

**EFFECTS OF NON-ESSENTIAL HEAVY METAL BIOREMEDIATION ON
ENZYMES ACTIVITIES IN SOILS IRRIGATED WITH TREATED WASTEWATER**

BY

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DECLARATION

I declare that the mini dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Agriculture (Soil Science) has not previously been submitted by me for a degree at this or any other university; that it is my work in design and execution, and all material therein has been duly acknowledged.

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28/01/2025

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Date

DEDICATION

I dedicate this research project to my supportive parents (Mr. Papola Ephraim and Miss. Tlouamma Selina), including my caring siblings (Tlouamma Morongwa and Tlouamma Kopano).

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ABSTRACT

Treated wastewater (TWW) can serve as a substitute option for irrigation to decrease the water scarcity pressures in semi-arid regions of Limpopo Province, South Africa. However, prolonged use of TWW can degrade soil quality and enzyme activities due to heavy metal contamination. Bioremediation has been proposed as an efficient method to eliminate the contaminants from the soil using indigenous microbes. Thus, the objectives of this study were to evaluate the effectiveness of bioremediation of non-essential metals in soils irrigated with TWW and further assess the response of enzyme activities post-bioremediation of non-essential heavy metals. The TWW was collected from the University of Limpopo Experimental Farm (ULEF) at the night dam's exit point and analysed for pH, EC, and non-essential heavy metals (As, Cd, Cr (VI), and Pb). The quantified water parameters were compared against the Republic of South Africa (RSA) and Food and Agriculture Organization (FAO) thresholds, and the parameters were above the permissible levels except for pH, EC, and Pb. Soil samples for contamination were collected from ULEF and analysed for physicochemical properties, non-essential heavy metals, and enzyme activities. The selected isolated soil microbes were identified as *B. cereus*, *A. fumigatus*, and *E. cloacae*. The bioremediation study was arranged in a completely randomized design with four treatments which were control (without inoculation), and soils inoculated with three selected microbes for 12 weeks. The results revealed that irrigation with TWW led to higher levels of non-essential heavy metals, pH, and EC in the soil and inhibited enzyme activities. The study proved that selected microbes decreased the concentration of non-essential metals, pH, and EC levels ($P \leq 0.05$) post-contamination with TWW. Inoculation of *B. cereus* decreased the concentration of As and Pb by 20% and 56% respectively, while *E. cloacae* decreased Cr and Cd by 50% and 15% respectively. Additionally, inoculation of *B. cereus* increased phosphatase and arylsulfatase activities by 636% and 319% respectively. The control sample without inoculations, proved to bioremediate the soil and increase the urease activity by 1308%. Since the microbial inoculation had an insignificant effect on β -glucosidases, it was suggested that future research should be done to optimize bioremediation strategies by selecting suitable microbes that will stimulate β -glucosidases activity.

Keywords: Soil health, indigenous microbes, soil contamination, non-essential heavy metals, irrigation

CHAPTER 1

INTRODUCTION

1.1 Background

The sustainability of agriculture relies heavily on freshwater; however, it is compromised by escalating water shortages (Aydin *et al.*, 2015). Drought has emerged as a challenge to most farmers in Limpopo province, South Africa (Makhura *et al.*, 2004). The province is vulnerable to extreme climate events, marked by high evaporation rates and arid conditions, often leading to droughts (Botai *et al.*, 2016). In addition, it was estimated that over three million farmers in South Africa produce crops to feed their families, however, drought could worsen rural poverty in the province of Limpopo (Stats SA, 2007) leading to food insecurity. According to Chivangulula *et al.* (2023), drought effects can lead to significant drop in agricultural output and the economy within the region.

Water scarcity pressures can be lessened by substituting fresh water with treated wastewater (TWW) in agricultural irrigation systems (Ayoub *et al.*, 2016). Treated wastewater is a combination of industrial and domestic wastes, which may contain some toxic heavy metals such as Cd, As, Cu, Co, nickel (Ni) and Pb, some in high concentrations (Khan *et al.*, 2011). Moreover, TWW contains essential nutrients which can also increase fertility in agricultural soils (Urbano *et al.*, 2017; Lahlou *et al.*, 2021). In arid and semi-arid areas, TWW has become a viable alternative to freshwater for agricultural irrigation purposes (Aydin *et al.*, 2015; Cary *et al.*, 2015; Qureshi *et al.*, 2016). However, TWW is known to possess elevated concentrations of heavy metals, salts, and pathogens which may accumulate in agricultural soils and further affect human health after consuming contaminated crops (Ofori *et al.*, 2021; Wang *et al.*, 2017). Therefore, irrigating crops with treated or untreated wastewater would bring these toxins into the soil. Due to their harmful, persistent, and bio-accumulative characteristics, heavy metals are considered as the most dangerous categories of pollutants (Aydin *et al.*, 2015).

Globally, heavy metals contamination in agricultural soils and crops is considered as a major ecological concern (Javid *et al.*, 2018). Heavy metals can be available in various ways, including exchangeable adsorption, association with Fe/Mn oxides and

carbonates, and complexity with soil organic matter and sulphides (Hu *et al.*, 2016; Ma *et al.*, 2016). Microbes primarily excrete soil enzyme activities, playing an essential role in biogeochemical cycles of nutrient cycling such as carbon, nitrogen, phosphorus, and sulphur (Cui *et al.*, 2018; Cui *et al.*, 2020). Different strategies have emerged to maximize wastewater's potential for reuse. Bioremediation is an advanced technology and eco-friendly method that utilizes microorganisms to eliminate and neutralize contaminants in the soil (Ajona and Vasathi, 2021). Using organic contaminants as a source of food and energy, this biological method relies on the catabolic activities of the organisms and their capacity to aid in the decomposition of organic contaminants (Pilon-Smits, 2005). The use of soil bioremediation techniques such as mycoremediation and bioaugmentation is an alternative to aid the effective usage of treated wastewater during crop irrigation. The efficiency of bioremediation is influenced by several factors, such as the availability of microbes and the concentration of contaminants, that need to be considered when selecting a treatment that offers the water quality required by crops. Therefore, this research aimed to investigate the effects of non-essential heavy metal bioremediation on enzyme activities in soils irrigated with treated wastewater.

1.2 Problem statement

The use of TWW may reduce water scarcity; however, prolonged use may result in high quantities of bioavailable heavy metals in soils. Generally, heavy metals are available in low quantities in the soil, but due to anthropogenic pollution and irrigation with treated wastewater, their levels may rise dramatically (Camposeo *et al.*, 2015). Mahmood and Malik (2014) discovered that wastewater irrigation altered the soil's chemical and physical characteristics and caused the plants to absorb heavy metals. According to Koundouras and Nikolaou (2022), non-essential heavy metals such as arsenic (As), chromium (Cr), copper (Cu), and lead (Pb) are frequently found to contaminate agricultural soils and adversely affect plant growth in excessive amounts. Bioremediation is one promising strategy for the amelioration of polluted soils due to the presence of heavy metals. A microbial remediation approach has been used to decrease the concentrations of toxic elements in soils, with promising outcomes that were conducted through preliminary studies that examined the application of bioremediations (Hasan, 2014). This bioremediation process can either have a

negative effect on soil health, such as limited effectiveness and reduced toxicity respectively, mainly associated with the enzymes that can be released during the mineralization process in the soil (Hasan, 2014). Other remediation strategies have been proposed before; however, they have various limitations compared to bioaugmentation such as contaminant complexity. This method employs specialized microorganisms and accelerates biodegradation rates; hence it is recommended for this study.

1.3 Rationale of the study

Several technologies such as physical remediation are available to eliminate contaminants from water sources (Dixit *et al.*, 2015). However, microbial remediation techniques are becoming more popular because of scientific community support due to their efficacy (Fawole, 2017). Developing remediation strategies such as bioaugmentation could be an effective way of restoring the soils contaminated with heavy metals caused by TWW irrigation. Bioremediation is a non-disturbing, effective, and cost-efficient form of soil remediation. Elimination of non-essential heavy metals from soils with the use of microbes has been demonstrated to have significant potential (Phadu and Kgopa, 2022). Irrigating with TWW is a significant and efficient solution to the challenge of water scarcity (Trigui, 2015). Therefore, this study investigated the effectiveness and impact of the bioremediation process on soil health indicators such as enzymatic activities on soils irrigated with treated wastewater, as a sustainable strategy for safer use of treated wastewater.

1.4 Purpose of the study

1.4.1 Aim

This study aimed to investigate the effects of non-essential heavy metal bioremediation on enzyme activities in soils irrigated with treated wastewater.

1.4.2 Objectives

The objectives of the study were to:

- (i) Investigating the bioremediation of non-essential heavy metals (As, Cd, Cr (VI), Pb) in soils irrigated with treated wastewater.

- (ii) Assessing the post-bioremediation effects and response of enzyme activities in soils irrigated with treated wastewater.

1.4.3 Hypotheses

The null hypotheses of this study were:

- (i) Bioremediation has no influence on the non-essential heavy metals (As, Cd, Cr (VI), Pb) in soils irrigated with treated wastewater.
- (ii) The enzymatic activities are not affected post-bioremediation of non-essential heavy metals in soils irrigated with treated wastewater.

1.5 Significance of the study

This scientific research provides supporting evidence that bioremediation can be used as a strategy to reduce or remove toxic non-essential metals from soils irrigated with TWW. Therefore, at the same time, providing a solution to ameliorate the high levels of heavy metals in polluted agricultural soils. Furthermore, promoting the use of TWW as an alternative water source to address the water scarcity challenges. So, the effectiveness of the bioremediation will help to maintain the health of the soils irrigated with TWW. Additionally, this study also assessed the response of enzyme activities post-bioremediation of non-essential heavy metals in soils irrigated with treated wastewater. As such, an increase in enzyme activities post-bioremediation indicates improved soil health, as it suggests enhanced microbial activity, nutrient cycling, and pollutant degradation.

1.6 Structure of mini dissertation

The mini dissertation is comprised of 5 chapters in total. Chapter 1 outlines the background of the study, the research problem, the motivation, as well as the purpose of the study. Then chapter 2 outlines the literature review of the study, which unpack the work done and work not done on the problem statement and the possible research gaps. Chapter 3 focuses on achieving objective 1, whereas chapter 4 outlines objective 2 of the study. Chapter 5 provides the study's summary findings, including limitations and recommendations. The style of referencing used in the mini dissertation is the Harvard referencing style, which is adopted from the University of Limpopo postgraduate policy.

CHAPTER 2

LITERATURE REVIEW

2.1 The use of treated wastewater for irrigation in agricultural soils

Wastewater is generated from domestic, commercial, industrial and agricultural wastes as sources which contain contaminants and toxic elements, and harmful microbes (Rajohan *et al.*, 2014). The reuse of TWW is a critical necessity worldwide due to the ongoing water crisis. Agriculture relies heavily on freshwater sources, using approximately 70% of rivers and groundwater supplies, and accounting for nearly 92% of global water consumption (Rout and Kattumuri, 2022; WWAP-UNESCO World Water Assessment Programme, 2019; World Resources Institute, 2020). South Africa is a semi-arid region with irregular rainfall patterns (Kriel, 1992). The country's climate has an annual water deficit because evaporation exceeds rainfall (Snyman and Fouchè, 1991). Low rainfall, sporadic droughts, and non-perennial surface water resources make it challenging to grow food crops in some communities. Thus, it becomes crucial to use water as efficiently as possible (Nhamo *et al.*, 2020).

Recently it was reported that TWW has been used to irrigate vineyards in South Africa and Mexico (Mendoza-Espinosa *et al.*, 2019). The use of TWW is gaining international attention and is a great way to address the problem of water scarcity (Contreras *et al.*, 2017; Huertas *et al.*, 2008). Ungureanu *et al.* (2018) reported that 1.6–6.3% of TWW worldwide is utilized for agricultural land irrigation. World Health Organization (2006) documented that approximately 10% of the population in the world, consumes crops irrigated with TWW. Researchers revealed that the utilization of TWW is a valuable strategy for sustaining agricultural productivity, particularly in regions with limited water resources (Dery *et al.*, 2019; Gargouri *et al.*, 2022). According to global estimates, approximately 20 million hectares of land rely on treated and wastewater for irrigation purposes (Sato *et al.*, 2013), and this area is anticipated to expand in the future (Mendoza-Espinosa *et al.*, 2019). These estimates vary greatly, but they generally indicate that treated wastewater makes up 1.5–6.6% of the 301 million hectares of irrigated land worldwide (Sato *et al.*, 2013).

2.2 Accumulation of heavy metals in agricultural soils through irrigation of treated wastewater

Several studies suggested that prolonged use of TWW may increase the pollutants in the soil such as heavy metals (Houda *et al.*, 2016; Vanryckeghem *et al.*, 2018; Eckert *et al.*, 2018). Mineral salts and heavy metal traces found in TWW harm agricultural soils, crop quality, and yield, and can affect human health (Jiménez and Asano, 2008; Elgallal *et al.*, 2016; Khawla *et al.*, 2019). There have been observed effects including heavy metal deposition, salinization, and sodicity in soils irrigated with TWW, and these factors may lead to low soil productivity and irreversible soil deterioration (Jalil *et al.*, 2022). Due to their inability to decompose, heavy metals may lead to soil degradation and poor health, which results in low productivity (Rezapour *et al.*, 2019). Jalil *et al.* (2022) reported that crops cultivated in soils contaminated with heavy metals tend to accumulate in edible components to unacceptable levels.

Previous research has shown that heavy metals that can be absorbed in the soil following irrigation by TWW include Cd, Zn, Pb, and Cu (Xue *et al.*, 2019; El-Hassanin *et al.*, 2020). According to other findings, the presence of high heavy metals levels in soils irrigated TWW can pose a significant health risk to humans and the environment (Awan *et al.*, 2019; Khawla *et al.*, 2019; Kamanina *et al.*, 2023). Additionally, water sources may become contaminated with heavy metals if treated wastewater is released into receiving water bodies. In Morogoro, Tanzania, it was also noted that vegetables and soils irrigated with TWW water had high levels of Cu and Pb (Chove *et al.*, 2006). Mohammad Rusan *et al.* (2007) discovered that ten years of TWW irrigation raised heavy metals in the soil, specifically Fe, Mn, and Cu, during the first two years and then decreased over time. According to other research, soil containing higher levels of Pb, Cd, and Ni was the result of using treated effluent for irrigation for over 20 years (Singh *et al.*, 2010). In addition, the use of TWW may lead to structural degradation and salinization in soils, due to high sodicity and salinity compared to freshwater (Wu *et al.*, 2020a). The literature reveals inconsistent findings on the severity of these effects, likely due to variations in TWW quality, irrigation methods, soil parameters, and crop-specific factors (Chen *et al.*, 2013a; Wang *et al.*, 2017).

2.3 The effects of treated wastewater on soil physicochemical properties

TWW irrigation has the potential to alter the physicochemical characteristics of soils under arid or semi-arid regions. High evaporative demand, which rapidly concentrates the mineral components and other pollutants introduced by TWW, may worsen this effect (Le *et al.*, 2009; Sandri *et al.*, 2009; Sebastian *et al.*, 2009). Soil salinization, alkalization, and structural alterations are the main negative effects.

Soil pH: Since soils often have a high buffer against pH changes, TWW irrigation causes changes in soil pH gradually over time (Ayers and Westcot, 1985). The TWW irrigation may affect soil pH, influenced by the pH level and composition of the TWW employed. Treated wastewater irrigation was discovered in several studies to either (i) increase soil pH (Hermon, 2011; Lado *et al.*, 2012); (ii) decrease soil pH (Keser, 2013; Abunada and Nassar, 2015); or (iii) have no effect at all (Duan *et al.*, 2010; El-Nahhal *et al.*, 2013). According to Rusan *et al.* (2007), this is consistent with irregular patterns in the pH of soils irrigated with TWW for varying lengths of time. The different buffer capacities of the soils are probably the cause of the irregular pH reactions.

Electrical conductivity: Salinity issues brought on by the build-up of water-soluble salts in the roots zone may result in lower crop yields (Ayers and Westcot, 1985). The most common metric used to estimate soil salinity is electrical conductivity (EC). According to Rhodes *et al.* (1999), it can be used as an indicator to determine the concentration of total ionized salt in aqueous samples. Most studies that investigated how TWW irrigation affected soils found that it significantly increased EC (Bedbabis *et al.*, 2015; Andrews *et al.*, 2016; Ganjegunte *et al.*, 2017).

In the literature, several indicators have been employed to assess how TWW irrigation affects soil quality: Salinity is measured by soil electrical conductivity, whereas sodicity is measured by exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR) (Hopmans *et al.*, 2021a, 2021b). In general, higher soil EC values were linked to high salt concentrations and total dissolved solids (TDS) in the TWW irrigation; in certain instances, there was no significant difference in soil EC when the EC of freshwater and TWW used for irrigation were similarly low (El-Nahhal *et al.*, 2013); however, irrigation with treated municipal effluent that had a low salinity level significantly reduced the EC (Hassanli *et al.*, 2008). The accumulation of salts transported from treated wastewater is influenced by soil type. Heavy, clayey soils

experienced a more noticeable increase in EC when irrigated with TWW as opposed to light-textured soils (Adrover *et al.*, 2017). Because it can decrease the availability of soil water to crops and negatively impact yield production, salt leaching from the root zone is especially important when low-quality water is used for irrigation (Ayers and Westcot, 1985).

2.4 The effects of heavy metals on soil enzymes activities and microbial diversity

According to Moreno *et al.* (2006), microorganisms generate enzymes that function as biological catalysts, accelerating chemical reactions and metabolic pathways which degrade organic contaminants and generate vital chemicals for both microbial and plant growth. However, when soils are irrigated with TWW, the microbial dynamics may progressively shift. Soil enzymatic activity has been proven to be a more sensitive biological indicator of human-induced disturbances than plants and animals (Hinojosa *et al.*, 2004). The impact of heavy metals on soil's biological activity is influenced by soil characteristics, including humic content. However, concentrations and the types of pollutants or enzymes involved also play a role (Moreno *et al.*, 2001). Wyszowska and Kucharski (2003) observed that elevated levels of most heavy metals inhibited the soil enzyme activities. Enzymatic activities are primarily affected by organic matter, clay minerals, heavy metals availability, and other inorganic constituents such as metal ions (Tyler, 1981). According to Sethi and Gupta's (2015) research, adding zinc reduced the amount of protein and protease in soil sample treatments throughout incubation. The levels of exchangeable zinc showed a negative correlation with protease activity. Furthermore, Zn exhibited the strongest inhibitory effect on the activity of protease, followed by Cu, Cd, and Fe. Effron *et al.* (2004) discovered that heavy metals suppressed the soil's arylsulfatases, urease, and protease activity. According to Renella *et al.* (2005), zinc also had the strongest inhibitory effect on the activity of urease, followed by Cr, Cd, Co, and Fe. Cadmium was also shown to inhibit arylsulfatase and protease. Lorenz *et al.* (2006) discovered that arylsulfatase activity was greatly impacted by pollution, and that Cd contamination negatively impacted arylsulfatase, phosphatase, urease, and protease activities. According to Shen *et al.* (2005), the order of Cr > Cd > Zn > Mn > Pb (Zheng *et al.*, 1999) frequently led to reduction in inhibitory effects on urease activity. In the same study, Beta-glucosidase activity was most inhibited by Cd, followed by Zn, Fe, Cu, and Co. Enzymatic indexes

have emerged as effective indicators for evaluating the short-term impacts of organic amendments (Shi *et al.*, 2018), pollutants (Sanchez-Hernandez *et al.*, 2017), bioremediation efforts (Garaiurrebaso *et al.*, 2017), and land-use changes on soil biological activity (Schmidt *et al.*, 2018).

2.5 A developing strategy to remove heavy metal contamination in agricultural soils

The biogeochemical cycle of metals and the bioremediation of soils affected by heavy metals depend heavily on microorganisms (Spain and Alm, 2003). Bioremediation mostly involves microbial processes or microorganisms that can break down and change environmental pollutants into less harmful forms (Garbisu and Alkorta, 2003). Heavy metals are absorbed by microbes either passively (adsorption) or actively (bioaccumulation) (Hussein *et al.*, 2001). Phadu and Kgopa (2022) assert that the bioremediation process has been demonstrated to function more efficiently and successfully than other remedial techniques described in the literature. According to Siddiquee *et al.* (2015), the process of bioremediation is a good approach to eliminating various toxins in the soil by utilizing the ecosystem's inherent biological activity. It is mediated by plants or microbes, such as *Aspergillus fumigatus*, *Enterobacter cloacae*, and *Bacillus cereus*, to detoxify substances that are toxic to the environment and human health (Siddiquee *et al.*, 2015). Bioremediation involves transforming organic or inorganic waste into generally safe compounds through biological breakdown (Qazilbash, 2004). It is a dynamic and developing field that uses living organisms to purify contaminated environments (Nazir *et al.*, 2024). Therefore, it uses low-tech, low-cost techniques widely used on-site and have a high degree of public approval (Su, 2014). According to Shakoory *et al.* (2004), microorganisms that can thrive in soils polluted with heavy metals also have a high metal absorption capacity and are employed in bioremediation. Information regarding the selection of native microorganisms capable of breaking down heavy metals and triggering enzyme activity in the soil is lacking. According to Li *et al.* (2009), soil enzymes are essential biochemical components of the soil ecosystem's material and energy conversion processes, which include nutrient recycling, mineralization, and organic matter breakdown. Compared to other soil properties, their activities can react to fertilization, heavy metal pollution, and soil management more quickly (Bandick and Dick, 1999).

As a result, they may be utilized as possible indicators of soil quality and nutrient cycle processes (Gu *et al.*, 2009).

2.6 The use of bioremediation techniques in South Africa and their effects on soil microbial activities

The bioremediation approach has been used in South African contaminated soils (Jespersen *et al.*, 1993). It is, however, far less common than in Europe and America, where it is widely utilized to lessen the effects of pollution on the environment caused by undesired solids, liquids, and gases. In South Africa, bioremediation is not fully employed on a large scale, and recently there is scarcity of literature that focuses on bioremediation of non-essential metals on agricultural soils. Further research is also needed to determine the most effective bioremediation technique and treatment for pollutants and conditions in South Africa, as well as to speed up rates of degradation. Prolonged effects on soil health are unknown to many farmers, who utilize TWW to address water scarcity issues. Scientists and farmers should collaborate to solve these problems and implement bioremediation as an innovative method for eliminating pollutants or heavy metals from agricultural soils. Therefore, additional studies should be conducted on South African soils in arid and semi-arid locations using various microbes that can withstand toxic components in agricultural soils irrigated with TWW. The utilization of native microorganisms for bioremediation to boost soil enzyme activity has not received much attention in South Africa. Most research concentrated on the bioremediation of non-essential metals in agricultural soils, but they did not consider how the enzyme activities responded after the remediation. Thus, the current research aimed at investigating the effects of non-essential metals bioremediation on enzyme activities in soils irrigated with treated wastewater.

CHAPTER 3

BIOREMEDIATION OF NON-ESSENTIAL HEAVY METALS IN SOILS IRRIGATED WITH TREATED WASTEWATER

3.1 Introduction

Non-essential heavy metals pollution in agricultural soils is a critical environmental problem given that they are non-biodegradable (Radwan and Salama, 2006; Khan *et al.*, 2010; Muhammad *et al.*, 2011). Heavy metals such as Cu, Pb, Cd, and Zn, are mostly found in crops as contaminants (Kachenko and Singh, 2006). Residents who consume crops irrigated with TWW are at risk caused by concentration of heavy metals due to the use of this water. The present chemical and physical approaches to heavy metal treatment have two drawbacks which are, high costs and undesirable by-products (Ahmed *et al.*, 2021).

One important method or technique used to address this problem is bioremediation. It utilizes microorganisms and plants to transform environmental pollutants into less hazardous forms (Upadhyay *et al.*, 2016). Since it helps to return the ecosystem to its normal state following pollution, it is regarded as a viable solution to the problem. According to Dixit *et al.* (2015), it is cost-effective and provides long-term environmental benefits. It would be much better to adapt bioremediation technologies because they are economical, environmentally beneficial, and sustainable. Bioremediation efficacy is greatly influenced by the utilization and adaptation of microorganisms that have a proven ability to remediate and tolerate high toxicity. Thus, the importance of this objective was to investigate the bioremediation of non-essential heavy metals in soils irrigated with treated wastewater.

3.2 Materials and methods

3.2.1 Study area

The research study was conducted at the University of Limpopo (UL) Soil Science Laboratory (28° 0' 59.76" E; 25° 36' 54" S) in Limpopo Province, South Africa. Soil samples were collected from the fallowed land at UL Experimental Farm (ULEF) (-23° 50' 42.86" E; 29° 42' 44" S). The treated wastewater was collected at the ULEF night dam (Figure 3.1), which flows from the Mankweng Wastewater Treatment Plant (MWTP). Both the ULEF and MWTP are located in the Mankweng area, in Capricorn District Limpopo Province, South Africa. The farm is located in a semi-arid region that receives annual precipitation ranging between 250-500 mm, and the mean maximum temperatures reaching approximately 28°C. Various effluents are treated at MWTP, which are disposed from the surrounding areas of Mankweng including the hospital and the University of Limpopo. Physical, chemical, and organic contaminants from different sources may include food waste, hairs, paper fibres, plant materials, fuel, oils, medications, and faeces (Kgopa *et al.*, 2017). After the water has been treated from MWTP it flows to the night dam at the exit point where it gets released for irrigation at ULEF.



Figure 3.1: Exit point of the night dam at University of Limpopo Experimental Farm

3.2.2 Research design

An incubator experiment consisted of four treatments replicated twice, observed over 12 weeks, and arranged in a completely randomized design (CRD). The treatments were composed of inoculations of three indigenous toxic-metal-tolerant-microbes that were isolated from the same soil contaminated with the treated wastewater (two bacteria: *Bacillus cereus* and *Enterobacter cloacae* and one fungus: *Aspergillus fumigatus*), and one control sample without the inoculations of indigenous toxic metals tolerant microbes.

3.2.3 Treated wastewater and soil samples collection procedure

Soil samples for contamination and remediation were collected from a fallowed field at UL Experimental Farm at a depth of 0-30 cm, spade was used to clear the surface and demarcate the area before collection of samples. The soil samples were transferred into the sampling bags to form a composite sample and transported to UL Soil Science Laboratory for further analysis. The soil was air-dried and sieved with a 5 mm sieve to remove plant materials and rocks. The TWW was also collected from ULEF, at the exit point of the night dam which is flowing from MWTP using two 20 L bottles which were stored in the shade house, and the water was collected twice a week. The bottles were rinsed three times before collecting the treated wastewater. For the water analysis, the treated wastewater was collected using polyethylene containers which were rinsed several times with TWW before collecting the final water samples. Water samples were analysed for non-essential metals (As, Cr, Pb, and Cd) at the Limpopo Agro-Food Technology Station (LATS).

3.2.4 Procedure for contamination

The pots (4) for the experiment were washed using distilled water to prevent contamination and placed in the shade house next to the UL Soil Science laboratory. For contamination, 200 g of sieved soil sample was weighed and then transferred into the 20 cm (height) pots that were irrigated with TWW. Furthermore, they were irrigated to field capacity, and a soil moisture meter was used to monitor the moisture to avoid over-irrigation. The soils were irrigated based on the amount of moisture depleted by determining the weight difference before the irrigation of treated wastewater. The contamination was carried out for 12 weeks from 29th January – 21st April 2024.

Isolation of selected microorganisms

The culturing of microbes was done following the procedures by (Kastner *et.al*, 1998). Briefly, 1g of contaminated sample was suspended in 99ml of distilled water. The agars were prepared for different isolations, whereby the MYP Agar was used for *Bacillus Cereus*; Sabouraud Dextrose Agar was used for *Aspergillus Fumigatus* and the MacConkey agar was to isolate the *Enterobacter Cloacae*. The agars were used as the isolation mediums whereby MYP Agar required 46 g, Sabouraud Dextrose Agar required 65 g, and MacConkey Agar was 50 g. The agars were prepared inside 1L bottles filled with distilled water and were autoclaved for about 15-20 minutes. After being autoclaved, the bottles were left to cool down for a few minutes before being placed inside the petri dishes until the next day when they solidified. Morphological properties such as the colour of the agar, shape, and size were observed and identified on the isolated microbes (Figure 3.1).



A

B

Figure 3.2: Isolated microbes for bioremediation, A (*Enterobacter Cloacae*), B (*Bacillus Cereus*).

Bioremediation process

For the bioremediation process, 100 ml beakers were sterilized using ethanol and labelled according to the treatments. 50 g of contaminated soil was weighed into eight 100 ml glass beakers, then were inoculated with three identified inoculums (10^4 CFU/g) except for the control. The beakers were sealed to prevent contamination and incubated at 37°C for 12 weeks (Figure 3.2) (Fawole, 2017). Each beaker was replicated two times and labelled based on the inoculum, except the two beakers which were controls. During the remediation period, 20 ml of distilled water was used to irrigate the samples twice a week. Following 12 weeks, the soil's chemical properties, heavy metal concentrations, and soil enzyme activities were examined. The relative impact (R.I.) of inoculation of selected microbes was calculated using the following equations:

$$R.I. = [((\text{variate PB}/\text{variate BB})-1) \times 100] \dots\dots\dots \text{Equation 1}$$

Whereby PB is a variate post-bioremediation; BB is a variate before bioremediation.

The relative impact (R.I.) for post-irrigation was calculated using the following equation:

$$R.I. = [((\text{variate PI}/\text{variate BI})-1) \times 100] \dots\dots\dots \text{Equation 2}$$

Whereby PI is a variate for post-irrigation; BI is a variate before irrigation



Figure 3.3: Samples ready for incubation at 37°C for 12 weeks bioremediation

3.2.5 Data collection

Treated wastewater analysis

Treated wastewater was analysed for water pH using a pH meter, as well as the electrical conductivity using the water analysis probe on a conductivity meter. Non-essential metals (As, Cr (VI), Pb, and Cd) from the treated wastewater were determined using the EDTA method (Hesse, 1971), and the extracts were read using ICP-OES.

Soil analysis

For the pre-trial, the soil samples were analysed for soil texture using the hydrometer method (Bouyoucos, 1962). The samples for before and after contamination were used to analyse the soil pH (H₂O) and pH (KCl) using the pH meter (Reeuwijk, 2002). The electrical conductivity was also analysed in the very same samples using the electrode method (Rhoades, 1982). Non-essential metals in the soil (As, Cr (VI), Pb, and Cd) were determined using the EDTA method (Hesse, 1971) and analysed using ICP-OES.

3.2.6 Data analysis

Data for pre-trial, post-contamination, and treated wastewater were summarised using descriptive statistics, whereas post-remediation data was subjected to analysis of variance (ANOVA) using GenStat 20th Edition software. The descriptive analysis consists of mean, minimum, maximum and standard deviation. The mean separation for the significant data was done using Duncan's multiple range test at $P \leq 0.05$.

3.3 Results

3.3.1 The use and suitability of treated wastewater for irrigation

The chemical properties pH and EC were analysed for the TWW to verify the suitability of the water for irrigation purposes. Selected non-essential heavy metals which are As, Cr, Pb, and Cd were also quantified for the water samples. The water data obtained

from the treated wastewater was used for comparison against the South African thresholds in terms of the validity of the TWW for irrigation. All the quantified water properties were above the RSA and FAO thresholds except for pH, EC, and Pb. The results also revealed that As, Cr, and Cd were 37, 32, and 8 times higher than FAO standards respectively (Table 3.1).

Table 3.1: Summary of chemical properties of TWW against the Republic of South Africa (RSA) and Food and Agriculture Organization (FAO) thresholds

Water quality variables	Mean concentration \pm SD	RSA Threshold (DWAF 1996)	FAO Threshold (FAO, 2010)
pH (H ₂ O)	7.163 \pm 0.161	6.5-8.4	6.5-8.4
EC (mS/cm)	134 \pm 52.09	<540	400-600
As (mg/L)	3.733 \pm 0.578	2	0.1
Cr (mg/L)	3.2 \pm 0.6	1	0.1
Pb (mg/L)	0.363 \pm 0.0850	2	5
Cd (mg/L)	0.08 \pm 0.0361	0.05	0.01

EC = Electrical Conductivity; As = Arsenic; Cr = Chromium; Pb = Lead; Cd = Cadmium

3.3.2 The soil physicochemical properties for pre-contamination and post-contamination

The results for the texture of the soil particles for sand, clay, and silt were as follows: 90%; 8%; and 2% respectively. The texture of the soil was identified or classified as Loamy Sand using the soil textural triangle. According to the results below in Table 3.2, the chemical properties such as pH and EC showed an increase post-contamination with treated wastewater. Initially the pH (H₂O) mean was 6.06 before the irrigation, then post-contamination the pH mean increased 2.31 units to 8.42. Additionally, the pH (KCl) also increased by 2.16 units from 6.26 to 8.42. The EC also increased from 248 mS/cm to 2569 mS/cm, which indicates that the treated wastewater has increased the salt concentration in the soil after irrigation. Lastly, the

non-essential metals also showed an increase, for As from 19.1 mg/kg to 25.57 mg/kg, Cr from 10.53 mg/kg to 13.8 mg/kg, Pb from 35.1 mg/kg to 73.7, then lastly Cd from 1.517 mg/kg to 2.387 mg/kg.

Table 3.2: Descriptive statistics results analysed for soil chemical properties pre- and post-contamination of treated wastewater.

Soil parameters	Pre-contamination with TWW				Post-contamination with TWW			
	Min	Max	Mean	SD	Min	Max	Mean	SD
pH (H ₂ O)	6.02	6.1	6.06	0.06	8.33	8.41	8.37	0.06
pH (KCl)	6.21	6.3	6.26	0.06	8.34	8.5	8.42	0.12
EC (mS/cm)	244	252	248	5.66	2518	2620	2569	72.12
As (mg/kg)	19.1	19.8	19.5	0.36	23.7	26.7	25.57	1.63
Cr (mg/kg)	10.3	10.7	10.53	0.21	13.3	14.1	13.8	0.44
Pb (m/kg)	33.9	37.3	35.1	1.91	66.9	77.4	73.7	5.90
Cd (mg/kg)	1.48	1.55	1.52	0.04	1.91	3.02	2.39	0.57

pH (H₂O) = Active Acidity; pH (KCl) = Reserve Acidity; EC = Electrical Conductivity; As = Arsenic; Cr = Chromium; Pb = Lead; Cd = Cadmium

3.3.3 The morphological characteristics of the identified microorganisms that were used for bioremediation of non-essential heavy metals

The three selected microbes that were isolated and used for bioremediation of non-essential heavy metals were *Bacillus cereus*, *Aspergillus fumigatus*, and *Enterobacter cloacae*. Each microbe was identified based on the morphological properties as presented in Table 3.3.

Table 3.3: Morphological characteristics of selected microbes

Name of microbes	Morphological characteristics	Shapes identified	Gram staining
<i>Bacillus cereus</i>	Light-brown colony	Small rods	Positive
<i>Enterobacter cloacae</i>	Cream-white colony	Medium rods	Negative

Aspergillus fumigatus Brown colony Medium rods -

Bacillus cereus = Bacteria; *Aspergillus fumigatus* = Fungi; *Enterobacter cloacae* = Bacteria

3.3.4 The effects of selected microbial inoculums on non-essential metals in soils irrigated with TWW

Results from the analysis of variance discovered that the selected microbial inoculums that are tolerant toxic elements had a significant effect ($p < .001$) on the non-essential heavy metals which are As, Cr, Pb, and Cd as presented in Table 3.4. The post-contaminated samples showed the highest accumulation of non-essential heavy metals. However, after 12 weeks of incubation the concentrations of non-essential heavy metals started to decline. *Bacillus cereus* proved to be a microbe with the highest potential to reduce As (20.53 mg/kg) and Pb (32.07 mg/kg) in the soil as compared to other microbes. *Enterobacter cloacae* also had the highest potential to reduce Cr to 6.94 mg/kg, while the sample without inoculation followed by *A. fumigatus* showed to have the lowest concentrations of Cd at 2.01 mg/kg and 2.02 mg/kg respectively.

Arsenic distribution

The post-contaminated sample had a relative impact of 31% which was a higher concentration for Arsenic before bioremediation. The heavy metal started to decrease following the inoculation of selected microbes which were having high tolerance against Arsenic. The distribution of Arsenic for each selected microbes was as follows: *B. cereus* (20%); *A. fumigatus* (1%); then *E. cloacae* (2%). The results showed a highly significant difference ($p < .001$) of the Arsenic distribution in the soil. As such the trend from highest to lowest concerning the reduction of Arsenic concentration in the soil and the potential of microbes was as follows: *B. cereus* > *E. cloacae* > *A. fumigatus* (Table 3.4).

Chromium distribution

The post-contaminated sample had a relative impact of 31% which was a higher concentration for Chromium before bioremediation. The heavy metal started to decrease following the inoculation of selected microbes which were having high tolerance against Chromium. The distribution of Chromium for each selected microbes

was as follows: *B. cereus* (39%); *A. fumigatus* (37%); then *E. cloacae* (50%). The results showed a highly significant difference ($p < .001$) of the Chromium distribution in the soil. As such the trend from highest to lowest in terms of the reduction of Chromium concentration in the soil and the potential of microbes was as follows: *E. cloacae* > *B. cereus* > *A. fumigatus* (Table 3.4).

Lead distribution

The post-contaminated sample had a relative impact of 110% which was a higher concentration for Lead before bioremediation. The heavy metal started to decrease following the inoculation of selected microbes which were having high tolerance against Lead. The distribution of Lead for each selected microbes was as follows: *B. cereus* (56%); *A. fumigatus* (45%); then *E. cloacae* (5%). The results showed a highly significant difference ($p < .001$) of these microbes in the Lead distribution in the soil. As such the trend from highest to lowest concerning the reduction of Lead concentration in the soil and the potential of microbes was as follows: *B. cereus* > *A. fumigatus* > *E. cloacae* (Table 3.4).

Cadmium distribution

The post-contaminated sample had a relative impact of 57% which was a higher concentration for Cadmium before bioremediation. The heavy metal started to decrease following the inoculation of selected microbes which were having high tolerance against Cadmium. The distribution of Cadmium for each selected microbes was as follows: *B. cereus* (3%); *A. fumigatus* (6%); then *E. cloacae* (15%). The results showed a highly significant difference ($p < .001$) of the Cadmium distribution in the soil. As such the trend from highest to lowest concerning the reduction of Lead concentration in the soil and the potential of microbes was as follows: *E. cloacae* > *A. fumigatus* > *B. cereus* (Table 3.4).

Table 3.4: The analysis of variance for bioremediation of non-essential heavy metals using selected microbes for inoculation and the relative impact

Treatments	As (mg/kg)	R.I. (%)	Cr (mg/kg)	R.I. (%)	Pb (mg/kg)	R.I. (%)	Cd (mg/kg)	R.I. (mg/kg)
Pre-irrigation	19.5	-	10.53	-	35.1	-	1.52	-
Post-irrigation	25.57	31	13.8	31	73.7	110	2.39	57
Control (W.I.)	24.87 ^b	-3	7.91 ^b	-43	43.50 ^c	-41	2.01 ^a	-15
<i>B. cereus</i>	20.53 ^a	-20	8.48 ^c	-39	32.07 ^a	-56	2.31 ^b	-3
<i>A. fumigatus</i>	25.30 ^b	-1	8.68 ^c	-37	40.43 ^b	-45	2.24 ^b	-6
<i>E. cloacae</i>	25.03 ^b	-2	6.94 ^a	-50	69.87 ^d	-5	2.02 ^a	-15
p-value	<.001		<.001		<.001		<.001	

R.I. (Relative Impact) = $[(\text{variate post bioremediation}/\text{variate before bioremediation}) - 1] \times 100\%$; W.I. = Without Inoculum; As = Arsenic; Cr = Chromium; Pb = Lead; Cd = Cadmium. Small letters on columns indicate the means separation done on the Duncan Multiple Range Test at ($p < 0.05$).

3.3.5 Response of microbial counts to the remediation of non-essential heavy metals

Analysis of variance results showed that the transformed microbial counts had a significant effect ($p < .001$) on the selected treatments for bioremediation. The microbial counts indicate the number of colonies observed to eliminate the non-essential metals in the soil. The microbial counts for *B. cereus* showed to be higher than *E. cloacae* and *A. fumigatus* with 7.41 and a relative impact of 17%. The lowest counts were observed from *B. cereus* with relative effects of 11% (Table 3.5).

Table 3.5: The analysis of variance for transformed microbial counts and the relative impact on the treatments used for bioremediation

Treatments	<i>B. cereus</i>	R.I (%)	<i>A. fumigatus</i>	R.I (%)	<i>E. cloacae</i>	R.I (%)
Pre-irrigation	6.40	-	6.27	-	6.21	-
Post-irrigation	6.46	1	6.48	3	6.35	2
Control (W.I.)	6.01 ^a	-7	6.02 ^a	-7	6.01 ^a	-5
<i>B. cereus</i>	7.20 ^c	11	6.15 ^{ab}	-5	6.43 ^b	1
<i>A. fumigatus</i>	6.31 ^b	-2	7.37 ^c	14	6.33 ^b	-0
<i>E. cloacae</i>	6.22 ^b	-3	6.27 ^b	-3	7.41 ^c	17
p. value	<.001		<.001		<.001	

R.I. (Relative Impact) = $[(\text{variate post bioremediation}/\text{variate before bioremediation}) - 1] \times 100\%$; W.I. = Without Inoculum; Small letters on columns indicate the means separation done on Duncan Multiple Range Test at ($p < 0.05$).

3.3.6 The effects of selected microbial inoculums on soil pH and EC

The outcomes from analysis of variance (Table 3.6) discovered that the selected microbial inoculations had a significant effect ($p < .001$) on the soil's chemical properties specifically the pH and EC. The post-contaminated sample showed an increase in soil pH and EC, which were recorded as 8.37 and 2569 mS/cm respectively. This indicated a significant increase of the pH by 38% and EC by 935% following the irrigation by TWW (Table 3.6). Post bioremediation of non-essential heavy metals proved that there was a decline in the pH (H₂O and KCl) and EC values. *A. fumigatus* had a lower pH (H₂O) of 6.04 as compared to other microbes, whereas *B. cereus* also had the lowest pH (KCl) as compared to other selected microbes. Additionally, the electrical conductivity also declined post bioremediation, however, *B. cereus* was the selected microbe that showed to reduce the salts concentration since it was the lowest with 107 mS/cm.

Table 3.6: The analysis variance of the relative impact of the selected microbes on the soil chemical properties

Treatments	pH (H ₂ O)	R.I (%)	pH (KCl)	R.I (%)	EC (mS/cm)	R.I (%)
Pre-irrigation	6.06	-	6.26	-	248	-
Post-irrigation	8.37	38	8.42	35	2569	935
Control (W.I.)	7.02 ^c	-16	7.12 ^c	-15	1207 ^c	-53
<i>B. cereus</i>	6.06 ^a	-28	6.08 ^a	-28	107 ^a	-95
<i>A. fumigatus</i>	6.04 ^a	-28	6.14 ^a	-27	169 ^b	-93
<i>E. cloacae</i>	6.24 ^b	-25	6.23 ^b	-26	116 ^a	-95
p-value	<.001		<.001		<.001	

R.I. (Relative Impact) = $[(\text{variate post bioremediation}/\text{variate before bioremediation}) - 1] \times 100\%$; W.I. = Without Inoculum; As = Arsenic; Cr = Chromium; Pb = Lead; Cd = Cadmium. Small letters on columns indicate the means separation done on Duncan's multiple range test at ($p < 0.05$).

3.4 Discussions

3.4.1 The use and validity of treated wastewater for irrigation in agricultural soils

pH and EC of treated wastewater

The quality of TWW is primarily affected by the type of chemical waste and the effectiveness of the treatment facilities (Pedrero *et al.*, 2010). Based on the findings presented in Table 3.1, the water pH and EC analysed from the TWW were recorded as 7.16 and 134 mS/cm respectively, of which the pH falls under the suitable range for the RSA threshold (DWAF, 1996) and FAO threshold (FAO, 2010) since the acceptable range is 6.5-8.4 (pH), while the EC is below the limits as the recommended range is <500 mS/cm. In comparison, Kgopa *et al.* (2018) found that the pH values in both wastewater and borehole sources were within permissible levels based on FAO limits for vegetable production and SA water quality guidelines, and it was suggested that the water could be used for agricultural irrigation. Ideally, when the EC value is below the limits for treated wastewater it indicates that the water had few dissolved substances, chemicals, and minerals. This can lead to more efficient treatment processes and better water quality. Since the average value of pH was 7.16, it

indicates that the treated wastewater is slightly alkaline. Therefore, irrigating with water that has high alkalinity may result in trace element deficiencies and imbalances such as Ca^{2+} and Mg^{2+} in the soil. This is supported by the observed soil texture in this study since it was identified as loamy sand, and as such most crops grow effectively at a pH of 6 in this type of soil. Electrical conductivity reflects the total dissolved solids (TDS) in treated wastewater; therefore, it is a good indicator of salinity hazards to crops and the most essential factor in determining whether water is suitable for irrigation. So, since the measured EC value (134 mS/cm) was below the threshold it means that the treated wastewater is permissible for irrigation and can be widely used without tempering the soil health and crop quality.

Non-essential heavy metals

Non-essential heavy metals in TWW from MWTP had concentrations that were above the RSA and FAO thresholds except for Pb. From the observed levels of non-essential metals in the TWW, it showed that As was the highest with 3.73 mg/L, while the lowest heavy metal inside the water was Cd with 0.08 mg/L. The overall trend for the non-essential metals in the TWW was $\text{As} > \text{Cr} > \text{Pb} > \text{Cd}$. According to a study by Kgopa *et al.* (2017), the TWW from MWTP contains non-essential metals, and the primary source of heavy metals could be medications from Mankweng Hospital. Additionally, Kgopa *et al.* (2018) reported the results where they show the maximum concentrations of heavy metals from TWW collected from ULEF night-dam exit point whereby Cr was 0.7 mg/l, Pb 0.45 mg/l, Cd 0 mg/l, and as 0.49 mg/l. However, based on the present study the concentration of these non-essential metals in TWW has increased compared to the previous findings. Elevated concentration levels may be due to different materials or substances received from MTWP which receives effluents coming from different areas such as UL and Mankweng Hospital. So, irrigating with this TWW in agricultural soils can also add these non-essential metals which might harm other soil properties and other processes such as enzyme activities.

3.4.2 The effects of treated wastewater for irrigation on soil pH and EC

According to Penn and Camberato (2019), pH is regarded as the "master variable" of soil chemistry because of its significant influence on many chemical processes involving vital plant nutrients. The current study discovered that TWW irrigation had a

significant effect on soil pH and EC levels since the concentration of salts increased in the soil after irrigation (Table 3.2). The acceptable range for the pH in soil for crop production is 6.5-8.4, of which the current pH level increased to 8.41 after the irrigation with TWW, which is alkaline and falls within the acceptable limit. Additionally, the acceptable EC in soil for crop production ranges from 250 mS/cm to 750 mS/cm and the EC increased to 2569 mS/cm after the irrigation of TWW which was above the permissible limit. These findings are in contrast with research conducted by Jahany *et al.* (2020), that discovered that using irrigating with TWW led to an increased soil pH ranging from 0.3 to 0.45 units in comparison with the control sample. An increase in soil pH is associated with alkalinity which in turn may reduce the soil quality.

Other studies proved that irrigating with TWW consistently alters the soil properties such as pH and EC (Rezapour and Samadi, 2011, Aydin *et al.*, 2015). According to Achiba *et al.* (2009), carbon mineralization and the release of OH-ions through ligand exchange are two mechanisms linked to organic matter loading that may be responsible for the observed increase in pH in all TWW-irrigated soils in this study. A further explanation might be the introduction of basic cations (calcium, magnesium, and sodium) through the TWW itself. An important measure of soil productivity is soil salinity. According to Adiento-Borbe *et al.* (2006), soil processes and productivity may be affected by any increase in EC. The findings of the soil EC showed that there were high salinity levels in soils irrigated with TWW compared to soils irrigated with freshwater.

3.4.3 The effects of treated wastewater for irrigation on soil non-essential metals

The findings shown in Table 3.2 revealed that the non-essential metals increased post-irrigation with TWW. Initially, the non-essential concentrations for As, Cr, Pb, and Cd were recorded as 19.5 mg/kg, 10.53 mg/kg, 35.1 mg/kg, and 1.517 mg/kg respectively which proved to be above the threshold limits, and the implications on soil quality might be reduced fertility and contaminants accumulation. The irrigation of treated wastewater in the soil led to a significant increase of the above-mentioned non-essential heavy metals whereby As increased to 25.57 mg/kg, Cr 13.8 mg/kg, Pb 73.7 mg/kg, then lastly Cd 2.387 mg/kg. The trend of the increased non-essential metals after the irrigation of treated wastewater from highest to lowest was Pb > As > Cr >

Cd. As such, these values obtained after the irrigation of TWW also indicate that they are above the permissible threshold in terms of the suitability of TWW for irrigation as presented in Table 3.1. This is substantiated by the research of Haroon *et.al* (2019) where it was discovered that Pb concentration was highest among the heavy metals in soils irrigated with TWW. The extractability of Pb varied considerably depending on TWW irrigation. Exchangeable Pb ranges from 28 to 72 mg/kg among the sampling sites irrigated with TWW (Haroon *et.al*, 2019). According to Kirkham (2006) and Kaonga *et al.* (2010), heavy metals are frequently introduced into soils through fertilizers, composts, or sludge (such as from treated wastewater).

Based on these observations, irrigating with TWW from MWTP results in the accumulation of non-essential heavy metals in the soil which ultimately affect the soil health and quality. In turn, these non-essential metals may also cause problems to the crops' tempering with the growth and the physiological characteristics when irrigated with TWW. In contrast, the study by Hechmi *et al.* (2020) found that plant toxicity issues could arise from irrigation water that contains non-essential metal concentrations over the threshold. Typically, toxicity causes impaired growth, plants to grow more slowly, changes in the morphology of the plant, reduced yield, or may even lead to death. Crop types, development stages, hazardous ion concentrations, soil and climate conditions, and other factors all affect the extent of damage (Hechmi *et al.*, 2020a).

3.4.4 The effects of selected microbes for bioremediation on non-essential heavy metals

The selected microbes used for bioremediation were *B. cereus*, *A. fumigatus*, and *E. cloacae* as presented in Table 3.4 The results indicated that these selected microbes inoculated for bioremediation, had a significant effect ($p < .001$) on the non-essential heavy metals which are As, Cr, Pb and Cd as presented on Table 3.3. Initially, the soil samples contaminated with TWW had the highest concentrations of non-essential metals and the relative impact (%). However, after the inoculation of the selected microbes for bioremediation, the non-essential metals started to decrease in concentration levels. The observed results for non-essential metals after 12 weeks of bioremediation were recorded as follows: Arsenic (*B. cereus* had reduced the concentration to 20.53 mg/kg – 20%, *E. cloacae* by 25.03 mg/kg – 2% and *A.*

fumigatus by 25.30 mg/kg – 1%). This proved that *B. cereus* is a resisting and tolerant gram-positive indigenous microbe since it was able to reduce the concentration of As compared to other microbes. According to Magar *et al.* (2022), *B. cereus* employs a variety of coping strategies to deal with As, so this could be the basis for the observed results of As reduction. Therefore, the bioremediation of non-essential heavy metals could be investigated using this bacterium due to its heavy metal hyper tolerance capabilities. The observations from Cr after 12 weeks of remediation revealed that *E. cloacae* was the effective microbe that reduced the Cr concentration to 6.94 mg/kg (50%) in the soil as compared to other selected microbes. The order in terms of reduction of Cr was *E. cloacae* (6.94 mg/kg – 50%), *B. cereus* (8.48 mg/kg – 39%), and *A. fumigatus* (8.68 mg/kg – 37%). *Enterobacter cloacae* is a gram-positive bacterium that is rod-shaped and can survive under mesophilic conditions with an ideal temperature of 37°C. These results are substantiated by the research conducted by Pattnaik *et al.* (2020), which indicated that *Enterobacter sp.* decreased 84.65% of Cr in 92 hours under ideal conditions and that the reduction rate increased to 94% when iron was present. Therefore, either plasmid or chromosomal DNA is responsible for *Enterobacter cloacae's* increased potential to withstand hazardous metals and antibiotics (Marzan *et al.*, 2017). According to Pattnaik *et al.* (2020), the native bacterial strain *E. cloacae* is a viable bio-agent for bioremediation in various polluted sites caused by heavy metals, and it is resistant to Cr concentrations. Consequently, it can be applied to sustain soil fertility for sustainable agriculture and to remediate soils contaminated with chromium. *Bacillus cereus* reduced the concentration of Pb by 56% based on the present study as compared to other microbes. The reduction trend for Pb was, *B. cereus* (32.07 mg/kg – 56%), *A. fumigatus* (40.43 mg/kg – 45%) and *E. cloacae* (6.87 mg/kg – 5%). Based on these findings it proved that *B. cereus* had a high tolerance and resistance to reduce Pb in the soil contaminated with TWW compared with other microbes. This contrasts with a study by Murthy *et al.* (2012) that found that *Bacillus cereus's* growth, protein content, and lead biosorption capacity all declined as lead concentration increased, demonstrating the harmful effect of lead. *Bacillus cereus* may be a potential microbe for the reduction of Pb from industrial effluents that could pollute the soil after irrigation because of its Pb resistance and biosorption ability.

The control sample without inoculations had a significant decrease in Cd concentration to 2.01 mg/kg which was the lowest compared to other treatments. Before bioremediation, the concentration of this treatment was recorded as 2.39 mg/kg, and post-bioremediation concentration declined to 2.01 mg/kg. Additionally, the control treatment also reduced the Cr concentration by 43% which was the second highest following *E. cloacae*. Interestingly, this treatment was only irrigated with distilled water like other treatments for 12 weeks, but the only difference was that there were no microbes inoculated. The possibility might be that if the contaminated soil is allowed to rest for some time and irrigated with fresh water without additional heavy metals can also be a solution to clean up the soil or reduce toxic elements such as Cr and Cd. These observations could be attributed to processes such as dilution and desorption, since irrigating with fresh water could lead to dilution of Cd and Cr ions from the soil making them less concentrated or being desorbed from soil particles and thus reduce their concentrations.

Additionally, *A. fumigatus* was the only microbe that falls under fungi species however based on the observations the bacteria (*B. cereus* and *E. cloacae*) used for bioremediation proved to reduce the concentrations of the non-essential heavy metals rapidly than *A. fumigatus* and of which made them effective. For all observed non-essential metals used in this study for bioremediation, the bacteria group showed high tolerance and high degrading capacity compared to *A. fumigatus* as a fungus. It can still be considered as a microbe to reduce non-essential metals since there was a slight difference in terms of reducing the identified non-essential heavy metals in the soil compared to the bacteria.

3.5 Conclusion

The present study has proved that the use of TWW collected from MWTP can increase the non-essential heavy metals and the salts concentration in the soil if utilized for irrigation purposes. Post-contamination using TWW increased the pH and EC levels as compared to pre-contaminated soil, so this shows that the TWW had adverse effects on chemical properties and non-essential heavy metals after irrigation. The treated wastewater had Cr, As and Pb concentrations that were above the permissible limits of the RSA and FAO thresholds and proved to have high toxicity in the soil.

Additionally, the soil pH and the EC started to increase post-irrigation with TWW which indicated high salts concentration. However, the selected microbes such as *B. cereus*, *A. fumigatus* and *E. cloacae* that were selected for bioremediation of non-essential heavy metals in the soil proved to be very much tolerant to those heavy metals as they were able to reduce their concentrations to lower levels and less toxicity. *Bacillus cereus* was the most effective indigenous microbe which remediated most harmful heavy metals such as Pb and As, and it was able to reduce the electrical conductivity to lower concentration compared to other selected microbes. As such, a gram-positive bacteria (*B. cereus*) performed the best during the bioremediation process due to its strength and tolerance against the non-essential metals. Therefore, the null hypothesis (i) is rejected since there was an influence on the non-essential heavy metals by selected indigenous microbes during bioremediation. Bioremediation has proven to be an effective method to eliminate toxic elements in the soil and salts concentration in contaminated soils, therefore farmers who might have challenges heavy metals pollution in their fields can adopt this method or strategy and use TWW for irrigation to solve water scarcity issues.

CHAPTER 4

ASSESSING ENZYME ACTIVITIES POST-BIOREMEDIATION OF NON-ESSENTIAL HEAVY METALS IN SOILS IRRIGATED WITH TREATED WASTEWATER

4.1 Introduction

Water quality variations may have an impact on the chemistry of the soil as well as physical characteristics, enzymes, and soil microbial activities. Enzyme activities specifically β -glucosidase, phosphatase, and urease which cycle carbon, phosphorus, and nitrogen, respectively, are used in biological assays (Adetunji *et al.*, 2017) and are sensitive indicators of soil ecosystem sustainability and soil health (Moebius-Clune *et al.*, 2016; Adetunji *et al.*, 2017). However, the soil enzyme activities do not reflect the overall biological activity because they are substrate-specific (Adetunji *et al.*, 2017).

Thus, a bioindicator of ecological health and soil quality is soil enzyme activity (Liu *et al.*, 2020b; Wahsha *et al.*, 2017). For instance, arylsulfatase with Pb, Zn, and Cd exhibits a linear decline in activity as heavy metal levels rise (Aponte *et al.*, 2020). Furthermore, most researchers have found no connection between heavy metal contamination and the activity of enzymes (Tripathy *et al.*, 2014; Zhang *et al.*, 2010). The organic matter content, and nutrient availability can all affect how the activities of the enzymes react to heavy metal stress. According to Kandziora-Ciupa *et al.* (2016), the pH of heavy metal-contaminated soils had a significant impact on β -glucosidase activity and soil acid phosphatase. Therefore, the importance of this chapter was to assess the enzyme activities following the bioremediation of non-essential heavy metals in soils irrigated with treated wastewater.

4.2 Materials and methods

4.2.1 Description of study area and research design

The description of the study area as well as the research design are the same as those described in Chapter 3 under section 3.2.

4.2.2 Data collection

Soil physicochemical analysis

Pre-trial, post-contamination and post-remediation soils were analysed for enzyme activities using colorimetric methods which were phosphatases, β -glucosidases, urease, and arylsulfatase. These enzymes were measured in the laboratory by adding a synthetic substrate linked to a fluorescent molecule, and their activities were determined by using biochemical assays.

Phosphatase's analysis

Phosphatase's activity was determined using Tabatabai and Bremner (1969) method. Briefly, 1g of dry soil was placed into a 50 ml conical flask. Afterwards, the conical flask was filled with 1 ml of 0,115 M p-nitrophenylphosphate (pNP) solution, and 4 ml of modified universal buffer was added, and 0.2 ml of toluene at a pH of 6.5 (Skujins, 1985). The flask was swirled gently and then incubated for 1 hour at a temperature of 37°C. Following 1 hour incubation, other solutions were added to the mixture which were 4 ml of 0.5 M sodium hydroxide and 1 ml of 0.5 M calcium chloride. The soil suspension was passed using the Whatman filter paper, and the spectrophotometer was used to measure the optical density of a filtrate at a wavelength of 430 nm. The blank was conserved the same however without the soil sample. Phosphatase activity was then calculated using the p-nitrophenol in water standard curve and it was quantified as mole of p-nitrophenol released per gram of dry soil per hour.

$$\text{Phosphatase activity} = \frac{\text{Final absorbance}}{\text{Concentration} \times \text{Incubation time} \times \text{sample dry mass}} \dots\dots\dots \text{Equation 1}$$

β-glucosidases analysis

A test tube equivalent to (16 mm x 257 mm) was used to place 0.5 g of moist soil, followed by addition of 0.1 ml of toluene. After 10 minutes, 0.6 ml of p-nitrophenyl β-D-glucoside (PNG) solution, 1.5 ml of McIlvaine buffer (which was prepared using dibasic sodium phosphate with citric acid solution at a pH of 4.8), and 0.9 ml of distilled water were added to the mixture (Hayano, 1973). Ten minutes later, the test tube was swirled for 10 seconds, and the filter paper was used to pass the soil suspensions. Following filtration, 2 ml of 2 M of Tris solution was prepared, whereby Tris (hydroxymethyl) aminomethane was dissolved in distilled water and added to the filtrate. Afterwards, test tube was swirled for few seconds and the solution was transferred to a cuvette to measure the yellow colour intensity using the spectrophotometer at a wavelength of 400 nm. The amount of p-nitrophenol in the filtrate was quantified using a calibration established from the results of standards at 0, 0.2, 0.4, 0.8, and 1.0 μmol of p-nitrophenol.

$$\beta \text{ (}\mu\text{mol of p - } = \frac{\textit{nitrophenol}}{g} - \text{dry weight soil/g)} = \frac{S-C}{DW} \dots\dots\dots\text{Equation 2}$$

Where S is the p-nitrophenol mean concentration in the sample, C is the p-nitrophenol concentration in the control; then DW is the dry mass of the soil sample.

Urease analysis

The urease activity was determined using the non-buffered method (Paulson and Kurtz, 1969). Five grams of soil was placed into an Erlenmeyer flask (100ml) and it was wetted with 2,5 ml of 0.08 M aqueous urea solution. The sealed flasks were placed in an incubator at a temperature of 37°C. The stoppers were removed after 2 hours incubation, and 50 ml of 1 N KCl to 0.01 HCl solution was added. Afterwards, mixtures were placed on a mechanical shaker for 30 minutes to mix them thoroughly. Following filtration of the resultant suspensions, dilution was done using 1 ml of filtrate diluted to 10 ml of distilled water. Additionally, 5 ml of Na salicylate and 2 ml of 0.1% Na dichlorisocynurate were also added. The filtrates were then subjected to a colorimetric technique for ammonia analysis. Sodium salicylate solution was prepared through mixing 100 ml 0.12 % Na nitroprusside with 100 ml 17 % Na salicylate and 100 ml

distilled water. After 30 minutes incubation, the optical density was measured at a wavelength of 425 nm.

$$\text{Urease activity } (\mu\text{mol NH}_4\text{-N g}^{-1}\text{h}^{-1}) = \frac{[(S-B) \times V \times 10]}{(2 \times \text{dwt})} \dots\dots\dots \text{Equation 3}$$

Where S is the concentration of ammonium-N in the sample; B is the concentration of ammonium-N in the blank; V is the total volume of the extract; 10 is the dilution factor; 2 is the incubation period; Dwt is the dry mass of the soil.

Arylsulfatase analysis

Arylsulfatase was determined by transferring 1 g of soil into a 50 ml Erlenmeyer flask, with the addition of 0.25 ml of toluene, 4 ml of acetate buffer and 1 ml of p-nitrophenyl sulphate solution. The contents were mixed by swirling the flask for few seconds. The flask was stoppered and incubated at a temperature of 37°C. Following 1 hour of incubation, the stoppers were removed and 1 ml of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH were added into the flask and swirled for few seconds. A filter paper was used to allow the soil suspensions to be released. The filtrates intensity with yellow colour was determined using a spectrophotometer at a wavelength of 410nm. The p-nitrophenol was calculated using the calibration curve set from the standard p-nitrophenol (Tabatabai and Bremner, 1970).

$$\text{Arylsulfatase activity} = \frac{(\text{pNP concentration } (\mu\text{mol/ml}) \times \text{reaction volume (ml)})}{(\text{Soil weight (g)} \times \text{Incubation time (h)})} \dots\dots\dots \text{Equation 4}$$

4.3 Results

4.3.1 The soil enzymes' activities for pre-contamination and post-contamination

The results in Table 4.1 show the minimum, maximum, means, and standard deviation values for the enzyme activities before and post-contamination with TWW. The pre-trial results revealed that phosphatases had an average of 2.93 μmol, β-glucosidases (0.68 μmol), urease (2.82 μmol) and arylsulfatase (4.08 μmol). However, after the contamination of soil using TWW, it was noted that there was a decline in the

percentage from the means for phosphatase, β -glucosidases, urease, and arylsulfatase and they were recorded as 87%, 91%, 91%, and 77% respectively. These observed changes indicated that the contamination of TWW influenced the performance of enzyme activities. The highest average observed for pre-trial was from arylsulfatase activity with 4.08 μmol , whereas the lowest activity was observed from β -glucosidases which was recorded as 0.68 μmol . The post-contamination results also revealed that arylsulfatase was higher than other enzyme activities with 0.93 μmol , and the lowest was β -glucosidases with 0.06 μmol . The overall trend in terms of the activity of enzymes from highest to lowest was arylsulfatase > phosphatases > urease > glucosidases.

Table 4.1: Descriptive statistics results analysed for soil enzyme activities before and post-contamination of treated wastewater.

Soil enzymes	Pre-contamination with TWW				Post-contamination with TWW			
	Min	Max	Mean	SD	Min	Max	Mean	SD
Phosphatases (μmol)	2.91	2.94	2.93	0.02	0.36	0.40	0.38	0.03
β - glucosidases (μmol)	0.65	0.70	0.68	0.04	0.03	0.10	0.06	0.05
Urease (μmol)	2.76	2.88	2.82	0.08	0.17	0.30	0.24	0.09
Arylsulfatase (μmol)	4.05	4.10	4.08	0.04	0.89	0.96	0.93	0.05

μmol = Micromole units; Min = Minimum; Max = Maximum; SD = Standard Deviation

4.3.2 The effects of microbial inoculation for bioremediation of non-essential heavy metals on soil enzyme activities

Analysis of variance revealed that the inoculation of selected indigenous microbes had a highly significant effect ($p < 0.001$) on phosphatases, however, they did not have any significant effect ($p > 0.05$) on β -glucosidases enzyme activity as presented in Table

4.2. The phosphatase enzyme activity declined by 87% when the soil was contaminated with TWW, however, the activity started to increase when *B. cereus* was inoculated, which led to an increase of 636%, which was the highest activity observed among the treatments. *A. fumigatus* resulted in the lowest activity, which influenced phosphatase activity by 18%. Inoculation of selected indigenous microbes had a highly significant effect ($p < 0.001$) on urease and arylsulfatase activities as presented in Table 4.2. Urease declined by 91%, however, the activity increased post-bioremediation on the control sample without inoculation with an R.I. value of 1308%. *Bacillus cereus* inoculation resulted in a lower urease activity by 29%. The arylsulfatase activity declined by 77% post-irrigation with TWW, however, the inoculation of microbes significantly increased the activity. *Bacillus cereus* was the highest inoculant with 3.90 μmol , whereas *E. cloacae* was the lowest inoculant which influenced phosphatase activity with 1.40 μmol .

Table 4.2: Relative impact of microbial inoculation for bioremediation of non-essential heavy metals on Phosphatases and β -glucosidases activities

Treatments	Phosphatases (μmol)	R.I. (%)	β -glucosidases (μmol)	R.I. (%)	Urease (μmol)	R.I (%)	Arylsulfatase (μmol)	R.I (%)
Pre-contamination	2.93	-	0.68	-	2.82	-	4.08	-
Post-contamination	0.38	-87	0.06	-91	0.24	-91	0.93	-77
Control (W.I.)	1.44 ^c	278	0.12 ^a	100	3.38 ^d	1308	2.58 ^c	-37
<i>B. cereus</i>	2.80 ^d	636	0.18 ^a	200	0.17 ^a	-29	3.90 ^d	319
<i>A. fumigatus</i>	0.45 ^a	18	0.17 ^a	183	0.70 ^b	192	1.98 ^b	113
<i>E. cloacae</i>	1.05 ^b	176	0.19 ^a	217	1.64 ^c	583	1.40 ^a	51
p-value	<.001		0.710		<.001		<.001	

R.I. (Relative Impact) = $[(\text{variate post bioremediation}/\text{variate before bioremediation})-1] \times 100\%$; W.I. = Without Inoculum; *B. cereus* = *Bacillus cereus*; *A. fumigatus* = *Aspergillus fumigatus*; *E. cloacae* = *Enterobacter cloacae*. Small letters on columns indicate the means separation done on Duncan Multiple Range Test at ($p < 0.05$).

4.3.3 Pearson`s correlation between soil chemical properties and enzymes

The Pearson correlation analysis (Table 4.4) discovered that pH (H₂O) was strong positively correlated with pH (KCl) where r=0.99, EC (r=0.98), urease activity (r=0.96), and was strongly negative correlated with phosphatase (r= -0.03), and glucosidase (r= -0.90). pH (KCl) strong positively correlated with EC (r= 0.99), urease (r=0.94), and strongly negative correlated with β -glucosidase (r= -0.94). Electrical conductivity was positively correlated with urease (r=0.90) and negatively associated with β -glucosidase (r= -0.97).

Table 4.3: Pearson correlation between soil chemical properties and enzymes

Parameters	pH (H ₂ O)	pH (KCl)	EC	Phosphatase	β -glucosidase	Urease	Arylsulfatase
pH (H ₂ O)	-						
pH (KCl)	0.99	-					
EC	0.98	0.99	-				
Phosphatase	-0.03	-0.08	-0.04	-			
β -glucosidase	-0.90	-0.94	-0.97	0.06	-		
Urease	0.96	0.94	0.90	-0.25	-0.80	-	
Arylsulfatase	-0.05	-0.04	0.05	0.89	-0.13	-0.32	-

pH (H₂O) = Active soil acidity; pH (KCl) = Reserve soil acidity; EC = Electrical conductivity. The bolded values indicate moderate to high positive correlations.

4.4 Discussions

4.4.1 The performance of soil enzyme activities for pre-trial and post-contamination

The average for phosphatases, β -glucosidases, urease, and arylsulfatase were 2.93 μ mol, 0.68 μ mol, 2.82 μ mol and 4.08 μ mol respectively before soil contamination with TWW. Based on these findings it was noted that arylsulfatase had the highest activity compared to the other soil enzymes. The lowest enzyme activity was from glucosidases compared to the other three soil enzymes. As such, the trend from highest to lowest activities of enzymes was arylsulfatase > phosphatases > urease > glucosidases. In chapter 3, it was discovered that the TWW collected from MWTP

introduced non-essential metals (Cr, Cd, Pb, and As) into the soil after irrigation. As such, based on the current observations the enzyme activities declined after the contamination of TWW where the average means were 0.38 μmol , 0.06 μmol , 0.24 μmol and 0.93 μmol for phosphatases, glucosidases, urease and arylsulfatase respectively. Phosphatases decreased by 2.55 units, β -glucosidases by 0.65 units, urease by 2.58, and arylsulfatase by 3.15 units. These findings proved that the contamination of TWW which contains non-essential metals had effects on the soil enzyme activities since they started to decline. The inhibition of phosphatases by non-essential metals is in comparison with research by Landi *et al.*, (2000) which discovered that phosphatase was significantly inhibited by Cd contamination in the soil. The reduction in β -glucosidase activity following TWW contamination is supported by other studies that found that Co, Cu, Fe, and Zn had the greatest inhibitory effect on β -glucosidase activity (Geiger *et al.*, 1998). Additionally, Shen *et al.* (2005) and Chanda Mallaiah (2014) discovered that the sequence of urease activity declined in the following order: Cr > Cd > Zn > Mn > Pb.

Lorenz *et al.*, (2006) also discovered that contamination significantly affected the activity of arylsulfatase as compared to the other enzyme activities. These findings proved that the occurrence of non-essential metals in the soil added through irrigation of TWW can inhibit the enzyme's activities and this in turn negatively affects the soil fertility. Additionally, the soil pH (8.37) and EC level (2569 mS/cm) were higher in the soil following the irrigation of TWW, which indicated the possibility of alkalinity and salt concentration accumulation in the soil which might have led to the inhibition of soil enzyme activities since they were under salinity conditions. Eivazi and Tabatabai (1990) found that in anthropogenic soils, β -glucosidase activity dropped as soil pH increased from 4.5 to 8.5 and from 4.3 to 7.4 (Xiao-Chang and Qin, 2006). For evaluating environmental changes caused by soil acidification, the sensitivity of β -glucosidase to pH variations can be a biochemical indicator (Acosta-Martinez and Tabatabai, 2000). Numerous studies have demonstrated that salts accumulation limits agricultural output by negatively affecting enzyme and biochemical activities (Rietz and Haynes, 2003; Karlen *et al.*, 2008) (Rady, 2011). Since urease activity is susceptible to excessive salinity and sodicity, it has been proposed that urease activity can serve as an indicator for soil quality (Zhang *et al.*, 2014). These findings are

supported by the observations (Table 4.4) where it was revealed that urease and EC had a strong positive correlation.

4.4.2 The effects of microbial inoculation for bioremediation of non-essential metals on soil enzyme activities

The enzyme (phosphatase, urease, and arylsulfatase) activities were significantly affected ($p < 0.001$) by the microbial inoculations used for the bioremediation of non-essential metals in the soil (Table 4.2). However, it was also revealed that microbial inoculation did not have any significant effect ($p > 0.05$) on β -glucosidase activity. The microbial inoculations used for bioremediation were *B. cereus*, *A. fumigatus*, and *E. cloacae*. Initially, the enzyme activities for phosphatase, β -glucosidase, urease, and arylsulfatase declined by 87%, 91%, 91%, and 77% respectively when the soil was contaminated with TWW. This indicated that the presence of non-essential metals (As, Cr Cd, and Pb) derived from TWW inhibited the enzyme activities in the soil, hence they declined. The microbial inoculation showed the potential to increase the phosphatase activity post-bioremediation whereby *B. cereus* had a higher R. I value of 636%, *E. cloacae* (176%), and *A. fumigatus* by 18%. Therefore, the trend for the microbes to increase the phosphatase activity was *B. cereus* > *E. cloacae* > *A. fumigatus*.

These observations could explain that during the bioremediation process, the selected microbes might have solubilized phosphorus from insoluble sources, making it available and leading to increased phosphatase activity. The most important factors that contribute to the significant effect of microbial inoculation on enzyme activities are the inoculum type and quality. *Bacillus cereus* and *E. cloacae* are examples of phosphorus-solubilizing bacteria (Chen *et al.*, 2006), so since they were used as a microbial inoculant during bioremediation, they increased the phosphorus availability in the soil which also led to increased phosphatase activity. This contrasts with research by Shams El-Deen *et al.* (2020), which found that an increase in soil phosphatase activity is caused by inoculation with bacterial strains either alone or in combination with fungi. Similar outcomes of the synergistic interaction between the fungus *Glomus intraradices* and the phosphate-solubilizing bacteria *Bacillus subtilis* were reported by Kohler *et al.* (2007). These interactions led to increased phosphatase

activity in the soil. Czarnes *et al.* (1999) reported that, the extracellular enzyme activities of phosphatases were increased by the combined inoculation of fungi and phosphate-solubilizing bacteria, improving the quality of the soil.

One of the enzymes that control the mineralization factor and the cycling of sulphur in the soil is arylsulfatase activity. Based on the observations, the selected microbes increased the activity whereby *B. cereus* was 319%, *A. fumigatus* (113%), and *E. cloacae* (51%). Since the microbial inoculations had a significant effect on the activity of arylsulfatase, the reason for this observation could be that sulphur-oxidizing microbes increased the sulphate availability in the soil which enhanced the arylsulfatase activity. The sulphur-oxidizing microbes in this present study were *B. cereus* and *A. fumigatus* which could have degraded organic sulphur compounds and released inorganic sulphur compounds that were utilized by the arylsulfatase enzyme. Urease activity also showed a significant increase when microbes were inoculated for bioremediation, however, it has been observed that the control sample which was not inoculated with microbes had the highest relative impact (1308%) as compared to other treatments. A possible reason for this observation could be that the native microbes that already existed in the soil before the inoculation were able to remediate the non-essential metals and increase the nitrogen content which is associated with urease activity. *Bacillus cereus* had the lowest R.I. value (29%) compared to other treatments, and this could have been caused by the competition for resources such as nutrient availability (nitrogen, phosphorus, and potassium) and aeration between inoculated microbes and native microbes. However, these findings proved that the microbial inoculants chosen for this current study were favourable and had the potential to induce enzyme activities hence significant effects were observed. Since the microbial inoculation did not have any significant effect on β -glucosidase activity, the possibility for this observation might be that the selected inoculants were not optimized for stimulating the β -glucosidase. Additionally, native microbes might have dominated the β -glucosidase activity, as well as competition for resources which reduced the significant impact of inoculated microbes. Further research could be done on how to optimize the bioremediation strategies in terms of suitable microorganisms to stimulate β -glucosidase activity, nutrient amendments, and how to prevent competition between the existing and the inoculated microbes to improve the

effectiveness of the complex relationship between bioremediation and β -glucosidase enzyme activity.

4.5 Conclusion

The results revealed that before the contamination, the performance of enzymes was favourable, however after the irrigation of TWW the enzyme activities declined significantly. The findings proved that the bioremediation of non-essential metals had significant ($p < 0.001$) effects on phosphatase, urease, and arylsulfatase activities whereas it had non-significant ($p > 0.05$) effect on β -glucosidase. The bioremediation process was mediated by microbial inoculants which were *B. cereus*, *A. fumigatus*, and *E. cloacae* to stimulate the enzyme activities. Urease activity was also affected by microbial inoculation; however, it also revealed that contaminated soil without any inoculation can have the potential to increase the activity done by native or existing microbes in the soil. Since β -glucosidase was not significantly affected by any microbial inoculation, further research could be done on how to optimize the bioremediation strategies in terms of suitable microorganisms to stimulate β -glucosidase activity. Additionally, future studies could explore how to prevent competition between the existing and the inoculated microbes to improve the effectiveness of the complex relationship between bioremediation and β -glucosidase enzyme activity. There is a need for repetition of the experiment to understand why the control sample (without inoculations) had the highest relative impact % which led to increased urease activity.

CHAPTER 5

SUMMARY OF FINDINGS, SIGNIFICANCE OF FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

5.1 Summary of findings

The use of treated wastewater can reduce water scarcity issues semi-arid regions such as Limpopo province, South Africa. However, short-term use of TWW can affect the soil negatively by the addition of non-essential metals through irrigation. Hence, this study focused on the use of indigenous microbes that are tolerant to harsh conditions against toxic heavy metals used for bioremediation of soils contaminated by irrigation with TWW. Furthermore, to assess whether these selected microbes would decrease the levels of non-essential heavy metals in the soil irrigated with TWW. The use and validity of TWW for irrigation in agricultural soils were assessed, including the effects on soil chemical parameters, and to observe the effects of selected microbes on non-essential metals during bioremediation. Additionally, the effects of microbial inoculation on enzyme activities were assessed to verify whether the selected microbes had the potential to remediate the soil and increase the activities of soil enzymes.

The findings of the study proved that the TWW collected from MWTP was polluted with non-essential metals such as Cr, Cd, As, and Pb. The non-essential metals from TWW were above the permissible limits of the RSA (DWAF, 1996) and FAO (FAO, 2010) thresholds except for Pb. This proved that the toxicity of these metals negatively affected the soil following irrigation with TWW, and the degree of toxicity was high and above recommended levels. The water parameters measured were also compared with RSA and FAO limits, and it was revealed that the water pH and EC were within the permissible limits. The contamination of TWW in the soil increased the non-essential metals and significant change was observed in the soil chemical parameters. The selected indigenous microbes (*B. cereus*, *A. fumigatus*, and *E. cloacae*) were used to bioremediate the soil contaminated with TWW. The results revealed that these indigenous microbes had the potential to reduce the concentration of non-essential heavy metals in the soil contaminated with TWW. The observations indicated that microbial inoculation led to a decrease in non-essential heavy metals which were As,

Cr, Cd, and Pb. Interestingly, *B. cereus* was the most effective indigenous microbe compared to others since it was able to remediate the most harmful heavy metals (Pb and As) and reduce the EC level to a lower concentration. Another compelling observation was made on a control sample without inoculation which revealed that there was a significant decrease in Cr which showed the lowest concentration as compared to other treatments for the experiment. This observation suggests that contaminated soil can be allowed to rest for some time and be irrigated with non-contaminated water can be a solution to reduce non-essential heavy metals in the soil. The irrigation of TWW also tempered soil chemical properties, however microbial inoculations were able to reduce the pH and EC to optimal levels. Overall, bioremediation was a good strategy to reduce the non-essential heavy metals in the soil irrigated with TWW.

Additionally, the study assessed the enzyme activities post-bioremediation of non-essential heavy metals, to verify if the microbial inoculation could have any significant effects on soil enzymatic activities. The study revealed that the irrigation of TWW affected the soil enzyme activities since a significant decline was observed. The observed soil enzymes for this present study were *phosphatase*, *urease*, β -glucosidase, and arylsulfatase. However, the microbial inoculation for bioremediation revealed that they had a significant effect on phosphatase, urease, and arylsulfatase except for β -glucosidase. An interesting observation was that a contaminated sample without any inoculation had the highest urease activity compared to other treatments, and this could be that the native microbes that already existed in the soil had the potential to remediate the soil and improve urease activity. However, the microbial inoculations did not have any significant effect on β -glucosidase activity, but an increase in enzyme activities was observed during the bioremediation period. This might be because, during the bioremediation process, there was a competition between native and inoculated microbes, or the microbes chosen for this study were not suitable to produce β -glucosidase activity. Overall, the microbial inoculation was a successful strategy to stimulate enzyme activities in the soil contaminated with TWW.

5.2 Significance of findings

The findings of the present study revealed that the indigenous toxic-metal-tolerant microbes have a high potential to reduce the concentrations of As, Cr, Cd, and Pb in soil contaminated with TWW. The selected microbes were able to survive the harsh conditions against the non-essential metals when inoculated in the soil. *Bacillus cereus* as a gram-positive microbe was able to degrade harmful non-essential metals (Pb and As) effectively as compared to other selected microbes. *Enterobacter cloacae* as a gram-negative microbe also could reduce the concentration of Cr in the soil. The study revealed that the TWW carried these non-essential heavy metals and were further accumulated in the soil through contamination. However, bioremediation proved to be a good strategy since it was able to reduce the concentrations of non-essential metals in the soil using the selected indigenous microbes. These findings revealed that a choice of bacteria for bioremediation is important taking into consideration the strength and the capacity to reduce the concentration of non-essential metals to lower and optimal levels. Lastly, the present study proved that the microbial inoculation for bioremediation on non-essential heavy metals could be a better strategy to increase the enzyme activities in the soil since they were affected following the contamination of TWW. The microbial inoculation had a significant effect to improve the urease, phosphatase and arylsulfatase activities since they are good indicators for soil health. However, the microbial inoculation did not have any significant effect on β -glucosidase activity. In general, bioremediation could be adopted as a sustainable and cost-effective strategy to clean-up non-essential heavy metals in agricultural soils.

5.3 Conclusion

In conclusion, this study proved that TWW contain non-essential metals, and they can accumulate in the soil through irrigation. However, the indigenous microbes; *B. cereus*, *E. cloacae* and *A. fumigatus* showed that they have a potential to reduce the non-essential metals in the soil. The chemical properties (pH and EC) and enzymes activities were also affected by non-essential heavy metals concentrations in the soil, however the selected microbes proved to have a capacity to remediate the soil and increase the enzyme activities. Overall, the study was successful, and the use of

bioremediation was an effective and efficient method as it proved to mitigate toxic elements in the soil following irrigation with TWW.

5.4 Recommendation

The study suggests that farmers should utilize TWW for irrigation in their agricultural fields as a way of solving water scarcity issues, however, they should be aware of the long-term effects of TWW on soil health and microbial diversity. The TWW should meet quality standard before it could be used for irrigation since it can damage crops and reduce soil quality if there is presence of toxic elements. Furthermore, the selected indigenous microbes; *B. cereus*, *A. fumigatus*, and *E. cloacae* could be used for bioremediation to decrease the non-essential metals in the soil caused by irrigation of TWW. However, since these microbes are pathogenic there can be exposure to toxins caused by indigenous microbes which may pose health risks to humans and further affect the crops in the fields. So, if this strategy is used by farmers on a larger scale, there should be proper containment and handling procedures to avoid environmental issues. Additionally, irrigating with TWW should be assessed before use to verify if the essential parameters (EC and pH) are permissible. The study further suggests that the enzyme activities during bioremediation should be monitored effectively since they help identify limiting factors in the soil such as nutrient deficiencies and inadequate aeration that may hinder the bioremediation process, and if there is a need for any organic amendments in the soil. Overall, monitoring enzyme activities and chemical properties during bioremediation are essential for evaluating process efficiency, predicting outcomes, and adjusting environmental conditions to optimize microbial activity. The proposed future study could be an evaluation of the potential microbes and organic amendments to enhance soil health and enzyme activities in contaminated agricultural soils. Sterilising the soil or microbial characterization to assess the native microbes is recommended in the control treatment for the bioremediation, before the contamination experiment. Additionally, there is a need for the analysis of the essential nutrients that may influence the microbes to effectively remediate the contaminated soil.

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APPENDICES

Appendix 3.1: ANOVA for As following irrigation with treated wastewater for 12 weeks.

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	2	2.4267	1.2133	4.69	
Treatment	3	46.5267	15.5089	59.91	<.001
Residual	6	1.5533	0.2589		
Total	11	50.5067			

Appendix 3.2: ANOVA for Cd following irrigation with treated wastewater for 12 weeks.

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	2	0.009800	0.004900	3.80	
Treatment	3	0.208967	0.069656	54.04	<.001
Residual	6	0.007733	0.001289		
Total	11	0.226500			

Appendix 3.3: ANOVA for Cr following irrigation with treated wastewater for 12 weeks.

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	2	0.11120	0.05560	1.99	
Treatment	3	5.48700	1.82900	65.40	<.001
Residual	6	0.16780	0.02797		
Total	11	5.76600			

Appendix 3.4: ANOVA for Pb following irrigation with treated wastewater for 12 weeks.

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	2	1.412	0.706	0.36	
Treatment	3	2400.367	800.122	410.73	<.001

Residual	6	11.688	1.948
Total	11	2413.467	

Appendix 3.5: ANOVA for *Bacillus cereus* counts as remediating microbe

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.002450	0.002450	0.70	
Treatment	3	1.655400	0.551800	156.91	<.001
Residual	3	0.010550	0.003517		
Total	7	1.668400			

Appendix 3.6: ANOVA for *Aspergillus fumigatus* counts as remediating microbe

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.007813	0.007813	1.58	
Treatment	3	2.316038	0.772013	156.09	<.001
Residual	3	0.014838	0.004946		
Total	7	2.338688			

Appendix 3.7: ANOVA for *Enterobacter Cloacae* counts as remediating microbe

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.009800	0.009800	2.60	
Treatment	3	2.194450	0.731483	194.20	<.001
Residual	3	0.011300	0.003767		
Total	7	2.215550			

Appendix 3.8: ANOVA for soil pH (H₂O) following irrigation with treated wastewater for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.0036125	0.0036125	20.16	
Treatment	3	1.3033375	0.4344458	2424.81	<.001

Residual	3	0.0005375	0.0001792		
Total	7	1.3074875			

Appendix 3.9: ANOVA for soil pH (KCl) following irrigation with treated wastewater for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.0008000	0.0008000	2.00	
Treatment	3	1.4341500	0.4780500	1195.13	<.001
Residual	3	0.0012000	0.0004000		
Total	7	1.4361500			

Appendix 3.10: ANOVA for EC following irrigation with treated wastewater for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	200.0	200.0	1.63	
Treatment	3	17443259.0	581086.3	4750.02	<.001
Residual	3	367.0	122.3		
Total	7	1743826.0			

Appendix 4.1: ANOVA for Phosphatases following soil bioremediation for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.019207	0.019207	8.57	
Treatment	3	5.978462	1.992821	889.31	<.001
Residual	3	0.006723	0.002241		
Total	7	6.004392			

Appendix 4.2: ANOVA for Glucosidases following soil bioremediation for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.003281	0.003281	0.70	
Treatment	3	0.006925	0.002308	0.50	0.710
Residual	3	0.013963	0.004654		
Total	7	0.024168			

Appendix 4.3: ANOVA for Urease following soil bioremediation for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.01505	0.01505	1.11	
Treatment	3	11.89318	3.96439	292.43	<.001
Residual	3	0.04067	0.01356		
Total	7	11.94890			

Appendix 4.4: ANOVA for Arylsulfatase following soil bioremediation for 12 weeks

Source	D.F.	S.S.	M.S.	V.R.	F pr.
Rep stratum	1	0.001565	0.001565	0.25	
Treatment	3	6.904398	2.301466	364.58	<.001
Residual	3	0.018938	0.006313		
Total	7	6.924902			