Bioavailability and bioremediation of heavy metals and nutrients in cultivated and fallowed soils following irrigation with treated wastewater

Master of science in
Agriculture (Soil Science)

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RECOMMENDATIONS AND CONCLUSIONS

DECLARATION

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

Candidate: Moedisha Lorraine Phadu ________________ Signature __________________ Date

DEDICATION

I dedicate this work to my late father,

Christopher M. Phadu and my lovely

mother, Sylviah M. Phadu.

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ABSTRACT

Global shortage of fresh quality water has led to the use of treated wastewater in arid and semi-arid regions. Although, the treated wastewater has proven to be the best solution in ameliorating pressures brought by water shortage, it contains toxic heavy metals, some in high concentrations that could possibly pose health risks and degrade soil quality. Therefore, the objectives of the study were to determine the vertical and horizontal distribution of bioavailable heavy metals on virgin, cultivated and fallowed fields and to investigate the bioremediation abilities of selected soil microbes on non-essential heavy metals in cultivated and fallowed soils following irrigation with treated wastewater at University of Limpopo (UL) Experimental Farm. Three fields, namely, virgin field (VF), cultivated field (CF) and fallowed field (FF), each being 6.4 ha, were each divided into 40 equal grids, equivalent to 40 m × 40 m, which were used in vertical assessment of heavy metals.

Soil profiles were established inside each grid and soil samples collected at 0-20; 20-40 and 40-60 cm soil depth for further laboratory analysis. The soil samples were analyzed for basic soil physico-chemicals, namely, particle size distribution, soil pH (H_20 and KCl), electrical conductivity (EC), reduction potential (Eh), organic carbon (OC) and cation exchange capacity (CEC). Five essential heavy metals namely zinc (Zn), iron (Fe), copper (Cu), cobalt (Co), manganese (Mn) and five non-essential heavy metals, namely, arsenic (As), chromium (Cr), lead (Pb), aluminium (Al), and cadmium (Cd), were also extracted from the soil samples. Heavy metal resistant Gram-negative (–) and Gram-positive (+) bacteria were isolated from the soil and identified as *Providencia rettgeri* (–), *Enterobacter cloacae* (–), *Bacillus cereus* (+) and *Arthrobacter aurescens* (+).

The isolated bacteria were cultured and inoculated in heavy metal-contaminated soils and incubated for 12 weeks to bioremediate the non-essential heavy metals. Results obtained suggested that the treatments had no significant ($P \leq 0.05$) effects on vertical distribution of all the essential and non-essential heavy metals among the three fields. However, on average Co was above the permissible level at 53 mg/kg in CF at 0-20 cm and although all the other essential heavy metals increased, they were still within the permissible levels. The concentration of As was also above the permissible levels in CF with an average concentration of 4.30 mg/kg. Cadmium levels were also above the permissible levels in CF with an average concentration of 1.146 mg/kg in CF and this increased by 0.46 units from VF which had an average value of 1 mg/kg. However, fallowing reduced Cd to 0.51 mg/kg which was below or within the expected limits in soil previously irrigated with treated waste water.

Gram-positive bacteria reduced more concentrations of non-essential heavy metals separately and combined, especially in the fallowed field. Irrigation with treated wastewater has shown to have both negative and positive effects on the concentration of essential and non-essential heavy metals in cultivated and fallowed fields. Bioremediation coupled with fallowing has been proven to be the best solution in ameliorating heavy metal toxicity while naturally improving the quality of the soil.

CHAPTER 1

RESEARCH PROBLEM

1.1 Background

Global shortage of fresh quality water has led to increased use of treated wastewater for irrigation purposes mainly in arid- and semi-arid regions in South Africa (FAO, 2015). The removal of heavy metals from contaminated domestic or industrial effluents which form part of wastewater is reported to be difficult following the fact that heavy metals are persistent and non-biodegradable (Mico *et al*., 2006). Therefore, the treated wastewater may contain some toxic heavy metals such as Cd, As, Cu, Co, nickel (Ni) and Pb, some in high concentrations (Khan *et al*., 2011). Continuous use of treated wastewater for irrigation purposes results in bioaccumulation of these toxic heavy metals in soils and groundwater until they are in excess amounts (Deepti *et al*., 2013).

Heavy metal pollution has lately become one of the most problematic environmental concerns affecting soil and water quality, eventually leading to health concerns (USGS, 2006). Soil serves as a sink for nutrients and heavy metals (Mico *et al*., 2006). The transfer of toxic materials is through absorption of toxic materials by the roots, to the edible parts of crops, (Rajohan *et al*., 2014) leading to health problems such as damage of the kidneys, nervous system, cancer, organ damage, and cause cell mutations and in extreme cases could cause death (Vijayanand and Divyashree, 2015). Accumulation of heavy metals in different organs of the human body such as kidneys, bones and liver may result in severe side effects (Singh *et al*., 2010). In reports by Radwan and Salama (2006) and Singh *et al*. (2010), diseases like gastrointestinal disorders, diarrhoea, stomatitis, tremor, ataxia and paralysis may be caused by Pb, As, mercury (Hg), Zn and Cu metals when in excess amounts in the human body. Soil and groundwater contamination of heavy metals pose a more serious concern because they severely have a threat to the food chain, human health and the ecosystem (Rajohan *et al*., 2014). Statistically, it was estimated that over 1 billion people are currently exposed to elevated concentrations of toxic metals and metalloids in the environment and several million people suffer from sub-clinical metal poisoning (Syed and Chintala, 2015).

Heavy metal contaminated soils limit plant habitats due to toxicity, resulting in ecological, evolutionary, and nutritional problems (Ayangbenro and Babalola, 2017). Excess amounts of toxic heavy metals in the soil reduces plant growth by decreasing seed germination and inhibiting photosynthesis, resulting in reduced yield and economic problems for farmers (Vijayanand and Divyashree, 2015). The excess in heavy metals reduces yields by causing most crops to perish and therefore farmers tend to harvest fewer yields, therefore farmers increase the price of the crops or else they would have spent more than their profit (Vijayanand and Divyashree, 2015).

Heavy metal contamination not only affected crops or human health, but also negatively affected the microbial population by altering their natural habitats (Chipasa, 2003). Soil microbes are the most important component of fundamental foundation affecting soil health (USDA, 2017). Therefore, by affecting microbial population size, diversity and activities as well as microbial genetic structures, heavy metal toxicities affect soil health indirectly (Chipasa, 2003).

Although heavy metals are naturally present in the soil, geologic and anthropogenic activities increase the concentration of these elements to amounts that are harmful to both plants and animals (Chibuike and Obiora, 2014). In most cases and environments, heavy metal pollution is caused by anthropogenic activities such as contaminated waste disposal, agricultural activities including the application of excessive agrochemicals that contain heavy metals, mining, sludge from treatment plants, waste from hospitals, pharmaceutical companies and filling stations. The mentioned wastes could be part of constituents in wastewater that is used for irrigation of agricultural fields (Kiziloglu *et al*., 2007).

In previous studies, heavy metal polluted soils were remediated using the process of phytoremediation which refers to the planting of crops with the aim that they will absorb the heavy metals through their roots while removing them from the soil and groundwater (Alkorta *et al*., 2006; Flipps, 2008). Phytoremediation has a challenge of coming up with a solution to get rid of those crops after they have absorbed the heavy metals from the soil (Alkorta *et al*., 2006). Thus, the use of microbes such as bacteria, fungi and actinomycetes can serve as a better solution to remediate soils from heavy metal pollution. Bioremediation is one of the most cost effective, efficient and environmentally friendly technology to use in order to address the problem of heavy metal bioaccumulation in soil and water (AL-Jaboobi *et al*., 2014). Bioremediators not only have the ability to remove toxic metals from the soil but they can also serve other functions in the soil such as nutrient cycling. Moreover, this method can be used while crops are planted, it does not require the farm to be left bare while remediation takes place (Rosch, 2015).

The study investigated the bioavailability of heavy metals and the effects of microbial communities on their bioremediation capabilities in cultivated and fallowed soils following irrigation with treated wastewater.

1.2 Problem statement

Preliminary studies showed high concentrations of toxic heavy metals such as Cd, Ni, As, Pb, Co, Cu and Zn in cultivated soils at the University of Limpopo Experimental Farm. The toxic heavy metals were most likely introduced to the soil by irrigation with treated wastewater that receives effluents from heavy metal polluted areas such as filling stations, agricultural fields, the hospital and run-offs across Mankweng area. Although the use of treated wastewater reduces the pressure brought by water shortage, its continuous use could lead to high bioavailable heavy metal concentrations in the soil. The researcher explored the use of bioremediators as a strategy to reduce heavy metal concentrations in soils.

1.3 Rationale of the study

The use of treated wastewater for irrigation serves as an important and efficient way of overcoming the problem of water scarcity in arid to semi-arid regions like Limpopo. However, treated wastewater can lead to increased bioavailability of heavy metals in the soil. Although purification systems that this water was obtained from might not be efficient enough in purifying all the pollutants brought by various effluents. There are alternative methods such as bioremediation that can reduce heavy metal concentrations in contaminated soils. The main focus of this study was to explore bioremediation using species of bacteria indigenous to the fields, as a biological strategy to on improve the health of the soil and to benefit farmers who are faced with problems such as heavy metal pollution. The strategy would not only improve soil and crop quality, it is also efficient, eco-friendly, cheaper and can be well adopted by small scale farmers.

1.4 Purpose of the study

1.4.1 Aim

The aim of this study is to investigate the distribution of heavy metals and the role of different microbial communities responsible for bioremediation of heavy metals in cultivated and fallowed soils following irrigation with treated wastewater at the University of Limpopo (UL) Experimental Farm.

1.4.2 Objectives

The objectives of the study are:

1. To determine the vertical and horizontal distribution of bioavailable heavy metals on cultivated and fallowed soils following irrigation with treated wastewater at UL Experimental Farm.
2. To investigate the bioremediation abilities of selected soil microbes on non-essential heavy metals in cultivated and fallowed soils following irrigation with treated wastewater at UL Experimental Farm.

1.5 Reliability, validity and objectivity

Reliability was ensured by the use of statistical levels of significance as derived through the use of analysis of variance; validity was achieved through replication of samples during analysis for Objectives 1 and 2; and in space for Objectives 1 to 2 during sampling. Objectivity was achieved by ensuring that the findings were discussed on the basis of empirical evidence, as shown in the statistical analyses, in order to eliminate all forms of subjectivity.

1.6 Bias

Bias was minimised by ensuring that the experimental error in each experiment was reduced through replications in all treatments.

1.7 Scientific significance of the study

The study was undertaken to investigate the negative effects that come with the use of treated wastewater use for irrigation of agricultural crops. Furthermore, the heavy metal bioremediation techniques would benefit the agricultural industry in improving the health and quality of soils, and in turn improving production levels that could elevate food security in our country.

1.8 Structure of the mini-dissertation

The mini-dissertation is made up of 5 chapters. Chapter 1 outlines the description and the details of the research problem; Chapter 2 is the literature review clearly outlining the work undertaken and gaps regarding the problem statement. Chapter 3 and Chapter 4 are aligned to Objective 1 and Objective 2, respectively. Chapter 5 includes the summary of all findings of the research in the current study, the conclusion and integration of all chapters to provide the significance of the findings and recommendations for future research. The referencing style followed in the mini-dissertation is Harvard referencing format, following the author-alphabet, in both text and reference list, as approved by the University of Limpopo Senate.

CHAPTER 2

LITERATURE REVIEW

2.1 Work done on problem statement

2.1.1 Bioavailability of heavy metals in cultivated and fallowed soils following irrigation with treated wastewater

<u>Presence of heavy metals in agricultural soils</u>: Heavy metals are considered as dense metals with a density greater than that of water; they are fixed, non-biodegradable and also referred to as transition metals on the periodic table (Afifi *et al*., 2011). Heavy metal accumulation results from either weathering of parent materials in acidic condition or anthropogenic sources (Rajohan *et al*., 2014). Wuana and Okieimen (2011) reported that soils are the main reserve of heavy metals, and unlike other chemicals, they do not undergo chemical or microbial degradation easily.

Soil heavy metal pollution pose health risks and hazards to human beings and the ecosystem through direct ingestion or contact with contaminated soil and to the food chain that starts form the soil to the plants that are grown on the contaminated soil (Jain *et al*., 2012). The major contribution to human exposure to heavy metals is through food consumption of contaminated food products like vegetables and fruits more than to dermal contact (Wuana and Okieimen, 2011). Heavy metals can accumulate in the soil through agricultural practices like continuous application of agrochemicals such as the application of herbicides, pesticides, insecticides and with continuous irrigation with heavy metal contaminated water (Rajohan *et al*., 2014).

Treated wastewater as a source of heavy metals agricultural soils: Wastewater is water that has been adversely affected in quality by anthropogenic influence (Tilley *et al*., 2011). Wastewater is made from a combination of domestic, industrial, commercial or agricultural waste, surface run-off or storm water and from sewer inflow or infiltration; all these sources contain all kinds of pollutants, including toxic metals, chemicals and harmful microbes (Erakhrumen, 2007; Rajohan *et al*., 2014). Pollution of freshwater environments by heavy metals, such as at the Msundi river and two of its tributaries, the Bayne’s Spruit and Slangspruit in KwaZulu Natal, forms part of the global and local crisis due to the toxic nature of metals (Shozi, 2015).

Studies on effects of treated wastewater demonstrate that heavy metals could be added in higher concentrations in agricultural soils (Kayastha, 2014), with detrimental effects on the ecosystem. Abedi-Koupai *et al*. (2006) conducted a study investigating the effect of treated wastewater on soil chemical and physical properties in an arid region. In their conclusion, they reported that the accumulation of Pb, manganese (Mn), Ni and Cu in the soil increased significantly in the wastewater treatment as compared to the groundwater treatment.

The long-term or rather continuous irrigation with wastewater leads to high accumulation of metals particularly on the top soil and the level of heavy metals decreases as soil depth increases (Afifi *et al*., 2011). According to Abedi-Koupai *et al*. (2006), movement of heavy metals in soils irrigated with treated wastewater is slow and more than 90% become accumulated at a depth of 10-15 cm, which is mainly where most crop roots are found.

Galavi *et al*. (2010) studied the effect of different treatments of irrigation and compared them with irrigation with wastewater, on absorption and accumulation of some heavy metals and nutrients and their possible contamination in sorghum crop and soil. Their findings were that irrigation with wastewater increased the percentage of organic matter, total nitrogen, potassium (K), phosphorus (P), calcium (Ca), sodium (Na), EC and sodium adsorption ratio (SAR) than in the control. Rusan *et al*. (2007) reported that Pb and Cd content in barley crops increased with wastewater irrigation.

The findings indicated the dangers of irrigating with wastewater, as human exposure to very low contents of Cd may result in kidney damage, bone defects and fractures (Ciura *et al*., 2005). According to Mojiri and Jalalian (2011), irrigation with treated wastewater increased EC, P and metals such as Cd, Zn, Fe and As but caused a decrease in soil pH. In a field accumulation risk assessment of heavy metal done in Saudi Arabia, wastewater irrigated fields contained the highest amounts of heavy metals in amounts that are above permissible levels set by WHO. The concentrations of Ni, Cd and Cr in the soil were above the safe limit by 90%, 28% and 83%, respectively (Balkhair and Ashraf, 2015).

<u>Heavy metal effects on living forms in agricultural soils</u>: Different heavy metals have different functions in the soil and affect the microbes differently, and each metal is known to have unique features and physico-chemical properties that complement its specific toxicological mechanisms of action (Jarup, 2003). Metals such as Co, Cu, Mn and Zn serve as micronutrients and are used for reduction-oxidation processes to stabilize molecules through electrostatic interactions, as components of various

enzymes and for regulation of osmotic pressure (Jarup, 2003). Some metals like Cd, Pb and Hg have no biological role, are non-essential but toxic to microorganisms (Rhodes, 2014). In biological systems, heavy metals have been reported to affect cellular organelles and components such as cell membrane, mitochondria, lysosome, endoplasmic reticulum, nuclei and some enzymes involved in metabolism, detoxification, and damage repair (Salem *et al*., 2000).

Cadmium is categorised as one of the most phytotoxic metal pollutants (Nagajyoti *et al*., 2010), and this follows its characteristic of being highly mobile, especially in soils that have low CEC as well as low pH (Fashola *et al*., 2016). Therefore, it is easily transferred to higher trophic levels in the food chain (Tchounwou *et al*., 2012). It is widely distributed in the earth's crust at an average concentration of about 0.1 mg/kg with its highest level of accumulation being in sedimentary rocks, and marine phosphates with about 15 mg/kg of Cd (Tchounwou *et al*., 2012).

Enrichment of Cd in soil can change soil physical and chemical properties, reduce the richness, diversity, activity of soil microorganisms, and inhibit soil respiration, subsequently affecting soil fertility (Li *et al*., 2016). While it is being transported, it can pollute soils and water bodies and it is toxic even at very low concentrations (Tchounwou *et al*., 2012). Cadmium has an ability to strongly adsorb to organic matter, thus can remain in the soil for a long time (Ramya *et al.*, 2014). Its availability in the soil makes it more dangerous because that is when it is rapidly absorbed by crops (Rosch, 2015).

Natural levels of As in soil before irrigation with wastewater usually range from 1 to 40 mg/kg (Hood, 2010). A strong association between As exposure and increased risks of both carcinogenic and systemic health effects has been reported (ATSDR, 2017). In one study, it was reported that long-term exposure to high levels of As might cause brain damage, cardiovascular and respiratory disorder, conjunctivitis, dermatitis, skin cancer in human beings (Andreas, 2012). Arsenic can damage cell membrane of plants, inhibits roots extension and proliferation (Trouba *et al*., 2011). It also interferes with critical metabolic processes, thus resulting in loss of fertility, yield and fruit production. Oxidative stress, physiological disorders, deactivation of enzymes are also a result of As impacts (Bissen and Frimmel, 2003).

Zinc availability often increases as pH decreases, and Zn toxicity is most common when plants are grown in acidic soil and when there is excess magnesium in the soil (Leitao, 2009). Excess amount of Zn in the soil affects photosynthesis by inhibiting growth rate of plants, reducing their chlorophyll content, slowing germination rate and reducing plant biomass (Mortvedt *et al*., 2000). Toxic effects of Zn to human beings include ataxia, depression, gastrointestinal irritation, hematuria, icterus, impotence, kidney and liver failure, lethargy, macular degeneration, metal fume fever, prostate cancer, seizures and vomiting (Kabata-Pendias and Mukherjee, 2007; Leitao, 2009). Microbe exposure to Zn may reduce microbial biomass by inhibiting their growth and causing death (Chibuike and Obiora, 2014).

Copper and Ni toxicity are usually seen in sandy soils usually at concentrations above 600 mg/kg and 400 mg/kg, respectively, that are alkaline in pH (Salem *et al*., 2000). Copper’s toxicity effects in micro-organisms include the disruption of cellular function and the inhibition of enzyme activities (Dixit *et al*., 2015). Copper may cause retarded growth in crops by causing chlorosis of leaves. Excess amounts of Ni can impede the uptake of other essential nutrients especially iron (FAO, 2012; Kabata-Pendias and Mukherjee, 2007). In microorganisms, Ni toxicity may result in the disruption of cell membranes, inhibition of enzyme activities and may cause oxidative stress (Dixit *et al*., 2015).

Exposure to Pb in children causes reduced intelligence, impaired development and an increased risk of cardiovascular disease; in adults, it can cause anorexia, chronic nephropathy, damage to neurons, high blood pressure, hyperactivity, insomnia, learning deficits, reduced fertility, renal system damage and risk factor for Alzheimer’s disease (Nagajyoti *et al*., 2010). In plants, it affects photosynthesis and growth, causes chlorosis and inhibits enzyme activities and seed germination. Microorganisms that are exposed to Pb toxicity have denatured nucleic acid and protein, have their enzymes activities inhibited and have become transcribed (Fashola *et al*., 2016; Mupa, 2013).

Cobalt is a trace element in plants and it is a component of a number of enzymes in legumes. It is important for nitrogen fixation by the bacteria that associate with legumes (Rani *et al*., 2010). High levels of Co can reduce the amount of Cd that is taken up by plants (Hood, 2010). On average, 1 kg of soil contains about 8 mg of Co though this amount varies widely around the Earth from 0.1-70 mg/kg. Ideally, for healthy and productive soil, the concentration of Co should at least be between 1‐2 mg/kg. If the concentration of Co in the soil is greater than 100 mg/kg, toxicity effects might prevail (FAO, 2012; Kabata-Pendias and Mukherjee, 2007).

Bioavailability of heavy metals in the soil: Bioavailability is referred to as the percentage of the total amount of a metal in a specific environment, within a given period, being either available or can be made available for uptake by microorganisms at the immediate location of the organism (Olaniran *et al*., 2013). The physiological and toxic effects of heavy metals to the biological system are determined by the metal speciation (Ayangbenro and Babalola, 2017) and the bioavailability of those heavy metals and not by the total amount of heavy metal (Rajohan *et al*., 2014). Bioavailability of heavy metals in the soil was affected by soil properties such as soil pH and organic matter (Shozi, 2015). Organic matter content has a strong influence on CEC, buffering capacity as well as on the retention of heavy metals in the soil.

Therefore, soils that have high amounts of organic matter had a smaller amount of bioavailable heavy metals (Chipasa, 2003; Olaniran *et al*., 2013). At high pH, metals tend to form insoluble metal mineral phosphates and carbonates, whereas at low pH they tend to be found as free ionic species or as soluble organo-metals and are more bioavailable in the soil (Shozi, 2015). At low pH, metals were less likely to form insoluble precipitates with phosphates because much of the phosphate has been protonated (Huges and Poole, 2001). A small change in pH can change metal solubility and bioavailability by several orders of magnitude (Rhodes, 2014). Although some complexes were formed due to basic conditions of the soil, it differs according to different heavy metals in which complexes are soluble and which are not. For example, those that were formed with Cd, Ni, and Zn are soluble, while those formed with Cr and iron are insoluble, and this occurs due to the difference in the nature of metals (Hoffman and Sandrin, 2007).

The bioavailability of Zn, Pb and Cu from soil decreases with increasing pH (Rhodes, 2014). The decreased availability of metals was affected by higher adsorption and precipitation in alkaline and neutral environments (Takac *et al*., 2009). Takac *et al.* (2009) reported on a positive correlation between pH of the soil and the bioavailable metals in a bioavailability study undertaken at Central Spis region.

The occurrence and bioavailability of heavy metals as well as their movement in soils and sediments are regulated by physicochemical processes such as natural weathering, periodical deposition of river sediments in the floodplain, oxidation/reduction, adsorption/desorption, pH, organic matter and CEC of the soil in question (Rajapaksha *et al*., 2004; Rajohan *et al*., 2014). Heavy metals are mostly enriched in clay zones that have a low pH, and this strongly means that the distribution of heavy metals in a certain field are effectively regulated by soil texture and their reductive dissolution (Rajohan *et al*., 2014). Fine textured soils in their experiment on distribution of heavy metals in different soil types retain more metals and thus increase their concentration due to clay soil's large surface area and factors such as sorption, co-precipitation and complex formation (Olaniran *et al*., 2013).

In water-logged soils such as wetlands, the soil redox potential becomes very low and at the same time, the pH will be neutral to alkaline. This will affect the mobility of the heavy metals (Rhodes, 2014; Shozi, 2015). The redox potential controls the chemical speciation, bioavailability, toxicity and mobility of many major and trace metals; it may also provide new opportunities for engineered remediation strategies such as *in situ* microbial degradation of organic solvents and reductive sequestration of compounds (Chibuike and Obiora, 2014).

Speciation is defined as the identification and quantification of the different, defined species, forms, or phases in which an element occurs (Ashraf *et al*., 2011). Many important redox sensitive components in particular, trace metal undergo redox transformations and the reactivity, mobility, toxicity and bioavailability of these metals frequently depend on their redox state (Olaniran *et al*., 2013). At the UL Experimental farm, bioavailability is of great importance as it will clearly depict the effects that continuous irrigation with treated wastewater have on the soil and due to its effect on other soil properties such as soil pH this will affect the mobilisation of heavy metals thus causing high bioavailability and toxicity effects.

2.1.2 Bioremediation of non-essential heavy metals in cultivated and fallowed soils following irrigation with treated wastewater

Bioremediation of heavy metals in the soil: Bioremediation is a waste management technique that involves the use of microorganisms to remove or neutralize pollutants in contaminated sites (Singh and Ward, 2013). The bioremediation process is environmentally friendly and cost effective (Torreta, 2015) and can either be *in situ* or *ex situ* (Joutey *et al*., 2013). The Environmental Protection Agency (2012) reported that heavy metals are mostly non-degradable; therefore, during bioremediation, heavy metals are transformed from one organic complex or oxidation state to another. Due to a change in their oxidation state, these metals can be transformed to become less toxic (Vijayanand and Divyashree, 2015). The heavy metals could also be easily volatilized, become more water soluble which allows them to be leached out or less soluble which could encourage precipitation and become easily removed from the environment (Imborvungu *et al*., 2010; Leitao, 2009).

Bioremediation is a natural process that works well when pollution-eating organisms have access to food source, oxygen and other conditions such as enough moisture that encourage their rapid growth (Ayangbenro and Babalola, 2017; Singh and Tripathi, 2007). The micro-organisms performing the remediation process would then be able to break down the pollutant at a correspondingly faster rate (Vijayanand and Divyashree, 2015). According to Bahafid *et al*. (2013), microorganisms that participate in bioremediation are referred to as bioremediators, and common examples are bacteria (*Bacillus subtilis, Pseudomonus putida, Enterobacter cloacae)* and fungi (*Penicillium, Aspergillus, Rhizopus*); they are potential microbial agents for the removal of heavy metals from aqueous solutions or in contaminated soils (Leitao, 2009). Microorganisms behave differently during soil remediation due to the metal uptake rate, and this depends on factors such as the type of metal, temperature change and pH (Singh and Tripathi, 2007).

Heavy metal toxicity affects microbial population size, diversity, and activities, as well as the genetic structure (Ayangbenro and Babalola, 2017). Kgopa *et al*. (2017) reported that heavy metals have a negative impact on soil microorganisms through alteration of their genetic make-up. Fungi and bacteria are the main components of the soil microbial biomass. However, it had been reported that fungi were more tolerant to heavy metals as a group than bacteria (Rajapaksha *et al*., 2004; Shozi, 2015). In a study by Karigar and Rao (2011), a two-way defence mechanism by fungi was reported. The mechanism comprised the initial production of degradable enzymes to target the pollutants and then making themselves resistant to heavy metals. The fungi species used the chemicals for their growth and development through biosorption or bioaccumulation and thus removing the toxins from either the soil or water (Kota *et al*., 2014). The uptake was assisted by the microbial cell walls that consisted of polysaccharides, lipids and proteins that gave off functional groups that could bind heavy metal ions (Chibuike and Obiora, 2014).

The industry businesses have been growing rapidly, with more heavy metals being released into the environment (CSIR, 2010). Microbes were previously used to clean up or remove toxic heavy metals, for example, when oil spills up, bacteria could be introduced to the area of the spill where to break down the hydrocarbons of the oil into carbon dioxide (Das and Chandran, 2011). According to a study by Chipasa *et al*. (2003), *Bacillus* species used biosorption as a remediation process for heavy metals. Biosorption simply refers to the ability of bacterial cells to adsorb, chelate or precipitate metal ions in the solution into insoluble particles or aggregates which could be removed either by sedimentation or filtration from the solution. Syed and Chintala (2003) reported that all the isolates of *Bacillus* were able to adsorb Pb at a concentration of 1000 ppm, with Cu and Cd concentration being the least to be adsorbed. Five microbes were able to remove 93.18% Zn, 84.13% Pb and 87.9% Cr when incubated at 37^0 C for 72 hours (Vijayanand and Divyashree, 2015). Generally, with increased incubation time, more metals were removed.

2.2 Work not yet done on problem statement

2.2.1 Bioavailability of heavy metals in cultivated and fallowed soils following irrigation with treated wastewater

Vertical distribution of bioavailable heavy metals in wastewater irrigated soils has not been studied in agricultural soils. However, there is a need to substantiate the assumption that irrigation with wastewater has an effect on the vertical distribution of bioavailable heavy metals. Bioavailability studies done in South Africa mostly focused on determining heavy metals in soils in relation to human health; and not on soil health and effects on soil microfauna (Jarup, 2003; Yalala, 2015).

2.2.2 Bioremediation of non-essential heavy metals following irrigation with treated wastewater

Bioremediation using diverse microorganisms indigenous to cultivated and fallowed soils contaminated with heavy metals following irrigation with treated wastewater has not been investigated yet. The research aimed at identifying specific microbes that were indigenous to the soils in question then used as inoculants for bioremediation. The most tolerant microbes were isolated, identified and cultured from each field, then used for remediation of heavy metals in soil.

CHAPTER 3

BIOAVALABILITY OF HEAVY METALS IN CULTIVATED AND FALLOWED SOILS FOLLOWING IRRIGATION WITH TREATED WASTEWATER

3.1 Introduction

Heavy metals, also referred to as trace metals, could be introduced into agricultural soils from various sources, including atmospheric deposition of metal/metalloid-bearing particles, application of sewage sludge, phosphate fertiliser, pig slurry and pesticides and irrigation with reused water (Hariprasad and Dayananda, 2013). Heavy metals in soils exist in several chemical forms such as the ionic forms (inorganic forms) or the organic forms (Efremova and Izosimova, 2017), depending on the chemical state of the elements in the contaminating material (Osakwe *et al*., 2012). Bioavailability of heavy metals in the soil refers to that portion of the total amount of a metal in a specific environmental compartment that, within a given period, is either available or can be made available for uptake by microorganisms from the direct surrounding of the organism (Olaniran *et al*., 2013). The increased bioavailability results in toxicity risks of heavy metals to other life forms.

Risks associated with polluted soils are contamination of the food chain by the bioavailable toxic elements (Balkhair and Ashraf, 2015). The risks of heavy metal transfer into the food chain are dependent on the mobility of the heavy metal species and their availability in the soil (Wuana and Okieimen, 2011). Studies, globally, have raised concerns on the availability of heavy metals as a result of treated wastewater irrigation (Balkhair and Ashraf, 2015; Hariprasad and Dayananda, 2013). Therefore, this chapter explores the bioavailability of heavy metals in fields that were irrigated with treated wastewater.

3.2 Materials and methods

3.2.1 Description of the study site

The study was carried out at the University of Limpopo (UL) Experimental Farm (23°83'31''S, 29°69'46''E) located west of Mankweng in the Capricorn District of Limpopo Province. The area is semi-arid with an estimated annual rainfall of 400-500 mm per annum, experiencing hot summers and cool winters. Rainfall is only received during the summer months between November and January. Three fields of 6.4 ha in size were identified for this study. The first one was virgin field (VF) and has never been cultivated or irrigated. The second was a cultivated field (CF) in its third year of onion cultivation and irrigated with treated water, then the last was fallowed field (FF) which has been fallowed for 5 years following 3 years of cultivation and irrigation with treated wastewater (Figure 3.1). The soil in this area was classified as Bainsvlei soil form, developed from a granite parent material (Soil Classification Working Group, 1991). The soils of the study area had clay increasing with depth, and most of the profiles were dominated by dark colour while some had red colour representing high concentrations of iron.

Treated wastewater used for irrigation in these fields was received from the Mankweng WasteWater Treatment Plant (MWTP) (23°85'82''S, 29°70'82''E) which is located adjacent to the UL Experimental Farm. The main focus sampling points at the MWTP were the exit of pond 16 that transfers water into the furrow conveying water to the night dam at UL Experimental Farm, the entry and exit sites of the night-dam. The MWTP received effluent from various places across Mankweng area (23°87'84''S, 29°71'37''E), namely, University of Limpopo (23°88'71''S, 29°73'84''E), Mankweng hospital (23°87'90''S, 29°72'61''E), two local

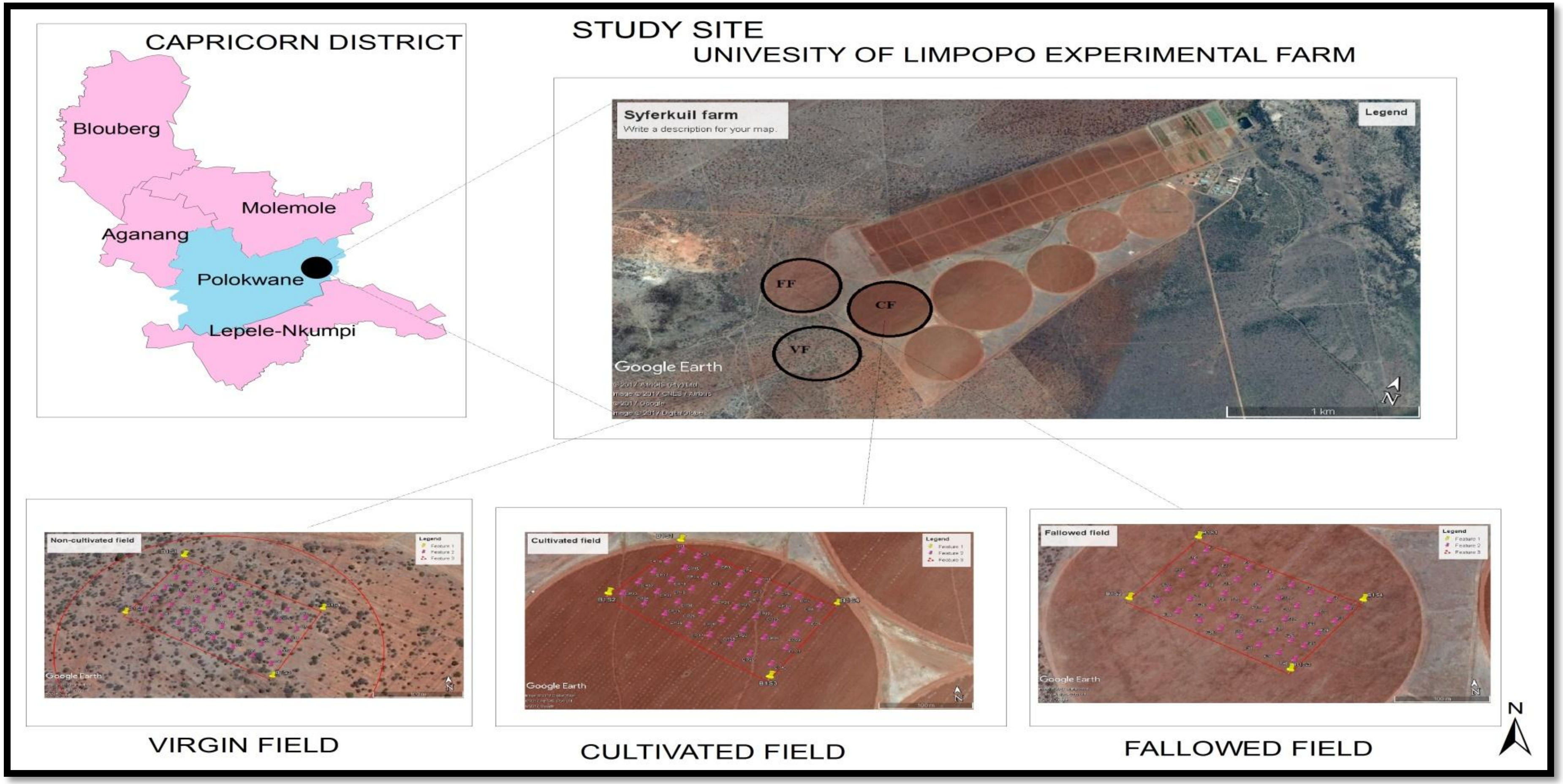

Figure 3.1 Map of the study site showing the three selected fields.

shopping centres, filling stations, human settlements and from runoff water from buildings and agricultural fields. The effluent went through physical, biological and chlorine treatments prior to disposal into the furrow before being passed on to the night-dam as treated wastewater.

3.2.2 Research design and sampling

The study comprised of two factors, fields (VF, CF, and FF) and soil sampling depths (0-20, 20-40 and 40-60 cm) and each factor had three treatments. Field boundaries of the study sites were demarcated using differential global positioning system (DGPS) (Trimble Navigation Ltd., Sunnyvale, CA) and mapped with ArcGIS software (ESRI, Redlands, CA). Grids of 40m × 40 m each were created, and soil profiles were opened in each grid for assessment of vertical distribution.

3.2.3 Data collection

Soil samples were analysed for particle size distribution using the hydrometer method (Bouyoucos, 1962). Soil pH (H_2O) and pH (KCl) were analysed using a pH meter (Reeuwijk, 2002). Electric Conductivity (EC) and reduction potential (Eh) were determined using the electrode method whereby EC and Eh meters where used, respectively, (Rhoades, 1982). Heavy metals from the soil and treated wastewater (Co, Cd, Cu, Cr, Al, Pb, Zn, Fe, Mn and As) were extracted and the use of EDTA method (Hesse, 1971) and extracts were read with ICP-OES.

3.2.4 Data analysis

Data were summarized using descriptive statistics and then subjected to factorial analyses of variance (ANOVA) through Stata 12.0 version. Descriptive statistics were used to produce mean, standard deviation, minimum and maximum values for heavy metals in order to assess the level of contamination in the soil.

3.3 Results

3.3.1 Chemical quality of wastewater

Treated wastewater samples were analysed to determine their quality as they were the major contaminants of the soil. The average value of soil pH (H_2O) was 6.62, with a high EC value of 237.5 mS/m. The concentrations of heavy metals obtained were 45.28 mg/l for Zn, 4.83 mg/l for Cu, 0.33 mg/l for Pb, 4.46 mg/l for As, 12.65 mg/l for Al, 1.44 mg/l for Cr and 0.01 mg/l for Cd (Table 3.1). Bioavailable Zn, Cu, Cr, Al and As were all above the threshold set by FAO.

Table 3:1 Summary of the chemical quality of the wastewater.

Average concentrations of water quality variables	Average concentrations	FAO Threshold
pH (H_20)	6.62	6.5-8
EC (mS/m)	23.75	400-600
Zn (mg/l)	45.28	2.0
Cu mg/l	4.83	0.2
Pb mg/l	0.33	5.0
As mg/l	4.46	0.10
Al mg/l	12.65	5.0
Cr mg/l	1.44	0.10
Cd mg/l	0.01	0.01

3.3.2 Distribution of soil physicochemical properties

Descriptive statistics of soil physicochemical properties in the three fields at a depth of 0-20 cm: The mean values of clay and sand in VF were 35.1 ± 15.19 and 55.1 ± 15.34%, respectively. Soil pH (H_2O) ranged between 4 - 7.16, with an average of 5.7 ± 0.93. Soil pH (KCl) was observed to range between a minimum of 4.04 with a maximum of 6.29, at an average of 4.8 ± 0.52. Electrical Conductivity values ranged between 4.52 – 51.31 mS/m. Soil OC % ranged between 3.62% - 8% with an average of 5.7 ± 0.99. Cation exchange capacity ranged between 0.22 – 1.73 mS/m, with an average of 0.8 ± 0.22 (Table 3.2).

Clay percentage of CF had a range of 4% - 24% and an average of 10.90 ± 5.66. Sand % ranged from 4% to as high as 84%. Soil pH varied from strongly acidic to alkaline throughout the field (5.88- 7.58), with an average of 6.42 ± 0.46 (Table 3.2). Soil pH (KCl) also showed a low potential acidity with a minimum value of 4.55 ranging up to 6.75. Soil pH (KCl) had an average of 5.35 ± 0.44. Soil EC had very high values that could lead to salinity hazards if proper management is not applied.

Soil EC varied from 44.57 to 322 mS/m, with an average of 148.55 ± 69.72. Organic carbon percentage and CEC had ranges between 0.04 – 1.1.2 and 1.0 -1.86, respectively. Soil OC% had an average 0.49% ± 0.27% and CEC had an average of 1.43 ± 0.16 (Table 3.2).

Both the percentage of clay and sand at FF were reported to be high with their minimum values of 15.2 and 33.6, respectively, and their maximum values of 51.2 and 73.6. Mean values were 33.15 ± 6.99 and 53.60 ± 8.15, respectively. Soil pH

(H_20) and pH (KCl) values ranged between minimum of 4.27 and 4.92 with maximum values of 8.94 and 6.61. Soil pH (H_2O) and pH (KCl) had average values of 7.67 ± 0.64 and 5.54 ± 0.43. Soil EC values ranged between 50.03 and 217.9 with an average value of 100.88 ± 37.39. Reduction potential was recorded between -17.20 to 8.21 with an average of -5.95 ± 6.68. Soil OC % and CEC had minimum values of 0.46 and 1.73 and maximum values of 4.87 and 2.44, respectively. Their average values were 2.15 ± 0.67 and 2.07 ± 0.18 (Table 3.2).

Table 3.2. Descriptive statistics for soil physicochemical properties in the three fields at 0-20 cm.

Basic soil properties	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
%Clay	8	76	35,1	15,19	4,00	24,00	10,90	5,66	15,20	51,20	33,15	6,99
%Sand	17	75	55,1	15,34	4,00	84,00	63,20	20,80	33,60	73,60	53,60	8,15
pH (H_20)	4	7,16	5,7	0,93	5,58	7,58	6,42	0,46	4,27	8,94	7,67	0,64
pH (KCl)	4,04	6,29	4,8	0,52	4,55	6,75	5,35	0,44	4,92	6,61	5,54	0,43
EC(mS/m)	4,52	51,31	18,3	10,56	44,57	322,00	148,55	69,72	50,03	217,90	100,88	37,39
Eh(mV)	1,5	38,9	15,6	10,49	3,00	59,20	32,53	13,99	-17,20	8,20	-5,95	6,68
OC%	3,62	8	5,7	0,99	0,04	1,12	0,49	0,27	0,46	4,87	2,15	0,67
CEC	0,22	1,73	0,8	0,22	1,10	1,86	1,43	0,16	1,73	2,44	2,07	0,18

Min = minimum, Max = Maximum, St Dev = Standard deviation, EC=electric conductivity; Eh=reduction potential; OC=organic carbon; CEC=cation exchange capacity

Descriptive statistics of soil physicochemical properties in the three fields at a depth of 20-40 cm: The mean values of clay and sand in VF were 29.06 ± 10.88 and 55.81 ± 17.36%, respectively. Soil pH (H_2O) ranged between 3.95 - 7.16, with an average of 5.50 ± 0.90. Soil pH (KCl) was observed to range between a minimum of 3.54 with a maximum of 5.86, at an average of 4.82 ± 0.59. Electrical Conductivity values ranged between 4.66 – 34.48 mS/m with an average of 18.54 ± 10.73. Soil OC % ranged between 2.15% - 9.15% with an average of 5.77 ± 1.35. Cation exchange capacity ranged between 0.32 – 1.55 mS/m, with an average of 0.72 ± 0.20 (Table 3.3).

Clay percentage of CF had a range of 4% - 28% and an average of 13.90 ± 5.20. Sand % ranged from 0% to as high as 84%. Soil pH varied from acidic to alkaline throughout the field (5.71 - 7.56), with an average of 6.27 ± 0.33 (Table 3.3). Soil pH (KCl) also showed a high potential acidity with a minimum value of 4.60 ranging up to 6.69. Soil pH (KCl) had an average of 5.30 ± 0.43. Soil EC had very high values that could lead to salinity hazards if proper management is not applied.

Soil EC varied from 39.12 to 235 mS/m, with an average of 119.24 ± 42.42. Soil reduction potential ranged from 6.20 mV to 57. 70 mV with an average of 31.87 ± 12.97 Organic carbon percentage and CEC had ranges between 0.07 – 1.21 and 1.25 -1.62, respectively. Soil OC% had an average 0.44% ± 0.27% and CEC had an average of 1.43 ± 0.09 (Table 3.3).

Clay% and sand% at FF were reported to have minimum values of 23.20 and 41.60, respectively, and maximum values of 47.20 and 69.60. Mean values were 33.50 ±

5.79 and 54.20 ± 7.05, respectively. Soil pH (H_20) and pH (KCl) values ranged between minimum of 7.20 and 4.75 with maximum values of 8.55 and 6.31. Soil pH (H_2O) and pH (KCl) had average values of 7.65 ± 0.23 and 5.17 ± 0.29. Soil EC values ranged between 39.76 and 169.10 with an average value of 87.51 ± 31.62. Reduction potential was recorded between -22.20 to 12.10 with an average of -5.56 ± 7.36. Soil OC % and CEC had minimum values of 0.69 and 1.64 and maximum values of 2.80 and 2.48, respectively. Their average values were 1.71 ± 0.51 and 2.00 ± 0.17 (Table 3.3).

Descriptive statistics of soil physicochemical properties in the three fields at a depth of 40-60 cm: The mean values of clay and sand in VF were 31.53 ± 11.11 and 53.85 ± 15.72%, respectively. Soil pH (H_2O) ranged between 3.33 – 7.16, with an average of 5.33 ± 1.05. Soil pH (KCl) was observed to range between a minimum of 3.77 with a maximum of 6.12, at an average of 4.86 ± 0.53. Electrical conductivity values ranged between 4.52 – 299 mS/m with an average of 25.35 ± 46.11. Soil Eh had arrange of 2.5 – 32.7 with average of 17.26 ± 10.59. Soil OC % ranged between 2.63% - 8.6% with an average of 5.52 ± 1.15. Cation exchange capacity ranged between 0.22 – 2.25, with an average of 0.75 ± 0.29 (Table 3.4).

Clay percentage of CF had a range of 4% - 32% and an average of 15.25 ± 7.68. Sand % ranged from 4% to as high as 84% with an average of 48.60 ± 23.54. Soil pH varied from strongly acidic to alkaline throughout the field (3.27 – 6.94), with an average of 6.08 ± 0.56 (Table 3.4). Soil pH (KCl) also showed a low potential acidity with a minimum value of 4.51 ranging up to 6.61. Soil pH (KCl) had an average of 5.48 ± 0.54. Soil EC varied from 29.29 to 298.1 mS/m, with an average of 105.96 ±

52.23. Soil Eh at CF had a range of 10 - 56.7 with average of 29.46 ± 11.37. Organic carbon percentage and CEC had ranges between 0.07 – 1.00 and 0.95 -1.78, respectively. Soil OC% had an average 0.38% ± 0.25% and CEC had an average of 1.36 ± 0.15 (Table 3.4).

Clay and sand percentages at FF were reported to have minimum values of 23 and 38, respectively, and their maximum values of 47 and 66. Mean values were 33.51 ± 5.97 and 52.16 ± 7.14, respectively. Soil pH (H_20) and pH (KCl) values ranged between minimum of 4.01 and 4.53 with maximum values of 8.84 and 6.75. Soil pH (H_2O) and pH (KCl) had average values of 7.40 ± 0.71 and 5.22 ± 0.38. Soil EC values ranged between 22.76 and 166.20 with an average value of 79.55 ± 33.19. Reduction potential was recorded between -22.4 to 8.30 with an average of -6.79 ± 7.15. Soil OC % and CEC had minimum values of 0.46 and 1.39 and maximum values of 2.71 and 2.93, respectively. Their average values were 1.75 ± 0.64 and 1.95 ± 0.26 (Table 3.4).

Table 3.3. Descriptive statistics for soil physicochemical properties in the three fields at 20-40 cm.

Basic soil properties	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
%Clay	14	67	29,06	10,88	4	28	13,90	5,20	23,20	47,20	33,50	5,79
%Sand	12	83	55,81	17,36	0	84	57,78	20,21	41,60	69,60	54,20	7,05
pH (H_20)	3,95	7,16	5,50	0,90	5,71	7,56	6,27	0,33	7,20	8,55	7,65	0,23
pH (KCl)	3,54	5,86	4,82	0,59	4,60	6,69	5,30	0,43	4,75	6,31	5,17	0,29
EC (mS/m)	4,66	34,48	17,51	8,42	39,12	235,30	119,24	42,42	39,76	169,10	87,51	31,62
Eh(mV)	2,83	35,8	18,54	10,73	6,20	57,70	31,87	12,97	-22,20	12,10	-5,56	7,36
OC%	2,15	9,15	5,77	1,35	0,07	1,21	0,44	0,27	0,69	2,80	1,71	0,51
CEC	0,32	1,55	0,72	0,20	1,25	1,62	1,42	0,09	1,64	2,48	2,00	0,17

Min = minimum, Max = Maximum, St Dev = Standard deviation, EC=electric conductivity; Eh=reduction potential; OC=organic carbon; CEC=cation exchange capacity

Table 3.4. Descriptive statistics for soil physicochemical properties in the three fields at 40-60 cm.

Basic soil properties	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
%Clay	12	56	31,53	11,11	4	32	15,25	7,68	23	47	35,51	5,97
%Sand	20	73	53,85	15,72	4	84	48,60	23,54	38	66	52,16	7,14
pH (H_20)	3,33	7,16	5,33	1,05	3,265	6,94	6,08	0,56	4,01	8,84	7,40	0,71
pH (KCl)	3,77	6,12	4,86	0,53	4,51	6,61	5,48	0,54	4,53	6,75	5,22	0,38
EC (mS/m)	4,52	299	25,35	46,11	29,29	298,1	105,96	52,23	22,76	166,20	79,55	33,19
Eh(mV)	2,5	32,7	17,26	10,59	10	56,7	29,46	11,37	-22,40	8,30	-6,79	7,15
OC%	2,63	8,6	5,52	1,15	0,07	1,00	0,38	0,25	0,46	2,71	1,75	0,64
CEC	0,22	2,25	0,75	0,29	0,95	1,78	1,36	0,15	1,39	2,93	1,95	0,26

Min = minimum, Max = Maximum, St Dev = Standard deviation, EC=electric conductivity; Eh=reduction potential; OC=organic carbon; CEC=cation exchange capacity

Field (A) × soil depth (B) interaction and field were each highly significant ($P \leq 0.01$) on clay, contributing 1 and 96% in total treatment variation (TTV) of the variable, respectively (Table 3.3). However, the soil depth alone had no significant effects on clay (Table 3.3). The blocking effects had highly significant effects on clay content, although the effects were negligent at 2%. In contrast, A × B interaction and soil depth were each significant ($P \leq 0.05$) on sand, contributing 24 and 37% in TTV of the variable, respectively, whereas field type had no effects on the variable. The blocking effects were highly significant on sand, thus contributing 17% in TTV of the respective variables (Table 3.5).

Table 3.5: Total treatment variation (TTV) in a field × soil depth factorial experiment on percentage clay and percentage sand under irrigation with treated wastewater.

Source	DF	Clay		Sand	
		MSS	TTV (%)	MSS	TTV (%)
Rep	39	162.03	2^{***}	444.67	17***
Field (A)	2	15484.06	96^{***}	321.88	12^{ns}
Depth (B)	2	123.04	1^{ns}	986.65	37**
A × B	4	235.51	1^{***}	646.12	24**
Error	312	64.75	0	240.74	10
Total	359	16069.39	100	2640.06	100

TTV (%) = Total Treatment Variation = (MSS/TOTAL) × 100

***Highly significant at $P \leq 0.01$; **Significant at $P \leq 0.05$, ^{ns}Not significant at $P \leq 0.05$.

Field (A) × depth (B) interaction was significant on pH (KCl) and EC at $p \leq 0.05$ contributing 5% and 2% in total treatment variation (TTV) of the variable, respectively. However, A × B interaction was not significant for pH (H_20), Eh, OC and CEC (Table 3.6) with the blocking effects that were significant and negligent at 1%, 1%, 0% and 0%, respectively. The blocking effects had highly significant effects on soil pH (KCl) and EC, although the effects were negligent at 4 and 1% respectively and in contrast, blocking effects were not significant for OC. Field type was highly significant ($P \leq 0.01$) on soil pH (H_20), pH (KCl), EC, Eh, OC and CEC, with each contributing 96%, 85%, 93%, 99% and 99% in total treatment variation (TTV) of the variable, respectively. Soil depth was not significant for Eh (Table 3.6).

Clay percentage

Relative to VF at depth 0-20 cm, clay reduced by 69% at CF and increased by 1% at FF (Table 3.7). At depth 20-40 cm clay soil decreased by 57% for CF and increased by 1% for fallowed field, both in relation to VF. As for depth 40-60 cm, relative to VF clay decreased by 46% in CF and increased by 15% in FF (Table 3.7).

Sand percentage

Relative to VF, sand increased by 15% in CF at the depth of 0-20 cm, and FF decreased by 5%. An increase of 5% at 20-40 cm for CF and a decrease of 1% at FF were observed. At 40-60 cm depth, sand decreased by 9% in CF and increased by 0.4% in FF all in relation to VF (Table 3.8).

Table 3.6: Total treatment variation (TTV) in a field × soil depth factorial experiment on basic chemical properties of soil under irrigation with treated wastewater.

Source	DF	pH ($H_2$0)		pH (KCl)		EC		Eh		OC		CEC	
		MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Rep	39	0.78	1^{**}	0.53	4^{***}	3310.86	1^{***}	218.86	1^{***}	0.76	0^{ns}	0.06	0^{***}
Field (A)	2	131.93	96^{***}	10.68	85^{***}	334256.29	93^{***}	43009.28	99^{***}	895.64	100^{***}	47.98	99^{***}
Depth (B)	2	2.85	2^{***}	0.57	5^{**}	12676.15	4^{***}	119.88	0^{ns}	2.51	0^{**}	0.15	0^{**}
A × B	4	0.06	0^{ns}	0.67	5^{***}	5866.17	2^{***}	14.42	0^{ns}	0.58	0^{ns}	0.04	0^{ns}
Error	312	1.22	1	0.18	1	1487.26	0	96.55	0	0.66	0	0.358	1
Total	359	136.84	100	12.63	100	357596.73	100	43458.99	100	900.15	100	48.59	100

TTV (%) = Total Treatment Variation = (MSS/TOTAL) × 100,

***Highly significant at $P \le 0.01$; **Significant at $P \le 0.05$, ^{ns}Not significant at $P \le 0.05$., EC=electric conductivity; Eh=reduction potential; OC=organic carbon; CEC=cation exchange capacity.

Table 3.7: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on clay percentage relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	35.13^{ab}	−	32.07^{b}	−	28.0^{ab}	−
Cultivated field$^+$	10.9^{c}	− 69	13.9^{c}	− 57	15.25^{c}	− 46
Fallowed field$^+$	35.6^{a}	1	32.4^{b}	1	32.1^{b}	15

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 3.8: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on sand percentage relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	55.08^{ab}	−	55.11^{ab}	−	53.2^{ab}	−
Cultivated field$^+$	63.2^{a}	15	57.78^{ab}	5	48.6^{b}	−9
Fallowed field$^+$	52.3^{ab}	−5	54.3^{ab}	−1	53.4^{ab}	0.4

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Soil pH (H_2O) and pH (KCl)

Table 3.9 shows the relative impact of the different cultivation intensities on soil pH (H_2O) with VF being the basis of comparison. In relation to VF, soil pH (H_2O) increased by 33% in the CF and by 35% in the FF at a depth 0-20 cm. An increase of 14% at CF and of 1% at FF were obtained at a depth 20-40 cm. Relative to VF, soil pH (H_2O) increased by 15% in CF and 41% in FF.

Relative to VF, soil pH (KCl) at depth 0-20 cm increased in both CF and FF by 11% and 12%, respectively. Moreover, at depths 20-40 and 40-60 soil pH (KCl) increased by 11% and 13% for CF, respectively, and increased again by 7% and 7% in FF (Table 3.10).

Table 3.9 Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on soil pH (H_2O) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	5.66^{cd}	–	5.52^{d}	–	5.30^{d}	–
Cultivated field$^+$	7.55^{a}	33	6.27^{b}	14	6.08^{b}	15
Fallowed field$^+$	7.67^{a}	35	7.56^{a}	37	7.49^{ab}	41

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 3.10: Effects of cultivated fields irrigated with (+) and without (-) treated

wastewater on pH (KCl) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field^{-}	4.83^{d}	–	4.80^{d}	–	4.85^{cd}	–
Cultivated field^{+}	5.35^{ab}	11	5.30^{ab}	11	5.48^{ab}	13
Fallowed field^{+}	5.41^{a}	12	5.34^{bc}	7	5.17^{ab}	7

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100

Electrical conductivity and reduction potential

Table 3.11 illustrates how EC was relatively affected in different depths of different fields with varying cultivation intensities. Relative to VF, EC in CF increased by 710% at 0-20 cm, and again by 512% at 20-40 cm and lastly, increased by 337% at 40-60 cm depth. As for FF, EC also increased at very high percentages of 426% at 0-20 cm, 337% at 20-40 cm and 259% at 40-60 cm, and all these impacts are in relation to VF.

Virgin field served as the basic of comparison or the control factor for all variables. Relative to VF, an increase of Eh in CF was obtained for all depths by 108% for 0-20 cm, 72% for 20-40 cm and 53% for 40-60 cm. The fallowed field showed a decrease of Eh for all depths in relation to VF and its corresponding three depths. It decreased by 140% at 0-20 cm, 125% at 20-40 cm and 139% at 40-60 cm depths (Table 3.12).

Table 3.11: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on electrical conductivity (EC) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field^{-}	18.34^{d}	−	19.48^{d}	−	24.24^{d}	−
Cultivated field^{+}	148.55^{a}	710	119.24^{b}	512	105.96^{bc}	337
Fallowed field^{+}	96.47^{bc}	426	85.04^{c}	337	86.96^{c}	259

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 3.12: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on reduction potential (Eh) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field^{-}	15.64^{b}	−	18.60^{b}	−	19.20^{b}	−
Cultivated field+	32.52^{a}	108	31.87^{a}	72	29.46^{a}	53
Fallowed field+	-6.25^{c}	−140	-4.63^{d}	−125	-7.40^{c}	−139

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Organic carbon and cation exchange capacity

Organic carbon decreased in all the depths and different fields in relation to VF. A decrease of 91% at 0-20 cm, 93% at 20-40 cm and 93% at the 40-60 cm was observed in CF in relation to VF and the corresponding depths, respectively. In FF, there was also a decrease in all depths with 63% at 0-20 cm, 68% at 20-40 cm and 71% at 40-60 cm (Table 3.13).

Table 3.13: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on organic carbon (OC) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	5.67^{ab}	–	5.94^{a}	–	5.56^{ab}	–
Cultivated field$^+$	0.49^{c}	−91	0.44^{c}	−93	0.38^{c}	−93
Fallowed field$^+$	2.09^{b}	−63	1.90^{b}	−68	1.63^{b}	−71

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Cation exchange capacity increased at all depths and different fields. It was observed that in relation to VF, CF increased by 89% at depth 0-20 cm, 90% at depth 20-40 cm and by 86% at depth 40-60 cm. Furthermore, in FF, an increase of 170% at 0-20 cm, 171% at depth 20-40 cm and 167% at depth 40-60 cm was observed (Table 3.14).

Table 3.14: Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on cation exchange capacity (CEC) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	0.76^{c}	–	0.75^{c}	–	0.73^{c}	–
Cultivated field$^+$	1.43^{b}	89	1.42^{b}	90	1.36^{b}	86
Fallowed field$^+$	2.05^{a}	170	2.02^{a}	171	1.96^{a}	167

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

3.3.3. Descriptive statistics for essential and non-essential bioavailable heavy metals at a depth of 0-20 cm. Concentrations of Zn ranged between 0.01 – 17.9 mg/kg with an average value of 5.34 mg/kg ± 7.46 at VF, ranged between 36.60 – 73.60 with an average of 51.41 ± 6.65 at CF and lastly ranged between 52.5 – 182.98 with an average of 109.5 ± 35.18 at FF High concentrations of Fe were observed in CF and FF with ranges of 200 - 816 mg/kg and 280 – 1220, respectively, whereas in VF it ranged between 0.8 – 19.6 mg/kg. Manganese values ranged from 0.01 – 24.9 mg/kg with an average of 6.46 ± 9.62 in VF, in CF it ranged between 0.04 – 126 mg/kg with an average of 44.95 ± 28.17 and in FF it ranged between 9.12 – 84 mg/kg with an average of 53.61 ± 22.2.

Cobalt values increased from VF through to FF with ranges of 0.27 mg/kg – 22.3 mg/kg in VF; 15.56 – 34.92 in CF and 30.1 – 74.98 in FF (Table 3.15). The average concentrations of Cu in the three fields were 2.83 mg/kg ± 5.11 in VF; 6.74 mg/kg ± 0.78 and 12.75 ± 4.21 in FF.

Aluminium was observed to have the highest concentration among all the non-essential heavy metals, reaching its highest at CF. Its concentrations ranged between 1.65 – 22.80 mg/kg of soil with an average of 11.58 mg/kg ± 5.84 in VF, 141.80 – 389.60 mg/kg with an average of 270.32 ± 48.37 in CF and in FF the range was 12.23 – 33.75 with an average of 24.83 ± 5.48. The concentrations of As in the three fields are as follows, 0.28 – 19.80 (5.11mg/kg ± 4.64) in VF, 0.09 – 18.60 (4.50 mg/kg ± 4.30) in CF and 0.05 – 16.25 (4.43 ± 3.70) in FF. The concentrations of Cr in the three fields are as follows, 0 – 47 (6.46 mg/kg ± 8.77) in VF, 0.64 – 8 (3.01 mg/kg ± 2.10) in CF and 0.41 – 99.50 (16.73 ± 27.07) in FF. The concentrations of Cd in the three fields are as follows, 0.05 – 13.10 (1mg/kg ± 2.03) in VF, 2.10 – 3.90

(1.15 mg/kg ± 1.37) in CF and 0.24 – 1.91 (0.64 ± 0.54) in FF. The concentrations of Pb in the three fields are as follows, 0.11 – 6.10 (0.79 mg/kg ± 0.98) in VF, 0.03 – 18.60 (2.89 mg/kg ± 4.09) in CF and 0.02 – 14.75 (2.14 ± 3.49) in FF (Table 3.15).

Table 3.15. Descriptive statistics of essential and non-essential heavy metals in the three fields at 0-20 cm.

	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
Essential heavy metals (mg/kg)												
Co	0,27	22,3	6,46	5,97	15,56	34,92	29,65	3,26	30,1	74,98	50,66	11,36
Cu	0	27,2	2,83	5,11	3,34	8,20	6,74	0,78	6,65	25,48	12,75	4,21
Fe	0,8	19,6	4,49	3,47	200,00	816,00	375,05	141,96	280	1220	733,44	200,89
Mn	0,01	24,9	6,46	9,62	0,04	126,00	44,95	28,17	9,12	84	53,61	22,2
Zn	0,01	17,9	5,34	7,46	36,60	73,60	51,41	6,65	52,5	182,98	109,5	35,18
Non-essential heavy metals (mg/kg)												
Al	1,65	22,80	11,58	5,84	141,80	389,60	270,32	48,37	12,23	33,75	24,83	5,48
As	0,28	19,80	5,11	4,64	0,09	18,60	4,50	4,30	0,05	16,25	4,43	3,70
Cr	0,00	47,00	6,46	8,77	0,64	8,00	3,01	2,10	0,41	99,50	16,73	27,07
Cd	0,05	13,10	1,00	2,03	2,10	3,94	11,46	13,71	0,24	19,10	0,64	0,54
Pb	0,11	6,10	0,79	0,98	0,03	1,860	2,89	4,09	0,02	1,475	2,14	3,49

Min = minimum, Max = Maximum, St Dev = Standard deviation, As = arsenic, Cr = chromium, Pb = lead, Al = aluminium, Cd = cadmium, Zn = Zinc, Fe = iron, Cu= copper, Co = cobalt, Mn = manganese

Descriptive statistics for essential and non-essential bioavailable heavy metals at a depth of 20 – 40 cm. Concentrations of Zn ranged between 0.00 – 17.75 mg/kg with an average value of 5.37 mg/kg ± 7.50 at VF, ranged between 25.40 – 64 with an average of 49.93 ± 6.84 at CF and lastly ranged between 32.90 – 177.50 with an average of 101.60 ± 37.50 at FF. High concentrations of Fe were observed in CF and FF with ranges of 177 - 928 mg/kg and 494.75 – 1575, respectively, whereas in VF it ranged between 1.49 – 20.30 mg/kg. Manganese values ranged from 0.01 – 24.10 mg/kg with an average of 6.11 ± 9 in VF, in CF it ranged between 26.68 – 171.20 mg/kg with an average of 49.02 ± 30.30 and in FF it ranged between 8.63 – 86.50 mg/kg with an average of 55.13 ± 20.82.

Cobalt values increased from VF through to FF with ranges of 0.97 mg/kg – 30.40mg/kg in VF; 24.40 – 35.88 in CF and 37.80 – 69 in FF (Table 3.16). The average concentrations of Cu in the three fields were 2.56 mg/kg ± 3.82 in VF; 6.87 mg/kg ± 0.52 and 13.29 ± 3.76 in FF.

Aluminium was observed to have the highest concentration among all the non-essential heavy metals, reaching its highest at CF. Its concentrations ranged between 1.45 – 19.95 mg/kg of soil with an average of 11.95 mg/kg ± 4.87 in VF, 182 – 383.20 mg/kg with an average of 283.25 ± 51.70 in CF and in FF the range was 16.70 – 41.75 with an average of 27.64 ± 4.87. The concentrations of As in the three fields are as follows, 0.61 – 20.2 (4.90 mg/kg ± 3.76) in VF, 1.43 – 14.13 (5.26 mg/kg ± 2.88) in CF and 0.66 – 15.53 (6.51 ± 4.05) in FF. The concentrations of Cr in the three fields are as follows, 0.13 – 21.90 (4.98 mg/kg ± 4.45) in VF, 0.43 – 7.90 (2.63 mg/kg ± 1.85 in CF and 1.20 – 121.50 (19.26 ± 28.05) in FF. The concentrations of Cd in the three fields are as follows, 0.08 – 2.85 (0.97 mg/kg ±

0.70) in VF, 2.87 – 6.56 (4.50 mg/kg ± 1.10) in CF and 0.36 – 9.91 (3.37 ± 5.65) in FF. The concentrations of Pb in the three fields are as follows, 0.01 – 8.54 (0.83 mg/kg ± 1.35) in VF, 0.17 – 9.52 (3.75 mg/kg ± 6.51) in CF and 0.07 – 15.13 (2.77 ± 4.12) in FF (Table 3.16).

Descriptive statistics for essential and non-essential bioavailable heavy metals at a depth of 40 - 60 cm. Concentrations of Zn ranged between 0.01 – 18.20 mg/kg with an average value of 5.74 mg/kg ± 7.50 at VF, ranged between 35.40 – 63.60 with an average of 50.46 ± 6.77 at CF and lastly ranged between 45.55 – 170 with an average of 107.56 ± 36.29 at FF. High concentrations of Fe were observed in CF and FF with ranges of 214 - 936 mg/kg and 297 - 1135, respectively, whereas in VF it ranged between 1.49 – 11.20 mg/kg. Manganese values ranged from 0.01 – 26.20 mg/kg with an average of 6.56 ± 9.56 in VF, in CF it ranged between 5.76 – 104 mg/kg with an average of 41.56 ± 17.05 and in FF it ranged between 6 – 109.5 mg/kg with an average of 61.54 ± 24.49.

Cobalt values increased from VF through to FF with ranges of 0.59 mg/kg – 21.80 mg/kg in VF; 26.12 – 36.28 in CF and 42.1 – 75.5 in FF (Table 3.17). The average concentrations of Cu in the three fields were 3.30 mg/kg ± 5.92 in VF; 6.96 mg/kg ± 0.46 and 14.09 ± 5.34 in FF.

Aluminium was observed to have the highest concentration among all the non-essential heavy metals, reaching its highest at CF. Its concentrations ranged between 1.94 – 21.30 mg/kg of soil with an average of 11.31 mg/kg ± 5.24 in VF, 211 – 444 mg/kg with an average of 290.33 ± 55.39 in CF and in FF the range was 19.74 – 37.75 with an average of 28.97 ± 4.91. The concentrations of As in the three fields are as follows, 0 – 22.04 (5.58 mg/kg ± 4.89) in VF, 0.11 – 14.33 (6.33 mg/kg ± 4.01) in CF and 0.07 – 16.03 (8.33 ± 4.93) in FF. The concentrations of Cr in the three fields are as follows, 0 – 14.80 (4.71 mg/kg ± 3.92) in VF, 0.14 – 7.9 (2.95 mg/kg ± 2.04) in CF and 20.83 – 29.36 (1.70 ± 1.09) in FF. The concentrations of Cd in the three fields are as follows, 0.11 – 6.10 (0.79 mg/kg ± 1.04) in VF, 0.04 – 26.84 (2.66 mg/kg ± 4.75) in CF and 2.77 – 4.33 (0.02 ± 1.14) in FF. The concentrations of Pb in the three fields are as follows, 0.04 – 23.40 (1.35 mg/kg ± 3.78) in VF, 3.15 – 40.4 (12.93 mg/kg ± 14.69) in CF and 4.43 – 6.17 (0.28 ± 2.15) in FF (Table 3.17).

Table 3.16. Descriptive statistics of essential and non-essential heavy metals in the three fields at 20-40 cm.

	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
Essential heavy metals (mg/kg)												
Co	0,97	30,40	7,52	6,13	24,40	35,88	30,06	2,38	37,80	69,00	52,13	8,61
Cu	0,01	22,30	2,56	3,82	5,32	8,32	6,87	0,52	3,50	21,08	13,29	3,76
Fe	1,49	20,30	5,41	3,77	177,00	928,00	350,83	154,40	494,75	1575,00	841,74	191,79
Mn	0,01	24,10	6,11	9,00	26,68	171,20	49,02	30,30	8,63	86,50	55,13	20,82
Zn	0,00	17,75	5,37	7,50	25,40	64,00	49,93	6,84	32,90	177,50	101,60	37,50
Non-essential heavy metals (mg/kg)												
Al	1,45	19,95	11,95	4,87	182,00	383,20	283,25	51,70	16,70	41,75	27,64	4,87
As	0,608	20,4	4,90	3,76	1,43	14,13	5,26	2,88	0,66	15,53	6,51	4,05
Cr	0,13	21,90	4,98	4,45	0,43	7,90	2,63	1,85	1,20	121,50	19,26	28,05
Cd	0,08	2,85	0,97	0,70	2,87	6,56	2,50	14,10	0,36	19,91	3,79	5,65
Pb	0,01	8,54	0,83	1,35	0,17	9,52	3,75	6,51	0,07	15,13	2,77	4,12

Min = minimum, Max = Maximum, St Dev = Standard deviation, As = arsenic, Cr = chromium, Pb = lead, Al = aluminium, Cd = cadmium, Zn = Zinc, Fe = iron, Cu= copper, Co = cobalt, Mn = manganese

Table 3.17. Descriptive statistics of essential and non-essential heavy metals in the three fields at 40-60 cm.

	Virgin field				Cultivated field				Fallowed field			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
Essential heavy metals (mg/kg)												
Co	0,59	21,80	6,01	5,27	26,12	36,28	30,15	1,79	42,1	75,5	55,06	9,73
Cu	0,00	27,20	3,30	5,92	6,34	8,36	6,96	0,46	3,92	28,5	14,09	5,34
Fe	1,49	11,20	5,04	3,23	214,00	936,00	378,70	162,05	297,5	1135	805,67	175,95
Mn	0,01	26,20	6,56	9,56	5,76	104,00	41,56	17,05	6	109,5	61,54	24,49
Zn	0,01	18,20	5,74	7,50	35,40	63,60	50,46	6,77	107,56	36,29	45,55	170
Non-essential heavy metals (mg/kg)												
Al	1,94	21,30	11,31	5,24	211,2	444	290,33	55,39	19,738	37,75	28,97	4,91
As	0	22,04	5,58	4,89	0,11	14,33	6,33	4,01	0,07	16,03	8,33	4,93
Cr	0	14,80	4,71	3,92	0,14	7,9	2,95	2,04	20,83	29,36	1,70	1,09
Cd	0,109	6,10	0,79	1,04	0,04	26,84	2,66	4,75	2,77	4,33	0,02	14,75
Pb	0,04	23,40	1,35	3,78	3,152	40,4	12,93	14,69	4,43	6,17	0,28	21,5

Min = minimum, Max = Maximum, St Dev = Standard deviation, As = arsenic, Cr = chromium, Pb = lead, Al = aluminium, Cd = cadmium, Zn = Zinc, Fe = iron, Cu= copper, Co = cobalt, Mn = manganese

3.3.4. Distribution of bioavailable essential heavy metals

Field (A) × soil depth (B) interaction was significant ($P \leq 0.05$) on Fe and Cu with each contributing 0% in total treatment variation (TTV) of the variable, respectively. However, A × B interaction was not significant for Zn, Co and Mn (Table 3.18). Blocking effects were significant for Fe and Cu although the effects were negligent at 0% and 1%. However, blocking effects were not significant for Zn, Co and Mn. Field type was highly significant ($P \leq 0.01$) on Zn, Fe, Cu, Co and Mn with each contributing 99%, 99%, 98%, 100% and 98% in total treatment variation (TTV) of the variable, respectively. Soil depth was not significant for all the selected essential heavy metals (Table 3.18).

Table 3.18: Total treatment variation (TTV) in a field × soil depth factorial experiment on essential bioavailable heavy metals under irrigation with treated wastewater.

Source	DF	Zn		Fe		Cu		Co		Mn	
		MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Rep	39	587.15	0^{ns}	28207.17	0^{**}	23.90	1^{**}	54.27	0^{ns}	231.35	0^{ns}
Field (A)	2	305537.10	99^{***}	18674163.50	99^{***}	3231.87	98^{***}	63366.91	100^{***}	83847.58	98^{***}
Depth (B)	2	319.95	0^{ns}	30967.82	0^{ns}	11.41	0^{ns}	74.06	0^{ns}	107.79	0^{ns}
A × B	4	198.30	0^{ns}	52156.30	0^{**}	9.47	0^{**}	78.51	0^{ns}	557.76	1^{ns}
Error	312	459.34	0	18803.33	0	14.51	0	45.35	0	444.35	0.52
Total	359	307101.84	100	18804298.13	100	3291.17	100	63619.11	100	85188.82	100

TTV (%) = Total Treatment Variation = (MSS/TOTAL) × 100,

***Highly significant at P ≤ 0.01; **Significant at P ≤ 0.05, ^{ns}Not significant at P ≤ 0.05. Zn = zinc, Fe = iron, Cu = copper, Co = cobalt, Mn = manganese

3.3.5 Distribution of bioavailable non-essential heavy metals

Field (A) × soil depth (B) was significant for all the bioavailable non-essential heavy metals (Table 3.19). Blocking effects were highly significant on Cr, Pb, Al and Cd but were only significant for As, each heavy metal contributed 6%, 7%, 0%, 21% and 11% in total treatment variation (TTV) of the variable, respectively. The bioavailable non- essential heavy metals further contributed 90%, 91%, 100%, 70% and 20% in total treatment variation (TTV) of field type, respectively. Soil depth was only significant for As and Al with total treatment variation (TTV) contribution of 48% and 0%, respectively and depth was not significant for other heavy metals (Table 3.19).

Table 3.19: Total treatment variation (TTV) in a field × soil depth factorial experiment on non-essential bioavailable heavy metals under irrigation with treated wastewater

Source	DF	As		Cr		Pb		Al		Cd	
		MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Rep	39	28.15	11**	590.98	6***	321.52	7***	1636.66	0***	52.01	21***
Field (A)	2	50.51	20**	8903.60	90***	4064.94	91***	2750106.18	100***	177.09	70***
Depth (B)	2	118.82	48***	7.08	0^{ns}	21.24	1^{ns}	2407.87	0**	8.68	3^{ns}
A × B	4	33.97	14^{ns}	107.72	1^{ns}	7.15	0^{ns}	1011.32	0^{ns}	4.44	2^{ns}
Error	312	15.74	6	238.77	2	48.73	1	826.11	0	10.04	4
Total	359	247.20	100	9848.16	100	4463.58	100	2755988.15	100	252.28	100

TTV (%) = Total Treatment Variation = (MSS/TOTAL) × 100,

***Highly significant at P ≤ 0.01; **Significant at P ≤ 0.05, ^{ns}Not significant at P ≤ 0.05. As = arsenic, Cr = chromium, Pb = lead, Al = aluminium, Cd = cadmium

Factor A (field) showed high significance among the essential heavy metals and contributed the most in TTV% and thus mean separation was done. Relative to VF, the combined effects of treated wastewater and cultivation (hereafter referred to as the combined effects) in CF and FF increased Co, 338%, 669%; Cu by 120% and 330%; Fe by 76% and 67% and Zn by 11% and 134%, respectively. However, Mn only increased in CF by 624% and decreased by 99% in FF (Table 3.20).

Relative to VF, the combined effects of treated wastewater and cultivation in CF and FF increased Cd by 297% and 227% and Pb by 279% and 163%, respectively, for the fields. However, Cr increased by 239% in CF and reduced by 49% in FF (Table 3.21).

Aluminium showed significance for different fields and depth, thus mean separation was done. Relative to VF, the combined effects of treated wastewater and cultivation in CF and FF increased the concentration of Al at 0-20 cm by 122% and 193%; at 20-40 cm by 344% and 405% and at 40-60 cm by 469% and 442% (Table 3.22).

Table 3.20 Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on cobalt (Co), copper (Cu), manganese (Mn), iron (Fe) and zinc (Zn) relative to the virgin field

Treatment	Co	R.I. (%)	Cu	R.I. (%)	Mn	R.I. (%)	Fe	R.I. (%)	Zn	R.I. (%)
Virgin field$^-$	6.84^c	−	3.11^c	−	6.24^c	−	95.37^c	−	45.42^c	−
Cultivated field$^+$	29.95^b	338	6.85^b	120	45.18^b	624	168.2^b	76	50.48^b	11
Fallowed field$^+$	52.58^a	669	13.37^a	330	3.78^a	−99	159.68^a	67	106.24^a	134

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 3.21 Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on cadmium, chromium, and lead relative to the virgin field.

Treatment	Cd		Cr		Pb	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	0.78^b	–	5.58^b	–	3.4^b	–
Cultivated field$^+$	3.1^a	297	18.9^b	239	12.9^a	279
Fallowed field$^+$	2.55^a	227	2.86^b	−49	8.94^b	163

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 3.22 Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on aluminium (Al) relative to the virgin field.

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	9.11^{cd}	–	5.24^{cd}	–	3.34^d	–
Cultivated field$^+$	20.25^{ab}	122	23.25^{ab}	344	19.02^c	469
Fallowed field$^+$	26.71^a	193	26.46^a	405	18.11^c	442

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Relative to VF, the combined effects of treated wastewater and cultivation in CF, FF concentration of As at 0-20 cm decreased by 99% for both fields. At 20-40 cm, it decreased by 26% and 19%, respectively, and at depth 40-60 cm, it decreased by 99% on both fields (Table 3.23).

Table 3.23 Effects of cultivated fields irrigated with (+) and without (-) treated wastewater on arsenic (As) relative to the virgin field

FIELDS	0-20 cm		20-40 cm		40-60 cm	
	Variable	R.I. (%)	Variable	R.I. (%)	Variable	R.I. (%)
Virgin field$^-$	5.58^{bc}	−	9.84^{a}	−	6.61^{b}	−
Cultivated field$^+$	4.8^{d}	−99	7.26^{ab}	−26	5.33^{bc}	−99
Fallowed field$^+$	4.93^{d}	−99	7.99^{ab}	−19	5.79^{bc}	−99

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

3.4. Discussion

3.4.1 Soil pH, electric conductivity, reduction potential, organic carbon and cation exchange capacity of the soil in the three fields

Results from the current study observed an increase of soil pH from VF to FF, it was observed that the soil pH(H_20) increased with depth in both VF and FF but remained high in all depths of CF. With their specified averages outlined in tables 3.2 to 3.4 it can be concluded that the soil at VF is moderately acidic, soil is CF is slightly acidic and soil in FF is slightly alkaline following pH ranges by Oliveira *et al*.(2016). According to Bauder *et al*. (2009), the acceptable amount of soil pH should range from 6.5 - 8.4 coupled with an EC range of 250-750 mS/m, to avoid salinity problems. The average pH did not fall outside this range. The soil pH at CF implies that the bioavailability of heavy metals will be increased as they prefer an acidic pH to be mineralised and mobilized but at FF, they will return to the immobile state. Oliveira *et al*. (2016) reported an increase in soil pH during their experiments after irrigation with treated wastewater under semi-arid climate.

The electrical conductivity (EC) was the highest in CF, followed by FF, with the lowest in VF and in terms of depth, EC increased with depth in both VF and FF and was high in all depths in CF. Although the observed EC values fall within the acceptable ranges of EC in the soil, the results showed that with continuous irrigation with treated wastewater, EC values could increase to levels that could pose salinity hazards. A study conducted by Jahantigh (2008) proved that EC increased in soil irrigated with recycled or rather treated wastewater in an arid region and this followed a drastic increase in salts that was carried in the water. Jimoh and Mahmud (2014) reported a decrease in EC of the soil when the concentration of heavy metals increased during their research. Jimoh and Mahmud (2014) classified electrical conductivity of soil as non – saline < 200; moderately saline 200 – 1200; very saline >1200. Most of the soil samples in this study were moderately saline while some were non-saline. Fallowed field and VF were mostly non-saline while CF was saline, indicating that fallowing decreased the risk of salinity in the soil.

Soil Eh fluctuates normally between −300 and + 900 mV (Husson, 2013). Waterlogged soils have an Eh below +350 to +250 mV while dry soils above +380 to +400 mV (Husson, 2013; Seo and DeLaune, 2010). The more positive the potential, the greater the species attraction for electrons and tendency to be reduced. This means that when the soil has more positive Eh values, the more likely it is to be concentrated with ion of heavy metals, resulting in toxicity (Seo and DeLaune, 2010). Four main classes of soil conditions can be determined according to Eh values. These are aerated soils that have an Eh over +400 mV; moderately reduced soils having between +100 and +400 mV; reduced soils with values between −100 and +100 mV; and highly reduced soils with −100 and −300 mV. Interestingly, pH and Eh are negatively correlated in soils (Husson, 2013; Macías and Arbestain, 2010). The average values of Eh in all the depths of VF qualified the soil condition as reduced. The Eh value of CF was 31.28 mV and that of FF was −6.09 mV, also qualifying them to be reduced soils, but variation was observed at the three fields which led to a high significance (Pezeshki, 2001). Because CF shows a possibility of increasing its Eh with irrigation of treated wastewater, this will also make heavy metals more bioavailable in this field than the other two fields, and the opposite will happen to FF. Borch *et al*. (2010) reported that redox processes have a major control over the chemical speciation, bioavailability, toxicity and mobility of major and trace elements.

Organic carbon and CEC decreased from VF to CF, and this could be due to the constant disturbance of the soil through cultivation and increased at F. In terms of depth CEC and OC kept same trend of increasing with depth in both VF and FF while no trend was observed in CF and this could be the result of constant cultivation of the soil. Chipasa (2003) investigated the effect of wastewater on heavy metals availability in a treatment system; what was reported was that high amounts of organic matter reduced the concentration of heavy metals by immobilizing them. This includes soils containing minerals with a high specific surface and high CEC decreasing the solubility of heavy metals. Therefore, this justifies the increasing concentrations of some heavy metals observed in CF. An increase in soil organic matter leads to a decrease of soil Eh, meaning that in soils rich in easily decomposable organic matter, oxidation processes consume large amounts of oxygen, which leads to the formation of organic compounds with reducing properties (Husson, 2013).

3.4.2 Essential heavy metals of the soil in the three fields

The essential heavy metals employ biochemical and physiological functions in plants and animals. They are important constituents of several key enzymes and play important roles in various oxidation-reduction reactions (Wuana and Okieimen, 2011). In decreasing order in terms of quantity, the essential metals in VF ranged from Co > Mn > Zn > Fe > Cu with a decrease in the 2nd depth and an increase in the 3rd depth and in both FF and CF, the range were Fe > Zn > Mn > Co > Cu. The maximum permissible levels of Fe, Zn, Mn, Co and Cu in soil set by WHO are 50000 mg/kg, 300 mg/kg, 2000 mg/kg, 50 mg/kg and 100 mg/kg, respectively (Chiroma *et al*., 2014). Cobalt was reported to be above the permissible level at 53 mg/kg in CF;

although all the other elements increased, they were still within the permissible levels. The vertical distribution of the essential heavy metals was not significant, meaning that there was no defined trend that their concentrations followed. This could be because of their ionic status of 2 +. Brady and Weil (2010) reported that elements with the highest valence electrons are not easily moved from the exchange sites and by that, the elements could be accumulated at the depth with the highest clay content.

The United States Department of Agriculture report (USDA, 2017) showed that high soil pH resulted in high concentrations of anionic metals while low soil pH resulted in high concentrations of cationic metals. Cobalt concentration increased from VF with an average concentration of 6.84 mg/kg then went up to 30 mg/kg in CF and reached its highest value of 53 mg/kg in FF. Bioavailable Zn, Cu and Mn also increased from VF to CF and reached their highest concentration in FF, but Fe concentrations were higher in CF but lower in FF.

The change in soil pH and Eh had a major effect on the change in concentrations of these elements. According to Brady and Weil (2010), a decrease in Eh results in increased bioavailability of Mn, and this justifies its increase in the fields with corresponding decrease Eh concentration. This happens because when soil has negative Eh values; its affinity to electrons decrease and thus heavy metals may be available but not in a solution. Frohne *et al*. (2011) reported that deficiency is more likely to happen for Cu at a high pH and low Eh. According to Khaskhoussy *et al*. (2015), Cu concentration is not affected by irrigation with treated wastewater. Its fluctuation is solely based on the general change in soil physico-chemicals conditions. They also reported that Cu is stabilised by clay minerals, organic matter and oxides of Fe and Mn.

The concentration of Zn was affected by the application of treated wastewater as it has shown to drastically increase from VF to FF. It was reported that Zn deficiency in soil is more likely to happen when the pH is below 4 and above 8 coupled with high Eh (Frohne *et al*., 2011). Therefore, because the pH of the soil in this study was within that range, Zn bioavailability increased throughout VF to CF and then FF. The observed results were different from the study conducted by Khaskhoussy *et al*. (2015) in which they reported that the Zn concentration did not change due to irrigation with treated wastewater but was initially available in the soil and only leached and displaced to the deeper depths of the profile.

3.4.3 Non-essential elements of the soil in the three fields

From the results obtained, the amount of non-essential metals varied significantly and showed a decreasing trend of As > Cr > Al > Pb > Cd in VF, Al > Pb > As > Cd > Cr in CF and Cr > As > Pb > Al > Cd in FF. It was observed that the concentration of As was above the permissible levels in CF, with an average concentration of 11.679 mg/kg. Cadmium levels were above the permissible levels (WHO, 2001) in CF with an average concentration of 3.1 mg/kg in CF, on increased by 2.33 units from VF which had an average value of 0.77 mg/kg. Furthermore, it was found that with fallowing the amount of Cd reduced to 2.55 mg/kg which is below or within the expected limits. Chromium was only above the permissible level in FF with an average concentration of 18.9 mg/kg. Lead was also above the permissible level in CF with a concentration of 30 mg/kg. An increase in soil pH and decrease in soil Eh could have led to an increase in Al from VF to CF. This contradicts a study by

Olafisoye *et al*. (2013) whereby an increase in soil pH led to a decrease in Al availability as these two parameters clearly showed opposing trends. The results obtained in the study showed that concentrations in soils were generally higher for lead and lowest for Cd metal and similar to the findings by Olafisoye *et al*. (2013). Heavy metals are classified within a category of environmental toxins and the investigations of these metals place special importance on environmental safety (Kumar *et al*., 2013).

Non- essential metals show toxicity at very low concentrations, usually below 10 ppm (Chiroma *et al*., 2014). Arsenic, Pb and Hg are the first, second and third hazards on the priority list of heavy metal pollutants as designated by the United States Agency for Toxic Substances and Disease Registry (ATSDR, 2017). The maximum allowable concentrations of As, Cd, Cr and Pb in soil by guidelines of South Africa are 5.8 mg/kg, 3 mg/kg, 6.5 mg/kg and 20 mg/kg, respectively (Kamunda *et al*., 2016).

Cadmium and Pb concentrations were low at a low Eh and rose when the Eh increased, which can be attributed to interactions with dissolved organic carbon, manganese and precipitations such as sulfides (Frohne *et al*., 2011; Stepniewska *et al*., 2009). This trend was observed when Eh increased in CF and the amounts of Cd and Pb also increased. Cadmium solubility decreased with organic matter inputs because of the induced decrease in Eh and the increase in pH (Kashem and Singh, 2001). Conversely, the concentrations of As sharply decreased when the soil Eh increased, indicating that low Eh promotes the mobility of this element (Frohne *et al*., 2011). The presence of one metal is reported to have an effect on the availability of other metals. Bioavailable Cu was reported to increase the toxicity of Zn (Chibuike and Obiora, 2014). When varying cations were adsorbed equally by soil components the ratio of any two cations in soil solution would be approximately the same as the ratio at the exchange sites (Rieuwerts *et al*., 2015). However, mainly due to differences in the charges and hydrated radii of cations, selectivity of different metals by adsorbents occurs. Therefore, the presence of Cd as a soil contaminant may absolutely affect the bioavailability of Pb and Zn (Rieuwerts *et al*., 2015).

3.5 Conclusion

Virgin field was used as the base of comparison to rate whether irrigation with treated wastewater lead to heavy metal contamination. It was found that some toxic non-essential heavy metal such as Pb were in high concentration before irrigation with treated wastewater. Soils of cultivated field were contaminated with heavy metals, especially the non-essential heavy metals which were found to be over the recommended standard values following irrigation with treated wastewater.

Irrigation with treated wastewater had both positive and negative impacts on the soil physicochemical properties and bioavailability of heavy metals; it increased the bioavailability of most of the heavy metals in the soil. Fallowing, in this regard, has shown to be effective in reducing the toxic heavy metals and in some cases, increasing some essential heavy metals. The reduction in the concentration of the heavy metals concentration in fallowed field could be that treated wastewater was no longer used for irrigation. Microorganisms were also given a chance to remediate the soil and the basic soil physicochemical properties also changed such that bioavailability of heavy metals had to reduce. For example, soil pH has increased to levels that are reported to be able to make heavy metals immobile. Another reason could be that the weeds that grew on the fallowed field had absorbed the bioavailable heavy metals. Interestingly, this treated wastewater affected the bioavailability of heavy metals such that they were significant among different fields. Fallowing is then recommended for farmers that have the same problem of increased heavy metal pollution in their agricultural soils. For further studies, one could focus on suppressing the bioavailability of heavy metals from the irrigation water to reduce the pollution in the soil.

CHAPTER 4

BIOREMEDIATION OF NON-ESSENTIAL HEAVY METALS IN CULTIVATED AND FALLOWED SOILS FOLLOWING IRRIGATION WITH TREATED WASTEWATER.

4.1 Introduction

Industries are rapidly expanding and improving and while that happens, great amounts of toxic wastes such as heavy metals get released and spread in the environment and water sources (USDA, 2000). These heavy metals are then transferred into wastewater through runoffs. After purification, the treated wastewater is used for irrigation in agricultural fields. The treated wastewater containing large amount of heavy metals results in heavy metal pollution in soil (Reshma *et al*., 2011). There are several techniques that have been used to remove these heavy metals from water and soil and this includes chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical inoculum (Prathusha and Suneetha, 2011). Most of these techniques become unsuccessful when the concentrations of heavy metals are less than 100 mg/l (Olaniran *et al*., 2013). Some of the heavy metals such as Cd and As have toxic effects at very low concentrations of 0.01 ml/l and also the cleaning methods currently used are expensive, time consuming and still releases some other toxic wastes after removal of heavy metals (Ahluwalia and Goyal, 2007). Most heavy metal salts are water-soluble and get dissolved in wastewater, which means they cannot be separated by physical separation methods (Hussein *et al*., 2004).

The use of microorganisms such as bacteria and fungi for remediation purposes is thus a possible solution for heavy metal pollution since it includes maintainable remediation technologies to rectify and restore the natural condition of soil without giving off any more toxic substances (Dixit *et al*., 2015). Bioremediation refers to the use of microorganisms such as bacteria and fungi to detoxify heavy metals by either absorbing them or converting them into carbon oxide, energy or methane (Garima and Singh, 2014). Bioremediation relies on microbes that live naturally in soil and groundwater, and these microbes pose no threat to people at the site or in the community (EPA, 2012). Therefore, the objective of this study was to investigate the bioremediation abilities of Gram-positive and Gram-negative bacteria on non-essential heavy metals in treated wastewater irrigated fields.

4.2 Materials and methods

4.2.1 Description of the study site

The experiment was carried out in the Soil Science laboratory of University of Limpopo (28° 0' 59.76" E; 25° 36' 54" S), South Africa. Soil samples for the experiment were collected from a cultivated field (CF) and fallowed field (FF), as described in Chapter 3, Figure 3.1 at a depth of 0-20 cm.

4.2.2 Research design

The experiment was a 2 × 8 factorial study in completely randomised design. The first factor was the two fields which were CF and FF and the second factor was the microorganism inoculants. The second factor was made up of a before-inoculation sample (BI), control with no inoculant (0I); two Gram-negative microorganisms (*Providencia rettgeri* (A) and *Enterobacter cloacae* (B)); the combination of the two Gram-negative microorganisms (AB); two Gram-positive microorganisms (*Bacillus cereus* (C) and *Arthrobacter aurescens* (D) and the combination of two Gram-positive microorganisms (CD).

4.2.3 Data collection

Heavy metal analysis

Five non-essential heavy metals (As, Al, Cd, Cr and Pb) were extracted from the soil samples through the use of the EDTA method as described in Chapter 3 (Hesse, 1971).

Isolation of pure cultures of microorganisms

From the samples collected at 0-20 cm depth, 1 g of soil was weighed and suspended in 99 ml of distilled water to make soil suspension. Serial dilution was made up to 10^{-3} dilution factor. An aliquot of 1 ml from each dilution was taken and spread evenly over nutrient agar plates for growth of bacteria species. The plates were incubated at 37°C for the bacteria species for 7 days. A streaking plate was used to obtain single colonies of pure culture of bacteria species. Gram staining of the microorganisms was done to distinguish between the Gram-positive and Gram-negative microorganisms (Figure 4.1) (Zhang *et al*., 2010).

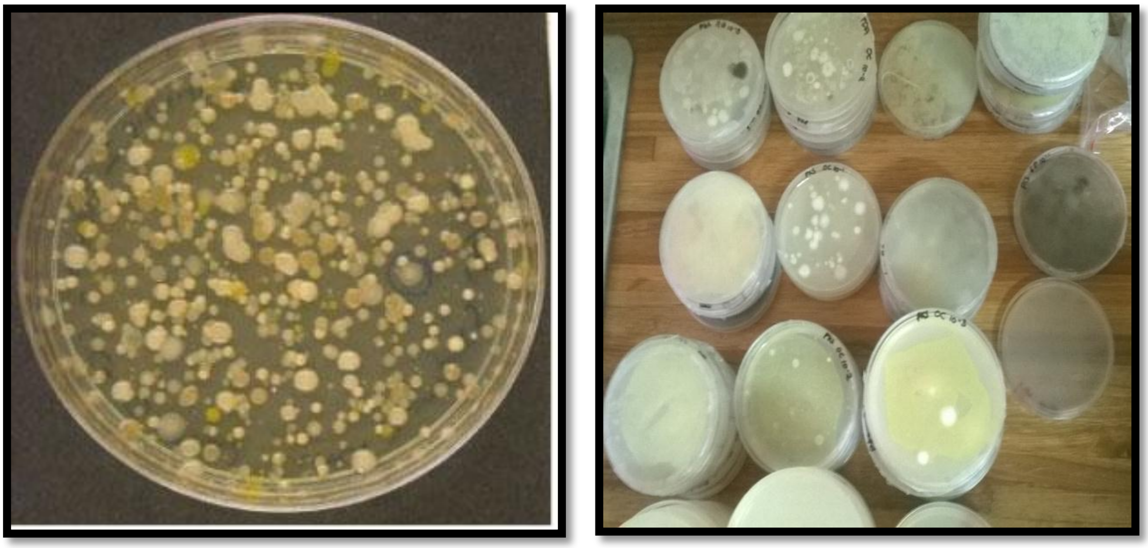

Figure 4.1: Nutrient agar plates showing different bacterial colonies from the soil samples during the process of isolation.

Identification of isolated pure cultures of microorganisms

Microorganisms were identified using a Maldi Biotyper through formic acid extraction method by Singhal *et al*. (2015), at the Biotechnology Unit, University of Limpopo. Fourteen Eppendorf tubes were sterilised and labelled according to the inoculums. Three hundred µl of deionized water was pipetted and transferred into each of the Eppendorf tubes. A quantity of pure culture biological material grown on agar plates (Figure 4.2) (between one colony and 5-10 mg) was transferred into the tubes in accordance with the labels and the respective samples and mixed thoroughly by vortexing. Nine hundred µl of alcohol was added into the tubes and mixed thoroughly. The samples were then centrifuged at maximum speed (15 000 rpm) for 2 minutes. The supernatant was decanted, and the samples were centrifuged again until the remaining alcohol was removed without disturbing the pellet. The alcohol-pellets were allowed to dry at room temperature for 2-3 minutes. Ten (10) ml of 70% formic acid was then added to the pellets and then mixed thoroughly by vortexing.

Ten ml of acetonitrile was then added to the samples, and the samples were mixed thoroughly by vortexing. Samples were then centrifuged at 15 000 rpm for 2 minutes. One µl of the supernatant was transferred onto a Maldi target plate and allowed to dry at room temperature. The samples on the Maldi targets were then overlayed with 1 µl of α-Cyano-4-hydroxycinnamic acid (HCCA) solution within 1 hour and allowed to dry at room temperature before being placed into the Maldi-tof for identification (Singhal *et al*., 2015).

Figure 4.2: Pure culture of a bacteria sample used for identification (*Bacillus cereus*).

Microbial culturing of the identified microorganisms

Prior to inoculation, four microorganisms were cultured following a method by Kastner *et al*. (1998). Briefly, four Erlenmeyer flasks (500 ml) were sterilized and labelled as per organism. Cultures of the microorganisms were transferred into the flasks filled with 250 ml nutrient broth. The flasks were incubated on a shaker at 150 rpm at 25^0 C for 72 hours. After 72 hours the samples were transferred into sterile centrifuge tubes and centrifuged at maximum speed in order to obtain single pellets.

The supernatant was then discarded without disturbing the pellets. Following protocol B, 5 ml of deionized water was added into the centrifuge tubes and mixed thoroughly. The samples were then ready to be used as inoculants (Kastner *et al*., 1998) (Figure 4.3 A).

Bioremediation process

Composite samples were made from all the samples collected from 0-20 cm with respect to each field (CF and FF). Eight 50 g of soil was weighed from composite samples of CF and FF and transferred into 16 100 ml sterile glass beakers. Each of the glass beakers were replicated 3 times and labelled according to the inoculums of the microorganisms. Each glass beaker was inoculated with the prepared samples in the centrifuge tubes except for the control. The samples were incubated at 37^0 C for 12 weeks and irrigated two times a week with 20 ml of distilled water. After 12 weeks the soils were analysed again for bioavailable heavy metals (Figure 4.3 B) (Fawole *et al*., 2017).

Figure 4.3: Inoculated samples (A) ready for incubation, and (B) incubated at 37^0 C

4.2.4 Data analysis

All data were subjected to factorial analyses of variance (ANOVA) through Statistix 10.0 version. Mean separation was done for significant means using Tukey’s multiple range test at $P \leq 0.05$.

4.3 Results

4.3.1 Morphological, microscopical and Gram staining characteristics of the microorganisms used for the process of bioremediation

The morphological, microscopical and gram staining characteristics of the microorganisms used for the process of bioremediation are presented in Table 4.1. The first bacterium was identified as *P. rettgeri* which developed as a cream colony with medium rods, and it tested negative on gram stain. The second Gram-negative bacterium was identified as *E. cloacae* which grew as cream white colony with medium sized rods. The two Gram-positive bacteria were *B. cereus* which grew as small rods, light brown colony and the other one was *A. aurescens* grew as pink colony with big rounds (Table 4.1).

Table 4.1: Identified microorganisms that were used for bioremediation non-essential heavy metals on cultivated and fallowed fields

Sample names	Morphological characteristic	Shapes under microscope	Gram staining results
Providencia rettgeri	Cream colony	Medium rods	Negative
Enterobacter cloacae	Cream-white colony	Medium rods	Negative
Bacillus cereus	Light-brown colony	Small rods	Positive
Arthrobacter aurescens	Pink colony	Big rounds	Positive

4.3.2 Non-essential heavy metal distribution following bioremediation

The field × inoculation effects were significant on Cd, Cr and Pb contributing 35%, 12% and 40% in TTV of the respective variables, but were not significant on Al and As (Table 4.1). Factor A (field) was not significant for all the selected non-essential heavy metals. Factor B (Inoculum) was highly significant on Al, As, Cd, Cr and Pb, contributing 76%, 55%, 50%, 74%, and 29% in TTV% of the respective variables (Table 4.2).

Distribution of cadmium: Relative to the reference sample in CF, OI reduced Cd by 67% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *P. rettgeri*, *E. cloacae*, *B. cereus*, and *A. aurescens* reduced Cd concentration by 77%, 95%, 73%, 80%, 58% and 90%, respectively. Relative to the reference sample in FF, OI reduced Cd by 54% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *E. cloacae*, *B. cereus*, and *A. aurescens* reduced Cd concentration by 3%, 26%, 60%, 14% and 0%. *P. rettgeri* increased Cd concentration by 8%, respectively (Table 4.3).

Distribution of chromium: Relative to the reference sample in CF, OI reduced Cr by 89% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *P. rettgeri*, *E. cloacae*, *B. cereus*, and *A. aurescens* reduced Cr concentration by 84%, 93%, 100%, 76%, 98% and 63%. Relative to the reference sample in FF, OI reduced Cr by 64%, whereas *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *E. cloacae*, *B. cereus*, *A. aurescens* and *P. rettgeri* reduced Cd concentration by 64%, 98%, 46%, 65%, 73% and 83%, respectively (Table 4.4).

Table 4.2 Total treatment variation (TTV) in a field × soil depth factorial experiment on non-essential heavy metals under irrigation with treated wastewater.

Source of variance	DF	Al		As		Cd		Cr		Pb	
		MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Rep	2	5.04	7	0.94	14	0.21	3	2.43	9	0.63	10
Field (A)	1	0.12	0^{ns}	1.2	18^{ns}	0.46	7^{ns}	0.07	0^{ns}	0.81	13^{ns}
Inoculation (B)	7	55.07	76^{***}	3.68	55^{***}	3.19	50^{***}	20.37	74^{***}	1.8	29^{*}
A × B	7	4.98	7^{ns}	0.22	3^{ns}	2.28	35^{***}	3.19	12^{*}	2.5	40^{***}
Error	30	7.04	10	0.71	11	0.29	5	1.36	5	0.57	9
Total	47	72.25	100	6.75	100	6.43	100	27.42	100	6.31	100

TTV (%) = Total Treatment Variation = (MSS/TOTAL) × 100,

***Highly significant at P ≤ 0.01; **Significant at P ≤ 0.05, ^{ns}Not significant at P ≤ 0.05.

Table 4.3 Response of cadmium (Cd) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria.

Inoculum	CF		FF	
	Cd	R.I. (%)	Cd	R.I. (%)
Before inoculum (BI)	4.24^{a}	–	1.94^{bc}	–
No inoculum control (I0)	1.38^{bcd}	− 67	0.89^{bcd}	− 54
P. rettgeri	1.13^{bcd}	− 73	2.09^{b}	8
E. cloacae	0.86^{bcd}	− 80	0.78^{bcd}	− 60
P. rettgeri + *E. cloacae*	0.99^{bcd}	− 77	1.87^{bc}	− 3
B. cereus	1.78^{bcd}	− 58	1.66^{bcd}	− 14
A. aurescens	0.44^{cd}	− 90	1.94^{bc}	0
B. cereus + *A. aurescens*	0.22^{d}	− 95	1.43^{bcd}	− 26

BI= Before inoculum=Reference sample.

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Table 4.4: Response of chromium (Cr) in cultivated field (CF) and fallowed field following bioremediation with selected bacteria.

Inoculum	CF		FF	
	Cr	R.I. (%)	Cr	R.I. (%)
Before inoculum (BI)	7.48^{a}	–	4.63^{ab}	–
No inoculum control (0I)	0.82^{c}	− 89	1.67^{bc}	− 64
P. rettgeri	0.01^{c}	− 100	0.76^{c}	− 83
E. cloacae	1.79^{bc}	− 76	2.48^{bc}	− 46
P. rettgeri + *E. cloacae*	1.18^{bc}	− 84	1.68^{bc}	− 64
B. cereus	0.15^{c}	− 98	1.62^{bc}	− 65
A. aurescens	2.80^{bc}	− 63	1.23^{bc}	− 73
B. cereus + *A. aurescens*	0.52^{c}	− 93	0.08^{c}	− 98

BI=Before Inoculum=Reference sample

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Distribution of lead: Relative to the reference sample in CF, OI reduced Pb by 80% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *P. rettgeri*, *E. cloacae*, *B. cereus*, and *A*. *aurescens* reduced Cd concentration by 77%, 80%, 80%, 83%, 83% and 82%, respectively. Relative to the reference sample in FF, OI reduced Cr by 79% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *E. cloacae*, *B. cereus*, *A. aurescens* and *P. rettgeri* reduced Cd concentration by 79%, 80%, 83%, 83%, 83% and 82%, respectively (Table 4.5).

Table 4.5: Response of lead (Pb) in cultivated field (CF) and fallowed field following bioremediation with selected bacteria.

Inoculum	CF		FF	
	Pb	R.I. (%)	Pb	R.I. (%)
Before inoculum (BI)	3.82 ^{a}	−	3.63 ^{a}	−
No inoculum control (0I)	0.76 ^{b}	− 80	0.76 ^{b}	−79
P. rettgeri	0.75 ^{b}	− 80	0.62 ^{b}	− 83
E. cloacae	0.66 ^{b}	− 83	2.06 ^{b}	− 43
P. rettgeri + *E. cloacae*	0.86 ^{b}	− 77	0.75 ^{b}	− 79
B. cereus	0.67 ^{b}	− 83	0.77 ^{b}	− 79
A. aurescens	0.68 ^{b}	− 82	0.63 ^{b}	− 83
B. cereus + *A. aurescens*	0.77 ^{b}	− 80	0.68 ^{b}	− 81

BI=Before inoculum=Reference sample

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Distribution of aluminium: Relative to the reference sample, OI reduced Al by 61% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *P. rettgeri*, *E. cloacae*, *B. cereus*, and *A. aurescens* reduced Cd concentration by 46%, 62%, 63%, 60%, 61% and 59%, respectively (Table 4.6).

Table 4.6: Response of aluminium (Al) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria.

Inoculum	Al	R.I. (%)
Before inoculum (BI)	14.07^{a}	−
No inoculum control (0I)	5.42^{b}	− 61
P. rettgeri	5.23^{b}	− 63
E. cloacae	5.66^{b}	− 60
P. rettgeri + *E. cloacae*	7.58^{b}	− 46
B. cereus	5.43^{b}	− 61
A. aurescens	5.75^{b}	− 59
B. cereus + *A. aurescens*	5.35^{b}	− 62

BI=Before inoculum=Reference sample

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

Distribution of arsenic: Relative to the reference sample, OI reduced As by 89% whereas, *P. rettgeri* + *E. cloacae*, *B. cereus* + *A. aurescens*, *P. rettgeri*, *E. cloacae*, *B. cereus*, and *A. aurescens* reduced Cd concentration by %, 80%, 95%, 67%, 75% and 74%, respectively (Table 4.7).

Table 4.7: Response of arsenic (As) in cultivated field (CF) and fallowed field following bioremediation with selected bacteria.

Inoculum	As	R.I. (%)
Before inoculum (BI)	2.60 ^{a}	–
No inoculum control (0I)	0.27 ^{b}	– 89
P. rettgeri	0.13 ^{b}	– 95
E. cloacae	0.86 ^{b}	– 67
P. rettgeri + *E. cloacae*	0.55 ^{b}	– 79
B. cereus	0.65 ^{b}	– 75
A. aurescens	0.68 ^{b}	– 74
B. cereus + *A. aurescens*	0.32 ^{b}	– 88

BI=Before inoculum = Reference sample

Relative impact [R.I. (%)] = [(Field/Virgin Field) – 1] ×100.

4.4 Discussion

4.4.1 Inoculum A (*Providencia rettgeri*)

Providencia rettgeri is a Gram-negative bacterium that is commonly found in both water and land environments (Triverdi *et al*., 2015). A study by Hassen *et al*. (1998) reported that a strain of *P. rettgeri* was isolated from wastewater and solid water compost in Tunisia, and it showed tolerance to chromium, copper and other heavy metals. Likewise, it could be obtained in polluted effluents, as reported by Foti *et al*. (2009). Since it is an ubiquitous microorganism, it could have been in the soil naturally, brought by run off or even brought by the treated wastewater during irrigation at the study site.

The trend of heavy metals was Cr > As > Pb > Cd > Al at CF and As > Pb > Cr > Al > Cd in order of the most reduced heavy metals to the least reduced by *P. rettgeri.* It was able to reduce 83% of Cd at its highest bioremediation compared to the other microorganisms. Thacker *et al*. (2006) reported that *P. rettgeri* could grow and reduce chromate to 100% at a concentration ranging from 100–300 mg/l and 99.31% at a concentration of 400 mg/l, pH 7 and temperature 37 °C. The finding by Thacker *et al*. (2006) was better than what the current research results. This could be due to the fact that the pH at both CF and FF was not kept constant at 7 like that of the report. Bestawy *et al*. (2013) reported that among other microorganisms, *P. rettgeri* was highly resistant to high concentrations of cadmium, copper and cobalt in polluted activated sludge.

4.4.2 Inoculum B *(Enterobacter cloacae)*

Enterobacter cloacae is a rod-shaped Gram-negative bacterium that live in mesophilic environments with an optimal temperature of 37^0 C. It is aerobic and facultatively anaerobic. *Enterobacter cloacae* is a human pathogen that can cause infections but can also act as a bioremediator. Under anaerobic conditions, it is able to convert toxic selenite in water sources that come from fossil fuel combustion to elemental, insoluble and non-toxic selenium. The trend of the bioremediated heavy metals was Pb > Cd > Cr > Al > As in CF and in FF was As > Al > Cd > Cr > Pb in order of the most reduced heavy metal to the least reduced heavy metal. It was able to reduce 90% of the bioavailable Cd in CF but generally, it performed better in FF during remediation of all the other bioavailable non-essential heavy metals.

Its least reduction was with Cd in FF whereby it didn't reduce the bioavailable Cd at all. Maximum resistance was tested against *E. cloacae* with increasing concentrations of silver (Ag), Pb, and Cd. The maximum biosorption capacities of *E. cloacae* to the heavy metals were reported to be 65% at 200 mg/kg, 54.28% at 100 mg/kg and 74.46% at 300 mg/kg (Bharathiraja and Rajasekaran, 2013). In a polluted soil bioremediation study by Banerjee *et al*. (2015), *E. cloacae* bioaccumulated 95.25% of Pb, followed by 64.17% of Cd then by 36.77% Ni after 72 h of inoculation. The results of this research were more successful than the study by Banerjee *et al*. (2015), and this could be because the microorganism was not given enough time to remediate the heavy metals that were bioavailable in the soil.

4.4.3 Inoculum C (*Bacillus cereus*)

Bacillus cereus is said to be aerobic and facultatively anaerobic. This means that it makes adenosine try-phosphate (ATP) by aerobic respiration if oxygen is present but is capable of switching to fermentation or anaerobic if oxygen is absent (Rohini and Jayalakshmi, 2015). It is also motile and commonly found in soil and food (Nath *et* *al*., 2012). *Bacillus cereus* is widely reported as a soil bacterium and also occurs in the rhizosphere of some plants (Vilain *et al*., 2006) and some strains of *B. cereus* produce antibiotics able to suppress fungal diseases of the rhizosphere (Syed and Chinthala, 2015). *Bacillus cereus* and *Bacillus thuringiensis* have been reported to increase extraction of Cd and Zn from soil and soil polluted with effluent from metal industry (Chibuike and Obiora, 2014).

Ghalib *et al*. (2009) reported a 70% decrease of chromium from the soil by two strains of *B. cereus*. From the results of this study, it was observed that *B. cereus* was able to remediate Cr > Pb > As > Al > Cd in CF and in FF the trend was Pb > As >Cr >Al > Cd in order of the most reduced heavy metal to the least reduced. Based on the results obtained from this research, *B. cereus* was able to reduce 100% of the bioavailable Cr in CF, which was a huge improvement from the previous study. Its least performance was on Cd whereby it increased it by 8% in FF. One study indicated that *B. cereus* was tolerant to a minimum level of 100ppm to the metals, Cd and Co (Garima and Singh, 2014; Rohini and Jayalakshmi, 2015), and this was in contrast with the current study as it could not bioremediate Cd efficiently.

4.4.4 Inoculum D (*Arthrobacter aurescens*)

Arthrobacter aurescens are basic soil bacteria that are able to fix nitrogen in the soil (Mongodin *et al*., 2006) and perform several important functions of removal of toxic chemicals (Singh and Kumar, 2006). It has been reported that *A. aurescens* can reduce hexavalent chromium, which can cause severe irritations to humans, and they are also known to degrade agricultural pesticides in the soil (Fu *et al*., 2014). Hexavalent chromium is 100 times more toxic than trivalent chromium because of its oxidation state, and is also much more soluble in water, allowing it to seep into groundwater very easily (Fu *et al*., 2014). This research revealed that *A. aurescens* was able to reduce heavy metals in CF at a trend of Cd>Pb>As>Cr>Al in CF and in FF, it was Pb > As > Cr > Al > Cd in order of the most reduced heavy metal to the least reduced. The results obtained in the research shows that *A.* aurescens reduced Cr by 98% and this was the highest remediated heavy metal among the other 4 heavy metals.

Very few organisms can grow in the presence of hexavalent chromium, but it has been recently discovered that *A. aurescens* cannot only grow in the presence of hexavalent chromium; it can also reduce it to trivalent chromium, its less toxic form. Maximum tolerated concentrations for the above metals were found to be 37, 525, 348, 1530 and 369 µM, respectively (Bafana *et al*., 2009). A similar degree of performance in bioremediation abilities was observed in this study whereby it showed high reduction of the bioavailable heavy metals although it didn’t perform well with Cd in FF.

4.4.5 Combination of Gram-positive and Gram-negative bacteria

From the results obtained, the combination of the Gram-positive bacteria (*B. cereus* + *A. aurescens*) always had the highest reduction of heavy metals than the combination of Gram-negative bacteria (*P. rettgeri* + *E. cloacae*). To add, the reduction was always highest at FF than at CF, meaning that bioremediation in this case was highly favourable at conditions of FF than that of CF. Although *B. cereus* + *A. aurescens* generally performed the best in comparison with *P. rettgeri* + *E. cloacae,* it was observed to have the lowest reduction of Cd in FF. Even when the individual microbes were used, they still had a poor performance. This could be because the Gram-positive bacteria used are not resistant to high levels of Cd. The trend of the non-essential heavy metals in CF was Cr > As > Cd > Pb > Al for *P. rettgeri* + *E. cloacae* and Cd > Cr > As > Pb > Al in for *B. cereus* + *A. aurescens* in the order of the most reduced heavy metal to the least. In FF the trends were As > Pb > Cr > Al > Cd for *P. rettgeri* + *E. cloacae* and Cr > As > Pb > Al > Cd for *B. cereus* + *A. aurescens* in the order of the most reduced to the least reduced heavy metal.

Both Gram-negative and Gram-positive bacteria have their cell wall charged with a negative charge. This is due to carboxyl, hydroxyl and phosphyl groups, thus in the presence of positive heavy metal cations, these groups are very important in cation sorption (Pires, 2015). Metals and metalloids get attached to these ligands on cell surfaces, which displace essential metals from their normal binding sites (Ayangbenro and Babalola, 2017). Once the metal and metalloid are bound, microbial cells can transform them from one oxidation state to another, thus reducing their toxicity (Gupta *et al*., 2012; Lesmana *et al*., 2009). By so saying, this defines the act of bioremediation observed in the study whereby the concentrations of non-essential heavy metals reduced in the soil.

4.5 Conclusion

Gram-positive bacteria performed the best individually and as a combination in bioremediation of the bioavailable non-essential heavy metals. Generally, this was mostly observed at FF than at CF. This means that fallowing of soils helps in bioremediation process, and this could be because the soil conditions are not constantly changed through the irrigation with treated wastewater. All the identified microbes were able to reduce the heavy metal concentration in the soil at different conditions of CF and FF, but worrying observations were seen with low reduction of concentrations of Cd at FF such that *P. rettgeri* increased it by 8% and *A. aurescens* could not even reduce it at all. For further studies, these microorganisms must be screened for Cd resistance in soils of FF in order to understand the negative performance observed. More research must also be done bioremediation of these non-essential heavy metals on both treated wastewater and polluted soils, especially with varying bacteria strains. In conclusion, bioremediation using bacteria coupled with fallowing has shown to have great potential in the removal of non-essential heavy metals. Therefore, it could be recommended for adoption by farmers who experience heavy metal pollution in their fields and as well as those who use treated wastewater for irrigation purposes.

CHAPTER 5

SUMMARY OF FINDINGS, SIGNIFICANCE OF FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

5.1 Summary of findings

The study focused on the effect that treated wastewater has on the bioavailability of essential and non-essential heavy metals in two different fields on a vertical distribution. Furthermore, the effect of fallowing on the heavy metal polluted soils was investigated in order to prove whether it helps with the suppression of the heavy metals. Bioavailability results revealed that some non-essential heavy metals such as Pb, Cd and Al have been in high concentrations even before irrigation with treated wastewater. The results further revealed that irrigation with treated wastewater increased the concentrations of the remaining two non-essential heavy metals named chromium and arsenic such that they exceeded the permissible levels set by both FAO and WHO.

The concentrations of all the essential heavy metals also increased due to irrigation with treated wastewater, and this was beneficial as they did not exceed the permissible levels set by FAO and had uses in soil. Although there had been an increase and decrease of concentrations of heavy metals, no significant difference was observed in terms of depth. Significant differences were observed among the different fields, and this could have been brought by the fact that each field has varying basic soil physico-chemicals responding differently to the irrigation water, thus leading to varying bioavailability of heavy metals. Aluminium and As displayed significant differences among depth, and mean separation was done to show the variation.

The study further investigated the bioremediation abilities of two Gram-negative and two Gram-positive bacteria that were native to the soils on the different fields on the non-essential heavy metals. The individual Gram-positive treatments performed best or rather reduced the most concentrations of the selected heavy metals compared to the Gram-negative bacteria. Overall, the bioremediation process was more successful on the fallowed field than in the cultivated field. This could be because treated wastewater which was, in the first place, the cause of the problem is no longer applied on the fallowed field and its effect has prevailed. A very interesting discovery was made when the results revealed that in the fallowed field, all the microorganisms struggled to reduce higher concentrations of Cd, and one microbe increased the concentration of Cd. This could be because the microbes were tolerant to Cd toxicity but do not have the ability to bioaccumulate it or rather transform it into a less toxic form. Moreover, since Cd was high before irrigation with treated wastewater, it could have killed most microorganisms that could possibly act as bioremediators.

5.2 Significance of findings

The study showed the importance of understanding the effects brought by treated wastewater used for irrigation of agricultural produce. Although treated wastewater is beneficial in alleviation of water scarcity, the quality is still questionable, and farmers need to take precaution when using the water for irrigation. It also brought to attention how much this treated wastewater carries toxic and beneficial heavy metals that could pose health risks when induced in high concentrations. Although treated wastewater has been reported to cause accumulation of heavy metals in the soil, heavy metals in different fields never leached too deep into the lower depths of the profiles. There was no effect on the vertical distribution of the heavy metals in all the studied fields. The study further brought forward and proved the abilities of bacteria that could have been identified on treated wastewater irrigated fields, which could be a cost-effective solution that is easily adoptable and a non-disturbing way of removing unwanted heavy metals from the soil when they have accumulated in high concentrations.

5.3 Conclusions

In conclusion, fallowing has been proved to have beneficial effects not only on the physicochemical properties of the soil but also as a bioremediation facilitator. Bioremediation process has been proved to work effectively and efficiently than what literature reports about other remedial methods. Gram-positive bacteria proved to have better bioremediation abilities than Gram-negative bacteria and therefore could be used for successful results. It has been observed that irrigation with treated wastewater had both negative and positive effects on the bioavailability of heavy metals in the soil. It increased and decreased the concentrations of the essential and non-essential heavy metals. Irrigation with treated wastewater clearly showed that it has no effect on the bioavailability of heavy metals on both fields with varying physicochemical properties. Overall, the study was a success, and the hypothesis which stated that irrigation with treated wastewater has an effect on the distribution of heavy metals in cultivated and fallowed soils was agreed upon. Moreover, the second hypothesis that states that microbes will reduce the amount of heavy metals in cultivated and fallowed soils following irrigation with treated wastewater has been agreed upon too.

5.4 Recommendations

Municipalities in different regions should start a programme of collecting wastewater at homes, treat it and advise farmers to use it for irrigation purposes. This follows reports of an alarming increase of water shortage. Wastewater from industries, factories and mines that could rather contain harmful and toxic chemicals must be collected separately, analysed and treated with respect of what it contains. That way, there could be levels of purification that differs catering the contaminants carried along with the wastewater. People who are faced with this heavy metal problem could simply adopt this bioremediation process to overcome the problem. This bioremediation process could also be adopted in water purification systems as it has been proved to be efficient and effective. Further research has to be done by other scientists on the bioremediation abilities of other beneficial tolerant microbes to other varying toxins other than heavy metals because they are not the only pollutants in wastewater. Fallowing of soils also could be adopted by farmers because as it has been reported that there is more marginal land in South Africa, the only land that we have must be protected and preserved in a more natural way than amended with the addition of more chemicals.

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APPENDICES

Appendix 3.1 Analysis of variance for clay content at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	30968.12	2	818.77	239.13	0.00
S. point	6319.17	39	15484.06	2.50	0.00
Depth	246.08	2	162.03	1.90	0.15
Field #depth	942.05	4	235.51	3.64	0.00
Residual	20202.63	312	64.75		

Total	58685.03	359	163.47		

Appendix 3.2 Analysis of variance for sand at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	643.76	2	321.88	1.34	0.26
S. point	17342.23	39	444.67	1.85	0.00
Depth	1973.30	2	986.65	4.10	0.12
Field #depth	2584.48	4	646.12	2.68	0.03
Residual	75111.92	312	240.74		
Total	97670.19	359	272.06		

Appendix 3.3 Analysis of variance for soil pH(H_20) at three depths of cultivated and fallowed fields following irrigation with treated wastewater

Source	Partial SS	DF	MS	F	Prob>F
Field	263.86	2	131.93	298.83	0.00
S. point	30.58	39	0.79	1.78	0.00
Depth	5.70	2	2.85	6.46	0.00
Field #depth	0.25	4	0.06	0.14	0.96
Residual	137.74	312	0.44		
Total	436.63	359	1.22		

Appendix 3.4 Analysis of variance for soil pH(KCl) at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	21.36	2	10.68	59.66	0.00
S. point	20.72	39	0.53	2.97	0.00
Depth	1.14	2	0.57	3.17	0.04
Field #depth	2.67	4	0.67	3.73	0.01
Residual	55.86	312	0.18		
Total	102.04	359	0.28		

Appendix 3.5 Analysis of variance for soil EC at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	668512.59	2	334256.29	224.75	0.00
S. point	129123.69	39	3310.86	2.23	0.00
Depth	25352.30	2	12676.15	8.52	0.00
Field #depth	23464.69	4	5866.17	3.94	0.00
Residual	464023.92	312	1487.26		
Total	1311560.42	359	3653.37		

Appendix 3.6 Analysis of variance for soil Eh at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	86018.57	2	43009.28	445.46	0.00
S. point	8535.68	39	218.86	2.27	0.00
Depth	239.77	2	119.88	1.24	0.29
Field #depth	57.68	4	14.42	0.15	0.96
Residual	30123.7	312	96.55		
Total	124928.26	359	347.99		

Appendix 3.7 Analysis of variance for soil OC at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	1791.28	2	895.64	1358.72	0.00
S. point	29.69	39	0.76	1.1.5	0.25
Depth	5.02	2	2.51	3.81	0.02
Field #depth	2.32	4	0.58	0.88	0.48
Residual	205.66	312	0.66		
Total	2035.93	359	5.67		

Appendix 3.8 Analysis of variance for soil CEC at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	95.95	2	47.98	1338.31	0.00
S. point	2.58	39	0.07	1.84	0.00
Depth	0.30	2	0.15	4.25	0.02
Field #depth	0.15	4	0.04	1.07	0.37
Residual	11.18	312	0.04		
Total	110.34	359	0.31		

Appendix 3.9 Analysis of variance for soil Al at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	5500212.36	2	2750106.18	3328.97	0.00
S. point	63829.78	39	1636.66	1.98	0.00
Depth	4815.74	2	2407.87	2.91	0.06
Field #depth	4045.29	4	1011.32	1.22	0.30
Residual	257747.38	312	826.11		
Total	5832234.94	359	16245.78		

Appendix 3.10 Analysis of variance for soil As at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	101.03	2	50.51	3.21	0.04
S. point	1097.96	39	28.15	1.79	0.00
Depth	237.63	2	118.82	7.55	0.00
Field #depth	135.89	4	33.97	2.16	0.07
Residual	4910.78	312	15.74		
Total	6485.94	359	18.07		

Appendix 3.11 Analysis of variance for soil Co at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	126733.83	2	6366.91	37.29	0.00
S. point	2116.49	39	54.27	2.48	0.00
Depth	148.06	2	74.06	0.03	0.97
Field #depth	314.06	4	78.51	0.45	0.77
Residual	14149.93	312	45.35		
Total	143286.71	359	399.13		

Appendix 3.12 Analysis of variance for soil Cr at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	17807.20	2	8903.60	37.29	0.00
S. point	23048.16	39	590.98	2.48	0.00
Depth	14.16	2	7.08	0.03	0.97
Field #depth	430.88	4	107.72	0.45	0.77
Residual	74497.50	312	238.77		
Total	115730.32	359	322.37		

Appendix 3.13 Analysis of variance for soil Fe at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	37348327	2	18674163.5	993.13	0.00
S. point	1100079.7	39	28207.17	1.50	0.03
Depth	61935.65	2	30967.82	1.65	0.19
Field #depth	208625.21	4	52156.30		0.02
Residual	5866640.31	312	18803.33		
Total	44536839.2	359	124058.05		

Appendix 3.14 Analysis of variance for soil Mn at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	167695.16	2	83847.58	188.70	0.00
S. point	9022.46	39	231.35	0.52	0.99
Depth	215.57	2	107.79	0.24	0.78
Field #depth	2231.03	4	557.76	1.26	0.29
Residual	138637.08	312	444.35		
Total	317633.35	359	884.77		

Appendix 3.15 Analysis of variance for soil Pb at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	8129.88	2	4064.94	83.41	0.00
S. point	12539.20	39	321.52	6.60	0.00
Depth	42.48	2	21.24	0.44	0.65
Field #depth	28.61	4	7.15	0.15	0.96
Residual	15205.09	312	48.73		
Total	35960.45	359	100.17		

Appendix 3.16 Analysis of variance for soil Zn at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	611074.19	2	305537.10	665.16	0.00
S. point	22898.97	39	587.15	1.28	0.13
Depth	639.90	2	319.95	0.70	0.50
Field #depth	739.19	4	198.30	0.43	0.79
Residual	143314.72	312	459.34		
Total	779207.44	359	2170.49		

Appendix 3.17 Analysis of variance for soil Cd at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	354.18	2	177.09	17.63	0.00
S. point	2028.53	39	52.01	5.18	0.00
Depth	17.37	2	8.68	0.86	0.42
Field #depth	17.78	4	4.44	0.44	0.78
Residual	3133.74	312	10.04		
Total	5553.22	359	15.47		

Appendix 3.18 Analysis of variance for soil Cu at three depths of cultivated and fallowed fields following irrigation with treated wastewater.

Source	Partial SS	DF	MS	F	Prob>F
Field	6363.74	2	3231.87	222.70	0.00
S. point	932.27	39	23.90	1.65	0.14
Depth	22.82	2	11.41	0.79	0.45
Field #depth	37.88	4	9.47	0.65	0.63
Residual	4527.74	312	14.51		
Total	11966.47	359	33.33		

Appendix 4.1 Analysis of variance for Al at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species.

Source	DF	SS	MS	F	P
Rep	2	10.08	5.04		
Field	1	0.12	0.12	0.02	0.90
Trt	7	385.49	55.07	7.82	0.00
Field #Trt	7	34.88	4.98	0.71	0.67
Error	30	211.17	7.04		
Total	47	641.74			

Appendix 4.2 Analysis of variance As at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species.

Source	DF	SS	MS	F	P
Rep	2	1.89	0.94		
Field	1	1.20	1.20	1.69	0.20
Trt	7	25.75	3.68	5.17	0.00
Field #Trt	7	1.51	0.22	0.30	0.95
Error	30	21.37	0.71		
Total	47	51.71			

Appendix 4.3 Analysis of variance Cd at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species.

Source	DF	SS	MS	F	P
Rep	2	0.41	0.21		
Field	1	0.46	0.46	1.61	0.21
Trt	7	22.31	3.19	11.18	0.00
Field #Trt	7	15.98	2.28	8.01	0.00
Error	30	8.55	0.29		
Total	47	47.71			

Appendix 4.4 Analysis of variance Cr at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species.

Source	DF	SS	MS	F	P
Rep	2	4.86	2.43		
Field	1	0.07	0.07	0.05	0.82
Trt	7	142.60	20.37	14.98	0.00
Field #Trt	7	22.32	3.19	2.34	0.04
Error	30	40.81	1.36		
Total	47	210.65			