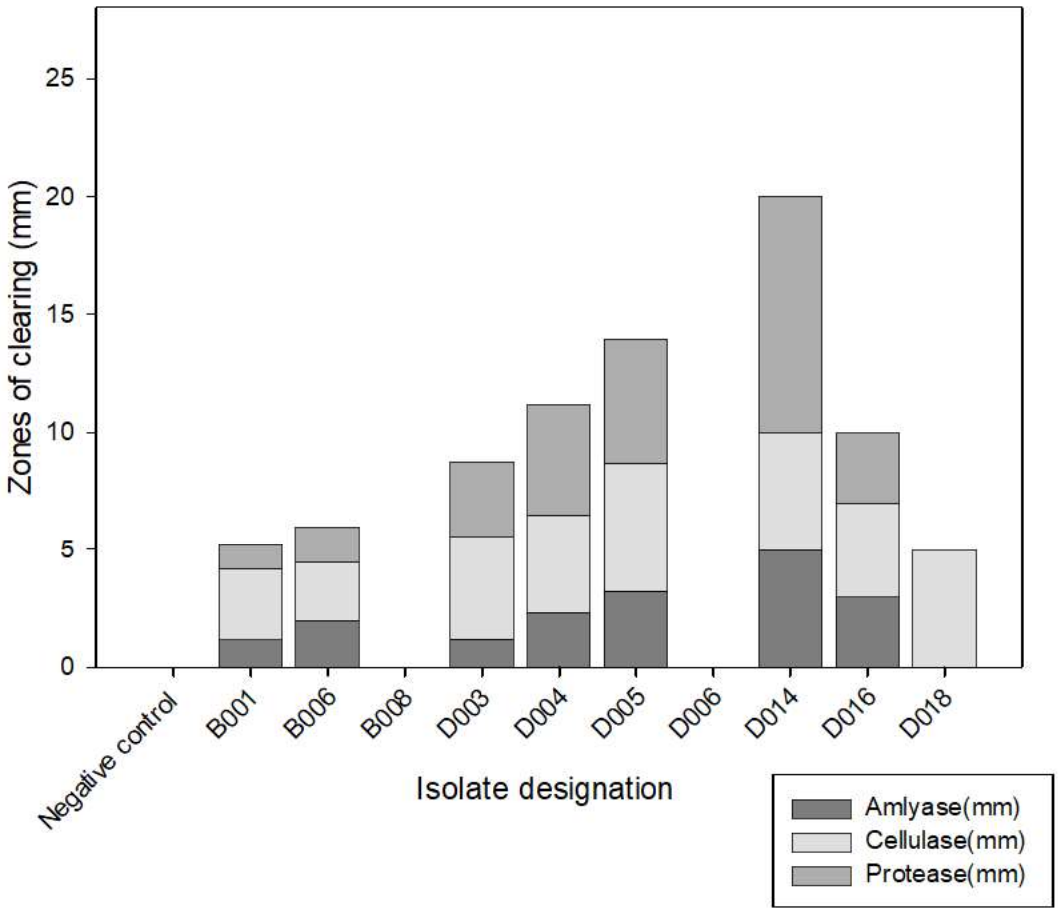

Figure 3:6: A graph illustrating zones of clearing measured in mm exhibited by all isolates

3.3.3 Selection of putative isolates based on performance against pre-defined bioremediation criteria

The bioremediation potential of the isolates varied. None of the isolates showed consistently high results across all the responses tested. Therefore, a microbial consortium encompassing more than one isolate with high bioremediation potential may perform better in comparison to individual strains. For these reasons a scoring matrix was developed to summarize all the results and give each isolate a percentage score befitting of the isolates bioremediation potential.

In this part of the study, all isolates were scored based on all the parameters that were analysed and the isolates that obtained a score above 50% in the scoring matrix were selected. Figure 3.7 illustrates the scoring matrix results for all the isolates. Of the 13 isolates that were screened only four obtained a score of above 50%. The isolate D014 displayed the highest score (91.3%) which can be attributed to the ability to remove ammonium and the production of amylase and protease enzymes at the highest rate. The isolate also achieved the second highest phosphate reduction rate and growth rate whilst obtaining the third highest nitrate reduction rate. Isolate D005 obtained the second highest score (75.1%) in the matrix owing to the highest growth rate and COD removal rate coupled with growth high cellulase production. Isolate D003 (68.2%) and B006 (50.6%) exhibited the third and fourth highest scores and were therefore selected for further analysis. The four isolates, B006, D003, D005 and D014, were then assessed for their ability to coexist as a consortium based on their growth profile.

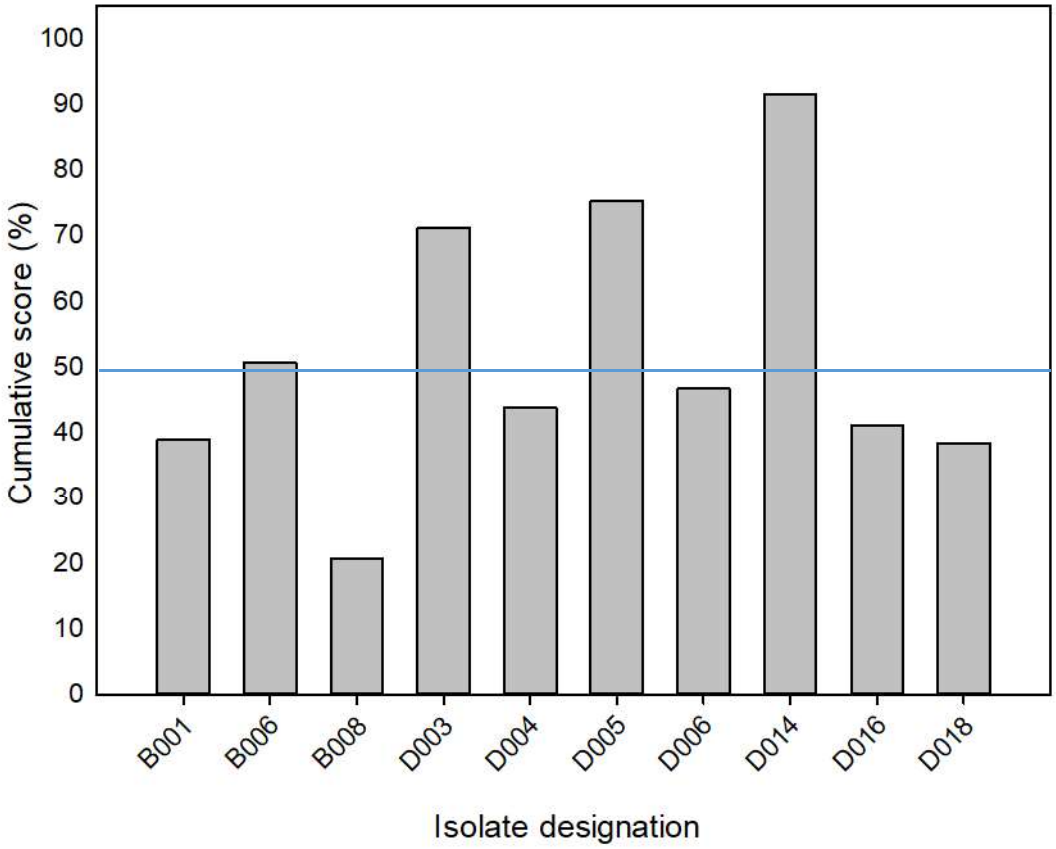

Figure 3:7: Bar graph illustrating the final cumulative score for all of the selection measures, of each individual isolate, presented in percentage (%). The horizontal line illustrates a 50% threshold.

3.3.3.1 Evaluating the ability of selected isolates to coexist as a consortium

The isolates in the consortium were evaluated for their ability to grow and coexist. Figure 3.8 A illustrates the spatial distribution of the four isolates after a cultivation period of 12 hours. Resultant data indicated that B006 and D005 dominated the consortium, resulting in proportions of 35 and 41% respectively. The other two isolates, D014 and D003 contributed a percentage of 15 and 9%, respectively, in the consortium. The isolate that was least dominant was D003, this could be attributed to its growth rate, as D003 had the slowest growth rate when compared to the other three isolates (Figure 3.3). In addition, isolate D003 did not obtain the highest waste ion removal rate, COD removal rate and production of

enzymes. For these reasons, D003 was omitted from the consortium leaving B006, D005 and D014.

The remaining three isolates were further evaluated for their ability to coexist and the results showed that B006 and D005 were still dominant in the consortium by 37 and 44% respectively, whilst D014 dominated by 19% (Figure 3.8 B). Although D014 did not dominate as much as B006 and D005, it was included in further studies because it scored the highest in the scoring matrix, had the highest ammonium removal rate and the highest production of amylase and protease. The ability of D014 to possess these attributes are valid reasons of including this isolate as ammonium, starch and proteins contribute immensely to the pollution fraction in wastewater. The results from this study indicate that the isolates were able to coexist which is advantageous as a single strains may only have the ability to metabolize a limited concentration of a particular compound whilst a consortium contains strains that will synergistically metabolise more than one compound (Romano et al., 2018). Another benefit of a consortium is that a broad spectrum of usage would be achieved as all the members in the consortium are selected due to their high performance with regards to their degradation ability (Patowary et al., 2016). Hibbing et al., (2010) proposed that the availability and individual demand for, and rate of consumption of nutrients will determine the predominance of different microorganism. Success or failure of a bioremediation strategy is dependent on the ability of the introduced microbes to survive and display their activities in the mixed culture (Mohan et al., 2005).

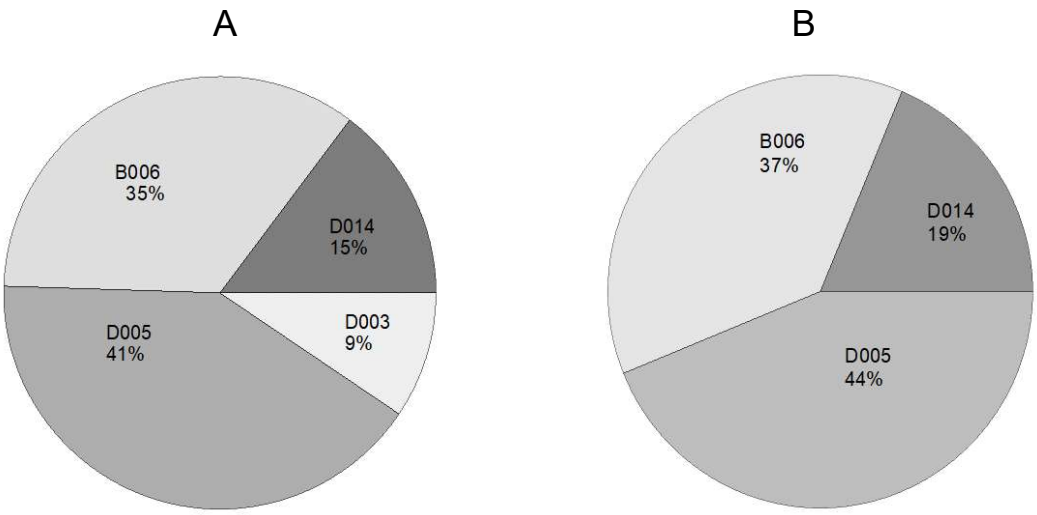

Figure 3:8: An illustration of the different proportions exhibited by the (A) four isolates and (B) the three isolates in a consortium

3.3.4 Determining the bioremediation potential of selected isolates in consortium

3.3.4.1 COD

The consortium comprising, B006, D005 and D014, was firstly, evaluated for the ability to grow and proliferate in SWW by measuring the flow rate. Secondly, the consortium was evaluated for the ability to reduce COD, ammonia, nitrates and phosphates and lastly the ability to produce enzymes of interest. The resultant data from the consortium trial was compared to the results obtained for individual isolates and displayed in Table 3.8. The consortium resulted in a growth rate of 0.910 h^{-1}, however, when comparing the consortium to the individual isolates, D005 showed the highest growth rate of 0.955 h^{-1}. When compared to the consortium the highest COD removal rate (55 $mg.L^{-1}.h^{-1}$) obtained by D005 whilst the consortium obtained a COD removal rate of 35 $mg.L^{-1}.h^{-1}$. The results show that there is a relationship between COD removal and growth, the ability to utilise COD at a higher rate could mean that this isolate easily utilises the constituents that contribute to COD for growth.

When comparing the consortium to the individual isolates, results showed that D014 had the highest nitrification ability by removing ammonium at a rate of 12.43 $mg.L^{-1}.h^{-1}$. The consortium, on the other hand, was able to remove ammonium at a rate of 7.33 $mg.L^{-1}.h^{-1}$. This could be attributed to the isolate not being able to proliferate and use nutrients as well as that of the other members in the consortium as evidenced in the population study (Section 3.3.3.1) where D014 showed the lowest population percentage after 12h of cultivation (Fig 3.8). With regards to nitrate removal, the consortium obtained the highest rate of removal (16.06 $mg.L^{-1}.h^{-1}$) followed by D014 (9.90 $mg.L^{-1}.h^{-1}$). This shows that the consortium has a nitrification-denitrification ability, with a stronger nitrification ability. Results obtained for phosphate removal showed that consortium had the third highest removal rate of 2.35 $mg.L^{-1}.h^{-1}$ and B006 had the highest overall removal rate of 3.29 $mg.L^{-1}.h^{-1}$. Evidently, in some instances the single isolates performed better than the consortium. One of the reasons that some of the isolates performed better individually than in a consortium could be related to the growth rate of the isolate. With regards to the consortium that was evaluated, D005 that had the highest growth rate individually, which means it is able to grow at a faster rate than the other isolates. Therefore, it uses nutrients at a faster rate than D014 with a slow growth rate. Cavaliere et al., (2017) stated that in a microbial community composed of different species, they compete using different strategies. Each of the individual isolates possess an ability that depends on its strategy and on the strategy of the individuals with whom it interacts. Individuals that use more successful strategies have higher chances to proliferate and their occurrence in the community increases. Members of the *Bacillus* genus are known to produce bacteriocins that can suppress the growth of other isolates from the same genus and not necessarily kill them (Abriouel et al., 2011). This ability of microorganisms to produce bacteriocins is considered a successful strategy to reduce numbers to obtain more nutrients. Since this strategy is to maintain population size and availability of nutrients, the more dominant isolates proliferate whilst the less dominant ones do not die but grow and use

nutrients at a slower pace. The slow growth rate and removal rate of D014 could be attributed to the above phenomenon.

Table 3.8: Growth rate and nutrient reduction rates obtained by single isolates and the consortium

Isolate	Growth rate h^{-1}	COD removal rate $mg.L^{-1}.h^{-1}$	Ammonium removal rate $mg.L^{-1}.h^{-1}$	Nitrate removal rate $mg.L^{-1}.h^{-1}$	Phosphate removal rate $mg.L^{-1}.h^{-1}$
B006	0.636	0.430	7.719	3.769	3.290
D005	0.955	55.00	3.690	6.120	2.330
D014	0.838	45.00	12.430	9.904	3.130
B006, D005, D014	0.910	35.00	7.333	16.062	2.351

The ability of the consortium to synthesise enzymes of interest was also determined and compared with results obtained from individual isolates. Table 3.9 displays resultant data emanating from the enzyme assay. The consortium had the highest overall amylase and cellulase production resulting in zones measuring 8.20 mm and 12.90 mm respectively, however, D014 had the highest overall protease production (10.00 mm). The ability to produce these key enzymes in consortium could be advantageous for bioaugmentation studies (Burgess and Pletschke, 2008).

Table 3.9: Enzyme activity illustrated as measurements of zones of inhibition obtained by single isolates and the consortium

Isolate	Amylase mm	Cellulase mm	Protease mm
B006	2.000	2.5000	1.500
D005	3.2330	5.4850	5.2560
D014	5.0000	5.0000	10.0000
B006, D005, D014	8.2000	12.9000	6.4000

3.3.5 Final selection of combination or single isolate for inclusion in a biological product

Resultant scores from the scoring matrix revealed that the consortium scored the highest, with a score of 76.4% followed by D014 (60.2%), D005 (53.5%) and lastly, B006 (32.9%) (Figure 3.9). Since the consortium scored the highest, it was selected as a bioaugmentation agent for further pilot scale studies. Data obtained from all the assessments that were conducted resulting in overall cumulative percentage scores substantiate the final selection as being the consortium. The benefits of using a consortium, rather than using one strain, were realised by Mukred et al., (2008) who discovered that their consortium degraded 98% of hydrocarbons. They concluded that each consortium member had a significant role and each one contributed differently whilst working to degrade hydrocarbons. Many species of microorganisms stably coexist by interacting with each other and effectively exert various functions (Kato et al., 2005). Sarkar et al., (2011) also used a consortium to degrade organic wastes and concluded that using a consortium reduces the time span of degradation.

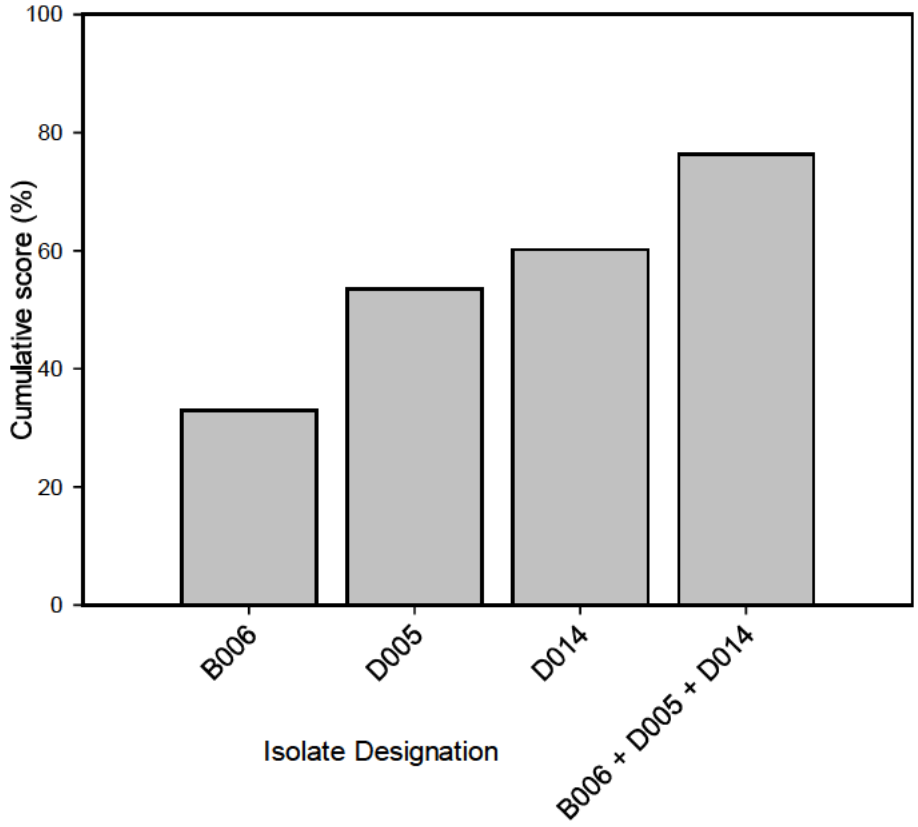

Figure 3:9: Bar graph with percentage scores for selecetd single isolates and selected isolates in a consortium

3.4 Chapter conclusion

- Thirteen *Bacillus* spp. were evaluated of which 10 were isolated from samples obtained from a treatment plant and three were obtained from the CSIR *Bacillus* database.
- All the isolates were successfully evaluated for their ability to remove COD, ammonium, nitrate and phosphate from synthetic wastewater and for production of pertinent enzymes such as amylase, cellulase, protease and lipase. The bioremediation potential and enzyme activity between the isolates varied.
- Isolates that exhibited high reduction rates of COD, ammonium, nitrates and phosphates along with enzyme activity were weighed using a scoring matrix where all key responses were measured and cumulatively scored. Isolates B006 (50.6%),

D005 (75.1%), D003 (68.2%) and D014 (91.3%) scored the highest in a scoring matrix.

- The selected isolates were evaluated for their ability to co-exist in a consortium, whereby, D003 was unable to compete with the other three isolates and was therefore omitted from further studies. The remainder of the isolates B006, D005 and D014 were then selected further evaluated in consortium studies.
- The three isolates in consortium were compared against results obtained from individual studies using the same bioremediation criteria. The results showed the potential of using the consortium over the individual isolates.
- It was concluded that selecting high performing isolates is paramount in determining the success of bioremediation.

Chapter 4: Molecular identification, safety, preservation and storage of selected isolates

4.1 Introduction

Bacillus spp. have been used as biological agents for decades to remediate waste, as plant growth promoting agents and as biocides and are set to become preferred hosts for the production of many new and improved products (Schallmey et al., 2004). When selecting a biological agent for these functions it is imperative to identify and establish its safety to animals, humans, plants, the environment and people end user.

Although most *Bacillus* spp. are non-pathogenic and beneficial, some strains are considered pathogenic, in particular isolates from the *B. cereus* group (Elshaghabee et al., 2017). Isolates that belong to this group are *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. anthracis.* Some members in this group can cause two distinct food poisoning syndromes: a rapid-onset emetic syndrome characterized by nausea and vomiting, and a slower-onset diarrhoea (Turnbull et al., 2004). Another pathogenic strain that causes the fatal systemic infections known as anthrax which is caused by exposure to *B. anthracis* (Koehler, 2009).

Although some members from the *B. cereus* species are known to be harmful, their benefits are far more significant. *B. cereus* isolates along with other *Bacillus* spp. have vast diversity and are exploited in biotechnological applications in various industries (Akram et al., 2014). Due to their benefits and the results obtained in Chapter 3, these isolates prove to be good bioremediation agents. Therefore, should any of the selected strains belong to the *B. cereus* group, it would be imperative that they be tested for the presence of the anthrax gene and the absence of *B. cereus* enterotoxin.

Traditional methods of identification include difference in morphology, growth profiles, enzymatic activity and metabolism based on biochemical tests, to define genera and species (Petti et al., 2005). Full and partial 16S rDNA gene sequencing methods have emerged in the last decade playing a pivotal role in the accurate identification of phenotypically aberrant microorganisms (Petti et al., 2005; Woo et al., 2008).

Identification of isolates belonging to the *B. cereus* species using 16S rDNA is difficult because of their genetic similarity. Demonstration of their high genetic relatedness has contributed to the suggestion that *B. anthracis, B. cereus* and *B. thuringiensis* are members of a single species (Rasko et al., 2005). These organisms have been traditionally differentiated based on their phenotypic characteristics, including pathogenic potential and they differ in 16S rDNA sequence by only nine nucleotides which has led to the assumption that these strains are the same (Ash et al., 1991; Carlson et al., 1994; Helgason et al., 2000b; Rasko et al., 2005). *B. anthracis* causes the acute fatal disease anthrax and is used as a potential biological weapon due to its high toxicity (Helgason et al., 2000b). An alternative or subsequent route, after identifying these microorganisms using 16S rDNA, would be to differentiate them from each other using the presence or absence of virulent genes and enterotoxins (Turnbull et al., 2004).

The two most common commercial kits available for the rapid detection of *B. cereus* enterotoxin, the RPLA kit and the TECRA kit (Rusul, 1995). The BCET-RPLA, which was used in this study, is a reverse passive latex agglutination (RPLA) test that enables soluble antigen such as bacterial toxins to be detected in an agglutination assay. The test may be used to demonstrate enterotoxin production by isolates belonging to the *B. cereus* group (BCET-RPLA toxin detection kit, Oxoid). The virulence of *B. anthracis* is based on the presence of two plasmids which are pX01 and pX02 (Jensen et al., 2003). To detect virulent

plasmids real time polymerase chain reaction (PCR) is used which amplifies and simultaneously quantifies a targeted DNA molecule (Wielinga et al., 2011).

After establishing the safety of the isolates that were selected in this study, preservation and storage was essential to ensure continuity in research, reproducible results and to avoid continuous sub-culturing, which could result in changes in the organisms of interest (Prakash et al., 2013). The aim of this part of the study was to determine the safety of the selected isolates for future research and potential commercial application in waste treatment as biological products. This aim was met by the following objectives; (a) identifying the selected isolates B006, D005 and D014, (b) assessing the biosafety of the isolates and (c) preserve, store and validate the cryo-cultures for future use.

4.2 7Methodology

4.2.1 Identification of isolates B006, D005 and D014 by 16S rDNA sequencing and analysis

4.2.1.1 Monoseptic cultivation of isolates B006, D005 and D014 for use in identification assay

Single colonies of each of isolates B006, D005 and D014 from monoseptic and purified plate cultures (> 3 passages), were re-streaked on fresh nutrient agar plates and incubated for 24 hours at 32 °C.

4.2.1.2 Identification of isolates by genetic evaluation – 16S rDNA sequence analysis

The isolates that were obtained from Section 4.2.1.1 were visually verified as monoseptic cultures and then sent to Inqaba Biotechnical Industries (Pty) LTD, Pretoria, South Africa for sequencing and analysis. At Inqaba, the DNA was extracted using the ZR Fungal/Bacterial DNA kit™ (Zymo Research). The 16S target region was amplified using the primers shown

in Table 4.1. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit), and sequenced in the forward and reverse directions on an ABI PRISM™ 3500 xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI).

Table 4.1: 16S rDNA primer sequence (Inqaba Biotechnical Industries, Pretoria, South Africa)

Name of primer	Target	Sequence (5' to 3')
27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

4.2.2 Biosafety assessment of selected microorganisms – B006, D014and D005

4.2.2.1 Anthrax toxin production by isolates identified as belonging to the *B. cereus* group of microorganisms

4.2.2.1.1. Monoseptic cultivation for anthrax detection assay

A colony from a monoseptic plate culture of each isolate was inoculated into 1000 ml Erlenmeyer flasks containing 100 ml TSB. The flasks were incubated at 32 °C for 24 hours on a rotary shaker (New Brunswick Scientific, New Jersey, USA). Thereafter, 2 ml from the culture flasks was aliquoted aseptically into sterile Eppendorf tubes and centrifuged for 10 minutes at 16060 *g* (Heraeus biofuge pico, Labcare, Buckinghamshire, England). The biomass was then stored at -20 °C for use in isolation of DNA for the anthrax detection assay.

4.2.2.1.2. Isolation of DNA from *B. cereus* isolates

Biomass obtained from section 4.2.2.1 cultivated for anthrax detection was used to isolate DNA using the High Pure PCR Template Purification kit (Roche, 11796828001), according to the manufacturer's instructions. The DNA obtained was visualised on a 0.8% agarose gel, containing ethidium bromide (Lee et al., 2012) and the concentration was determined using a ND-1000 spectrophotometer (Thermo scientific, Wilmington, USA). The two plasmids that are cause pathogenicity in *B. anthracis* are pX01 with toxic genes *pag*, *lef* and *cya* and pX02 with toxic genes *capA*, *capB* and *capC*. The sequences to the toxic genes that cause anthrax are illustrated in Table 4.2 adapted from (Patra and Garrigue, 1996; Ellerbrok et al., 2002; Luna et al., 2006).

Table 4.2: Sequences for toxic genes that are responsible for anthrax, pX01 and pX02 (Patra and Garrigue, 2018; Ellerbrok et al., 2002; Luna et al., 2006;)

Gene	Sequence (*5' to 3'*)	Length (bp)
Cya (pX01)	CAGCATGCGTTTTCTTTAGCCCCTTAGTTGAATCC GGTTTGGTTTAGTACCAGAACATGCCGGCTTCAAG ACCCC	1475
Lef (pX01)	GGATATGAACCCGTACTTGTAATAAATCCGCACCT AGGGTTGCCTTTTGCATATTATATCGAGCGAATCA CGAATATCAATTTGTAGC	1378
Pag (pX01)	CAGAATCAAGTTCCCAGGGGTCGGATAAGCTGCC ACAAGGCTACAGGGGATTTATCTATTCCATTGTTAC ATGATTATCAGCGG	898
CapA (p0X02)	CAACATTTGCAATCATGAATATTTATTACTTATAGT GTTGTCTCCACTGATACTTGATTTTCCGGATTATGG	1291

	TGCTAAGGGAACTAAAG-A-CGC-AGC-TAT-TAA-TATAACTGCGATAAG	
CapB (p0X02)	CTTAATAAGCAAGAAATCGAAAAGCAGGAAGGAGG CCTTTATTGTATCTTTAGTTCCCTTAGCAAGCGATG CATTATCTGGGAAGACCAGGAAGAACAAATTGGCA AAAAGC	2204
CapC (p0X02)	GGTCTTCCCAGATAATGCATCGCTTGAGGCCTTTA TTGTATCTTTAGTTCCCTTAGCAACATTTGCAATCA TGAATATTTATTACTTAT	1535

4.2.2.1.3. Real-time PCR

Reactions were set up with pX01 or pX02 specific primers (Luna et al., 2006), contained in two 10 × pre-mixes, TAM1 and TAM2 (Target Assay Mix) from the TaqMan *B. anthracis* detection kit (Applied Biosystems, part number 4382486). The primer sequences are illustrated in Table 4.3. Each TAM contains an internal positive control (IPC), as well as the TaqMan probe. IPCs avoid false negatives that may be obtained due to the presence of substances that inhibit PCR and demonstrates whether the PCR reagents are in working order, eliminating the need for a separate positive control and reducing the risk of cross-contamination.

Each 20 µl reaction mixture contained 2 µl 10 × TAM (1 or 2) pre-mix, 10 µl 2 × Fast Universal Master Mix (containing enzyme, dNTPs and buffer), and 1 µl *B. cereus* strain template of DNA. A negative control containing no template DNA was also included. Reaction mixtures were plated into a MicroAmp Fast optical 96-well reaction plate (Applied Biosystems, part number 4346906), and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, part number 4360954). The Applied Biosystems 7500 Fast Real-Time PCR System was

used for the amplification using the conditions as stated in the TaqMan® *Bacillus anthracis* detection kit (Applied Biosystems).

Table 4.3: Primer sequences used in the TaqMan® kit to detect anthrax specific target (Easterday et al., 2005)

Name of primer	Target	Sequence (5' to 3')
TAM 1	Anthrax specific targets	GAGTTTGATGTGAAGGT-GAGACATAATC
TAM 2	Anthrax specific targets	TTTGCATGACAAAGCGCTAA

4.2.2.2. Enterotoxin production by the isolates identified as belonging to the *B. cereus* group of microorganisms

4.2.2.2.1. Monoseptic cultivation for enterotoxin detection assay

Isolates that were identified as belonging to the *B. cereus* group were tested for enterotoxin production. A single monseptic colony, from Section 4.2.1.1, was used to inoculate 1000 ml Erlenmeyer flasks containing 100 ml Brain Heart Infusion Broth (Merck, Darmstadt, Germany) and incubated at 32 °C for 18 hours at 180 rpm on a shaker incubator (New Brunswick scientific, New Jersey, USA). After cell growth for 18 hours, a 2ml sample aliquot was centrifuged in Eppendorf tubes at 4 °C for 20 minutes at 3214 *g* (Eppendorf centrifuge 5810R, Hamburg, Germany). The supernatant was further filtered using a 0.2 µm low protein binding filter and thereafter used for the enterotoxin assay.

4.2.2.2.2. Confirmation of the absence of the *B. cereus* enterotoxin

The test was conducted using an OXOID BCET-RPLA kit (Oxoid TD0950, Thermo Scientific, UK) according to manufacturer's instructions. Supernatant obtained from *B. cereus* isolates

(Section 4.2.3.1) was used for this assay which also included a *B. cereus* enterotoxin producing positive control. The tests were conducted in two microtitre plates (Sigma Aldrich, USA) in triplicate (Figure 4.1). The isolate B006 was inoculated in the first three wells as illustrated in Figure 4.1A, row A, B and D, the isolate D005 was inoculated in rows E, G and H (Figure 4.1A). The positive and negative control were conducted in a separate micro titre plate in row A and row B respectively (Figure 4.1B). All wells inoculated with B006 and D005 were also inoculated with latex sensitised specific *B. cereus* anti-enterotoxin (rabbit IgG). Negative controls contained a latex suspension sensitized with non-immune rabbit globulins. The positive control contained lyophilized *B. cereus* enterotoxin which agglutinates the sensitized latex. Negative results are represented by the formation of a tight button settlement at the bottom of the wells. Positive results are shown by a diffuse layer at the bottom of the wells after agglutination occurs due to the formation of a lattice structure.

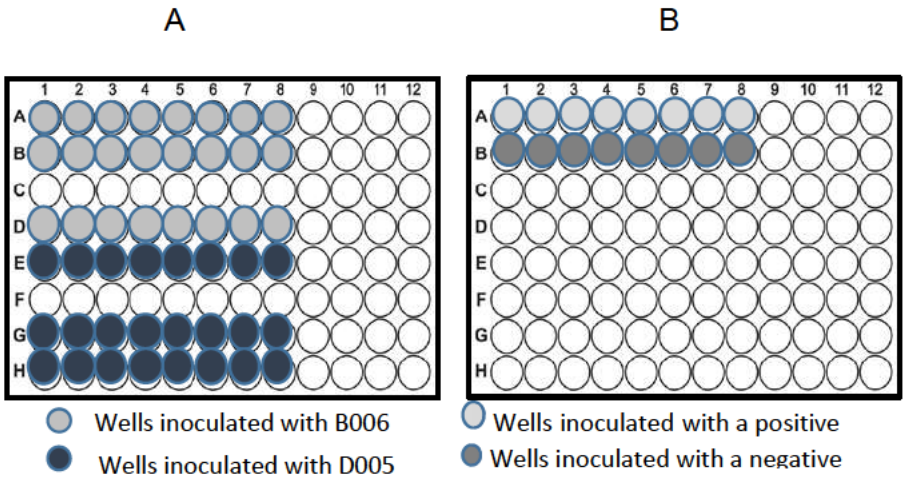

Figure 4:1: An overview of a micro titre plate and the wells that were occupied, for the toxin test by *B. cereus* strains, the positive control and negative control.

4.2.3 Isolate preservation, storage and viability assessment

4.2.3.1 Isolate preservation and storage: preparation of a master and working spore bank

After establishing safety of the selected isolates, the next step included preservation of the cultures. Isolates were streaked on nutrient agar plates and incubated overnight at 32 °C.

Resultant single colonies from each isolate were used to establish master spore banks. This was conducted by aseptically transferring a colony into triplicate 1800 mL Fernbach flasks containing 700 ml sterile SM (Chapter 3, Table 3.2). Flasks were incubated at 32 °C at 180 rpm on a rotary platform shaker (New Brunswick scientific, New Jersey, USA) until a sporulation efficiency of ~100% was achieved. Sporulation efficiency was calculated as per the equation below:

$$\% \, \boldsymbol{SE} = \frac{\boldsymbol{Mean\ count}_{(spore)}}{(\boldsymbol{Mean\ count}_{(cell)} + \boldsymbol{Mean\ count}_{(spore)})} \times \mathbf{100} \qquad \textit{(Eq.4.1)}$$

Three flasks were inoculated, of which two of the flasks were used to sample daily to check for the absence of contamination and to assess sporulation efficiency. Culture from the third flask was used to establish a master spore bank.

After reaching a sporulation efficiency of 100%, the cultures were ready for preservation. Broth from flask 3 (100 mL) was aseptically mixed with 100 mL sterile 50% $v.v^{-1}$ glycerol solution, under aseptic conditions. Homogenisation was conducted using a magnetic stirrer (Heidolph, Schwabach, Germany) under nominal speed (~250 rpm) for 5 minutes. The mixture was aseptically dispensed into 50 pre-labelled cryovials (Corning®, USA) under a laminar flow cabinet, which were then transferred to Nalgene Mr Frosty ™ cryo 1 °C freezing containers (Sigma Aldrich, USA) containing 250 mL isopropanol alcohol (IPA). The containers were then transferred to a -80 °C ultra-freezer (Thermo Scientific, North Carolina, USA) for 36 hours. Subsequent to storage, the cryovials were removed from the isopropanol and placed into labelled cryoboxes in an ultra-freezer (Thermo Scientific, North Carolina, USA) at -80 °C. These methods were adapted from Meza et al., (2004). A working spore bank (WSB), using cryovials from the MSB, was similarly prepared, however for the WSB, cryovials were used to inoculate triplicate flasks instead of a colony. The WSB comprised of 100 cryovials and was stored at -80 °C.

4.2.3.2 Viability assessment of cryopreserved cultures

4.2.3.2.1 Monoseptic status of cryopreserved isolates

To assess the aseptic status of the process all reagents used in the procedure were streaked onto NA plates to assess the sterility of each ingredient in the procedure. Each ingredient was streaked in triplicate and incubated at 32 °C for 24 hours and checked visually for the presence of contaminants. After a month of storage, the cryovials from the cryoboxes containing both master and working spore banks, were selected (10% of total number) spanning the start to end of the cell bank procedure and were similarly assessed for purity on NA plates. Cultures were also viewed at 1000× and 400× magnification using light microscopy (Olympus, Japan) and assessed for the presence of any potential contaminants.

The cell bank viable cell concentration was conducted by randomly selecting three cryovials from each cell bank. The preserved culture was serially diluted and thereafter plated on NA plates, which were incubated at 32 °C for 24 hours. After the incubation period, resultant colonies were counted and the viable cell number calculated.

4.2.3.2.2 Viability assessment

The monoseptic status of the master spore bank was confirmed and then stored in a separate ultra-freezer, only to be used to create new working spore banks, when the need arises. Therefore, only the working spore bank was assessed for viability, as these were the banks that would be used for the duration of the study, therefore, it was imperative to assess the viability of the cultures for the furtherance of the research. This was done by selecting three random cryovials form the working spore bank, which were each inoculated into separate 1800 mL Fernbach flasks containing 700 mL TSB. Following inoculation, an initial sample was taken from each flask to measure OD at a wavelength of 660 nm in triplicate. The flasks were then incubated at 32 °C on a rotary platform shaker (New Brunswick

Scientific, USA) with an agitation speed of 180 rpm. Flasks were sampled hourly and OD 660 nm was measured. During the study, the initial, intermediate and final samples were also assessed for monoseptic status by streaking on NA plates and by microscopic observation. The hourly samples were used to determine growth rate, doubling time and transfer time of the inoculum stage for use in future studies.

The ODs and Ln ODs were used to plot the growth curve of each culture. Maximum growth rate (μ_{max}) is an important parameter in modelling microbial growth under batch conditions and was therefore used to determine the growth characteristics of all three strains. To determine μ_{max}, the Ln OD was plotted against the culture age in hours to obtain the slope of the curve with a desirable regression coefficient exceeding 0.9. The maximum growth rate (μ_{max}) was hence determined from Ln OD measurements using the following equation:

$$\mu_{max} = \frac{d\ln(OD_{660nm})}{dt} \qquad \textit{(Eq.4.2)}$$

The doubling time (h) of the organism was calculated using the following equation:

$$Doubling\ time = \frac{\ln(2)}{\mu} \qquad \textit{(Eq.4.3)}$$

4.3 Results and Discussion

4.3.1 Identification of isolates B006, D005 and D014 by 16S rDNA sequencing and analysis

The molecular identity of the isolates were revealed using 16S rDNA sequence analysis. Figure 4.2 shows homology of D014 to database sequences, were it showed 99.9% similarity to *Bacillus subtilis and B. amyloliquefaciens, B. licheniformis*. Due to a very high

sequence homology in the ribosomal operons of *Bacillus* spp, it is often difficult to differentiate these closely related members (Jeyaram et al., 2011). It was reported that the classification based on 16S rDNA sequences is also limited by the databases used for sequence comparisons, hence it is accurate only up to genus level identification (Poretsky et al., 2014). Therefore, more than one identification method could be employed to distinguish these closely related *Bacillus* spp. down to their species level. Some of the methods that have been used to identify *Bacillus* spp. or as an addition to 16s rDNA are biochemical methods such as the API method, chromatographic analysis of whole cell fatty acids methyl ester (FAME) and molecular analysis such as gyrase B (gyrB) (Parvathi et al., 2009).

Since D014 was identified as belonging to the *B. subtilis* group a biosafety assessment were also conducted, however, according to the literature isolates belonging to this group are generally regarded as safe (de Boer Sietske and Diderichsen, 1991; Hong et al., 2008).

Sequences producing significant alignments:	Score (Bits)	E Value
gi|541990260|gb|KF535118.1| Bacillus subtilis strain BAB-448 ...	2572	0E00
gi|540362574|gb|KF052000.1| Bacillus amyloliquefaciens strain...	2572	0E00
gi|540362573|gb|KF051999.1| Bacillus licheniformis strain SQL...	2572	0E00
gi|540362572|gb|KF051998.1| Bacillus subtilis strain SQL 01 1...	2572	0E00
gi|540361039|gb|KF560370.1| Bacillus sp. BG-7-R5 16S ribosoma...	2572	0E00
gi|536590622|gb|KF499272.1| Bacillus sp. SP A9 16S ribosomal ...	2572	0E00
gi|533205856|gb|KF018234.1| Bacillus sp. WF-2 16S ribosomal R...	2572	0E00
gi|532809708|gb|KF495531.1| Bacillus subtilis strain BP7 16S ...	2572	0E00
gi|537360954|dbj|AB848923.1| Bacillus subtilis gene for 16S r...	2572	0E00
gi|532644178|gb|KF410851.1| Bacillus subtilis strain KIIT VSK...	2572	0E00
gi|532644177|gb|KF410850.1| Bacillus tequilensis strain KIIT ...	2572	0E00
gi|532529859|gb|KF471504.1| Bacillus subtilis strain Pb3 16S ...	2572	0E00
gi|532139510|gb|KF381492.1| Bacillus subtilis strain 16-5G 16...	2572	0E00
gi|532000542|gb|KF460566.1| Bacillus subtilis subsp. subtilis...	2572	0E00
gi|531038926|gb|KF306224.1| Bacillus subtilis strain 412 16S ...	2572	0E00
gi|530693224|gb|KF001839.1| Bacillus subtilis strain Jdm2 16S...	2572	0E00
gi|530331085|gb|KF478933.1| Bacillus subtilis strain RGRI1 16...	2572	0E00

Figure 4:2: Sequence Homology of the sequences obtained in this study against NCBI database (the first 17 matches)

Isolate B006 and D005 were classified as belonging to the *B. cereus* group. As per the 16S rDNA analysis, the identified isolates could be either *B. cereus* (99.63 %), *B. anthracis* (99.63%) or *B. thuringiensis* (99.63%) with both having identical sequence homology. The results from the 16s rDNA analysis also showed that only difference between the two strains was that B006 sequence homologies did not share any similarities with *B. mycoides* whilst matches between D005 and *B. mycoides* were present. The identification of organisms within the *B. cereus* group is difficult because of the genetic similarity between them (Helgason et al., 2000a; Lalloo et al., 2007). The *B. cereus* group comprises six separate species which are genetically similar but phenotypically very diverse (Ehling-schulz et al., 2019). Strains that belong to this group are *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. anthracis* (Priest, 1977; Lechner et al., 1998; Nakamura, 2016). Some researchers have used plasmids to differentiate closely related strains belonging to the *B. cereus* group, for example, *B. anthracis* harbours two plasmids, pX01 and pX02 that cause anthrax, some *B. cereus* strains harbour plasmid pCER270 encodes for the production of a toxin that causes food poisoning (Debuyser and Nguyen-the, 2010; Jeßberger et al., 2015).

Since *B. cereus* isolates showed close similarity to pathogenic strains that cause anthrax and enterotoxins, further studies were also conducted to confirm the absence of key virulent genes such as the *B. anthracis* lethal toxin complex (pX01) and poly-D-glutamic acid capsule (pX02) along with the absence of *B. cereus* enterotoxin (Okinaka et al., 1999). The identification of anthrax virulent genes were done using quantitative PCR and the production of enterotoxin was detected using an RPLA-BCET toxin detection kit. The ability of *B. cereus* microorganisms in removing utilising wastewater nutrients that pollute freshwater bodies was illustrated by Zhao et al., (2009). Through their research a *B. cereus* strain with denitrifying ability showed a total nitrogen removal of 68.6% and a COD removal of 71.7%.

According to the data presented in Chapter 3, B006 and D005, identified as belonging to the *B. cereus* group showed very good denitrification ability.

Sequences producing significant alignments:	(Bits)	Value
gi|451964251|gb|KC441811.1| Bacillus anthracis strain N34 16S...	2621	0E00
gi|451964215|gb|KC441775.1| Bacillus anthracis strain D40 16S...	2621	0E00
gi|451964208|gb|KC441768.1| Bacillus cereus strain D30 16S ri...	2621	0E00
gi|444304029|ref|NR_074453.1| Bacillus anthracis str. Ames st...	2621	0E00
gi|442539675|gb|KC201678.1| Bacillus thuringiensis strain Fh-...	2621	0E00
gi|442539674|gb|KC201677.1| Bacillus thuringiensis strain St-...	2621	0E00
gi|429490567|gb|JX941513.1| Bacillus sp. B31(2012) 16S riboso...	2621	0E00
gi|428186817|gb|JX993816.1| Bacillus cereus strain XX2010 16S...	2621	0E00
gi|426209261|gb|KC172044.1| Bacillus anthracis strain SH123 1...	2621	0E00
gi|425706809|gb|JX860335.1| Uncultured Bacillus sp. clone BVP...	2621	0E00
gi|353529011|gb|JN698042.1| Uncultured bacterium clone SHCB09...	2621	0E00
gi|405795162|gb|JX290089.1| Bacillus cereus strain KVP109 16S...	2621	0E00
gi|405795158|gb|JX290085.1| Bacillus cereus strain KVP105 16S...	2621	0E00
gi|401802751|gb|JX155766.1| Bacillus cereus strain JP44SK49 1...	2621	0E00
gi|401802746|gb|JX155761.1| Bacillus cereus strain JP44SK44 1...	2621	0E00
gi|401555340|gb|JX262401.1| Bacillus thuringiensis strain KW ...	2621	0E00
gi|400294694|gb|JX144724.1| Bacillus cereus strain JP44SK34 1...	2621	0E00
gi|400294687|gb|JX144717.1| Bacillus cereus strain JP44SK27 1...	2621	0E00

Figure 4:3: Sequence Homology of the sequences obtained in this study against NCBI database (the first 17 matches) for B006

Sequences producing significant alignments:	Score (Bits)	E Value
gi|449075095|gb|JX064565.1| Uncultured bacterium clone 11_K_1...	2611	0E00
gi|449075087|gb|JX064557.1| Uncultured bacterium clone 11_Am_...	2611	0E00
gi|559103294|gb|KF853117.1| Bacillus cereus strain BAB-2926 1...	2611	0E00
gi|559103292|gb|KF853115.1| Bacillus cereus strain BAB-2885 1...	2611	0E00
gi|557701631|gb|KF601916.1| Bacillus anthracis strain AN8 16S...	2611	0E00
gi|545006411|emb|HF911367.1| Bacillus sp. B5 HMK-2013 partial...	2611	0E00
gi|478686319|gb|JX519363.1| Bacillus cereus strain IAM 156125...	2611	0E00
gi|541990441|gb|KF535127.1| Bacillus cereus strain BAB-2832 1...	2611	0E00
gi|538283935|gb|KF254677.1| Bacillus sp. 47_(br20) 16S riboso...	2611	0E00
gi|531874549|gb|KF479667.1| Bacillus sp. D46 16S ribosomal RN...	2611	0E00
gi|531874496|gb|KF479614.1| Bacillus sp. C20 16S ribosomal RN...	2611	0E00
gi|531874456|gb|KF479574.1| Bacillus sp. B10 16S ribosomal RN...	2611	0E00
gi|531874439|gb|KF479557.1| Bacillus sp. A52 16S ribosomal RN...	2611	0E00
gi|525922698|gb|KF410593.1| Bacterium GX-2 16S ribosomal RNA ...	2611	0E00
gi|514251617|gb|KF054993.1| Bacillus mycoides strain IARI-JR-...	2611	0E00
gi|514251375|gb|KF054751.1| Bacillus thuringiensis strain IAR...	2611	0E00
gi|514081644|gb|KF053070.1| Bacillus thuringiensis strain BAB...	2611	0E00

Figure 4:4: Sequence Homology of the sequences obtained in this study against NCBI database (the first 17 matches) for D005

4.3.3.1. Anthrax toxin detection of isolates identified as belonging to the *B. cereus* group of microorganisms

Genomic DNA was successfully isolated from *Bacillus* isolates D005 and B006. Aliquots of 5 µl were used to visualise the quality of the extracted DNA on an agarose gel (Figure 4.5).

The concentration of the isolated DNA was assessed using a nano-drop (ThermoFisher Scientific, Massachusetts, USA).

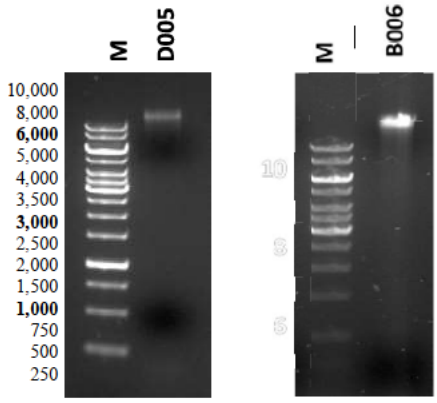

Figure 4:5: Agarose gel showing genomic DNA isolated from *Bacillus* D005 and B006. M is the O'Gene Ruler Plus 1 kb DNA Ladder (Thermo Scientific), with sizes of selected bands in Kb on the left side of each diagram.

Virulent forms of *B. anthracis* are known to harbour two plasmids, pX01 of 181 kb and pX02 of 93.5 kb which carry the virulent gene responsible for anthrax production (Luna et al., 2006). The quantitative PCR was designed to target the pX01 and pX02 plasmids using the TaqMan *B.* anthracis detection kit (Applied Biosystems). Real-Time PCR was carried out using 1 $ng.\mu l^{-1}$ DNA, with a final concentration of 50 $pg.\mu l^{-1}$ in the reaction mixture. Following analysis of the amplification signals, it was determined that the internal positive controls amplified successfully for tests done on both isolates which is illustrated in Figure 4.6 A and B. Figure 4.7 A and B illustrates that no amplification was observed using the primers that targeted the presence of pX01 and pX02 in isolates B006 and D005. The results confirmed the absence of the anthrax plasmid in both the isolates.

A B

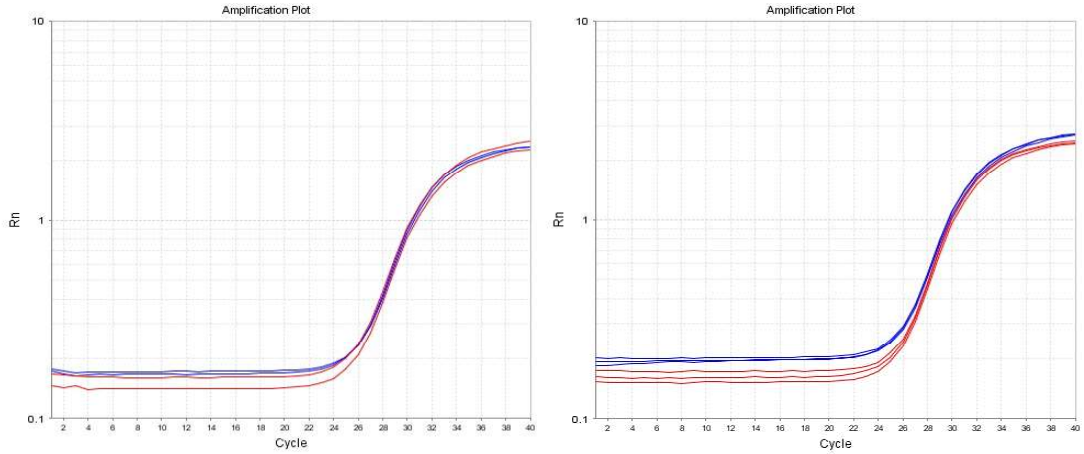

Figure 4:6: Amplification plots of Rnvs cycle number. Rn = normalised reporter (fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference). A and B Internal Positive Controls using the VIC reporter dye

A B

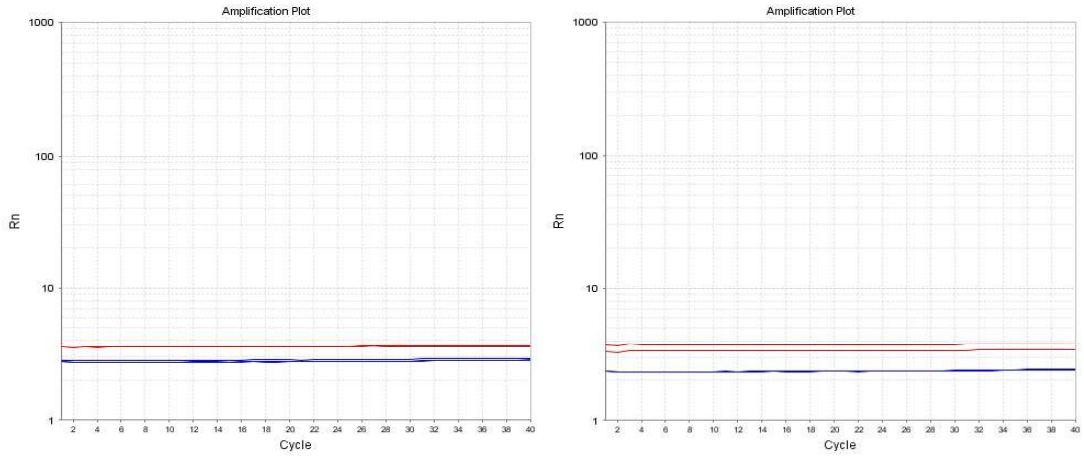

Figure 4:7: Amplification plots of Rn vs cycle number. pX01 and pX02 using the FAM reported dye. Rn = normalised reporter (fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference Red = TAM1; Blue = TAM2 (50 pg.µl^{-1} template). pX01 and pX02 using the FAM reported dye. A and B are amplifications resulting from B006 and D005 respectively

4.3.1.1 Confirmation of the absence of the *B. cereus* enterotoxin

Some *B. cereus* are known to produce enterotoxin which can gastrointestinal diseases (Senesi and Ghelardi, 2010). This part of the research therefore focused on evaluating the

strain for the production of enterotoxin using a commercial kit. A negative result is represented by the formation of a tight button settlement at the bottom of the wells as indicated in the BCET-RPLA toxin detection kit. A positive indication of the presence of the enterotoxin is indicated by a diffuse layer at the bottom of the wells after agglutination occurs due to formation of a lattice structure. Figure 4.8 illustrates the outcome of the assay and the results confirmed the absence of the *B. cereus* enterotoxin production by the isolates tested. *B. cereus* isolates B006 and D005 formed a tight round button settlement which represents the absence of the *B. cereus* enterotoxin. The absence of the enterotoxin confirms the safety of the organisms and renders them useable in their intended application as bioaugmentation agents.

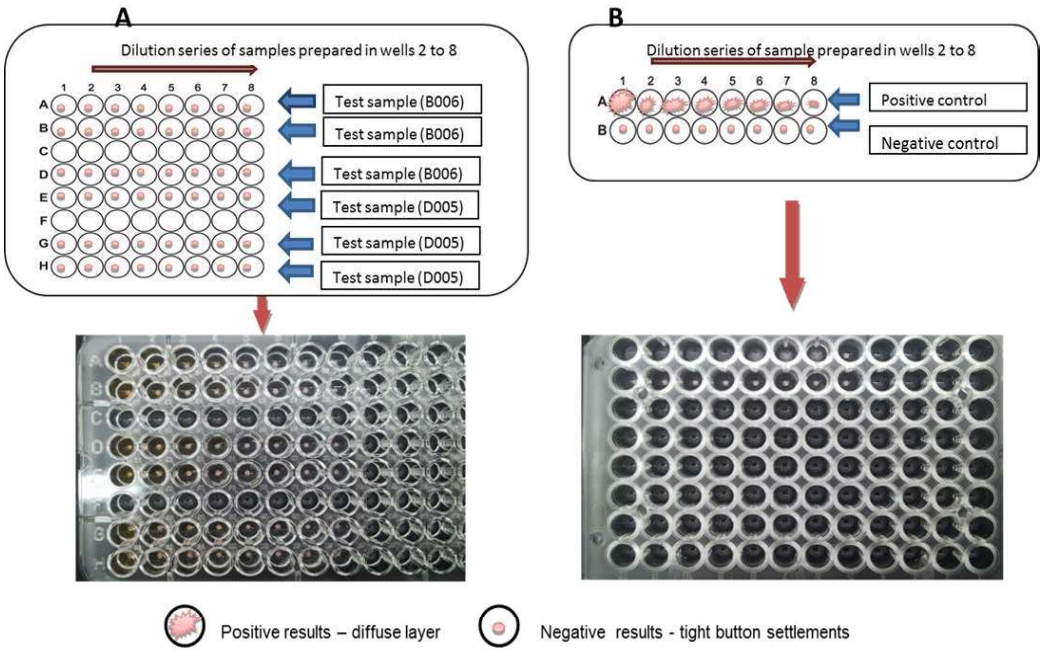

Figure 4:8: (A) Microtitre plate results accompanied by a schematic diagram illustrating the test samples B006 (rows A, B and D) and D005 (rows E, G and H). (B) An illustration of a positive control containing lyophilized *B. cereus* enterotoxin (row A) and a negative control depicted in row B of the microtiter plate.Isolate preservation, storage and viability

4.3.1.2 Isolate preservation and storage: preparation of a master and working spore bank

A master spore bank amounting to 50 cryovials and a working spore bank amounting to 100 cryovials of each *Bacillus* isolate, were successfully produced. Gao et al., (2007) reported that the viability of *Bacillus* spores was not influenced by freezing as they examined spores belonging to *B. megaterium* and discovered that the spores were still viable after freezing. For these reasons, opting to preserve our cultures as spores was a more feasible alternative to vegetative cells. The cryopreserved cultures (10%) were assessed for their monoseptic status by performing spread plates and streak plates. All the agar plates were monoseptic and no contamination was observed.

4.3.3.2. Viability assessment of cryopreserved cultures

The cryopreserved cultures were assessed for viability by growing the cultures in flasks containing media and allowing them to grow until stationary phase. The cultures were observed microscopically before inoculation to ascertain that they were in the spore form. Thereafter they were observed during the exponential phase of growth to ascertain viability. Figure 4.9, 4.10 and 4.11 show microscopic images of the isolates in their spore state and their vegetative state. The images show that the spores were viable and were able to germinate into vegetative cells. The images of the vegetative cells also show that a monoseptic culture was preserved as a uniform group of cells belonging to each respective isolate were observed.

B006 formed short, medium sized rods that clumped together, this isolate also formed short chains of two to three rods during the exponential phase of growth and after the exponential phase the rods detach to single rods. The sporulation efficiency obtained by this isolate was 85%, which is the percentage of spores that were in each cryovial. On the other hand, D005 and D014 obtained a sporulation efficiency of 95% whilst D005 had a similar cell morphology

as B006. D005 vegetative cells were observed as short medium sized rods that formed short chains during the exponential phase of growth. This is expected as the two isolates were identified as belonging to the *B. cereus* group. D014 cell morphology differed from the other two isolates, forming long thin chains that broke up into very short, thin rods. The difference in morphology is also expected as this isolate was identified as belonging to the *B. subtilils* family.

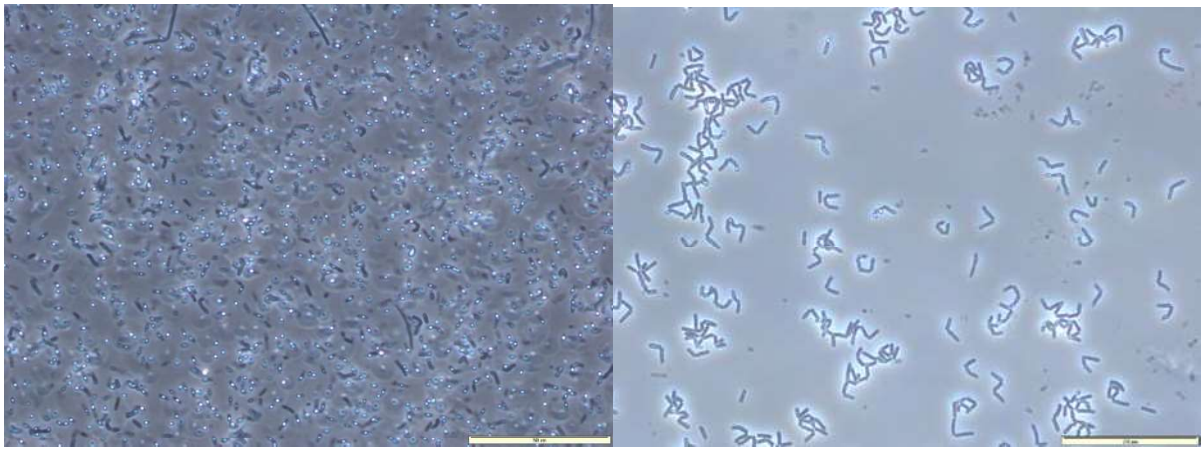

Figure 4:9: Vegetative and spore cell morphology of D005 observed microscopically at 400 × magnification.

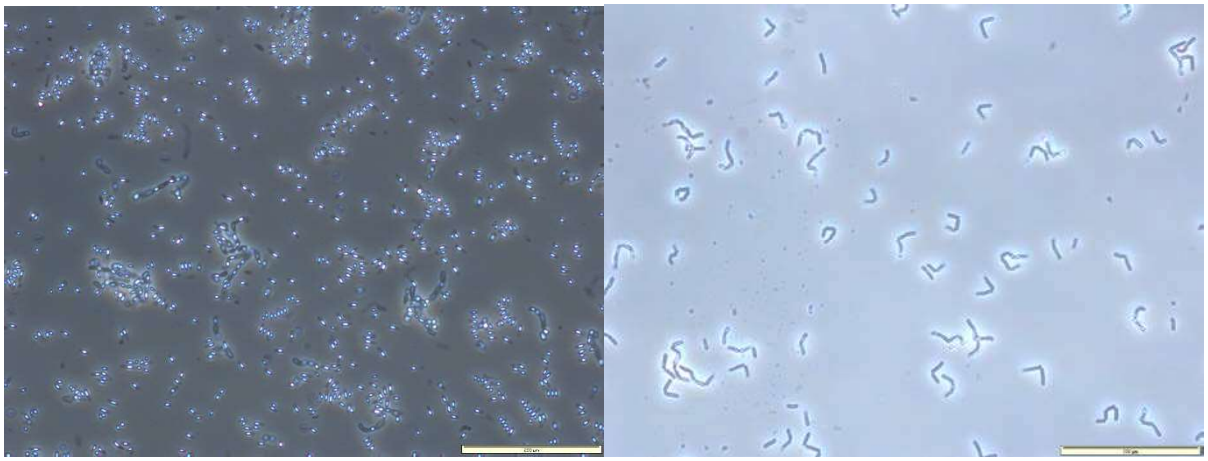

Figure 4:10: Vegetative and spore cell morphology of D005 observed microscopically at 400 × magnification.

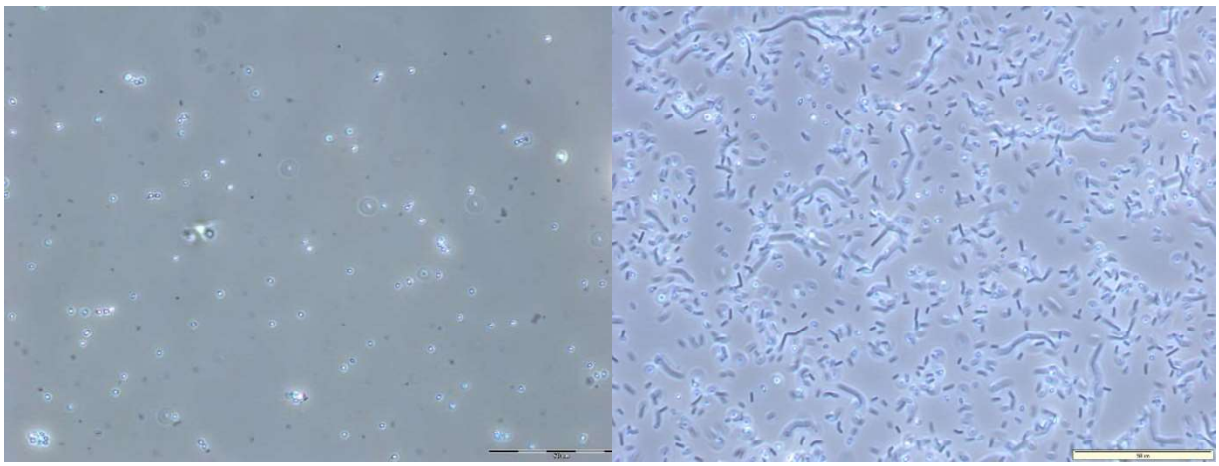

Figure 4:11: Vegetative and spore cell morphology of D014 observed microscopically at 400 × magnification

The viability assessment is used to assess the cryopreservation method by observing the ability of the cells to grow after a certain storage period. The preserved cultures (spore banks) were validated using growth studies to determine viability and consistency in cell concentration in each cryovial. Figure 4.12 illustrates the average growth profiles obtained by the three isolates cultivated in triplicate flasks, whilst the growth rate, doubling time and cell concentration at mid exponential phase is shown in Table 4.4. The average growth trend obtained by B006 exhibited a lag phase (1.5 h) and exponential phase (7 h). Although B006 and D005 were identified as belonging to the *B. cereus* group, they had completely different growth patterns. The lag phase observed from D005 growth trend was 4 h, followed by a 4 h lag phase and a 4 h growth phase. The growth rate, doubling time and cell concentration also differed between the two isolates as shown in Table 4.4. Isolate D014, exhibited the longest lag phase (6 h) followed by a 4 h exponential growth phase. The growth trend was used to measure the average growth rate and average doubling time of each isolate whilst the consistency in the growth trend obtained in triplicate flasks is demonstrated by error bars. The coefficient of variation for each of the parameters was <10% which is an indicator of good method execution.

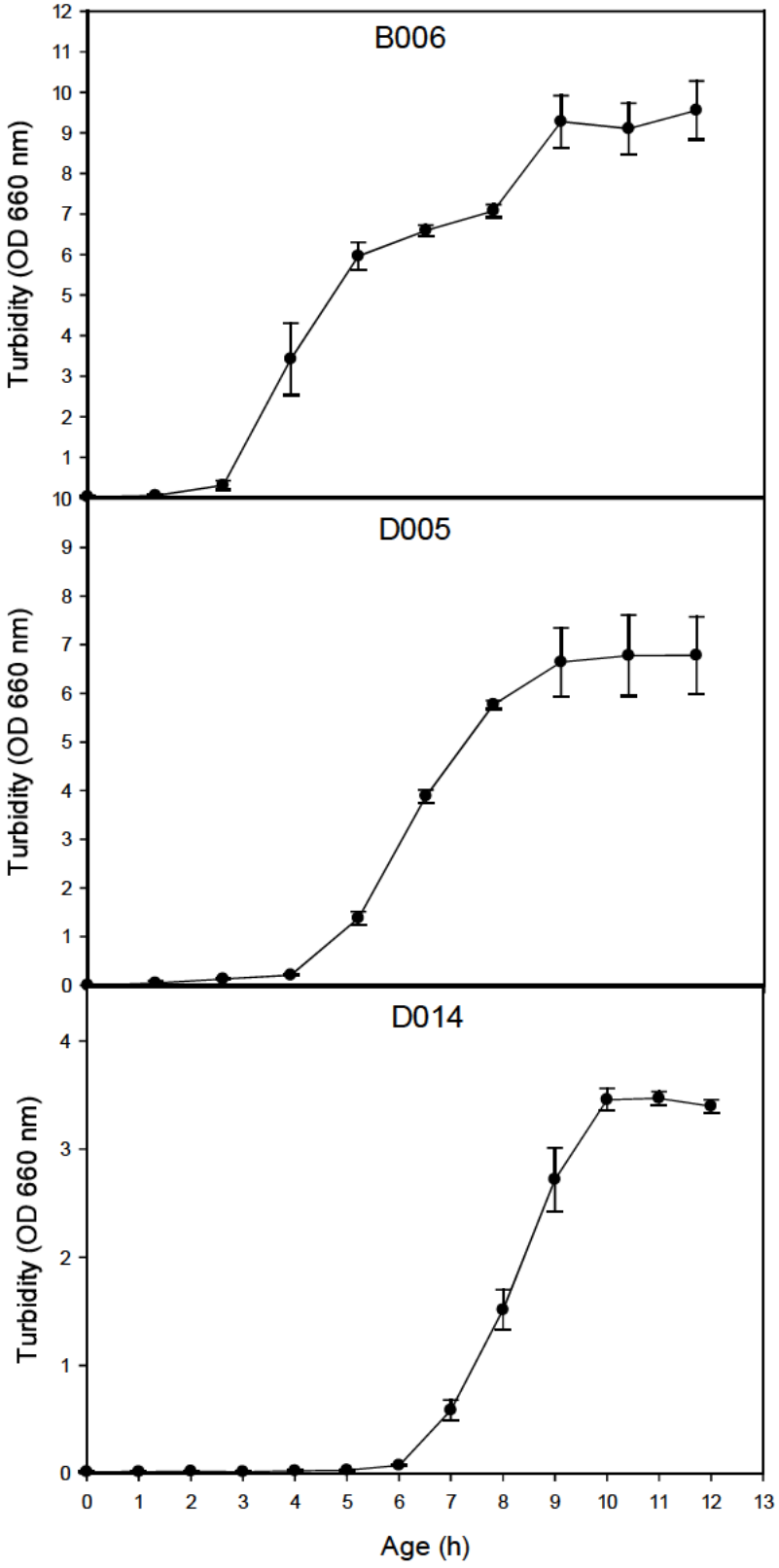

Figure 4:12: Average growth trend obtained by B006, D005 and D014 of time (age) vs OD including error bars.

Table 4.4: Average growth rate, doubling time and cell concentration obtained by B006, D005 and D014

	Unit	B006	D005	D014
Average growth rate (μ)	h^{-1}	1.652	1.132	1.205
Average doubling time	h	0.420	0.613	0.575
Viable cell concentration	$CFU.ml^{-1}$	1.45×10^{08}	3.69×10^{08}	6.99×10^{08}

4.4 Chapter conclusions

- The isolates that were selected were identified using 16S rDNA sequencing and the results showed that B006 and D005 are closely related to *B. cereus* group whilst D014 to the *B. subtilis* group.
- Biosafety assays further revealed that B006 and D005 are free of the anthrax causing plasmids. These isolates were rendered free of the *B. cereus* enterotoxin.
- The selected cryopreservation method was assessed for viability. The cultures were rendered viable after a month of storage by conducting a growth study, assessing cell concentration and the monoseptic status in each cryovial.

Chapter 5: Evaluation of bioaugmentation efficiency using a laboratory scale fluidized bed bioreactor system

5.1 Introduction

Wastewater treatment technologies are constantly evolving as there is always a greater need to treat wastewater to prevent environmental pollution. More efficient and cost-effective wastewater treatment options are therefore worthy of study (Bello et al., 2016). One of the key parameters to effective biological treatment is to maintain an active biomass which can either be suspended or fixed. Suspended growth options include ASP, aerobic digestion, high-rate anaerobic processes, anaerobic digestion, fermenters and mixed lagoons (Wik et al., 2009). Fixed film (attached growth) options include tricking towers, RBC, submerged biological contactors, packed bed and FBBR's. Fixed films have some advantages over suspended growth processes such as improved mass transfer, stable operation, long retention of biomass and good protection against toxic substances, therefore resulting in better treatment efficiencies (Metcalf and Eddy, 1991). A benefit of the improved efficiency is a smaller plant foot-print resulting in reduced capital and operating costs. FBBR's have a major advantages over other bioremediation systems, such as higher biomass concentrations, higher mass transfers, higher removal efficiency of pollutants, significantly reduced hydraulic retention times and reduction in process size (Ochieng, Odiyo, and Mutsago, 2003).

Most FBBR's developed for biological processes involving cells as biocatalysts include three phases; solid, liquid and gas (Godia and Sola, 1995). The liquid phase is the liquid or waste that will be treated, the gaseous phase is normally due to aeration from an external source and the solid phase is the solid support materials. In FBBR technology, biomass grows on solid support media and aeration is used to fluidize the bio-adsorbent material and mix the

wastewater. The bio-adsorbents should have a superficial velocity, sufficient to be suspended in the liquid column of the reactor. Because of the turbulence created by the aeration, the wastewater passes though the voids of the material, where the biomass is attached. The attached biomass can either be indigenous to that environment or have been added to the system to enhance the performance; the latter is termed bioaugmentation. Martín-Hernández, Suárez-Ojeda and Carrera (2012) used bioaugmentation with PNP-degrading bacteria to enhance wastewater treatment contaminated with shock loads of *p*-nitro phenol (PNP) in a sequencing batch reactor (SBR). Wakako et al., (2013) used *Pseudomonas stutzeri* to enhance treatment of piggery wastewater. In both studies, the researchers proved the effectiveness of bioaugmentation by using a single strain or a consortium of microorganisms.

The *Bacillus* isolates that were selected, based on wastewater bioremediation properties (Chapter 3), were further assessed for their ability to coexist and form biofilms, reduce pollutants using a lab scale FBBR. Studies have shown that some members of this genus attach to surfaces by forming extracellular polymeric substances (EPS) that hold the cell aggregates together forming a biofilm (Morikawa, 2006; Park et al., 2007; Flemming and Wingender, 2010; Vlamakis et al., 2013). The microorganisms produce EPS that forms a matrix providing the mechanical stability of biofilms, mediate adhesion to surfaces and also acts as an external digestive system attracting waste substrates and keeping extracellular enzymes close to the cells (Park et al., 2007). The FBBR technology exists on the phenomenon that the solid material will eventually be covered with biofilms because of the adhesion of microorganisms suspended in the wastewater (Lewandowski and Boltz, 2010).

This part of the study was conducted with the aim of evaluating the efficiency of selected *Bacillus* isolates as bioaugmentation agents in bioremediation of synthetic wastewater,

using a FBBR system. This was achieved by setting up of two FBBR systems; one bioaugmented (test) and the other non-bioaugmented (control). The bioaugmented and the non-bioaugmented FBBR's were packed with bio-adsorbent material and synthetic wastewater was allowed to flow into each of the bioreactors at varying flow rates. The test system was augmented with *Bacillus* isolates B006, D005 and D014 in equal proportions based on consortium studies (Chapter 3, Section 3.4.5).

5.2 Materials and Methods

5.2.1 FBBR system

5.2.1.1 Reactor design

The schematic of the FBBRs used in this study is illustrated in Figure 5.1, the design of the FBBR's was modified from reactor designs by Kawan et al., (2016) and Lariyah et al., (2016). Two FBBRs were constructed using 10.2 mm polyvinyl chloride (PVC) sheet with working volumes of ~17 L and dimensions of 660 × 180.11 × 200.87 mm. Each FBBR consisted of four ports. The first port situated at the bottom of the FBBR was to feed wastewater from the holding tank into the FBBR using 12 × 8 mm silicone tubing through a peristaltic pump (Watson-Marlow, UK). The second inlet at the bottom was to sparge air into the FBBR through a drilled (6.42 mm) stainless manifold (6 mm tube), controlled at 10 SLPM, using a rotameter. The third opening was an outlet to purge accumulated solids (once weekly) that had settled at the bottom of the FBBR. The fourth opening was at the top of the reactor for discharge of the treated effluent using a drilled (10.20 mm) PVC discharge tube (48.90 mm).

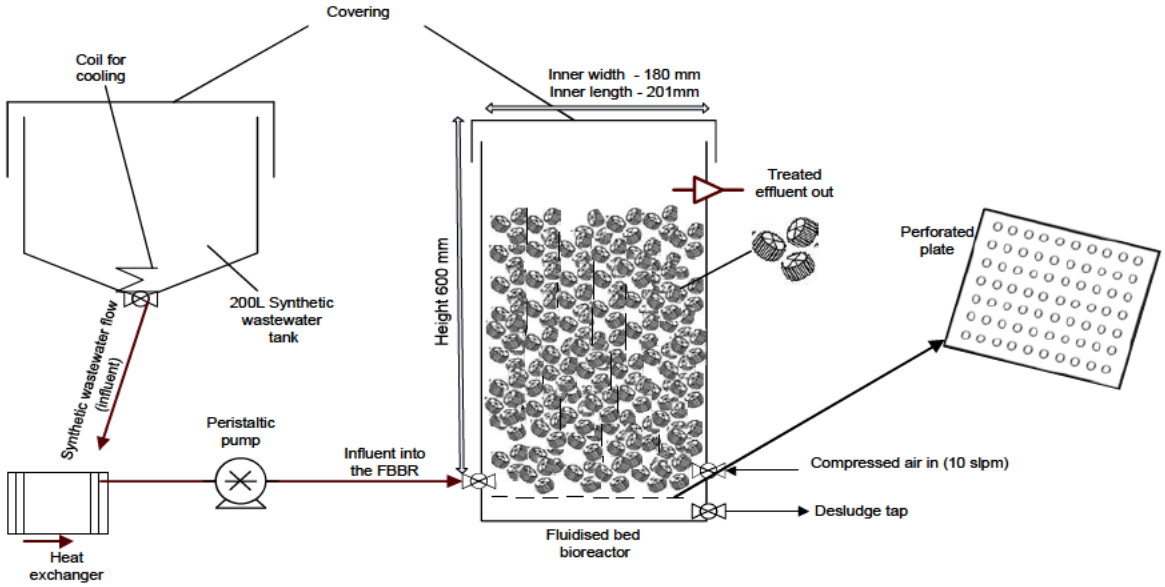

Figure 5:1: FBBR schematic design

5.2.1.2 Synthetic wastewater composition

SWW was made up according to the recipe shown in Table 5.1. This was made up as a 2000 x super concentrate which was re-constituted into a 200L holding tank every ~four days dependant on consumption from the holding tank at different flow rates. The SWW was maintained at 10 °C whilst in the tank using a cooling coil system, to minimise any microbial growth in the storage tank. The SWW was fed into the FBBR's at the desired flow rate using a variable speed peristaltic pump through a heat exchanger to pre-heat the influent to 25 °C which simulated ambient temperature conditions in the FBBR.

Table 5.1: Synthetic wastewater composition adapted from (Bracklow et al., 2007; Vorhees et al., 2014; Zafarzadeh et al., 2010a)

Compound	Unit	Amount
$NaHCO_3$	$mg.L^{-1}$	48
KH_2PO_4	$mg.L^{-1}$	22
NH_4Cl	$mg.L^{-1}$	100
CaCl	$mg.L^{-1}$	36
$MgSO_4.7H_2O$	$mg.L^{-1}$	35
K_2HPO_4	$mg.L^{-1}$	22
Yeast Extract	$mg.L^{-1}$	62.5
Glucose	$mg.L^{-1}$	62.5
Stock solution(minerals and trace metals)	ml	1

Minerals and trace metals: $CaCl_2$, 5 $mg.L^{-1}$; $FeSO_4.7H_2O$, 10 $mg.L^{-1}$; $CuCl_2.2H_2O$, 480 $ug.L^{-1}$; $Cr(NO_3)_3$ $.9H_2O$, 680 $ug.L^{-1}$; $MnSO_4.H_2O$, 100 $ug.L^{-1}$; $NiSO_4.6H_2O$, 300 $ug.L^{-1}$; $CoCl_2.6H_2O$, 50 $ug.L^{-1}$; $ZnCl_2$, 180 $ug.L^{-1}$; K_2MoO_4, 20 $ug.L^{-1}$; EDTA, 0.22 $ug.L^{-1}$

5.2.1.3 Bio-adsorbents physical properties

Both reactors (bioaugmented and non-bioaugmented) were filled with bio-adsorbent carrier material (BioZone, BioCentric Technologies) (Table 5.2) with a surface area of $650m^2.m^{-3}$. The loading of bio-adsorbent was 50% of FBBR reactor volume, which corresponded to an effective surface area of ~11 m^2 in each reactor. A photographic view of the bio-adsorbents is illustrated in Figure 5.2.

Table 5.2: Characteristics of the bio-adsorbent plastic media (Biozone, Biocentric Technologies)

Material	Polyethylene
Shape	Circle with six inner crosses and external fins
Diameter	12 mm
Specific surface area	650 $m^2.m^3$
Density	0.97 $g.cm^3$
Height	9mm

Figure 5:2: A photographic view of the bio-adsorbents used in this study

5.2.1.4 Inoculum preparation for augmentation

Cryopreserved cultures of B006, D005 and D014 were used to create an inoculum to augment the test FBBR. The inoculum was prepared by inoculating each of the three isolates into 1000 mL Erlenmeyer culture flasks containing 200 mL sterile SM respectively (Chapter 3, Table 3.2). The flasks were then incubated at 32 °C on a shaker incubator (New Brunswick Scientific, New Jersey, USA) at 180 rpm. Flasks were then observed daily until a 100% sporulation efficiency (SE) was observed microscopically (~5 days).

After a 100% sporulation efficiency was reached, the three organisms were blended in equal proportions and the mixture was normalised to 1 x 10^8 spores.mL^{-1} based on microscopic spore counts. The inoculum, containing the consortium, was stored in a sterile Erlenmeyer flask with silicone tube attached to a nipple on the bottom of the flask. This inoculum was stored at 4 °C and used as the additive for all of the FBBR studies. The inoculum was fed into the FBBR at the same time daily

The Erlenmeyer flask containing the inoculum was attached to the feed line using a T-piece connector (position 3 in Figure 5.3), after which 200 mL of the inoculum was pumped into the FBBR along with the SWW at the same feed rate.

5.2.1.5 FBBR conditioning

Influent was pumped continuously into each reactor at a flow rate of 1.5 $L.h^{-1}$ for 22 days to condition the reactor and to allow biofilm development on the bio-adsorbents on both the bioaugmented and non-bioaugmented FBBR. Naturally occurring microorganisms were allowed to form a biofilm in the non-bioaugmented FBBR. With regards to the bioaugmented FBBR, the inoculum mix (200 mL) containing B006, D005 and D014, prepared according to Section 5.2.1.5, was added into the inlet port of the FBBR (test) from the onset of the experiment and continued on a daily basis until the end of the experiment. The inoculum (200 mL) was added by attaching the flask to a T-piece connector (Figure 5.3) and pumped into the FBBR concurrently with the SWW at the same test flow rate as mentioned in Section 5.2.1.4. During the 22 day conditioning period, samples were taken and analysed but were not used to determine bioaugmentation efficiency as biofilms were still forming.

5.2.1.6 Initiation of FBBR trial

After the 22 day conditioning period, the flow rate of 1.5 $L.h^{-1}$ was continued for a further 22 days and samples were taken at the same time daily and analysed to determine bioremediation potential of *Bacillus* spp. The overall study was conducted for a period of 60 days, whereby samples were collected daily for 22 days per flow rate (three flow rates tested). The influent flow rates that were tested were 1.5, 2 and 3 $L.h^{-1}$.

Both the FBBR systems (bioaugmented and non-bioaugmented) were operated in parallel as illustrated in Figure 5.3. Dissolved oxygen (DO) was monitored using a DO meter and maintained between 30-50% for the duration of the study. The pH was maintained between 6.80 and 7.00 and was measured from each inlet and outlet sample. Deviations in pH were corrected in the synthetic wastewater tank using 10% sodium hydroxide or 10% hydrochloric acid.

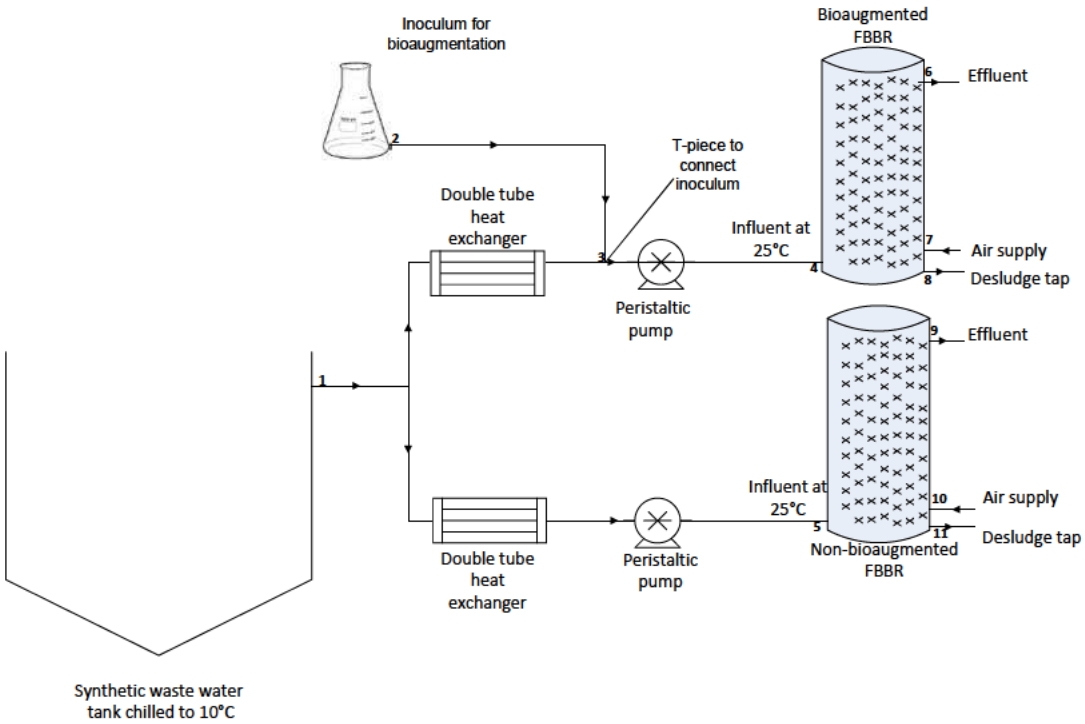

Figure 5:3: A schematic of the process showing the bioaugmented and non-bioaugmented FBBR experimental set-up.

5.2.2 Sampling and analysis (quantification of bioremediation activity to determine the removal of COD, ammonium, nitrates and phosphates)

Water samples were collected at a fixed time daily, from the inlet and outlet ports of the reactors. Samples were analysed for turbidity (by measuring OD at 660nm), pH, COD, ammonium, nitrate and phosphate according to methods outlined in Merck test kit inserts (catalogue numbers 1:00683.0001, 1:14848.0001, 1:14540.0001 and 1:14773.0001 respectively) as illustrated in Section 3.2.2.3.

Bioremediation efficiency was determined by 1) comparing daily percentage removal rate of COD, ammonium, nitrates and phosphates (equation 5.1) in the bioaugmented and non-bioaugmented FBBRs. 2) by evaluating the extent and overall improvement that occurred in the bioaugmented FBBR for COD, ammonium and phosphate removal in comparison to the control. 3) Statistical comparison of absolute waste ion removal percentages between the bioaugmented and non-bioaugmented FBBR systems and 4) by expressing the data to determine FFBR process efficiency. Results emanating from all efficiency evaluations were organised using the three flow rates tested. During statistical data analysis, points that deviated drastically from the mean (outliers) were eliminated. This meant that 18, 20 and 22 data points were used for flow rates of 1.5, 2 and 3 $L.h^{-1}$ respectively.

5.2.2.1 Comparison of daily waste removal of COD, ammonium and phosphates in bioaugmented and non-bioaugmented FBBRs

The effect of influent flow rate on daily percentage waste removal was determined on both the bioaugmented and the non-bioaugmented FBBR systems to compare waste removal efficiency at different flow rates. Daily waste removal percentages were calculated using the following equation:

$$Percentage\ removal\ (\%) = \left((Influent\ (mg.L^{-1}) - effluent(mg.L^{-1})) \div influent\ (mg.L^{-1})\right) \times 10$$

(Eq.5.1)

5.2.2.2 Evaluation of the extent of improvement that occurred in the bioaugmented FBBR in the removal of waste (COD, ammonium, nitrates and phosphates)

Overall percentage improvement in waste ion removal occurring in the bioaugmented versus the non-bioaugmented FBBR system was an indication of the average percentage improvement at each flow rate tested and was determined using the following equation:

$$Overall\ improvment\ (\%) = mean\ removal(\%)_{(bioaugmented)} - mean\ removal(\%)_{(non-bioaugmented)} \qquad \textit{(Eq.5.2)}$$

5.2.2.3 Statistical comparison of absolute waste removal percentages between bioaugmented and non-bio augmented FBBR systems

Absolute waste percentage removal was compared statistically to determine whether the augmented system performed significantly better than the non-augmented system, using the entire data sets for the two FBBR studies. This was done by using a two tailed t-test, to compare two populations where the p-value indicates the significance in difference between two data sets (Debella, 2000). The null hypothesis was that the percentage nutrient removal was significantly better in the augmented system than the non-augmented system.

5.2.2.4 Process efficiency of the bioaugmented FBBR

Process efficiency of absolute waste nutrient removal rates occurring in the bioaugmented FBBR were calculated, to infer performance measures of interest to the practical considerations for wastewater treatment.

Hydraulic retention time (HRT) was measured using equation 5.3:

$$\boldsymbol{HRT\ (h) = \frac{effective\ Volume\ inside\ the\ FBBR(L)}{Flow\ rate(L.h^{-1})}} \qquad \textbf{(Eq.5.3)}$$

The volumetric removal efficiency was calculated using equation 5.4:

$$\boldsymbol{Volumetric\ removal\ rate\ \left(mg.L^{-1}.L^{-}.h^{-1}\right) = \frac{average\ removal\ rate\ (mg.L^{-1}.h^{-1})}{FBBR\ volume\ (L)}}$$

(Eq.5.4)

5.3 Results and discussion

5.3.1 Evaluation of COD removal from synthetic wastewater in FBBR studies

The ability of the selected *Bacillus* spp. in removing COD was determined by evaluating the removal efficiencies in both the bioaugmented and non-bioaugmented FBBR. Figure 5.4 illustrates the percentage removal that occurred on a daily basis in each of the FBBR systems. Data at all the comparative points at different flow rates revealed that the highest percentage COD removal was 86.6% occurring in the bioaugmented FBBR, whilst in the non-bioaugmented FBBR, the highest removal was 78%. When evaluating the best case, with regards to the highest COD removal, in both reactors, the bioaugmented FBBR was better than the non-bioaugmented FBBR by 8.6%.

When evaluating the effect of influent flow rate on COD removal, the data revealed that at a flow rate of 1.5 $L.h^{-1}$, the bioaugmented FBBR performed better in 100% of the observations. At 2 $L.h^{-1}$ the bioaugmented FBBR performed better than the non-bioaugmented in 80% of the observations, whilst at 3 $L.h^{-1}$ the bioaugmented FBBR performed better in 91% of the observations. It can be deduced that there was better improvement in COD removal with a longer HRT (1.5 $L.h^{-1}$). Ladu and Lü, (2014) evaluated the impact of HRT on COD removal and concluded that there was a substantial difference in COD removal when the HRT was lowered.

A comparison of both the bioaugmented and non-bioaugmented FBBR's (Figure 5.8), with regards to the maximum percentage COD removal per flow rate, was also evaluated. At a flow rate of 1.5 $L.h^{-1}$, the highest percentage COD removal in the bioaugmented FBBR was 87% whilst the highest percentage removal in the non-bioaugmented FBBR was 73%. At 2 $L.h^{-1}$, the maximum COD removal in the bioaugmented and non-bioaugmented FBBRs was 81 and 78% respectively. Observations at 3 $L.h^{-1}$ revealed that the highest percentage COD removal in the bioaugmented FBBR was 66% whilst in the non-bioaugmented FBBR it was 56%. This was expected as at a longer there was a longer contact time between the substrate and the microorganisms. The importance of a longer HRT or contact time between the microorganisms and the substrate with regards to COD removal was realised by Ebrahimi et al., (2010) and Mohan et al., (2005). Ebrahimi et al., (2010) investigated the effect of HRT on COD removal using a RBC system and their results showed that there was more COD removal at a longer HRT , as 78 % of COD was removed after 16 h when compared to 53% in 8 h and 69% in 12 h. Mohan et al., (2005), also showed a 35% improvement in COD removal after bioaugmenting a SBR with sludge from a treatment plant at a HRT of 24 h.

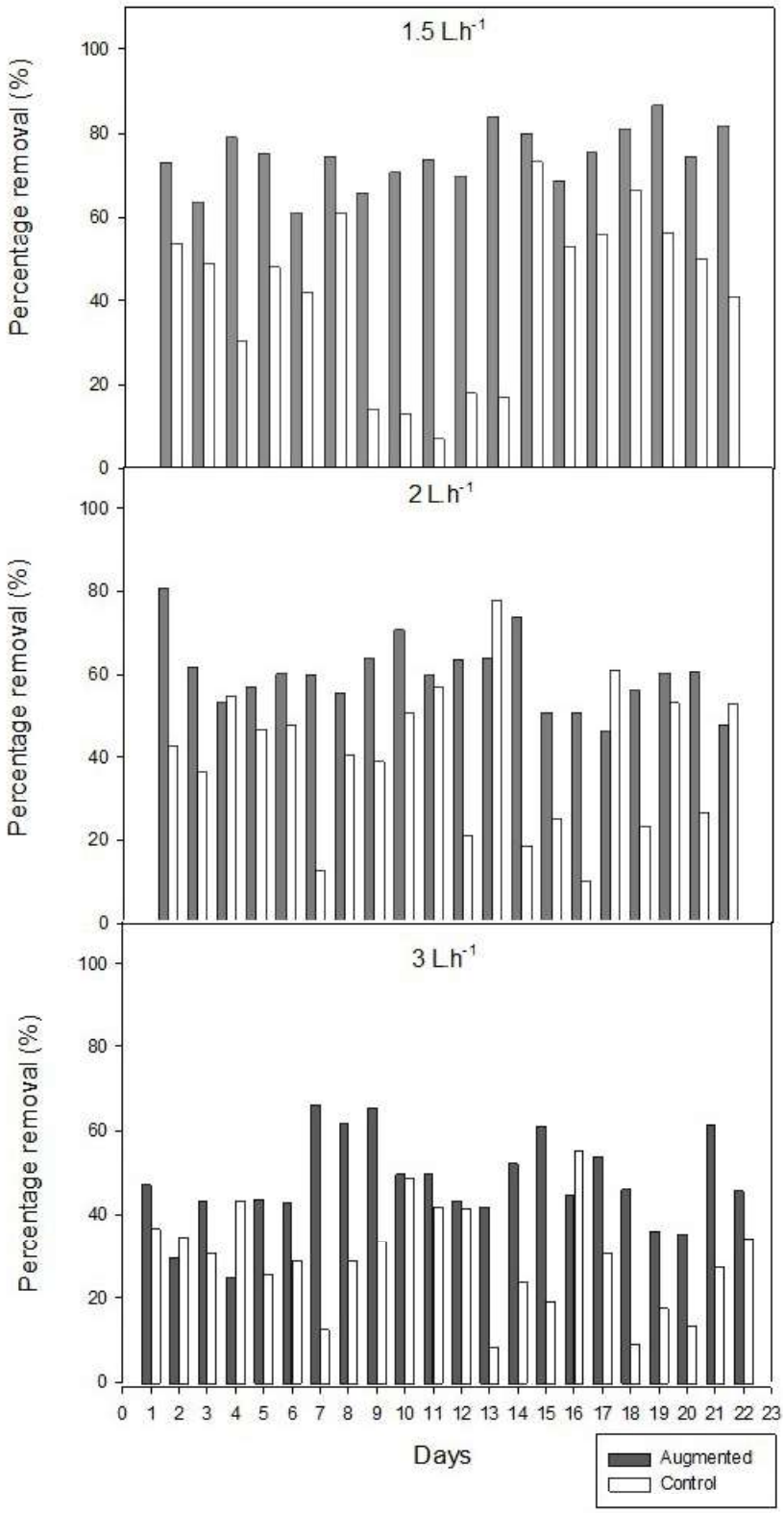

Figure 5:4: Daily COD percentage removal between bioaugmented and non-bioaugmented FBBRs operated at different flow rates

The overall percentage improvement in COD removal at different flow rates between the bioaugmented and the non-bioaugmented FBBR was determined (Figure 5.5). As expected, a higher improvement was observed at 1.5 $L.h^{-1}$ (32.59%) in comparison to 2 $L.h^{-1}$ (19.50) and 3 $L.h^{-1}$ (18.33%). Bioaugmentation was tested by Dadrasnia et al., (2017) who showed an improvement in the performance of a treatment system by removing 91.4% COD using *B. salmalaya*.

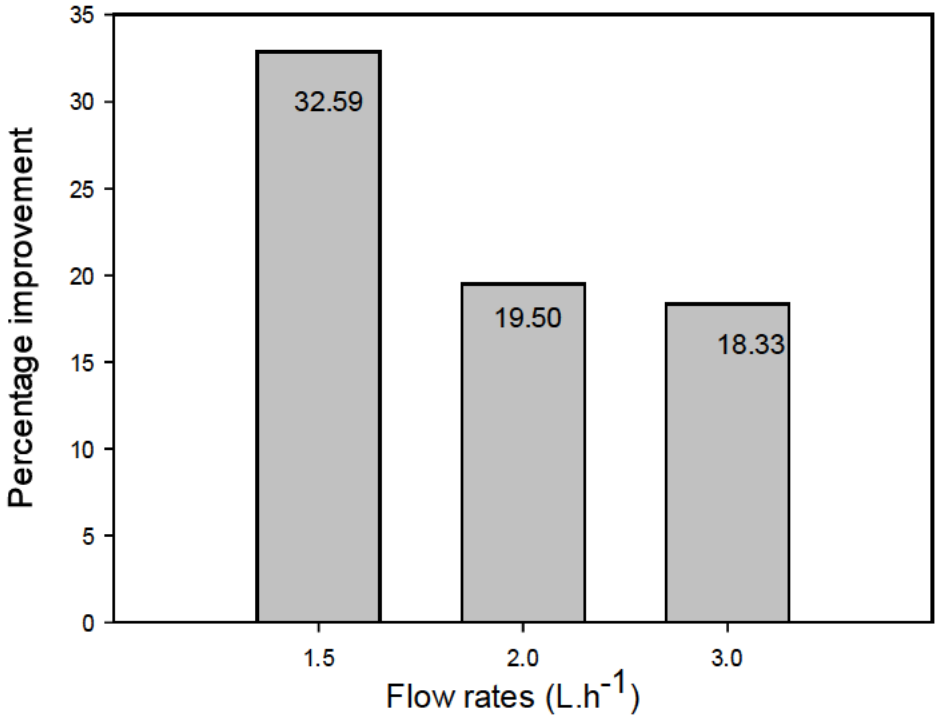

Figure 5:5: Overall percentage improvement in COD removal occurring in the bioaugmented over the non-bioaugmented FBBR at different flow rates.

Table 5.3 shows mean values of absolute COD removal percentages between the bioaugmented and non-bioaugmented FBBR at different flow rates. The results showed that there was a significant difference ($P<0.01$) in COD removal between the two FBBR systems at all flow rates (n=60) (Table 5.3). When comparing the mean absolute COD removal at each flow rate, results suggest that the highest COD removal occurred at 1.5 $L.h^{-1}$ (HRT 11.30 h).

Table 5.3: Statistical evaluation to test significance in improvement levels of absolute COD removal percentages in a bioaugmented vs non-bioaugmented FBBRs at different flow rates. The mean being the average in COD percentage removal

Flow rates $L.h^{-1}$	n	Mean values of absolute COD removal percentage (%)		p-value
		Bioaugmented	Non-bioaugmented	
1.5	18	74.46	32.89	P<0.01
2	20	59.78	40.27	P<0.01
3	22	47.61	29.29	P<0.01

Since the bioaugmentation efficiency of the *Bacillus* spp. was proven in improving COD removal, based on our observations, this part of the study focused on the efficiency of the bioaugmented FBBR with regards to volumetric removal rates.

In the bio-augmented FBBR, the average COD removal rate was highest the lowest flow rate of 1.5 $L.h^{-1}$ (4.23 $mg.L^{-1}.h^{-1}$) in comparison to 2 $L.h^{-1}$ (2.70 $mg.L^{-1}.h^{-1}$) and 3 $L.h^{-1}$ (3.96 $mg.L^{-1}.h^{-1}$). The flow rate has a direct correlation to nutrient loading and HRT, at lower HRT augmented bacteria have the longest contact time with the COD waste, therefore resulting the highest volumetric removal rate. Contrastingly, as flow rate increases the contact time of the bioaugmentation bacteria with the COD waste is also decreased, resulting in lower COD removal efficiencies. This indicates, that at the higher flow rates, the COD load exceeds the intrinsic capability of the bioaugmentation microorganism capacity for maximum volumetric COD removal.

Table 5.4: Process efficiencies at different flow rates showing COD removal in the bioaugmented FBBR.

Flow Rate	Hydraulic retention time	Volumetric removal rates
$L.h^{-1}$	h	$mg.L^{-1}.h^{-1}$
1.5	11.30	4.23
2	8.48	3.70
3	5.65	2.96

5.3.2 Evaluation of ammonium and nitrate removal from synthetic wastewater in FBBR studies

5.3.2.1 Evaluation of ammonium removal from synthetic wastewater in FBBR studies

In this section, daily percentage ammonium removal, between a bioaugmented and non-bioaugmented systems were compared. Figure 5.6 shows the daily ammonium percentage removal that occurred in both FBBR at different flow rates. Assessment of the data at all flow rates (percentage ammonium removal at 60 comparative points) indicated that the bioaugmented FBBR performed better than the non-bioaugmented FBBR 91% of the time. The data also revealed that the highest percentage ammonium removal was 96% in the bioaugmented test system, compared to only 76% in the non-bioaugmented control system. This indicates that under the best circumstances in our study, the augmented system was still 21% better at ammonium ion removal. This was expected because bioaugmentation entailed the seeding of a bio-adsorbent with a high performing consortium (as developed in Chapter 3), with the ability to perform heterotrophic nitrification. The ability of heterotrophic bacteria in removing ammonium has been demonstrated in the past (Joong et al., 2005; Ajao et al., 2011; Yang et al., 2007, 2017). Lalloo et al., (2007) further demonstrated the ability of *Bacillus* spp. in removing ammonium in aquaculture wastewater. According to previous research and this study, *Bacillus* spp. are capable of aerobic nitrification.

Figure 5.6 also shows that at a flow rate of 1.5 $L.h^{-1}$ and 2 $L.h^{-1}$, the bioaugmented system resulted in better ammonium ion removal than the non-bioaugmented control in all of the comparative data points. In addition, at a flow rate of 3 $L.h^{-1}$, the bioaugmented reactor performed better than the non-bioaugmented in 86% of the data points (Figure 5.4).

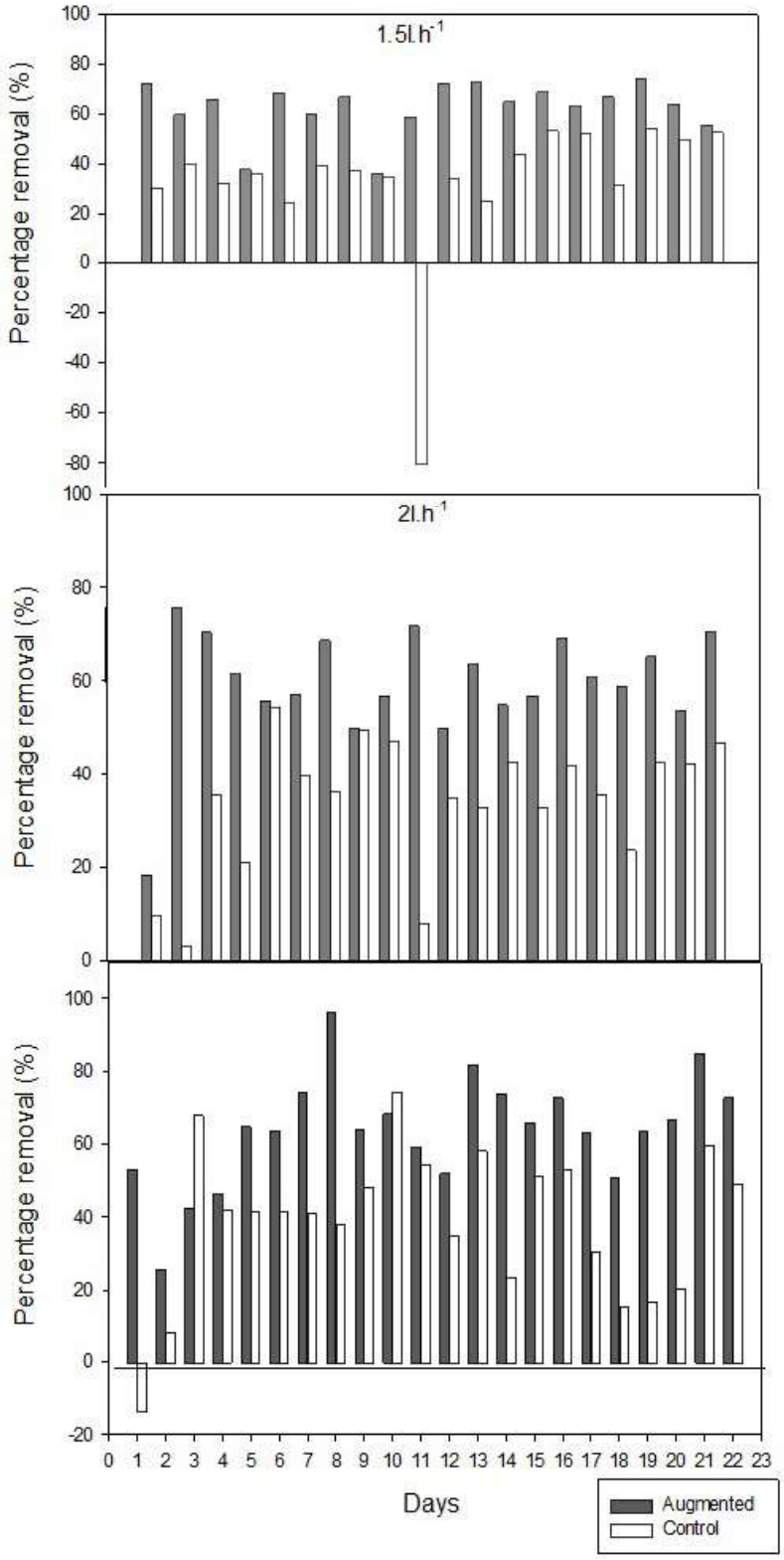

Figure 5:6: Daily ammonium percentage removal between a bioaugmented and non-bioaugmented FBBR presented at different flow rates.

Figure 5.7 displays the overall percentage improvement in ammonium removal occurred in the bioaugmented FBBR compared to the non-bioaugmented FBBR. Similar to the observation on COD removal, there was an overall improvement in ammonium removal in the bioaugmented FBBR at all the flow rates tested in comparison to the non-bioaugmented as observed in Figure 5.7. As expected, the highest overall percentage improvement occurred when the FBBR was fed at a flow rate of 1.5 $L.h^{-1}$ whilst the lowest improvement occurred at a flow rate of 3 $L.h^{-1}$. This is due to a higher HRT in the reactor that was operated at a lower flow rate of 1.5 $L.h^{-1}$ which allowed a longer contact time between the microorganisms and the substrates. Similar observation were previously reported by Cui et al., (2014), who evaluated the effect of bioaugmentation using a mixed culture from wastewater sludge and showed improved ammonium removal by 37%. *Bacillus* spp. have also been shown to remove ammonium in lab scale studies as reported by Yang et al., (2017). In their study, they have demonstrated this phenomenon using a sequencing batch reactor augmented with *Bacillus* spp. resulting in ammonium removal up to 95%. These studies show that the rationale for selecting specialised strains as bioaugmentation products is beneficial.

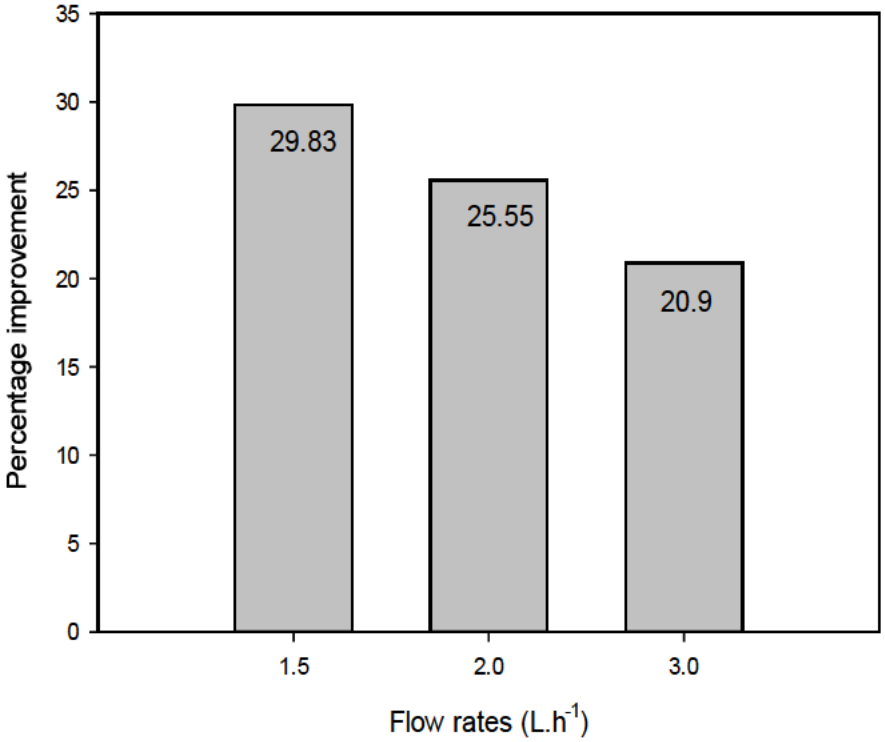

Figure 5:7: Overall percentage improvement in ammonium removal occurring in the bioaugmented over the non-bioaugmented FBBR at different flow rates.

Table 5.5 shows the mean value of absolute ammonium percentage removal between the bioaugmented and non-bioaugmented FBBR systems at different flow rates. The mean values depicting absolute ammonium ion removal percentage obtained from the bioaugmented and non-bioaugmented FBBRs were significantly different ($p<0.01$) at all flow rates ($n=60$). The bioaugmented FBBR showed better removal of absolute ammonium at all flow rates with improvement exceeding 20%. When comparing the mean absolute ammonium ion removal obtained at each flow rate in the augmented system, the results suggest that the most ammonium ion was removed at a flow rate of 1.5 $L.h^{-1}$ which was the longest HRT, although removal at the higher flow rates was not substantially different.

Table 5.5: Statistical comparison of absolute ammonium removal percentages in bioaugmented vs non-bioaugmented FBBR studies at different flow rates.

Flow rates $L.h^{-1}$	n	Mean values of absolute ammonium removal percentage (%)		p-value
		Bioaugmented	Non-bioaugmented	
1.5	18	62.65	32.82	P<0.01
2	20	58.95	33.40	P<0.01
3	22	60.64	39.74	P<0.01

Since the bioaugmentation efficiency of the *Bacillus* spp. was proven in improving ammonium removal, based on our observations, this part of the study focused on the efficiency of the bioaugmented FBBR with regards to volumetric removal rates. Table 5.6 displays the process efficiency of the bioaugmented FBBR with regards volumetric removal rates at different flow rates. The average ammonium removal rate at a flow rate of 3 $L.h^{-1}$ (HRT of 5.65 h) was higher (49.67 $mg.L^{-1}.h^{-1}$) as compared to the other flow rates tested. Similarity, there was a higher volumetric removal rate of 3.06 $mg.L^{-1}.L^{-1}.h^{-1}$ at this flow rate. At flow rates of 1.5 $L.h^{-1}$ (HRT 11.30 h) and 2 $L.h^{-1}$ (HRT 8.48 h) the average ammonium removal rates were 37.20 $mg.L^{-1}.h^{-1}$ and 35.30 $mg.L^{-1}.h^{-1}$ while the volumetric ammonium removal rates were 2.30 $mg.L^{-1}.L^{-1}.h^{-1}$ and 2.18 $mg.L^{-1}.L^{-1}.h^{-1}$ respectively, both lower than at the highest flow rate tested.

The results from this study suggest that ammonium removal is a play-off between higher residence time influencing more complete removal but hampering removal efficiency (speed of removal). This could be attributed to a better contact time between the wastewater and the bio-adsorbents with attached growth, which is governed by the flow rate of the wastewater passing through the bio-adsorbents and the rate at which the microorganisms utilise ammonium. This was evident form our results, with the highest flow rate (3 $L.h^{-1}$) resulted in the shortest residence time (5.65 h), but had the best ammonium removal rate

(volumetric removal rate). It has been thought that a longer HRT is more beneficial in removing wastewater ions (Liang et al., 2016), but more efficient technologies such as FBBRs bridge the gap between more complete removal and higher throughput. This observation can be beneficial for wastewater treatment plants as a shorter residence time results in a better ammonium removal as opposed to autotrophic nitrification where a longer HRT is preferred due to slow growth of nitrifiers. In the past ammonium removal from wastewater (nitrification) was thought to only to occur in the presence of autotrophic nitrifying bacteria. Research conducted by Joong et al., (2005); Lalloo et al., (2007); Xiao et al., (2011); Zhang et al., (2012); Chen et al., (2015) and Wan, He and Xue, (2017) has proven that removal of ammonium can be achieved by using heterotrophic bacteria. Our study supports this as heterotrophic *Bacillus* spp. were capable of enhancing ammonium removal. Furthermore, Moir et al., (1996); Daum et al., (1998) and Joo et al., (2005) conducted studies on the biochemical mechanisms of heterotrophic nitrification and discovered that the bacteria possess ammonia-oxidising and hydroxylamine-oxidising enzymes. Furthermore, conventional practise of using autotrophs for nitrification and heterotrophs for denitrification requires considerable cost, space and time and these organisms are also sensitive to process fluctuations (Kumar et al., 2010; Puyol et al., 2017). Therefore, the use of heterotrophs for nitrification promises to alleviate some of the current challenges of conventional nitrogen removal practises.

Table 5.6: Process efficiencies at different flow rates showing ammonium removal in the bioaugmented FBBR.

Flow Rate	Hydraulic retention time	Volumetric removal rates
$L.hr^{-1}$	h	$mg.L^{-1}.h^{-1}$
1.5	11.30	2.30
2	8.48	2.18
3	5.65	3.06

5.3.2.2 Evaluation of nitrate concentration in the bioaugmented and non-bioaugmented FBBR

Figure 5.8 displays the concentrations of nitrates in both the bioaugmented and non-bioaugmented FBBR. Nitrates are formed as a result of nitrification or oxidation of ammonium. Therefore, accumulation of nitrates in the bioaugmented FBBR would be an indicator that the *Bacillus* isolates were incapable of performing simultaneous nitrification-denitrification in an aerobic system. In our study, the nitrate concentration varied on a daily basis which was an indication of possible simultaneous nitrification-denitrification. Data obtained from the bioaugmented FBBR showed that at a flow rate of 1.5 $L.h^{-1}$ the nitrate concentration started decreasing on day nine and remained low until the flow rate was switched, however, on the last day there was a slight increase. When comparing ammonium percentage removal at this flow rate, it was observed that it was consistently above 60% for approximately 75% of the time. It is expected that during ammonium reduction nitrates are formed, if the consortium was unable to reduce ammonium or nitrates there would be an accumulation of both nutrients in the FBBR. The same pattern was observed when the bioaugmented FBBR was operated at a flow rate of 2 $L.h^{-1}$ the nitrate concentration fluctuated for the first 11 days, thereafter, the concentration was constantly low until the flow rate was switched to the subsequent test flow rate. At 2 $L.h^{-1}$ the ammonium percentage

removal in the bioaugmented FBBR was consistent and rarely decreased or fluctuated presenting a similar daily ammonium percentage removal. At a flow rate of 3 $L.h^{-1}$, the nitrate concentration increased from day 2 until day 6 after which it started decreasing for 8 days. There was drastic accumulation between days 13 and 15, thereafter, it decreased for the remainder of the study. The daily percentage ammonium removal displayed in the bioaugmented FBBR was above 50% for more than 60% of the days. The results obtained from the ammonium and nitrate data show that simultaneous nitrification-denitrification occurred in the bioaugmented FBBR. The fluctuation in nitrate concentration would occur during the switch to subsequent flow rates, thereafter the nitrate concentration would normalise and remain low for the duration of the study at that particular flow rate. The fluctuation could be attributed to acclimatization of the consortium and system as a whole. Another reason or ammonium and nitrate fluctuations, as mentioned by Zhao et al., (2010), could be caused by dissimilatory nitrate reduction to ammonium which is common in wastewater treatment systems using *Bacillus* spp. The phenomenon of ammonium accumulation was observed in their research, where they assessed the ability of *Bacillus* sp. LY in performing simultaneous nitrification and aerobic denitrification. They concluded that a possible reason for this phenomenon could be a high concentration of glucose. As in our study, glucose was also used as a carbon source and could have contributed to dissimilatory nitrate reduction to ammonium. Another reason is that the FBBRs were operated as an open system with varying bacterial population dynamic's.

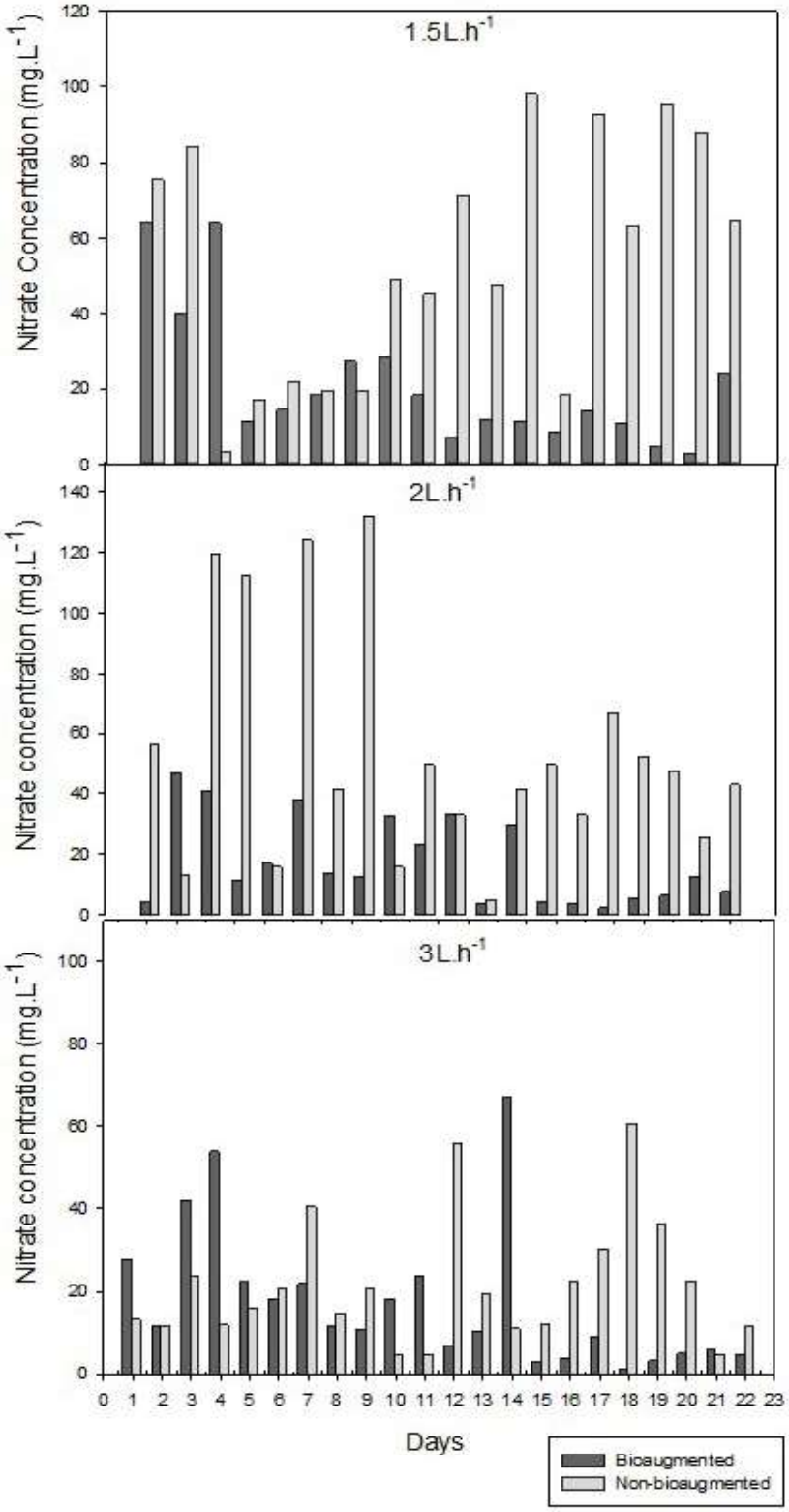

Figure 5:8: Nitrate concentration trends observed in the bioaugmented and non-bioaugmented FBBR at all flow rates

5.3.3 Evaluation of phosphate ion removal from synthetic wastewater in FBBR studies

This section focused on daily comparisons of percentage phosphate removal between the bioaugmented and non-bioaugmented FBBR systems. Figure 5.8 shows observation of all data points obtained between the bioaugmented and non-bioaugmented FBBR at different flow rates. The highest percentage phosphate removal at one data point was 89%, which occurred in the bioaugmented FBBR, whilst in the non-bioaugmented FBBR the highest percentage phosphate removal was 62%. This data indicated that even under the best circumstances, the bioaugmented system was better than the non-bioaugmented FBBR by 27%.

In order to determine the effect of influent flow rate on phosphate ion removal, data obtained from the bioaugmented and non-bioaugmented FBBR studies were compared at different flow rates. There was evidence of improved phosphate removal at all flow rates tested. A comparison of the data between the flow rates showed that at a flow rate of 1.5 $L.h^{-1}$, the bioaugmented system performed better in 61% of the observations and at 2 $L.h^{-1}$ the bioaugmented system was better in 65% of the observations. Interestingly, at 3 $L.h^{-1}$ the bioaugmented system performed better in 82% of the observations.

A comparison of the maximum percentage phosphate removal between the bioaugmented and non-bioaugmented FBBR's at 1.5 $L.h^{-1}$ were evaluated and shown to be 66.0 and 54.55% respectively. At 2 $L.h^{-1}$ the maximum phosphate removal in the bioaugmented and non-bioaugmented was 81.89 and 61.90% respectively. Observations at 3 $L.h^{-1}$ revealed that the highest percentage phosphate removal in the bioaugmented FBBR was 81.58% whilst in the non-bioaugmented FBBR the highest percentage phosphate removal was 58.33%. This trend shows the benefits of bioaugmentation, as phosphate removal efficiency was improved at all the flow rates. A similar observation was previously reported by Debroy

et al., (2013), who isolated a strain of *Bacillus* from a sewage canal and discovered that the strain removed 83% phosphate within 16 h from wastewater. Our study and the study conducted by DebRoy et al., (2013) give evidence of *Bacillus* spp. being capable of reducing phosphate concentration in wastewater.

In our study, glucose was used as a carbon source to make up SWW. Krishnaswamy et al., (2009) developed a consortium with *Bacillus* spp., *Pseudomonas* spp. and *Enterobacter* spp. and evaluated the ability of the isolates to individually remove phosphates using different carbon sources. The study showed that *Bacillus* spp. increased phosphate uptake when glucose was used as a carbon source. Glucose is a readily usable carbon source and for enhanced phosphate removal, the readily available carbon source plays a major role during the hydrolysis process. Our consortium consisting of only *Bacillus* spp. showed improvement in phosphate removal in the bioaugmented FBBR compared to the non-bioaugmented FBBR at all flow rates. This ability was displayed for COD, ammonium and nitrates, illustrating the use of *Bacillus* spp. as bioaugmentation agents.

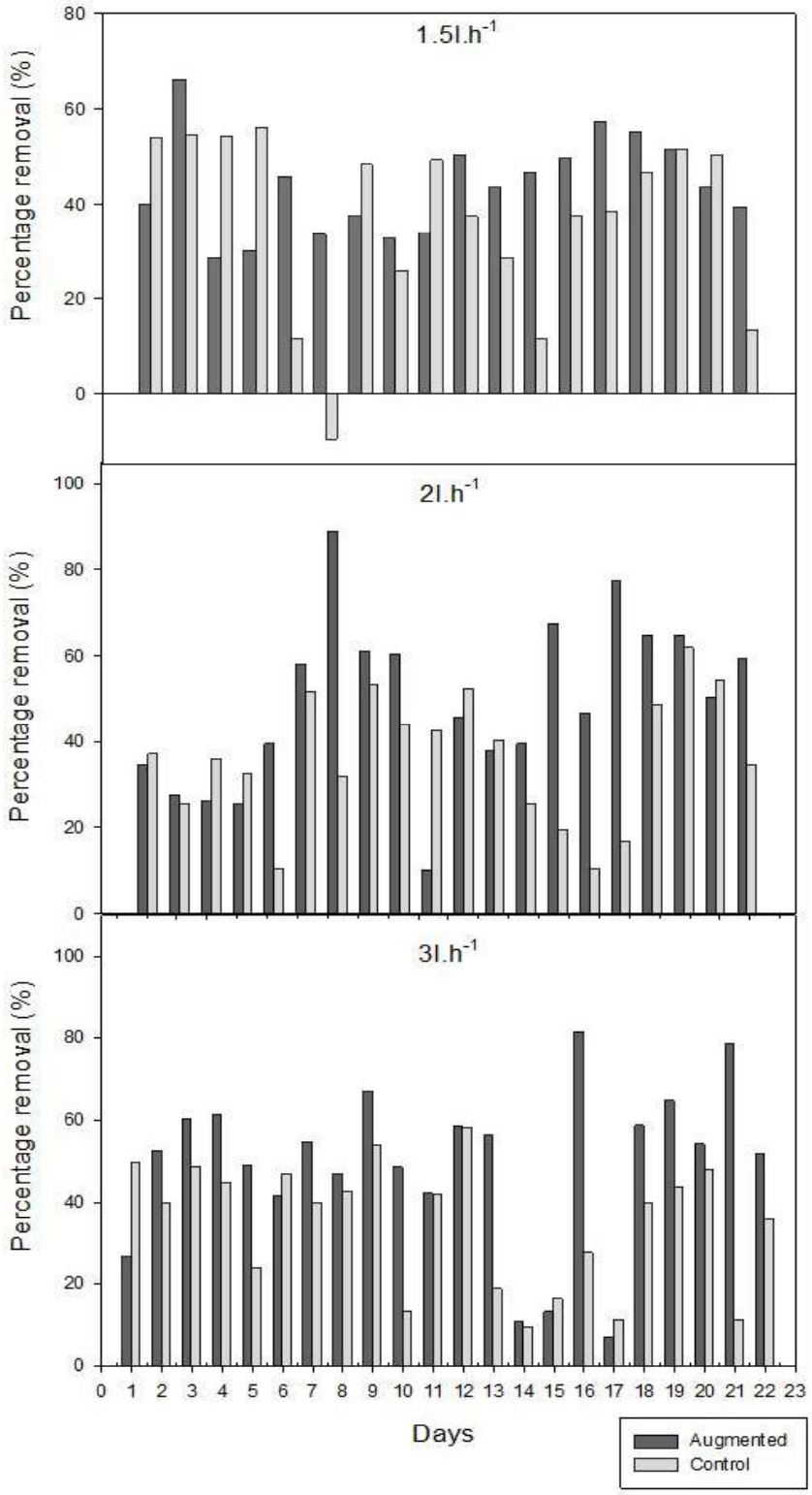

Figure 5:9: Daily phosphate percentage removal between a bioaugmented and non-bioaugmented FBBR presented at different flow rates

Figure 5.9 shows the overall percentage improvement in phosphate removal occurring in the bioaugmented versus the non-bioaugmented FBBR at different flow rates. In contrast to the improvement in COD and ammonium removal at different flow rates, a better improvement in phosphate removal was observed at a shorter HRT (3 $L.h^{-1}$) in comparison to the non-bioaugmented FBBR. Improved phosphate removal at a higher flow rate could be advantageous when applying at full scale. These results further confirm the usefulness of bioaugmentation in wastewater treatment, as there was improvement at all flow rates. Belkia and Smith, (2000) stated that when bioaugmentation is successful it can assist with plant start-up, organic and hydraulic loading and removal of toxic compounds.

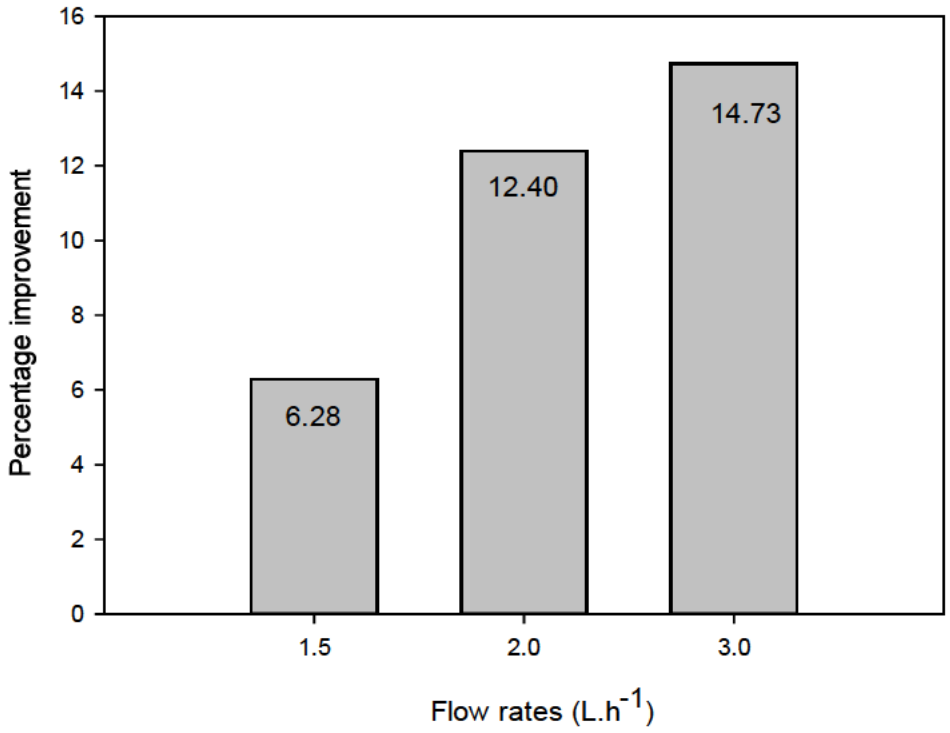

Figure 5:10: Overall percentage improvement in phosphate removal occurring in the bioaugmented over the non-bioaugmented FBBR at different flow rates.

This part of the study compared the bioaugmented and non-bioaugmented FBBR mean phosphate removal percentage. Table 5.7 shows the significant difference between the average percentage removal of the bioaugmented and non-bioaugmented FBBR systems.

The results obtained show that there was a significant difference (P<0.01) at flow rates of 2 and 3 $L.h^{-1}$. At a flow rate of 1.5 $L.h^{-1}$ there was no significant difference (P>0.01) between the bioaugmented and non-bioaugmented systems, which is simply ascribable to a lack of challenge on the systems due to excessive retention time.

Table 5.7: Statistical evaluation to test significance in improvement levels of absolute phosphate removal percentages in a bioaugmented vs non-bioaugmented FBBRs at different flow rates. The mean being the average in phosphate percentage removal.

Flow rates $L.h^{-1}$	n	Mean values of absolute phosphate removal percentage (%)		p-value
		Bioaugmented	Non-bioaugmented	
1.5	18	43.58	37.30	P>0.01
2	20	49.23	36.83	P<0.01
3	22	50.03	35.30	P<0.01

Percentage phosphate removal occurring in the bioaugmented FBBR was expressed in terms of process efficiency, which measured the impact of this study to wastewater treatment. Table 5.8 shows the average volumetric phosphate removal occurring in the bioaugmented FBBR only. The average phosphate removal rate at a flow rate of 3 $L.h^{-1}$ (HRT of 5.65 h) was higher (12.31 $mg.L^{-1}.h^{-1}$) as compared to the other flow rates tested. At flow rates of 1.5 $L.h^{-1}$ (HRT 11.30 h) and 2 $L.h^{-1}$ (HRT 8.48 h), the average phosphate removal rates were 10.74 $mg.L^{-1}.h^{-1}$ and 11.00 $mg.L^{-1}.h^{-1}$ respectively.

The results from this study suggest that there was an increase in overall aerobic phosphate removal by increasing the nutrient loading rate. This means by increasing the flow rate the concentration of phosphates in the FBBR also increased, therefore it is expected, that the phosphate volumetric removal rate increased with an increase in flow rate. Nutrient uptake

is solely dependent on the microorganism and their ability to utilise waste ions at a specific rate. Song et al., (2009) showed that decreasing HRT (higher flow rate) increased phosphorus removal efficiency because as HRT decreased, food-to-microorganism loading ratio increased and thus enhanced the biological ability and activity of bacteria. Our studies correlated strongly to the same phenomenon, confirming the relationship between nutrient loading and nutrient uptake. Krishnaswamy et al., (2009) also stated that heterotrophic bacteria were able to remove phosphates by accumulating them intracellularly and use the stored carbon reserves to produce energy for growth and to replenish phosphates. The ability of biofilm processes to remove phosphates in wastewater was studied by Lee et al., (2008) and concluded that biofilms were responsible for 65% removal of phosphates using a RBC as a treatment option. All of the findings in our study conclusively show that bioaugmentation enhances phosphate removal efficiency and in so doing improve wastewater treatment efficiency.

Table 5.8: Process efficiencies at different flow rates showing phosphate removal in the bioaugmented FBBR.

Flow Rate	**Hydraulic retention time**	**Volumetric removal rates**
$L.hr^{-1}$	**h**	**$mg.L^{-1}.h^{-1}$**
1.5	11.30	0.72
2	8.48	0.68
3	5.65	0.76

5.4 Chapter conclusion

- The ability of a consortium as a bioaugmentation agent comprising selected *Bacillus* spp. B006, D005 and D014 to remove wastewater pollutants (COD, ammonium, nitrates and phosphates) was successfully demonstrated in a laboratory scale FBBR system
- The results also revealed that the bioaugmentation with *Bacillus* species resulted in significant improvement in COD, ammonium, nitrates and phosphates removal at all flow rates.
- Improvement in percentage COD removal between the bioaugmented and non-bioaugmented FBBRs it was evident that at the lowest flow rate of 1.5 $L.h^{-1}$ (HRT of 11.30 h) there was better improvement compared to the other flow rates. At this flow rate, the bioaugmentation FBBR also showed the highest overall percentage removal and volumetric removal rate. Therefore, COD removal at a longer HRT was more effective than a shorter HRT using *Bacillus* spp. as bioaugmentation agents.
- Improvement in percentage ammonium removal between the bioaugmented and non-bioaugmented FBBRs it was evident that at the lowest flow rate of 1.5 $L.h^{-1}$ (HRT of 11.30 h) there was better improvement compared to the other flow rates. The overall removal percentage occurring in the bioaugmented FBBR was also higher at the same flow rate. However, with regards to volumetric removal rate, a higher removal rate was obtained at a shorter HRT of 5.65 h. For overall removal, a longer HRT was more effective in ammonium removal using *Bacillus* spp. as bioaugmentation agents.
- Improvement in percentage phosphate removal, between the bioaugmented and non-bioaugmented FBBRs, it was evident that at the highest flow rate of 3 $L.h^{-1}$ (HRT of 5.65 h) there was better improvement compared to the other flow rates. The overall removal percentage and volumetric removal rate occurring in the bioaugmented

FBBR were also higher at the same flow rate. For overall removal, a shorter HRT was more effective in phosphate removal using *Bacillus* spp. as bioaugmentation agents.

- The results show that there was a significant improvement between the bioaugmented and non-bioaugmented FBBR, the results obtained from the bioaugmented FBBR showed that there was a benefit to adding the selected *Bacillus* spp.
- Bacillus spp. were capable of reducing COD, ammonium, nitrates and phosphates in a single aerobic FBBR.
- The null hypothesis which is that heterotrophs such as *Bacillus* spp are not capable of nitrification – denitrification, The study has shown that the Bacillus isolates that were selected were capable of reducing ammonium, nitrates, phosphates and COD simultaneously. Therefore the null hypothesis for this study has been rejected.

Chapter 6: GENERAL CONCLUSION AND RECOMMENDATIONS

The introduction of exogenous microorganisms into environments (bioaugmentation) has been used in an attempt to accelerate bioremediation. It is desirable that the fate of an introduced organism be monitored in order to prove its contribution to pollutant degradation and to assess its influence on the ecosystem. This study was focused on the ability of indigenous *Bacillus* spp. as bioremediation agents to reduce pollutants such as COD, ammonium, nitrates and phosphates in wastewater.

The major conclusion drawn from this study were:

- Thirteen *Bacillus* spp. were evaluated of which 10 were isolated from samples obtained from a treatment plant and three were obtained from the CSIR *Bacillus* database. All the isolates were successfully evaluated for their ability to remove COD, ammonium, nitrate and phosphate from synthetic wastewater and for production of pertinent enzymes such as amylase, cellulase, protease and lipase. The bioremediation potential and enzyme activity between the isolates were found to be varied. Isolates that exhibited high reduction rates of COD, ammonium, nitrates and phosphates along with enzyme activity were weighed using a scoring matrix where all key responses were measured and cumulatively scored. Isolates B006 (50.6%), D005 (75.1%), D003 (68.2%) and D014 (91.3%) scored the highest in a scoring matrix.
- The selected isolates were evaluated for their ability to co-exist in a consortium, whereby, D003 was unable to compete with the other three isolates and was therefore omitted from further studies. The remainder of the isolates B006, D005 and D014 were then selected further evaluated in consortium studies. The three isolates in consortium were compared against results obtained from individual studies using the same bioremediation criteria. The results showed the potential of using the

consortium over the individual isolates. The results concluded that selecting high performing isolates is paramount in determining the success of bioremediation.

- The ability of the *Bacillus* spp. in reducing concentration of COD, ammonium, nitrates and phosphates was observed. Most importantly, simultaneous nitrification-denitrification was highlighted, as conventionally this occurs by a different set of bacteria.
- The isolates that were selected were identified using 16S rDNA sequencing and the results showed that B006 and D005 are closely related to *B. cereus* group whilst D014 to the *B. subtilis* group. Biosafety assays further revealed that B006 and D005 are free of the anthrax causing plasmids. These isolates were also rendered free of the *B. cereus* enterotoxin.
- The selected cryopreservation method was assessed for viability. The cultures were rendered viable after a month of storage by conducting a growth study, assessing cell concentration and the monoseptic status in each cryovial.
- The ability of a consortium as a bioaugmentation agent comprising selected *Bacillus* spp. B006, D005 and D014 to remove wastewater pollutants (COD, ammonium, nitrates and phosphates) was successfully demonstrated in a laboratory scale FBBR system. The results also revealed that there was an improvement in COD, ammonium, nitrates and phosphates removal at all flow rates. Statistical analysis also showed that the bioaugmented and non-bioaugmented FBBR were significantly different at all flow rates tested. There was correlation between overall percentage removal or percentage improvement and removal rates. With regards to ammonium and phosphates removal, the volumetric removal rate was higher at a shorter HRT. However, with regards to COD the volumetric removal rate is highest at the longest HRT.

- This study has demonstrated the application of *Bacillus* based bioaugmentation agents to enhance water treatment efficiency as a potential solution to water challenges in developing countries. Enhancing a wastewater system by introducing a consortium of microorganisms could cause an increase in the population density, ensuring rapid degradation of the nutrients. The introduction of indigenous microorganisms as bioaugmentation agents could emerge as one of only a few environmentally friendly techniques for pollution abatement.

Recommendations:

- The mechanism that *Bacillus* spp. use in removing ammonia, nitrates and phosphates could be further investigated.
- The shelf life of the preserved cultures could be determined and the benefits of preserving spores vs vegetative cells with regards to shelf life of both methods could be assessed
- The study was operated under a controlled environment and the chances of success were higher, therefore it would be beneficial to assess the ability of the isolates on actual wastewater effluent
- This technology can also be utilized for addressing the challenges of a wider range of different effluents such as agricultural and industrial wastewater.
- It is possible to scale up this technology therefore it would be recommended to assess the bioremediation potential of the isolates using a larger FBBR system that will treat larger volumes of actual effluent.

Chapter 7: REFERENCES

Abdulgader, M.E., Yu, Q.J., Williams, P., Zinatizadeh, A.A.L., 2007. A review of the performance of aerobic bioreactors for treatment of food processing wastewater 1131–1136.

Abriouel, H., Franz, C.M.A.P., Omar, N. Ben, Galvez, A., 2011. Diversity and applications of Bacillus bacteriocins. FEMS Microbiol. Rev. 35, 201–232.

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R.C., May, J.W., Vasiliadis, G., Seviour, R.J., 2007. Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor. Appl. Environ. Microbiol. 73, 2257–2270.

Ajao, A., Adebayo, G.B., Yakubu, S.E., 2011. Bioremediation of textile industrial effluent using mixed culture of Pseudomonas aeruginosa and Bacillus subtilis immobilized on agar-agar in a bioreactor. J. Microbiol. Biotechnol. Res. Sch. Res. Libr. J. Microbiol. Biotech. Res 1, 50–56.

Akpor, O.B., Muchie, M., 2010. Remediation of heavy metals in drinking water and wastewater treatment systems : Processes and applications. Int. J. 5, 1807–1817.

Akpor, O.B., Ogundeji, M.D., Olaolu, T.D., Aderiye, B.I., 2014. Microbial Roles and Dynamics in Wastewater Treatment Systems: An Overview. Int. J. Pure Appl. Biosci. 2, 156–168.

Akram, M., Shafaat, S., Bukhari, D.A., Rehman, A., 2014. Characterization of a thermostable alkaline protease from staphylococcus aureus S-2 isolated from chicken waste. Pak. J. Zool. 46, 1125–1132.

Al-Rekabi, W., Qiang, H., Qiang, W., 2007. Review on sequencing batch reactors. Pakistan J. Nutr. 6, 11–19.

Alariya, S.S., Sethi, S., Gupta, S., Lal, B., Lal, G.B., 2013. Amylase activity of a starch degrading bacteria isolated from soil. Sch. Res. Libr. Arch. Appl. Sci. Res. 5, 15–24.

Alisi, C., Musella, R., Tasso, F., Ubaldi, C., Manzo, S., Cremisini, C., Sprocati, A.R., 2009. Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Sci. Total Environ. 407, 3024–3032.

Amin, M., Rakhisi, Z., Zarei Ahmady, A., 2015. Isolation and Identification of Bacillus Species From Soil and Evaluation of Their Antibacterial Properties. Avicenna J. Clin. Microbiol. Infect. 2, 10–13.

Amin, M.T., Alazba, a a, Manzoor, U., 2014. A review on removal of pollutants from water / wastewater using different types of nanomaterials. Adv. Mater. Sci. Eng. 2014, ID 825910.

Anand, R.S., Yadav, D., Kumar, P., Pruthi, V., 2014. Microbiology of the Enhanced Biological 1, 25–29.

Anderson, E.I., 2003. An analytical solution representing groundwater-surface water interaction. Water Resour. Res. 39, 1–7.

Andreottola, G., Foladori, R., Ragazzi, M., Tatàno, F., 2000. Experimental comparison between MBBR and activated sludge system for the treatment of municipal wastewater. Water Sci. Technol. 41, 375–382.

Antony, S.P., Philip, R., 2006. Bioremediation in Shrimp Culture Systems. NAGA,WorldFishCenter Q. 29, 62–66.

Arana, I., Orruno, M., Barcina, I., 2013. Determination of the parameters defining the bacterial growth. Dep. Immunol. Microbiol. Parasitol.

Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E., Collins, M.D., 1991. Comparative Analysis of Bacillus-Anthracis, Bacillus-Cereus, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA. Int J Syst Bact 41, 343–346.

Baetens, D., 2001. Enhanced biological phosphorus removal-modelling and experimental design 1–261.

Belkia, E., Smith, P.., 2000. The bioaugmentation of a sequrencing batch reactor sludges for biological phosphorus removal. Water Sci. Technol. 35, 19–26.

Bello, M.M., Abdul Raman, A.A., Purushothaman, M., 2016. Applications of fluidized bed reactor in wastewater treatment – A review of the major design and operational parameters. J. Clean. Prod. 141, 1492–1514.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049–1055.

Bhargava, A., 2016. Activated Sludge Treatment Process. Int. J. Eng. Dev. Res. 4, 890–896.

Blackall, L.L., Crocetti, G.R., Saunders, A.M., Bond, P.L., 2002. A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 81, 681–691.

Boon, N., Goris, J., Vos, P. De, Verstraete, W., Top, E.M., 2000. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Comamonas testosteroni Strain , I2 gfp. Appl. Environ. Microbiol. 66, 2906–13.

Boopathy. R., 2000. Factors limiting bioremediation technology. Bioresour. Technol. 74, 63–67.

Bouwer, H., 2000. Integrated water management: emerging issues and challenges. Agric. Water Manag. 45, 217–228.

Bouwman, A.F., Beusen, A.H.W., Billen, G., 2009. Human alteration of the global nitrogen and phosphorus soil balances for the period 1970-2050. Global Biogeochem. Cycles 23.

Bracklow, U., Drews, A., Vocks, M., Kraume, M., 2007. Comparison of nutrients degradation in small scale membrane bioreactors fed with synthetic/domestic

wastewater. J. Hazard. Mater. 144, 620–626.

Burgess, J.E., Pletschke, B.I., 2008. Hydrolytic enzymes in sewage sludge treatment : A mini-review 34, 343–350.

Burghate, S.P., Ingole, N.W., 2013. Fluidized Bed Biofilm Reactor – A Novel Wastewater Treatment Reactor. Int. J. Res. Environ. Sci. Technol. 3, 145–155.

Butler, C.S., Boltz, J.P., 2013. Biofilm Processes and Control in Water and Wastewater Treatment. Compr. Water Qual. Purif. 3, 90–107.

Carlson, C.R., Caugant, D.A., Kolsto, A.B., 1994. Genotypic diversity among Bacillus cereus and Bacillus thuringiensis strains. Appl. Environ. Microbiol. 60, 1719–1725.

Cavaliere, M., Feng, S., Soyer, O.S., Jiménez, J.I., 2017. Cooperation in microbial communities and their biotechnological applications. Environ. Microbiol. 19, 2949–2963.

Chan, Y.J., Chong, M.F., Law, C.L., Hassell, D.G., 2009. A review on anaerobic-aerobic treatment of industrial and municipal wastewater. Chem. Eng. J. 155, 1–18.

Chávez-Crooker, P., Obreque-Contreras, J., 2010. Bioremediation of aquaculture wastes. Curr. Opin. Biotechnol. 21, 313–317.

Chen, Q., Ni, J., Ma, T., Liu, T., Zheng, M., 2015. Bioaugmentation treatment of municipal wastewater with heterotrophic-aerobic nitrogen removal bacteria in a pilot-scale SBR. Bioresour. Technol. 183, 25–32.

Chen, Y., Lin, C.J., Jones, G., Fu, S., Zhan, H., 2009. Enhancing biodegradation of wastewater by microbial consortia with fractional factorial design. J. Hazard. Mater. 171, 948–953.

Clarridge, J.E., Alerts, C., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17, 840–862.

Claus, D., 1992. A standardized Gram staining procedure. World J. Microbiol. Biotechnol.

8, 451–452.

Connell, D.W., Wu, R.S.S., Richardson, B.J., Lam, P.K.S., 2009. Chemistry of organic pollutants, including agrochemicals. Environ. Ecol. Chem. III, 1–10.

Correll, D.L., 1996. Phosphorus: A Rate Limiting Nutrient in Surface Waters. Poult. Sci. 78, 674–682.

Cox, H.H.J., Deshusses, M.A., 2001. CHAPTER 4 BIOTRICKLING FILTERS 99–100.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1–14.

Craun, G.F., Calderon, R.L., 2001. Waterborne disease outbreaks caused by distribution system deficiencies. J. / Am. Water Work. Assoc. 93, 64–75.

Cruz, H., Luckman, P., Seviour, T., Verstraete, W., Laycock, B., Pikaar, I., 2018. Rapid removal of ammonium from domestic wastewater using polymer hydrogels. Sci. Rep. 8, 1–6.

Cui, D., Li, A., Qiu, T., Cai, R., Pang, C., Wang, J., Yang, J., Ma, F., 2014. Improvement of nitri fi cation ef fi ciency by bioaugmentation in sequencing batch reactors at low temperature 8, 937–944.

Dadrasnia, A., Azirun, M.S., Ismail, S.B., 2017. Optimal reduction of chemical oxygen demand and NH 3 – N from landfill leachate using a strongly resistant novel Bacillus salmalaya strain 1–9.

Das, N., Chandran, P., 2010. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. Biotechnol. Res. Int. 2011, 1–13.

Daum, M., Zimmer, W., Papen, H., Kloos, K., Nawrath, K., Bothe, H., 1998. Physiological and molecular biological characterization of ammonia oxidation of the heterotrophic nitrifier Pseudomonas putida. Curr. Microbiol. 37, 281–288.

De-Bashan, L.E., Bashan, Y., 2004. Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). Water Res. 38, 4222–4246.

de Boer Sietske, A., Diderichsen, B., 1991. On the safety of Bacillus subtilis and B. amyloliquefaciens: a review. Appl. Microbiol. Biotechnol. 36, 1–4.

Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Sayem, S.A., 2013. Production and partial characterization of extracellular amylase enzyme from P-001. Springerplus 2, 1–12.

Debroy, S., Mukherjee, P., Roy, S., Thakur, A.R., Raychaudhuri, S., 2013. Draft Genome Sequence of a Nitrate- and Phosphate-Removing Bacillus sp., WBUNB009. Genome Announc. 1, 5–6.

DebRoy, S., Mukherjee, P., Roy, S., Thakur, A.R., Raychaudhuri, S., 2013. Draft genome sequence of a phosphate-accumulating Bacillus sp ., WBUNB004. Genome Announc. 1, 1–12.

Debuyser, M., Nguyen-the, C., 2010. Ability of Bacillus cereus Group Strains To Cause Food Poisoning Varies According to Phylogenetic Affiliation (Groups I to VII) Rather than Species Affiliation 48, 3388–3391.

Department of water and sanitation, 2018. National water and. Dep. water Sanit. Repub. South Africa 1.

Department of water and sanitation green drop certification, 2009. Green drop certification. Dep. water Aff. Repub. South Africa.

Díaz, E., 2004. Bacterial degradation of aromatic pollutants:a paradigm of metabolic versatility. Int. Microbiol. 7, 173–180.

Donnenfeld, Z., Crookes, C., Hedden, S., 2018. A delicate balance: Water scarcity in South Africa. South. Africa Rep. 13, 1–24.

Dudgeon, D., 2005. River rehabilitation for conservation of fish biodiversity in moonsonal Asia. Ecol. Soc. 10.

Dzionek, A., Wojcieszyńska, D., Guzik, U., 2016. Natural carriers in bioremediation: A review. Electron. J. Biotechnol. 23, 28–36.

Easterday, W.R., Ert, M.N. Van, Zanecki, S., 2005. Specific detection of Bacillus anthracis using a TaqMan ® mismatch amplification mutation assay.

Ebeling, J.M., 2006. Biofiltration - Nitrification Design Overview. Aquaculture.

Ebrahimi, A., Najafpour, G.D., Mohammadi, M., Hashemiyeh, B., 2010. Biological treatment of whey in an UASFF bioreactor following a three-stage BIOLOGICAL TREATMENT OF WHEY IN AN UASFF BIOREACTOR FOLLOWING A THREE-STAGE RBC. Chem. Eng. J. 16, 175–182.

Ebrahimi, Atiye, Najafpour, G.D., Mohammadi, M., Hashemiyeh, B., 2010. Biološka obrada surutke u postrojenju sa trostepenim rotirajućim biološkim kontaktorom i bioreaktorom uasff. Chem. Ind. Chem. Eng. Q. 16, 175–182.

Edokpayi, J. N., Odiyo, J.O., Durowoju, O.S., 2017. Impact of wastewater on surface water quality in developing countries: a case study of South Africa. Intech open Chapter 18, 64.

Edokpayi, Joshua N., Odiyo, J.O., Durowoju, O.S., 2017. Impact of Wastewater on Surface Water Quality in Developing Countries: A Case Study of South Africa. Water Qual.

Edwards, S.J., Kjellerup, B. V., 2013. Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. Appl. Microbiol. Biotechnol. 97, 9909–9921.

Effler, S.W., Brooks, C.M., Whitehead, K.A., 1996. Domestic waste inputs of nitrogen and phosphorus to Onondaga Lake, and water quality implications. Lake Reserv. Manag. 12, 127–140.

Ehling-schulz, M., Koehler, T.M., Lereclus, D., 2019. HHS Public Access.

Ekama, G.A., Dold, P.L., Marais, G.R., 1986. Procedures for determining influent COD fractions and the maximum specific growth rate of heterotrophs in activated sludge systems. Water Sci. Technol. 18, 91–114.

El Fantroussi, S., Agathos, S.N., 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr. Opin. Microbiol. 8, 268–275.

El, T., Wahbi, S., Mandi, L., Masi, S., Ouazzani, N., 2019. Journal of the Saudi Society of Agricultural Sciences Reuse study of sustainable wastewater in agroforestry domain of Marrakesh city. J. Saudi Soc. Agric. Sci. 18, 288–293.

Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B., Pauli, G., 2002. Rapid and sensitive identi ¢ cation of pathogenic and apathogenic Bacillus anthracis by real-time PCR. FEMS Microbiol. Lett. 214, 51–59.

Ellis, T.G., 2012. Chemistry of wastewater. Environ. Ecol. Chem. II, 1–25.

Elser, J.J., Bracken, M.E.S., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H., Ngai, J.T., Seabloom, E.W., Shurin, J.B., Smith, J.E., 2007. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecol. Lett. 10, 1135–1142.

Elshaghabee, F.M.F., Rokana, N., Gulhane, R.D., Sharma, C., Panwar, H., 2017. Bacillus as potential probiotics: Status, concerns, and future perspectives. Front. Microbiol. 8, 1–15.

Eriksson, E., Auffarth, K., Henze, M., Ledin, A., 2002. Characteristics of grey wastewater. Urban Water 4, 85–104.

Fábio, F., Barros, C., Paula, A., Simiqueli, R., Andrade, C.J. De, Pastore, G.M., 2013. Production of Enzymes from Agroindustrial Wastes by Biosurfactant-Producing Strains of Bacillus subtilis 2013.

Feng, X., Chu, K.H.H., 2004. Cost Optimization of Industrial Wastewater Reuse Systems. Process Saf. Environ. Prot. 82, 249–255.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.

Földes, T., Bánhegyi, I., Herpai, Z., Varga, L., Szigeti, J., 2000. Isolation of Bacillus strains from the rhizosphere of cereals and in vitro screening for antagonism against

phytopathogenic, food-borne pathogenic and spoilage micro-organisms. J. Appl. Microbiol. 89, 840–846.

Fujikawa, H., 1994. Diversity of the growth patterns of Bacillus subtilis colonies on agar plates 13, 159–167.

Gao, W., Smith, D.W., Li, Y., 2007. Effects of Freezing on the Survival of Escherichia coli and Bacillus and Response to UV and Chlorine After Freezing. Water Environ. Res. 79, 507–513.

Geissen, V., Mol, H., Klumpp, E., Umlauf, G., Nadal, M., van der Ploeg, M., van de Zee, S.E.A.T.M., Ritsema, C.J., 2015. Emerging pollutants in the environment: A challenge for water resource management. Int. Soil Water Conserv. Res. 3, 57–65.

Godia, F., Sola, C., 1995. Fluidized bed bioreactors. Biotechnol. programne 11, 479–497.

Gupta, V.K., Sadegh, H., Yari, M., Shahryari Ghoshekandi, R., Maazinejad, B., Chahardori, M., 2015. Removal of ammonium ions from wastewater A short review in development of efficient methods. Glob. J. Environ. Sci. Manag. 1, 149–158.

Haba, E., Bresco, O., Ferrer, C., Marques, A., Busquets, M., Maresa, A., 2000. Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate. Enzyme Microb. Technol. 26, 40–44.

Helgason, E., Caugant, D. a, Olsen, I., Kolstø, A., 2000a. Genetic Structure of Population of Bacillus cereus and B . thuringiensis Isolates Associated with Periodontitis and Other Human Infections Genetic Structure of Population of Bacillus cereus and B . thuringiensis Isolates Associated with Periodontitis and 38, 1615–1622.

Helgason, E., Økstad, O.A., Dominique, a, Johansen, H. a, Fouet, A., Hegna, I., Kolstø, A., Økstad, O.L.E.A., Caugant, D. a, Mock, L.E., Hegna, I.D. a, 2000b. Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis −− One Species on the Basis of Genetic Evidence One Species on the Basis of Genetic Evidence. Appl. Environ. Microbiol. 66, 2627–2630.

Hibbing, M.., Fuqua, C., Parsek, M.., Peterson, S.., 2010. Bacterial competition: surviving and thriving in teh microbial jungle. Nat Rev Microbiol 1, 15–25.

Hilton, J., O'Hare, M., Bowes, M.J., Jones, J.I., 2006. How green is my river? A new paradigm of eutrophication in rivers. Sci. Total Environ. 365, 66–83.

Holman, J.B., Wareham, D.G., 2005. COD, ammonia and dissolved oxygen time profiles in the simultaneous nitrification/denitrification process. Biochem. Eng. J. 22, 125–133.

Hong, H.A., Huang, J., Khaneja, R., Hiep, L. V, Urdaci, M.C., Cutting, S.M., 2008. The safety of Bacillus subtilis and Bacillus indicus as food probiotics 105, 510–520.

Hsueh, Y.H., Somers, E.B., Lereclus, D., Wong, A.C.L., 2006. Biofilm formation by Bacillus cereus is influenced by PlcR, a pleiotropic regulator. Appl. Environ. Microbiol. 72, 5089–5092.

Isaacs, S.., Henze, M., 1995. Controlled carbon source addition to an alternating nitrification-dentrification wastewater treatment process including biological removal. Water Resour. 29, 77–89.

Iwase, T., Tajima, A., Sugimoto, S., Okuda, K., Hironaka, I., Kamata, Y., Takada, K., Mizunoe, Y., 2013. A Simple Assay for Measuring Catalase Activity: A Visual Approach. Sci. Rep. 3, 3081.

Jena, H., Roy, G., Meikap, B., 2005. Comparative Study of Immobilized Cell Bioreactors for Industrial Wastewater Treatment. Proc. WMCI-2005 Ist and 2n.

Jensen, G.B., Hansen, B.M., Eilenberg, J., Mahillon, J., 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environ. Microbiol. 5, 631–640.

Jeon, J.S., Lee, S.S., Kim, H.Y., Ahn, T.S., Song, H.G., 2003. Plant Growth Promotion in Soil by Some Inoculated Microorganisms. J. Microbiol. 41, 271–276.

Jeßberger, N., Krey, V.., Rademacher, C., Bohm, M.., Mohr, A.., Ehling-Schulz, M., Scherer, S., Martlbauer, E., 2015. From genome to toxicity : A combinatory approach highlights the complexity of enterotoxin production in Bacillus cereus From genome to

toxicity : a combinatory approach highlights the complexity of enterotoxin production in Bacillus cereus. Front. Microbiol. 6, 1–15.

Jeyaram, K., Romi, W., Singh, T.A., Adewumi, G.A., Basanti, K., Oguntoyinbo, F.A., 2011. Distinct differentiation of closely related species of Bacillus subtilis group with industrial importance. J. Microbiol. Methods 87, 161–164.

Jiang, H.L., Maszenan, A.M., Tay, J.H., 2007. Bioaugmentation and coexistence of two functionally similar bacterial strains in aerobic granules. Appl. Microbiol. Biotechnol. 75, 1191–1200.

Jianlong, W., Xiangchun, Q., Libo, W., Yi, Q., Hegemann, W., 2002. Bioaugmentation as a tool to enhance the removal of refractory compound in coke plant wastewater. Process Biochem. 38, 777–781.

Jingjing Xiao, Changxiong Zhu , Dongyuan Sun, Ping Guo, Y.T., 2011. No Title. J. Environ. Sci. 23, 1279–1285.

Joo, H.-S., Hirai, M., Shoda, M., 2005. Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by Alcaligenes faecalis No. 4. J. Biosci. Bioeng. 100, 184–191.

Joo, H., Hirai, M., Ã, M.S., 2006. Piggery wastewater treatment using Alcaligenes faecalis strain No . 4 with heterotrophic nitrification and aerobic denitrification 40, 3029–3036.

Joong, K.K., Kyoung, J.P., Kyoung, S.C., Nam, S.W., Park, T.J., Bajpai, R., 2005. Aerobic nitrification-denitrification by heterotrophic Bacillus strains. Bioresour. Technol. 96, 1897–1906.

Jouanneau, S., Recoules, L., Durand, M.J., Boukabache, A., Picot, V., Primault, Y., Lakel, A., Sengelin, M., Barillon, B., Thouand, G., 2014. Science Direct Methods for assessing biochemical oxygen demand (BOD): A review. Water Res. 49, 62–82.

Kamara, A., Sally, H., 2003. Water for food, livelihoods and nature: Simulations for policy dialogue in South Africa. Phys. Chem. Earth 28, 1085–1094.

Karigar, C.S., Rao, S.S., 2011. Role of microbial enzymes in the bioremediation of pollutants: A review. Enzyme Res. 2011.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr. Microbiol. 57, 503–507.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M., Igarashi, Y., 2005. Stable coexistence of five bacterial strains as a cellulose-degrading community. Appl. Environ. Microbiol. 71, 7099–7106.

Kawan, J.A., Hasan, H.A., Suja, F., Jaafar, O., 2016. A REVIEW ON SEWAGE TREATMENT AND POLISHING USING MOVING BED. J. Eng. Sci. Technol. 11, 1098–1120.

Kearns, D.B., Chu, F., Branda, S.S., Kolter, R., Losick, R., 2005. A master regulator for biofilm formation by Bacillus subtilis. Mol. Microbiol. 55, 739–749.

Khanitchaidecha, W.. b, Nakaruk, A.. d, Koshy, P.., Futaba, K.., 2015. Comparison of Simultaneous Nitrification and Denitrification for Three Different Reactors. Biomed Res. Int. 2015, 1–7.

Khanitchaidecha, W., Nakaruk, A., Kazama, F., 2019. Heterotrophic nitrification and aerobic denitrification using pure-culture bacteria for wastewater treatment. J. water reuse Desalin. 10–18.

Kim, M., Jeong, S., Yoon, S.J., Cho, S.J., Kim, Y.H., Kim, M.J., Ryu, E.Y., Lee, S., 2008. Aerobic Denitrification of Pseudomonas putida AD-21 at Different C / N Ratios. J. Biosci. Bioeng. 106, 498–502.

Koehler, T.M., 2009. Bacillus anthracis physiology and genetics. Mol. Aspects Med. 30, 386–396.

Kornaros, M., Lyberatos, G., 2006. Biological treatment of wastewaters from a dye manufacturing company using a trickling filter. J. Hazard. Mater. 136, 95–102.

Kouker, G., Jaeger, K.E., 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53, 211–213.

Koul, S., Gauba, P., 2014. Bioaugmentation - A Strategy For Cleaning Up Soil. J. Civ. Eng. Environ. Technol. Print 1, 2349–8404.

Krishnaswamy, U., Muthusamy, M., Perumalsamy, L., 2009. Studies on the Efficiency of the Removal of Phosphate Using Bacterial Consortium for the Biotreatment of Phosphate Wastewater. Eur. J. Appl. Sci. 1, 6–15.

Kumar, A., Bisht, B.., Joshi, V.., Dhewa, T., 2011. Review on Bioremediation of Polluted Environment : A Management Tool. Int. J. Environ. Sci. 1, 1079–1093.

Kumar, A., Singh Bisht, B., Datt Joshi, V., 2010. Biosorption of Heavy Metals by four acclimated microbial species, Bacillus spp., Pseudomonas spp., Staphylococcus spp. and Aspergillus niger. J. Biol. Environ. Sci 4, 97–108.

Ladu, J.L.C., Lü, X., 2014. Effects of hydraulic retention time, temperature, and effluent recycling on efficiency of anaerobic filter in treating rural domestic wastewater. Water Sci. Eng. 7, 168–182.

Lalloo, R., Maharajh, D., Görgens, J., Gardiner, N., 2008. Functionality of a Bacillus cereus biological agent in response to physiological variables encountered in aquaculture. Appl. Microbiol. Biotechnol. 79, 111–118.

Lalloo, R., Maharajh, D., Görgens, J., Gardiner, N., Görgens, J.F., 2009. High-density spore production of a B. cereus aquaculture biological agent by nutrient supplementation. Appl. Microbiol. Biotechnol. 83, 59–66.

Lalloo, R., Moonsamy, G., Ramchuran, S., Görgens, J., Gardiner, N., 2010. Competitive exclusion as a mode of action of a novel Bacillus cereus aquaculture biological agent. Lett. Appl. Microbiol. 50, 563–570.

Lalloo, R., Ramchuran, S., Ramduth, D., Görgens, J., Gardiner, N., 2007. Isolation and selection of Bacillus spp. as potential biological agents for enhancement of water

quality in culture of ornamental fish. J. Appl. Microbiol. 103, 1471–1479.

Lariyah, M.S., Mohiyaden, H.A., Hayder, G., Hussein, A., Basri, H., Sabri, A.F., Noh, M.N., 2016. Application of Moving Bed Biofilm Reactor (MBBR) and Integrated Fixed Activated Sludge (IFAS) for Biological River Water Purification System : A Short Review Application of Moving Bed Biofilm Reactor (MBBR) and Integrated Fixed Activated Sludge (IF. Conf. Adv. Renew. energy Technol. 1–15.

Lawrie, R.A., Stretch, D.D., Perissinotto, R., 2010. The effects of wastewater discharges on the functioning of a small temporarily open/closed estuary. Estuar. Coast. Shelf Sci. 87, 237–245.

Lazarova, V., Manem, J., 1995. Biofilm characterization and activity analysis in water and wastewater treatment. Water Res. 29, 2227–2245.

Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiesser-Gunkel, E., STEWART, G.S.A.B., Scherer, S., 1998. Bacillus weihenstephanensis sp. nov. is a new psychrotolerant species of the Bacillus cereus group. Int. J. Syst. Bacteriol. 48, 1373–1382.

Lee, H., Choi, E., Yun, Z., Park, Y.K., 2008. Microbial structure and community of RBC biofilm removing nitrate and phosphorus from domestic wastewater. J. Microbiol. Biotechnol. 18, 1459–1469.

Lee, P.Y., Costumbrado, J., Hsu, C.-Y., Kim, Y.H., 2012. Agarose Gel Electrophoresis for the Separation of DNA Fragments. J. Vis. Exp. 1–5.

Lekang, O.I., Kleppe, H., 2000. Efficiency of nitrification in trickling filters using different filter media. Aquac. Eng. 21, 181–199.

Lewandowski, Z., Boltz, J.P., 2010. Biofilms in Water and Wastewater Treatment, Treatise on Water Science.

Liang, H., Liu, S., Li, X., Sun, X., Gao, D., 2016. Effects of HRT on biological nitrogen removal in single-stage autotrophic process. Appl. Environ. Biotechnol. 1–7.

Liu, Y., Villalba, G., Ayres, R.U., Schroder, H., 2008. Global phosphorus flows and environmental impacts from a consumption perspective. J. Ind. Ecol. 12, 229–247.

Lombardo, P., 2006. Phosphorus Geochemistry in Septic Tanks, Soil Absorption Systems, and Groundwater 87.

Loosdrecht, M. Van, Brdjanovic, D., 1997. Biological phosphate removal processes. Appl. microbiol biotechnol 289–296.

Loucks, D.P., van Beek, E., 2005. Water resource systems planning and management.

Lu, Z., Guo, W., Liu, C., 2018. Isolation , identification and characterization of novel Bacillus subtilis 427–433.

Luna, V.A., King, D.S., Peak, K.K., Reeves, F., Heberlein-Larson, L., Veguilla, W., Heller, L., Duncan, K.E., Cannons, A.C., Amuso, P., Cattani, J., 2006. Bacillus anthracis virulent plasmid pX02 genes found in large plasmids of two other Bacillus species. J. Clin. Microbiol. 44, 2367–2377.

Lyngwi, N.A., Joshi, S., 2014. Economically important Bacillus and related genera : a mini review Chapter 3 Economically important Bacillus and related genera : a mini review. Biol. Useful plants microbes 33–43.

Ma, B., Wang, S., Cao, S., Miao, Y., Jia, F., Du, R., Peng, Y., 2016. Bioresource Technology Biological nitrogen removal from sewage via anammox : Recent advances. Bioresour. Technol. 200, 981–990.

Mara, D., 2013. Domestic wastewater treatment in developing countries, Taylor and Francis.

Martín-Hernández, M., Suárez-Ojeda, M.E., Carrera, J., 2012. Bioaugmentation for treating transient or continuous p-nitrophenol shock loads in an aerobic sequencing batch reactor. Bioresour. Technol. 123, 150–156.

Matějů, V., Čižinská, S., Krejčí, J., Janoch, T., 1992. Biological water denitrification-A review. Enzyme Microb. Technol. 14, 170–183.

Mccarty, P.L., Bae, J., Kim, J., 2011. Domestic Wastewater Treatment as a Net Energy Producer À Can This be Achieved ? Environ. Sci. Technol. 7100–7106.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Naidu, R., 2011. Bioremediation approaches for organic pollutants: A critical perspective. Environ. Int. 37, 1362–1375.

Mével, G., Prieur, D., 2000. Heterotrophic nitrification by a thermophilic Bacillus species as influenced by different culture conditions. Can. J. Microbiol. 46, 465–73.

Meza, R.A., Monroy, A.F., Mercado, M., Poutou, A., Rodríguez, P., Pedroza, A.M., 2004. Study of the Stability in Real Time of. Univ. Sci. 9, 35–42.

Mino, T., van Loosdrecht, M.C.M., Heijnen, J.J., 1998. Review paper - Microbiology and biochemistry of the enhanced biological phosphate removal process. Wat. Res. 11, 3193–3207.

Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001. In situ bioremediation potential of an oily sludge-degrading bacterial consortium. Curr. Microbiol. 43, 328–335.

Mittal, A., 2011. Biological wastewater treatment. Water Today, Fulltide October, 32–44.

Mohan, S.V., Rao, N.C., Prasad, K.K., Sarma, P.N., 2005. Bioaugmentation of an anaerobic sequencing batch biofilm reactor (AnSBBR) with immobilized sulphate reducing bacteria (SRB) for the treatment of sulphate bearing chemical wastewater. Process Biochem. 40, 2849–2857.

Moir, J.W.B., Crossman, L.C., Spiro, S., Richardson, D.J., 1996. The purification of ammonia monooxygenase from Paracoccus denitrificans. FEBS Lett. 387, 71–74.

Morikawa, M., 2006. Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. J. Biosci. Bioeng. 101, 1–8.

Muga, H.E., Mihelcic, J.R., 2008. Sustainability of wastewater treatment technologies. J. Environ. Manage. 88, 437–447.

Mukred, A.M., Hamid, A.A., Hamzah, A., Yusoff, W.M.W., 2008. Development of Three

Bacteria Consortium for the Bioremediation of Crude Petroleum-oil in Contaminated Water. Online J. Biol. Sci. 8, 73–79.

Mulder, A., Graaf, A.A., Robertson, L.A., Kuenen, J.G., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16, 177–184.

Munch EV, Lant P, K.J., 1996. Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. Water Res 30, 277–284.

Naddafi, K., Hassanvand, M.S., Dehghanifard, E., Faezi Razi, D., Mostofi, S., Kasaee, N., Nabizadeh, R., Heidari, M., 2009. Performance evaluation of wastewater stabilization ponds in Yazd-Iran. Iran J. Environ. Heal. scince Eng. 6, 41–46.

Naddeo, V., Meriç, S., Kassinos, D., Belgiorno, V., Guida, M., 2009. Fate of pharmaceuticals in contaminated urban wastewater effluent under ultrasonic irradiation. Water Res. 43, 4019–4027.

Nakamura, L.K., 1998. Determine the Phylogenetic Relationship of the Group 2. Int. J. Syst. Bacteriol. 1031–1035.

Nelson, M.J., Nakhla, G., Zhu, J., 2017. Fluidized-Bed Bioreactor Applications for Biological Wastewater Treatment: A Review of Research and Developments. Engineering 3, 330–342.

Nemutanzhela, M.E., Roets, Y., Gardiner, N., Lalloo, R., 2014. The Use and Benefits of Bacillus Based Biological Agents in Aquaculture., Sustainable Aquaculture Techniques.

Nguyen, P.Y., Silva, A.F., Reis, A.C., Nunes, O.C., Rodrigues, A.M., Rodrigues, J.E., Cardoso, V.V., Benoliel, M.J., Reis, M.A.M., Oehmen, A., Carvalho, G., 2019. Bioaugmentation of membrane bioreactor with Achromobacter denitrificans strain PR1 for enhanced sulfamethoxazole removal in wastewater. Sci. Total Environ. 648, 44–55.

Nicholson, W.L., 2002. Roles of Bacillus endospores in the environment. Cell. Mol. Life Sci. 59, 410–416.

Nopens, I., Capalozza, C., Vanrolleghem, P. a, 2001. Stability analysis of a synthetic municipal wastewater. Tech. Rep. 32, 24.

Nozhevnikova, A.N., Simankova, M. V., Litti, Y. V., 2012. Application of the microbial process of anaerobic ammonium oxidation (ANAMMOX) in biotechnological wastewater treatment. Appl. Biochem. Microbiol. 48, 667–684.

Nzila, A., Razzak, S.A., Zhu, J., 2016. Bioaugmentation: An emerging strategy of industrial wastewater treatment for reuse and discharge. Int. J. Environ. Res. Public Health 13.

Oberholster, P.J., Ashton, P.J., 2008. State of the Nation Report An Overview of the Current Status of Water Quality and Eutrophication in South African Rivers and Reservoirs. State Nation Rep. 1–15.

Ochieng, A., Odiyo, J.O., Mustango, M., 2003. Biological treatment of mixed industrial wastewaters in a fluidised bed reactor. J. Hazard. Mater. 96, 79–90.

Ødegaard, H., 2006. Innovations in wastewater treatment: The moving bed biofilm process. Water Sci. Technol. 53, 17–33.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Res. 41, 2271–2300.

Okinaka, R.T., Cloud, K., Hampton, O., Hoffmaster, a. R., Hill, K.K., Keim, P., Koehler, T.M., Lamke, G., Kumano, S., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, # R., Jackson, P.J., 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J. Bacteriol. 181, 6509–6515.

Pant, G., Prakash, A., Pavani, J.V.P., Bera, S., Deviram, G.V.N.S., Kumar, A., Panchpuri, M., Prasuna, R.G., 2015. Production, optimization and partial purification of protease

from Bacillus subtilis. J. Taibah Univ. Sci. 9, 50–55.

Papirio, S., 2012. Fluidized-bed bioreactor applications for the treatment of metal-, sulfate- and nitrate-contaminated mine waters. Ing.Unicas.It.

Park, S.J., Yoon, J.C., Shin, K.-S., Kim, E.H., Yim, S., Cho, Y.-J., Sung, G.M., Lee, D.-G., Kim, S.B., Lee, D.-U., Woo, S.-H., Koopman, B., 2007. Dominance of endospore-forming bacteria on a Rotating Activated Bacillus Contactor biofilm for advanced wastewater treatment. J. Microbiol. 45, 113–21.

Parker, D.S., 2011. Introduction of New Process Technology into the Wastewater Treatment Sector. Water Environ. Res. 83, 483–497.

Parvathi, A., Krishna, K., Jose, J., Joseph, N., Nair, S., 2009. BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BACILLUS PUMILUS ISOLATED FROM COASTAL ENVIRONMENT IN COCHIN , INDIA 269–275.

Pasztor, I., Thury, P., Pulai, J., 2009. Chemical oxygen demand fractions of municipal wastewater for modeling of wastewater treatment 6, 51–56.

Patel, A., Zhu, J., Nakhla, G., 2006. Simultaneous carbon, nitrogen and phosphorous removal from municipal wastewater in a circulating fluidized bed bioreactor. Chemosphere 65, 1103–1112.

Patowary, K., Patowary, R., Kalita, M.C., Deka, S., 2016. Development of an Efficient Bacterial Consortium for the Potential Remediation of Hydrocarbons from Contaminated Sites 7, 1–14.

Patra, G., Garrigue, H., 1996. Identification and characterization of Bacillus an thracis by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA 145, 9–16.

Patra, G., Garrigue, H., 2018. Identification and characterization of Bacillus an thracis by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA 145, 9–16.

Patterson, R.A., 2003. NITROGEN IN WASTEWATER AND ITS ROLE IN CONSTRAINING ON-SITE PLANNING Future Directions for On-site Systems: Best Management Practice Proceedings of On-site ’03 Conference NITROGEN IN WASTEWATER AND ITS ROLE IN CONSTRAINING ON-SITE PLANNING 313–320.

Peter, J., Hoek, V. Der, Fooij, H. De, Struker, A., 2016. Resources , Conservation and Recycling Wastewater as a resource : Strategies to recover resources from Amsterdam ’ s wastewater. "Resources, Conserv. Recycl. 113, 53–64.

Petti, C. a, Polage, C.R., Schreckenberger, P., 2005. The Role of 16S rRNA Gene Sequencing in Identi cation of Microorganisms Misidenti ed by Conventional Methods. Society 43, 6123–6125.

Pfeiffer, T.J., Wills, P.S., 2011. Evaluation of three types of structured floating plastic media in moving bed biofilters for total ammonia nitrogen removal in a low salinity hatchery recirculating aquaculture system. Aquac. Eng. 45, 51–59.

Philips, S., Verstraete, W., Laanbroek, H.R., 2002. Origin, causes and effects of increased nitrite concentrations in aquatic environments. CEUR Workshop Proc. 1, 115–141.

Pisarevsky, A.M., Polozova, I.P., Hockridge, P.M., 2005. ENVIRONMENTAL PROBLEMS Chemical Oxygen Demand 78, 101–107.

Pochana, K., Keller, J., 1999. Study of factors affecting simultaneous nitrifictaion and denitrification (SND).

Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 9.

Prakash, O., Nimonkar, Y., Shouche, Y.S., 2013. Practice and prospects of microbial preservation. FEMS Microbiol. Lett. 339, 1–9.

Priest, F.G., 1977. Extracellular enzyme synthesis in the genus Bacillus. Bacteriol. Rev. 41, 711.

Puyol, D., Batstone, D.J., Hï¿½lsen, T., Astals, S., Peces, M., Krï¿½mer, J.O., 2017. Resource recovery from wastewater by biological technologies: Opportunities, challenges, and prospects. Front. Microbiol. 7, 1–23.

Radhakrishnan, R., Hashem, A., Abd Allah, E.F., 2017. Bacillus: A biological tool for crop improvement through bio-molecular changes in adverse environments. Front. Physiol. 8, 1–14.

Rahman, K.S.M., Rahman, J.T., Lakshmanaperumalsamy, P., Banat, I.M., 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour. Technol. 85, 257–261.

Rajasulochana, P., Preethy, V., 2016a. Comparison on efficiency of various techniques in treatment of waste and sewage water – A comprehensive review. Resour. Technol. 2, 175–184.

Rajasulochana, P., Preethy, V., 2016b. Comparison on efficiency of various techniques in treatment of waste and sewage water – A comprehensive review. Resour. Technol. 2, 175–184.

Raper, E., Stephenson, T., Anderson, D.R., Fisher, R., Soares, A., 2018. Industrial wastewater treatment through bioaugmentation. Process Saf. Environ. Prot. 118, 178–187.

Rasko, D.A., Altherr, M.R., Han, C.S., Ravel, J., 2005. Genomics of the Bacillus cereus group of organisms. FEMS Microbiol. Rev. 29, 303–329.

Robertsaon, G.P., Groffman, P.M., 2007. Nitrogen Transformations. Soil Microbiol. Ecol. Biochem. 341–364.

Robertson, G.P., Groffman, P.M., 2015. Nitrogen Transformations, 4th ed, Soil Microbiology, Ecology and Biochemistry. Elsevier Inc.

Rodgers, M., Zhan, X.M., 2003. Moving-medium biofilm reactors. Rev. Environ. Sci. Biotechnol. 2, 213–224.

Romano, S., Jackson, A.., Patry, S., Dobson, D.., 2018. Extending the “ One Strain Many Compounds ” (OSMAC) Principle to Marine Microorganisms. Mar. Drugs 16, 1–29.

Rossle, W.H., Pretorius, W.A., 2001. A review of characterization requirements for in-line prefermenters Paper 1: Wastewater characterisation. Water SA 27, 405–412.

Rout, S.K., 2013. Wastewater Treatment Technologies. Int. J. Water Resour. Environ. Sci. 2, 20–23.

Rusul, G., 1995. Prevalence of Bacillus cereus in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA 25, 131–139.

Salgot, M., Huertas, E., Weber, S., Dott, W., Hollender, J., 2006. Wastewater reuse and risk: Definition of key objectives. Desalination 187, 29–40.

Sarkar, P., Meghvanshi, M., Singh, R., 2011. Microbial Consortium: A New Approach in Effective Degradation of Organic Kitchen Wastes. Int. J. Environ. Sci. Dev. 2, 170–174.

Schallmey, M., Singh, A., Ward, O.P., 2004. Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50, 1–17.

Schroeter, R., Hoffmann, T., Voigt, B., Meyer, H., Bleisteiner, M., Muntel, J., Jürgen, B., Albrecht, D., Becher, D., Lalk, M., Evers, S., Bongaerts, J., Maurer, K.H., Putzer, H., Hecker, M., Schweder, T., Bremer, E., 2013. Stress responses of the industrial workhorse Bacillus licheniformis to osmotic challenges. PLoS One 8, 1–22.

Senesi, S., Ghelardi, E., 2010. Production, Secretion and Biological Activity of Bacillus cereus Enterotoxins 1690–1703.

Seviour, R.J., Mino, T., Onuki, B., 2003. The microbiology of phosphorus removal in activated sludge. FEMS Microbiol. Rev. 27, 99–127.

Shannon, M.A., Bohn, P.W., Elimelech, M., Georgiadis, J.G., Marĩas, B.J., Mayes, A.M., 2008. Science and technology for water purification in the coming decades. Nature 452, 301–310.

Sharma, S., Sachdeva, P., Virdi, J.., 2003. Emerging waterborne pathogens. Appl. Microbiol. Biotechnol. 61, 424–428.

Shon, H.K., Vigneswaran, S., Kandasamy, J., Cho, J., 2007. Characteristics of Effluent Organic Matter in Wastewater. UNESCO - Encycl. life Support Syst. Water wastewater Treat. Technol. 1–17.

Shukla, K.P., Singh, N.K., Sharma, S., 2010. Bioremediation: Developments, current practices and perspectives. Genet. Eng. Biotechnol. J. 2010, 1–20.

Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremediation. Trends Microbiol. 14, 389–397.

Singh, S., Kumar, B., 2017. Potential application spectrum of microbial proteases for clean and green industrial production. Energy, Ecol. Environ. 2, 370–386.

Smith, V.H., Tilman, G.., Nekola, J.C., 2016. Eutrophication : Impacts of Excess Nutrient Inputs on Freshwater , Marine , and Terrestrial Ecosystems marine , and terrestrial ecosystems 100.

Smith, V.H., Tilman, G.D., Nekola, J.C., 1998. Eutrophication: Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environ. Pollut. 100, 179–196.

Song, K.G., Cho, J., Ahn, K.H., 2009. Effects of internal recycling time mode and hydraulic retention time on biological nitrogen and phosphorus removal in a sequencing anoxic/anaerobic membrane bioreactor process. Bioprocess Biosyst. Eng. 32, 135–142.

Sonune, A., Ghate, R., 2004. Developments in wastewater treatment methods. Desalination 167, 55–63.

Stephenson, D., Stephenson, T.O.M., 1992. BIOAUGMENTATION FOR ENHANCING BIOLOGICAL WASTEWATER TREATMENT DIANNE STEPHENSON and TOM STEPHENSON School of Water Sciences, Cranfield Institute of Technology,

Cranfield, Bedford, U.K. 10, 549–559.

Su, J.-F., Zhang, K., Huang, T., Wen, G., Guo, L., Yang, S., 2015. Heterotrophic nitrification and aerobic denitrification at low nutrient conditions by a newly isolated bacterium , Acinetobacter sp . SYF26 829–837.

Su, J.., Zhang, K., Huang, T.., Wen, G., Guo, L., Yang, S.., 2015. Heterotrophic nitrification and aerobic denitrification at low nutrient conditions by a newly isolated bacterium, Acinetobacter sp. SYF26. Microbiol. (United Kingdom) 161, 829–837.

Su, J., Zhang, K., Huang, T., Wen, G., Guo, L., Yang, S., 2019. Heterotrophic nitrification and aerobic denitrification at low nutrient conditions by a newly isolated bacterium , Acinetobacter sp . SYF26 829–837.

Sulieman, A, M.E.., Yousif, A.W.., Mustafa, A.M., 2006a. Quality characteristics of wastewater of the Sudanese fermentation industry. Tenth Int. water Technol. Conf. 1091–1101.

Sulieman, A, M.E.., Yousif, A.W.., Mustafa, A.M., 2006b. QUALITY CHARACTERISTICS OF WASTEWATER OF THE SUDANESE FERMENTATION INDUSTRY. Tenth Int. water Technol. Conf. 1091–1101.

Sun, Z., Lv, Y., Liu, Y., Ren, R., 2016. Bioresource Technology Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a novel metal resistant bacterium Cupriavidus sp . S1. Bioresour. Technol. 220, 142–150.

Taylor, S.M., Yiliang, H.E., Bin, Z., Jue, H., 2009. Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium , Providencia rettgeri YL. J. Environ. Sci. 21, 1336–1341.

Tchobanoglous, G., Burton, F., Stensel, H.D., 1991. Wastewater engineering: An Overview. Wastewater Eng. Treat. Reuse 1–24.

Thaira, Y., Nasir, I.A., Usman, Y., 2014. Laboratory Perspective of Gram Staining and its Signifi cance in Investigations of Infectious Diseases 1, 3–6.

Thompson, I.P., Van Der Gast, C.J., Ciric, L., Singer, A.C., 2005. Bioaugmentation for bioremediation: The challenge of strain selection. Environ. Microbiol. 7, 909–915.

Turnbull, P.C.B., Sirianni, N.M., Lebron, C.I., Samaan, M.N., Sutton, F.N., Reyes, A.E., Peruski, L.F., 2004. MICs of Selected Antibiotics for 42, 3626–3634.

Tyagi, M., da Fonseca, M.M.R., de Carvalho, C.C.C.R., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22, 231–241.

Unicef and WHO, 2004. UNITED NATIONS POPULATION INFORMATION Population and Water Resources (contrib . by FAO). World Heal. Organ. 44, 1–4.

Van Der Gast, C.J., Whiteley, A.S., Starkey, M., Knowles, C.J., Thompson, I.P., 2003. Bioaugmentation strategies for remediating mixed chemical effluents. Biotechnol. Prog. 19, 1156–1161.

Vidali, M., 2001. Bioremediation . An overview * 73, 1163–1172.

Vijayaraghavan, P., Gnana, S., Vincent, P., 2013. A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. J. Biochem. … 4, 628–630.

Vlamakis, H., Chai, Y., Beauregard , P., Losick, R. and Kolter, R., 2013. NIH Public Access 6, 157–168.

Vogel, T.M., 1996. Bioaugmentation as a soil bioremediation approach. Curr. Opin. Biotechnol. 7, 311–316.

Vorhees, C. V, Williams, M.T., Rosario-Ortiz, F.L., Wert, E.C., Snyder, S.A., Barry, M.C., Hristovski, K., Westerhoff, P., Moon, H., Minakata, D., Crittenden, J., Fallis, A.., Von Gunten, U., Hoigne, J., Katsoyiannis, I.A., Canonica, S., Von Gunten, U., Rosenfeldt, E.J., Linden, K.G., Canonica, S., Von Gunten, U., Nopens, I., Capalozza, C., Vanrolleghem, P. a., ZHANG, Z. bin, ZHAO, J. fu, XIA, S. qing, LIU, C. qing, KANG, X. sheng, Omoike, A.I., Vanloon, G.W., Stoller, M., Dulekgurgen, E., Do??ruel, S.,

Karahan, ??zlem, Orhon, D., Levine, A.D., Tchobanoglous, G., Asano, T., Journal, S., Pollution, W., Federation, C., Jul, N., Hamilton, D., Rathnayake, U., Tanyimboh, T., Sophonsiri, C., Morgenroth, E., Corsi, S.R., Borchardt, M.A., Spencer, S.K., Hughes, P.E., Baldwin, A.K., 2014. Efficiency and energy requirements for the transformation of organic micropollutants by ozone, O3/H2O2 and UV/H2O2. Water Res. 40, 1–4.

Wagner, M., Rath, G., Amannt, R., Koops, H., 1995. In situ Identification of Ammonia-oxidizing Bacteria 264, 251–264.

Wakako, I.O., Miyahara, M., Kim, S.W., Yamada, T., Matsuoka, M., Watanabe, A., Fushinobu, S., Wakagi, T., Shoun, H., Miyauchi, K., Endo, G., 2013. Bioaugmentation of a wastewater bioreactor system with the nitrous oxide-reducing denitrifier Pseudomonas stutzeri strain TR2. Seibutsu-kogaku Kaishi 115, 37–42.

Wan, W., He, D., Xue, Z., 2017. Removal of nitrogen and phosphorus by heterotrophic nitrification-aerobic denitrification of a denitrifying phosphorus-accumulating bacterium Enterobacter cloacae HW-15. Ecol. Eng. 99, 199–208.

Wang, J., Yan, D., Dixon, R., Wang, Y.P., 2016. Deciphering the principles of bacterial nitrogen dietary preferences: A strategy for nutrient containment. MBio 7, 14–16.

Wei, Y., Van Houten, R.T., Borger, A.R., Eikelboom, D.H., Fan, Y., 2003. Minimization of excess sludge production for biological wastewater treatment. Water Res. 37, 4453–4467.

Welander, U., Mattiasson, B., 2003. Denitrification at low temperatures using a suspended carrier biofilm process. Water Res. 37, 2394–2398.

Whiteley, C.G., Lee, D.J., 2006. Enzyme technology and biological remediation. Enzyme Microb. Technol. 38, 291–316.

Wielinga, P.R., Hamidjaja, R.A., Ågren, J., Knutsson, R., Segerman, B., Fricker, M., Ehling-Schulz, M., de Groot, A., Burton, J., Brooks, T., Janse, I., van Rotterdam, B., 2011. A multiplex real-time PCR for identifying and differentiating B. anthracis virulent

types. Int. J. Food Microbiol. 145, S137–S144.

Wik, T.E.I., Lindén, B.T., Wramner, P.I., 2009. Integrated dynamic aquaculture and wastewater treatment modelling for recirculating aquaculture systems. Aquaculture 287, 361–370.

Woese, C., 1987. Bacterial evolution background. Microbiology 51, 221–271.

Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tse, H., Yuen, K.Y., 2008. Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin. Microbiol. Infect. 14, 908–934.

Xia, S., Guo, J., Wang, R., 2008. Performance of a pilot-scale submerged membrane bioreactor (MBR) in treating bathing wastewater. Bioresour. Technol. 99, 6834–6843.

Yan, L., He, Y.L., Kong, H.N., Tanaka, S., Lin, Y., 2006. Isolation of a new heterotrophic nitrifying Bacillus sp. strain. J. Environ. Biol. 27, 323–326.

Yang, J., Wang, Y., Chen, H., Lyu, Y., 2019. Bioresource Technology Ammonium removal characteristics of an acid-resistant bacterium Acinetobacter sp . JR1 from pharmaceutical wastewater capable of heterotrophic nitri fi cation-aerobic denitri fi cation. Bioresour. Technol. 274, 56–64.

Yang, Q., Peng, Y., Liu, X., Zeng, W., Mino, T., Satoh, H., 2007. Nitrogen removal via nitrite from municipal wastewater at low temperatures using real-time control to optimize nitrifying communities. Environ. Sci. Technol. 41, 8159–8164.

Yang, X.P., Wang, S.M., Zhang, D.W., Zhou, L.X., 2011. Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, Bacillus subtilis A1. Bioresour. Technol. 102, 854–862.

Yang, Y., Xie, L., Tao, X., Hu, K., Huang, S., 2017a. Municipal wastewater treatment by the bioaugmentation of Bacillus sp . K5 within a sequencing batch reactor 1–16.

Yang, Y., Xie, L., Tao, X., Hu, K., Huang, S., 2017b. Municipal wastewater treatment by the bioaugmentation of Bacillus sp. K5 within a sequencing batch reactor. PLoS One

12, 1–15.

Yao, Y., 2014. Use of Carbohydrate , Protein and Fat to Characterise Wastewater in Terms of its Major Elemental Constituents and Energy. Thesis.

Ye, Q., Li, K., Li, Z., Xu, Y., He, T., Tang, W., Xiang, S., 2017. Heterotrophic nitrification-aerobic denitrification Performance of Strain Y-12 under Low Temperature and High Concentration of Inorganic. Water 9, 835.

Zafarzadeh, A., Bina, B., Nikaeen, M., Attar, H.M., 2010a. Performance of Moving Bed Biofilm Reactors for Biological Nitrogen Compounds Removal From Wastewater By Partial Nitrification-Denitrification process. J. Environ. Heal. Sci. an Eng. 7, 353–364.

Zafarzadeh, A., Bina, B., Nikaeen, M., Attar, H.M., 2010b. Performance of Moving Bed Biofilm Reactors for Biological Nitrogen Compounds Removal From Wastewater By Partial Nitrification-Denitrification 7, 353–364.

Zanetti, L., Frison, N., Nota, E., Tomizioli, M., Bolzonella, D., Fatone, F., 2012. Progress in real-time control applied to biological nitrogen removal from wastewater. A short-review. Desalination 286, 1–7.

Zhang, B., Jiang, D.D., Zhou, W.W., Hao, H.K., Niu, T.G., 2009. Isolation and characterization of a new Bacillus sp. 50-3 with highly alkaline keratinase activity from Calotes versicolor faeces. World J. Microbiol. Biotechnol. 25, 583–590.

Zhang, Q.L., Liu, Y., Ai, G.M., Miao, L.L., Zheng, H.Y., Liu, Z.P., 2012a. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. Bioresour. Technol. 108, 35–44.

Zhang, Q.L., Liu, Y., Ai, G.M., Miao, L.L., Zheng, H.Y., Liu, Z.P., 2012b. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. Bioresour. Technol. 108, 35–44.

Zhao, B., He, Y.L., Zhang, X.F., 2010. Nitrogen removal capability through simultaneous heterotrophic nitrification and aerobic denitrification by Bacillus sp . LY 3330.

Zhao, S., Hu, N., Chen, Z., Zhao, B., Liang, Y., 2009. Bioremediation of reclaimed wastewater used as landscape water by using the denitrifying bacterium bacillus cereus. Bull. Environ. Contam. Toxicol. 83, 337–340.

Zheng, Z., Zhang, D., Li, W., Qin, W., Huang, X., Lv, L., 2018. Bioresource Technology Substrates removal and growth kinetic characteristics of a heterotrophic nitrifying-aerobic denitrifying bacterium , Acinetobacter harbinensis HITLi7 T at 2 ° C. Bioresour. Technol. 259, 286–293.

Zhou, G.-J., Ying, G.-G., Liu, S., Zhou, L.-J., Chen, Z.-F., Peng, F.-Q., 2014. Simultaneous removal of inorganic and organic compounds in wastewater by freshwater green microalgae. Environ. Sci. Process. Impacts 16, 2018.

Zhu, G., Peng, Y., Li, B., Guo, J., Yang, Q., Wang, S., 2008. Biological removal of nitrogen from wastewater Biological Removal of Nitrogen from Wastewater.

Zhu, K., Hï¿½lzel, C.S., Cui, Y., Mayer, R., Wang, Y., Dietrich, R., Didier, A., Bassitta, R., Mï¿½rtlbauer, E., Ding, S., 2016. Probiotic Bacillus cereus strains, a potential risk for public health in China. Front. Microbiol. 7.

Zhu, Y.G., Xue, X.M., Kappler, A., Rosen, B.P., Meharg, A.A., 2017. Linking Genes to Microbial Biogeochemical Cycling: Lessons from Arsenic. Environ. Sci. Technol. 51, 7326–7339.