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Tracing heavy metals in tomato plants watered with synthetic wastewater treated with a bacterial coated calcium alginate nanobiosorbent.

by

Haripriya Rama

(732066)

Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Dr. Kulsum Kondiah

Co-supervisor: Prof. Rob Veale

August 2019

Declaration

I, Haripriya Rama (732066), am a student registered for the degree of Master of Science (Diss) in Molecular and Cell Biology in the academic year 2019.

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Abstract

Heavy metals accumulate in soils and crops irrigated with acid mine decant (AMD) polluted water, posing environmental health risks. The dead bacterial biomass of *Micrococcus luteus* and *Enterobacter xiangfangensis* Pb204 were previously identified as effective biosorbents for the treatment of multi-metal systems under neutral conditions. The study aimed to treat AMD waters with free and immobilized bacterial biosorbent and determine the suitability of the treated water for agricultural purposes. Acid mine decants representative of the Central and Witwatersrand basin, Gauteng, South Africa were neutralized with limestone or sodium hydroxide. Limestone neutralized AMD samples were treated with *M. luteus* and *E. xiangfangensis* Pb204 individually or in combination to determine the most effective biosorbent. Subsequently, *M. luteus* was immobilized onto calcium alginate nanoparticles (CANPs) forming a nanobiosorbent for treatment of limestone neutralized AMD samples. The biosorption profile of the nanobiosorbent was compared to free CANPs, *M. luteus* and a mixture of *M. luteus* biosorbent with free CANPs, by quantifying metal concentrations using inductively coupled plasma optical-emission spectrometry (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS). Four groups of tomato plants (Rodade variety) were grown under controlled conditions and irrigated with tap water, untreated AMD water or AMD water treated with free biosorbent or nanobiosorbent, respectively. Plants from all groups that developed fruit (except for those irrigated with nanobiosorbent treated water) displayed stunted growth during the fruiting stage. Metal accumulation in the plant structures occurred in the order flowers>roots>stems>leaves=fruit. Most metals in the fruits exceeded the recommended maximum daily adult intake or the acceptable limits for human consumption even in the group irrigated with tap water. The commercial potting soil used to grow the plants contained elevated levels of Mn (1327.92 mg/kg) and Cu (17.11 mg/kg) which remained high in the soil from all treatment groups. These findings show that although treatment with *M. luteus* biosorbent or nanobiosorbent lowered metal concentrations in AMD, irrigating plants with the treated water resulted in the stunted development of fruit which was unfit for human consumption.

Keywords: Acid mine decant, biosorbent, calcium alginate, heavy metals, nanobiosorbent, tomato.

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Table of Contents

Declaration ii
Abstract iii
Acknowledgements iv
Research Outputs v
List of Abbreviations viii
List of Figures ix
List of Tables xi
Chapter 1: Introduction 1
1.1 Background 1
1.2 Research aim 3
1.3 Research objectives 3
1.4 Chapter outline 4
Chapter 2: Literature Review 5
2.1 Heavy metal contamination in South Africa 5
2.1.1 Heavy metal contamination by acid mine drainage 5
2.2 Heavy metal biotoxicity 6
2.3 Remediation of heavy metals from industrial wastewaters 8
2.3.1 Physicochemical treatment strategies 8
2.4 Bacterial biosorption 9
2.5 Enhancing biological treatment strategies with nanotechnology 14
2.5.1 Calcium alginate nanoparticles 15
2.6 Problem statement and research gap 16
2.7 Present study 16
2.8 Research hypothesis 17
Chapter 3: Biosorption of heavy metals from neutralized synthetic AMD wastewater 19
3.1 Introduction 19
3.2 Experimental procedure 20
3.2.1 Preparation of synthetic AMD 20
3.2.2 Preparation of biosorbent 21
3.2.3 Synthesis of CANPs 22
3.2.4 Synthesis of nanobiosorbent 22
3.2.5 Characterization and stability of the free CANPs and nanobiosorbent 23

3.2.6 Neutralization and biological treatment of the synthetic AMD samples ... 23
3.2.7 Statistical analysis of data ... 24
3.3 Results ... 24
3.3.1 Characterization and stability of CANPs and nanobiosorbent ... 24
3.3.2 Neutralization and treatment of AMD-1, AMD-2 and AMD-3 ... 26
3.4 Discussion ... 33
3.4.1 Structural formation and stability of CANPs and nanobiosorbent ... 34
3.4.2 Comparison of the neutralization strategies ... 34
3.4.3 Comparison of the bacterial biosorbents ... 36
3.4.4 Effect of immobilization ... 38
3.5 Conclusions ... 40
Chapter 4: Quantification of heavy metals in tomato plants watered with treated synthetic AMD wastewater ... 41
4.1 Introduction ... 41
4.2 Experimental procedure ... 42
4.2.1 Tomato plant pot trial ... 42
4.2.2 Sample preparation and heavy metal analysis ... 43
4.3 Results ... 44
4.3.1 Analysis of tomato plant growth ... 44
4.3.2 Heavy metal accumulation in tomato plant structures ... 49
4.3.3 Heavy metal quantification in water and soil samples ... 55
4.4 Discussion ... 57
4.4.1 Plant growth and development ... 57
4.4.2 Metal accumulation in tomato plants ... 60
4.5 Conclusions ... 63
Chapter 5: Conclusions and future recommendations ... 64
References ... 66
Appendices ... 79
Appendix A ... 79

List of Abbreviations

AMD	Acid mine decant
ANOVA	Analysis of variance
ARC	Agricultural research council
CANPs	Calcium alginate nanoparticles
C_a	Concentration after precipitate removal
C_b	Concentration before precipitate removal
C_{ex}	Concentration for experimental group
C_f	Final concentration
C_i	Initial concentration
C_{tp}	Concentration for tap water group
EDTA	Ethylenediaminetetraacetic acid
FeS_2	Pyrite
G residues	α-L-guluronic acid residues
HDS	High density sludge
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
LB	Luria Bertani
M residues	β-D-mannuronic acid residues
NC	Nanocrystal
PAR	Photosynthetic active radiation
SEM	Scanning electron microscopy

List of Figures

Figure 2.1. Acid mine drainage flowing from the Tweelopie Spruit catchment, Krugersdorp Nature Reserve……..6
Figure 2.2. Mechanisms of bacterial metal resistance..……..11
Figure 2.3. Calcium alginate gel formation……15
Figure 3.1. SEM images of free CANPs (a) and nanobiosorbent comprising *M. luteus* (b).……25
Figure 3.2. SEM images of free CANPs and nanobiosorbent that were stored for 8 weeks……26
Figure 3.3. The percentage reduction in metal concentrations after neutralization of AMD-1 (a), AMD-2 (b) and AMD-3 (c) with NaOH or limestone……..…..28
Figure 3.4. The percentage biosorption of metal from limestone neutralized AMD-1 (a), AMD-2 (b) and AMD-3 (c) treated with *M. luteus*, *E. xiangfangensis* Pb204, a combination of *E. xiangfangensis* Pb204 and *M. luteus* (2:1) and CANPs.....30
Figure 3.5. The percentage biosorption of metal from limestone neutralized AMD-1 (a), AMD-2 (b) and AMD-3 (c) treated with *M. luteus*, CANPs, nanobiosorbent and 1:1 mixture of free CANPs and *M. luteus*..……. ……...33
Figure 4.1. Tomato plants grown with tap water at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.……..……..45
Figure 4.2. Tomato plants grown with limestone neutralized AMD-3 water treated with *M. luteus* biosorbent at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.……...45
Figure 4.3. Tomato plants grown with limestone neutralized AMD-3 water treated with nanobiosorbent at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.…….46
Figure 4.4. Tomato plants grown with untreated AMD-3 water at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.……46
Figure 4.5. Plant height (a), leaf count (b) and leaf area (c) for each water treatment group over a period of 13 weeks……..……48

Figure 4.6. Development of tomato fruit in plants irrigated with tap water (a) and biosorbent treated limestone neutralized AMD-3 water (b) at 21 weeks…….....49
Figure 4.7. The graph represents the percentage of the metal concentrations of each experimental group relative to the tap water control group for the tomato plant roots (a), stems (b), leaves (c), flowers (d) and fruits (e)…..……..……..54
Figure 4.8. Metal concentrations of water (a) and soil (b) samples from each tomato plant treatment group……..…..56

List of Tables

Table 2.1. Heavy metal toxicity levels and associated health effects…….....7
Table 2.2. Advantages and disadvantages of physicochemical wastewater treatments……..9
Table 3.1. Elemental composition of synthetic AMD representative of the Central and Witwatersrand Basin…….....21
Table 4.1. Translocation factors of metals in tomato plants for all four treatments.50
Table 4.2. Elemental composition of tomato fruits produced by plants from different treatment groups in the present study……...51

Chapter 1: Introduction

1.1 Background

South Africa is currently facing severe water shortages as a result of urban industrialization (Ochieng, Seanego and Nkwonta, 2010). The water resources in Gauteng are constantly being polluted with mining and industrial effluent that contains elevated concentrations of heavy metals (McCarthy, 2011). The metal contaminated water flows into the surrounding environment accumulating in the soil and plants. Metal transfer then occurs through the food chain accumulating in animals and humans (Sharma and Agrawal, 2005). This poses chronic health risks to humans as metals induce biotoxicity when consumed in high concentrations (Duruibe, Ogwuegbu and Egwurugwu, 2007).

Currently, conventional treatment methods involve energy-demanding physical processes or the use of strong, toxic chemical reagents for the removal of heavy metals from wastewater (Barakat, 2011). Some of these physicochemical methods include chemical precipitation, membrane filtration, flocculation, flotation and ion exchange (Barakat, 2011). Physicochemical treatment strategies are very effective however, the treatment process involves high costs and secondary pollution is generated in the form of toxic sludge as a by-product (Semerjian and Ayoub, 2003). Biological treatments may be used as an alternative for the removal of heavy metals from wastewaters due to the eco-friendly, cheaper and efficient properties of biological materials (Fu and Wang, 2011).

Biological materials that have been studied for the treatment of heavy metal contaminated wastewater includes various plant materials and micro-organisms such as bacteria, algae and fungi (Wang and Chen, 2009). This study particularly focussed on the use of dead bacterial biomass for the removal of metals from wastewater. Some bacteria have a high affinity for metals as a result of adapting to environments containing high concentrations of metals (Etesami, 2018). Mechanisms such as biosorption, sequestration using metal binding proteins and enzymatic transformation are used by such bacteria to tolerate and survive metal toxicity (Etesami, 2018).

The present study exploited the passive process of biosorption in which bacteria are able to bind metal ions from the environment onto their cell surfaces as a treatment strategy for acid mine water. The positively charged metal ions bind to the negatively charged functional groups such as carboxyl, hydroxyl and phosphoryl groups that are present in the structural components of bacterial cell surfaces (Ayangbenro and Babalola, 2017). This process occurs irrespective of whether the cells are alive or dead. Dead bacterial cells were exploited as opposed to live cells in order to circumvent additional maintenance expenses involved in supplying the energy requirements of the cells for survival as well as averting the possibility of metal-induced cell toxicity that would lead to the termination of active metal uptake (Zabochnicka-Świątek and Krzywonos, 2014). Additionally, various factors such as pH, temperature, concentration of biomass and initial metal concentrations as well as the presence of competing metal ions affect the efficiency and selectivity of bacterial biosorbents (Ahalya, Ramachandra and Kanamadi, 2003). The treatment of heavy metal contaminated wastewater with bacterial biosorbents therefore requires optimization.

Dead bacterial cells can have low density, poor mechanical strength and poor rigidity. However, when immobilized onto polymeric materials such as calcium alginate or polyacrylamide, the structural stability, mechanical strength and the porosity is enhanced for metal biosorption purposes (Ahalya, Ramachandra and Kanamadi, 2003). Many studies have incorporated nanotechnology to improve the efficiency and selectivity of heavy metal contaminated wastewater treatment strategies (Qu, Alvarez and Li, 2013). The size of nanomaterials ranges between 1 and 100 nm. Properties associated with this size range includes reactivity, strong sorption, high surface to volume ratios and high specificity (Qu, Alvarez and Li, 2013). A study performed by Khezri *et al*. (2016) investigated the use of calcium alginate nanoparticles (CANPs) for the removal of lead (Pb) ions from aqueous solutions. The CANPs were found to successfully remove 99% of the Pb ions. Alginate is a biodegradable anionic polymer that may be cross-linked with calcium ions to form a porous gel matrix consisting of carboxylate groups which have an affinity for metal ions (Paques *et al*., 2014). CANPs can therefore be exploited for the treatment of metal contaminated wastewater.

The present study proposed to use the bacterial isolates *Enterobacter xiangfangensis* Pb204 and *Micrococcus luteus* originating from acid mine decant (AMD) on the West

Rand, Gauteng as biosorbents for the treatment of synthetic AMD wastewater. Studies performed by Vallabh (2014, 2017) found these two bacterial isolates to be highly tolerant of heavy metals and showed that the biosorption of Pb, nickel (Ni), cobalt (Co), manganese (Mn) and zinc (Zn) from multi-metal systems under neutral conditions at 25 °C was enhanced when the biosorbents were used as a consortium. Due to the properties of CANPs and their potential to enhance biosorption of heavy metals, the bacterial biomass was immobilized onto CANPs (nanobiosorbent) to determine whether metal removal further improved. The study further investigated the suitability of the biosorbent or nanobiosorbent treated synthetic wastewater for agricultural use. Tomato plants are widely grown by subsistence farmers in local communities and contribute 22 % to the vegetable production in South Africa (DAFF, 2018). Tomato plants were therefore selected to determine whether the metal transfer from the treated wastewater to the tomato fruits were within acceptable levels for safe consumption.

1.2 Research aim

The aim of this study was to determine if synthetic AMD wastewater treated with a bacterial biosorbent or nanobiosorbent was suitable for agricultural purposes.

1.3 Research objectives

To achieve the research aim, the following objectives were set:

1. To prepare synthetic AMD wastewater representative of the mining sector in Gauteng.
2. To treat neutralized synthetic AMD wastewater with either biosorbent composed of heat-killed *M. luteus* and/or *E. xiangfangensis* Pb204 or nanobiosorbent composed of bacterial biosorbent immobilized onto calcium alginate nanoparticles.
3. To grow tomato plants under controlled conditions using treated and untreated synthetic AMD wastewater for irrigation purposes.
4. To quantify the heavy metals in the roots, stems, leaves and fruits of the tomato plants by inductively coupled plasma optical-emission spectrometry (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS).

1.4 Chapter outline

This dissertation is structured as follows:

Chapter 1 gives a brief overview of the background to the study. It also highlights the aim and objectives set out to address the research gap identified in the area of study.

Chapter 2 provides an in depth review of current literature including aspects on heavy metal contamination and biotoxicity in South Africa, remediation strategies for their removal from industrial wastewaters with a focus on bacterial biosorption and the use of nanotechnology to enhance treatment.

Chapter 3 describes the preparation and treatment of synthetic AMD wastewater and its findings in relation to the levels of heavy metals that are acceptable for agricultural use. It further discusses the selection of the best biosorbent option for use in AMD wastewater treatment.

Chapter 4 outlines the watering and growth of the tomato plants using the treated synthetic AMD wastewater. Findings on the concentration of heavy metals in the different plant structures are reported and discussed with regard to safety limits in consumable fresh produce.

Chapter 5 provides a general conclusion to the study with recommendations for future investigation that can further the work reported here.

Chapter 2: Literature Review

2.1 Heavy metal contamination in South Africa

South Africa is currently facing a prominent case of water scarcity (Ochieng, Seanego and Nkwonta, 2010). The agricultural sector as well as the surrounding rural communities are severely affected by the depletion of potable water in South Africa (Ochieng, Seanego and Nkwonta, 2010). Many of the water resources including the West Rand, East Rand and Central Rand in Gauteng, South Africa are heavily contaminated with heavy metals as a result of urban industrialization (McCarthy, 2011; Durand, 2012). Industries involved in mining, electroplating, painting, textile as well as paper mill and pulp release toxic concentrations of non-biodegradable metals as effluent into the environment (Barakat, 2011; Pires *et al.*, 2011). The gold mining industry contributes approximately 221 million tons of mineral waste in South Africa (Oelofse *et al.*, 2007).

2.1.1 Heavy metal contamination by acid mine drainage

Acid mine drainage generated by the mining industry is the primary source of heavy metal contaminated wastewaters in Gauteng with an acidic pH of 2-3 and a sulphate concentration of approximately 3500 mg/L (McCarthy, 2011). Sulphuric acid and iron hydroxide are by-products that result from the oxidation reaction between pyrite (FeS_2), water and oxygen in the mines (Naicker, Cukrowska and McCarthy, 2003). This process is often facilitated by iron oxidizing and sulphur oxidizing bacteria (Baker and Banfield, 2003; Akcil and Koldas, 2006). The sulphuric acid generated by this reaction is able to dissociate other metals such as gold (Au), silver (Ag), aluminium (Al), Pb, iron (Fe), copper (Cu), Co, Ni, Zn, cadmium (Cd), calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), chromium (Cr) and Mn from the mine tailings.

Large amounts of groundwater are discharged from the karst aquifer through the mines which in turn releases elevated concentrations of the metals as AMD into the environment as shown in Figure 2.1 (Durand, 2012). Acid mine drainage leaches into other water bodies, soils, crops and surrounding rural developments which poses severe environmental health risks (Duruibe, Ogwuegbu and Egwurugwu, 2007; Kamunda, Mathuthu and Madhuku, 2016). An example of this occurrence includes the

decanting of AMD water originating from the West Rand nearby Krugersdorp where residents depend on boreholes as a source of drinking water (Oelofse *et al.*, 2007).



Figure 2.1. Acid mine drainage flowing from the Tweelopie Spruit catchment, Krugersdorp Nature Reserve. The AMD water contains iron appearing as thick yellow precipitation (Durand, 2012).

2.2 Heavy metal biotoxicity

Heavy metals are taken up into plant tissues as a result of AMD water contaminating soil that is used for agricultural purposes (Sharma and Agrawal, 2005). Through the consumption of contaminated water or vegetables, heavy metals bio-accumulate over time in animal and human tissues posing chronic health risks (Sharma and Agrawal, 2005). Metals induce biotoxicity in their soluble and stable oxidation states (Duruibe, Ogwuegbu and Egwurugwu, 2007). In this bioavailable state, metals are able to bind to biomolecules such as proteins and enzymes to form stable, biotoxic compounds (Duruibe, Ogwuegbu and Egwurugwu, 2007). These compounds hinder the functioning of the biomolecules resulting in the onset of various diseases. Some

metals such as Cu, Zn, Ni, Mn and Co are essential in the functioning of biochemical processes within cells (Nies, 1999). Other metals such as Pb are not essential for bodily functions. Uptake of high metal concentrations induces toxic effects on cellular components (Fashola, Ngole-Jeme and Babalola, 2016). For example, by binding to the sulphide groups present in most enzymes or other essential metals, toxic levels of heavy metals disrupt the physiology and functioning of the cells (Nies, 1999). Table 2.1 represents human health problems arising from the exposure to various high metal concentrations in water used for domestic purposes. The mitigation of heavy metal contamination is therefore of utmost importance due to their toxic environmental and health effects.

Table 2.1. Heavy metal toxicity levels and associated health effects.

Metal	Toxicity level in ppm (DWAF, 1996)	Health effect	References
Aluminium	0.5	Dermatitis, Alzheimer's disease, kidney problems and damage to the nervous system, brain and bones.	Jaishankar *et al.* (2014)
Cobalt	N/A	Rhinitis, asthma, risk of lung cancer and dermatitis.	Lauwerys and Lison (1994)
Copper	30	Liver damage.	Kamunda, Mathuthu and Madhuku (2016)
Iron	1	Risk of cancer, stricture development and ulcer formation in the GI tract.	Jaishankar *et al.* (2014)
Lead	0.01	Renal tumours, impairs functioning of joints, kidneys, nervous and reproductive systems.	Kamunda, Mathuthu and Madhuku (2016)
Manganese	5	Brain damage and neurodegeneration.	Crossgrove and Zheng (2004)
Nickel	N/A	Intestinal and oral cancer, kidney problems, haemorrhages, heart attacks and depression.	Kamunda, Mathuthu and Madhuku (2016)
Zinc	10	Impairment of growth and reproduction	Kamunda, Mathuthu and Madhuku (2016)

2.3 Remediation of heavy metals from industrial wastewaters

There are two strategies that are currently being utilized for the removal of heavy metals from wastewaters. Physicochemical processes employ physical or chemical approaches for removal and biological processes employ biological material such as biomolecules, microorganisms or plants for the bioremediation of heavy metal contaminated water (Fu and Wang, 2011). A combination of these two particular strategies is shown to be more effective than using a single strategy (Fu and Wang, 2011).

2.3.1 Physicochemical treatment strategies

Physicochemical treatment strategies are currently used to treat industrial wastewaters. However, these widely used treatments have respective advantages and disadvantages as shown in Table 2.2. Chemical precipitation is the most common method of treatment in South Africa whereby the metals are precipitated out in hydroxide forms when lime, limestone and other neutralizing reagents are applied (Akcil and Koldas, 2006). Other physicochemical treatments include ion exchange, membrane filtration, carbon adsorption, flocculation and flotation (Barakat, 2011; Rajasulochana and Preethy, 2016). Although highly efficient in removing the metals from the effluent, physicochemical treatments are expensive and produce secondary pollution in the form of solid waste that is also detrimental for the environment (Fu and Wang, 2011). The development of biological alternatives that are efficient, eco-friendlier and less costly are being explored.

Table 2.2. Advantages and disadvantages of physicochemical wastewater treatments.

Treatment	Description	Advantages	Disadvantages
Chemical precipitation (Johnson and Hallberg, 2005)	Neutralising agents such as lime, limestone or other coagulants are added to heavy metal contaminated effluents to form insoluble by-products such as hydroxides.	Simple and cheap.	Inefficient at low metal concentrations, produces large amounts of toxic solid waste that is costly to dispose of or recover.
Ion exchange (Fu and Wang, 2011)	An ion exchanger exchanges cations such as hydrogen ions on a solid resin with the metal cations in effluents.	Highly efficient in removing heavy metals and has a high capacity for treatment.	Very expensive when large volumes are involved, requires chemical reagents to form the resin generating secondary pollution.
Membrane filtration (Barakat, 2011; Fu and Wang, 2011)	Both organic and inorganic particles are excluded under high pressure through a porous membrane.	Highly selective in separating various particulates that range in size.	Expensive, prone to membrane fouling or the accumulation of particles on the porous membrane.

2.4 Bacterial biosorption

In order to survive and maintain homeostasis under toxic metal environments, micro-organisms like bacteria have developed several metal resistance mechanisms as shown in Figure 2.2. Biosorption is the metal-resistance process by which metal ions passively bind to biomass originating from microorganisms or plant material (Gupta, Nayak and Agarwal, 2015). In bacteria, the metal ions bind non-specifically to the structural components present in their cell walls (Zabochnicka-Świątek and Krzywonos, 2014). The Gram positive bacterial cell walls are composed of peptidoglycan, polymers of glycerol or ribitol, teichoic acids and lipoteichoic acids

(Gupta, Nayak and Agarwal, 2015). Components of the Gram negative bacterial cell walls include peptidoglycans, glycoproteins, lipoproteins, lipopolysaccharides and phospholipids (Gupta, Nayak and Agarwal, 2015). Anionic functional groups such as hydroxyl, sulfhydryl, carboxyl, amine and phosphate are present in these cell surface components and serve as metal-binding sites to external cationic metal ions (Etesami, 2018).

Ion exchange, complexation and adsorption are complex mechanisms involved in the uptake of metal cations onto the anionic functional groups present on the bacterial cell surface (Fashola, Ngole-Jeme and Babalola, 2016). Metal ions are replaced with other cationic functional groups present on the cell surface during the ion exchange process (Ayangbenro and Babalola, 2017). Anionic functional groups bind with metal ions forming complexes thus rendering the metal ions to a reduced or less toxic state (Ayangbenro and Babalola, 2017). During adsorption, metal ions bind to functional groups through van der Waals forces and electrostatic interactions such as covalent bonding, ionic bonding or hydrogen bonding (Zabochnicka-Świątek and Krzywonos, 2014). These mechanisms may be exploited for the bioremediation of heavy metals from wastewaters.

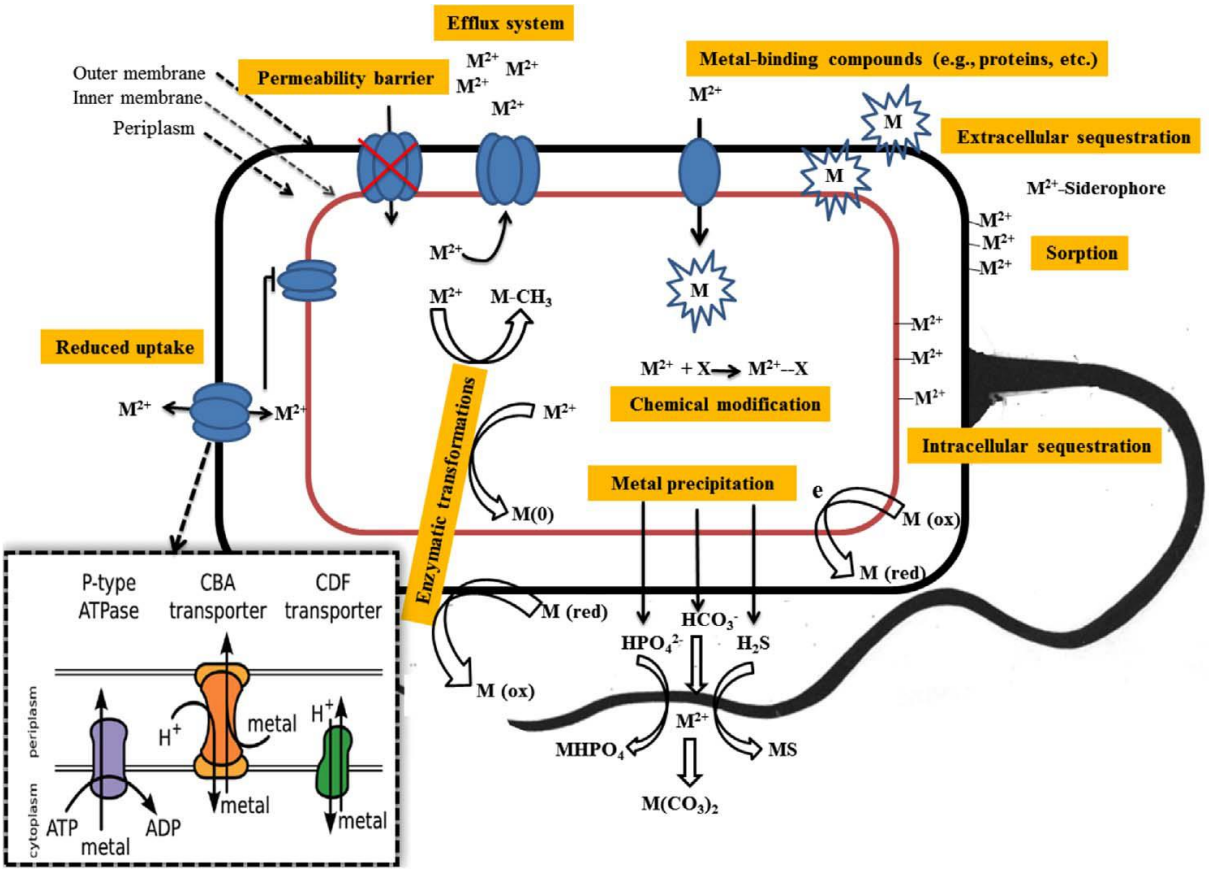


Figure 2.2. Mechanisms of bacterial metal resistance. Permeability barriers such as outer membrane proteins prevent metal ions from entering the cell due to conformational restrictions. Metal cations bind to the bacterial cell surface components through biosorption. Metal binding compounds such as metal-chelating proteins form complexes with metal ions in the extracellular space through extracellular sequestration. Within the cell, the metal cations may be reduced to less toxic forms through enzymatic transformation, complexation or chemical modification. Metal chelates or complexes from the intracellular environment is excluded into the extracellular space as metal precipitate. Metals may accumulate as aggregates when bound to metallo-chaperones within the cell through intracellular sequestration (Adapted from Etesami, 2018).

Schoeman and Steyn (2001) reported that the total capital costs (inclusive of pre-treatment, desalination and brine disposal) for the physicochemical treatment of mine water from the Blesbokspruit (Gauteng, South Africa) using reverse osmosis, electrodialysis or ion-exchange was approximately US$53 million, US$95.3 million and US$81.8 million, respectively. A recent report for the total cost for mine water treatment originating from the Witwatersrand Basin (Gauteng, South Africa) is

approximately R12 billion (Crowley and Henderson, 2016). Biosorbents have gained considerable attention in the field of heavy metal treatment technologies due to the high efficiency, cost-effectiveness, abundance, desorption and biodegradable properties (Michalak, Chojnacka and Witek-Krowiak, 2013). Biosorbents that are composed of dead or living biomass such as bacteria are widely considered to be low-cost adsorbents (Fomina and Gadd, 2014). While physicochemical treatment strategies involve the use of expensive chemicals, equipment or processing power, bacterial biosorbents are easily cultured and require little processing. The use of bacterial biosorbents under laboratory conditions for metal contaminated wastewater treatments have been extensively studied (Fomina and Gadd, 2014; Ayangbenro and Babalola, 2017).

Bacterial biosorbents may be prepared using either living or dead biomass. Bioaccumulation of metals using living biomass requires a system that facilitates the continual growth and survival of the live cells which increases the cost of application (Gaur *et al.*, 2014). Metal toxicity may also inhibit the growth of the live cells compared to dead cells (Michalak, Chojnacka and Witek-Krowiak, 2013). As such, dead biomass is preferred for bacterial biosorption of heavy metals due to the cost effectiveness, ease of metal sorption and recovery as well as reuse of the dead cells (Fomina and Gadd, 2014).

Several studies have compared biosorption and bioaccumulation for bioremediation of metal contaminated water using microorganisms. A study performed by Srinath *et al.* (2002) compared the bioaccumulation and biosorption efficiencies of *Bacillus coagulans* and *Bacillus megatarium* in the treatment of Cr (VI) contaminated effluent. The bioaccumulation and biosorption efficiencies for *B. coagulans* were 23.8 mg/g and 39.9 mg/g compared to 15.7 mg/g and 30.7 mg/g for *B. megaterium*, respectively. Biosorption using non-living cells displayed higher efficiency compared to the bioaccumulation treatment. In another study, Machalová *et al.* (2015) compared the biosorption and bioaccumulation of radionuclide $^{109}Cd^{2+}$ by *M. luteus* and *Kocuria palustris*. It was found that 85 % and 83 % of Cd had localized on the cell surface compared to the 15 % and 17 % of Cd that had localized in the cytoplasm for *M. luteus* and *K. palustris*, respectively. These studies showed better removal of metals through biosorption compared to bioaccumulation.

Major drawbacks of biosorption using dead bacterial biomass includes the poor strength, small size, low density and mechanical instability of the biosorbent (Velkova *et al.*, 2018). The immobilization or entrapment of dead biomass onto support matrices such as polyvinyl alcohol, polysulfone, alginate, polyacrylamide and glutaraldehyde stabilizes the biosorbent by imparting mechanical strength, porosity, rigidity, larger size and resistance of the chemical and microbial properties to physical degradation (Ahalya, Ramachandra and Kanamadi, 2003; Wang and Chen, 2009).

Many studies have been performed to enhance the biosorption process through immobilization. Rani *et al.* (2010) performed a biosorption study that compared the removal of Cu, Cd and Pb before and after immobilizing the dead cells of *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* sp. in 2 % calcium alginate and 10 % polyacrylamide gel beads. The immobilized *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* sp. had removed 69.34 % of Cu, 90.41 % of Cd and 84.27 % of Pb, respectively, compared to 44.73 % of Cu, 86.66 % of Cd and 79.22 % of Pb biosorption by the free dried dead cells. In another study, Kuhn and Pfister (1989) successfully treated solutions containing Cd, Zn, Mn, Pb, Cu and strontium (Sr) using *Zoogloea ramigera* cells that were immobilized into calcium alginate beads. The immobilized cells removed 99.9 % of Cd from Cd solutions and 95.9 % of the metals were removed from solutions containing a mixture of Cd, Zn, Mn, Pb, Cu and Sr. In a separate study by Ilamathi, Nirmala and Muruganandam (2014), a consortium of yeast, *Pseudomonas aeruginosa*, *B. subtilis* and *Escherichia coli* were immobilized in calcium alginate beads. The immobilized biosorbent removed 84.62% of Cu, 67.17% of Cd, 49.25% of Cr and 61.02% of Ni from synthesized metal solutions containing 150 mg/L of each metal. The findings from these studies indicate the high biosorption properties of microorganisms that were immobilized using calcium alginate.

The efficiency and selectivity of biosorbents in the removal of metals from wastewaters are affected by several factors. These factors include pH, temperature, the initial concentration of the metals, the concentration of biosorbent used as well as the affinity of metals to the biosorbent where there are multiple metal elements in solution (Abbas *et al.*, 2014; Vallabh, 2017). The pH affects the bioavailability of the metal ions, the competition between metal ions and protons for binding sites as well as the net ionic charge of the functional groups present in the biosorbent (Abbas *et al.*, 2014). Higher

temperatures tend to increase the kinetic energy and biosorption activity in binding metals however temperatures that are too high potentially damages the physical state of the biosorbent (Fomina and Gadd, 2014). As the initial metal concentration increases, the biosorption efficiency increases however metals may still remain in solution as a result of the saturation of metal binding sites (Fomina and Gadd, 2014). A biosorption study by Ansari, Masood and Malik (2011) showed that the initial concentration of Cd and Ni in solution affected the biosorption efficiency of *E. coli* WS11. The biosorption efficiency increased from 4.96 mg/g to 45.37 mg/g and 6.96 mg/g to 55.31 mg/g when the concentration of Cd and Ni ranged from 50 to 400 mg/L. High concentrations of biomass may cause cells to aggregate thus affecting the number of binding sites available for metals (Gadd, 1990). The presence of more than one metal ion may inhibit maximum biosorption as the ions compete for binding sites (Gadd, 2009). Hence, other major drawbacks of utilizing biosorbents includes poor selectivity of metal ions and saturation of metal binding sites (Vijayaraghavan and Yun, 2008). It is therefore necessary to enhance the biosorption process and investigate desorption processes for reuse of biosorbents.

2.5 Enhancing biological treatment strategies with nanotechnology

Nanotechnology is an emerging field that is being exploited for the improvement of current wastewater treatment strategies (Mahmoud, 2016). Nanoscale particles (1-100 nm) are associated with several properties such as high surface to volume ratios, reactivity and high adsorption capacity (Qu, Alvarez and Li, 2013; Gaur *et al.*, 2014). The properties of nanomaterials may be exploited to improve the efficiency of current wastewater treatments as well as the quantity and quality of water (Qu, Alvarez and Li, 2013). Nanomaterials that are currently being explored for adsorption studies include carbon nanotubes, graphene-based nanoadsorbents, nanoscale metal oxides, magnetic nanoadsorbents and nanofibers with core-shell structures (Anjum *et al.*, 2016). The challenges associated with large-scale implementation of such nanomaterials includes potential environmental health risks (Anjum *et al.*, 2016). There are limited technologies available for the detection of nanoscale particles under the circumstances that large amounts of the nanomaterials are discharged into the surrounding environments and water bodies (Fu and Wang, 2011; Qu, Alvarez and Li, 2013).

2.5.1 Calcium alginate nanoparticles

Khezri *et al.* (2016) showed that synthesized CANPs were highly efficient in the removal of 99 % of Pb from solutions. Calcium alginate is a gel matrix that is environmentally friendly as it is composed of a biodegradable, non-toxic and biocompatible anionic polymer (Paques *et al.*, 2014). Sodium alginate is an inexpensive material that is readily available. The alginate polymer is composed of α-L-guluronic acid (G) residues and β-D-mannuronic acid (M) residues that are linked via 1,4-glycosidic linkages (Paques *et al.*, 2014). Upon addition of calcium cations, the G residues of sodium alginate form an 'egg box' around the calcium ions thus forming a stiff, strong, porous gel matrix as shown in Figure 2.3. The exposed carboxylate functional groups of the G residues are potential metal binding sites therefore the calcium alginate gel may be used for treatment of heavy metal contaminated wastewaters (Khezri *et al.*, 2016). The size of the CANPs further provides a high surface to volume ratio thereby increasing the amount of available metal binding sites.

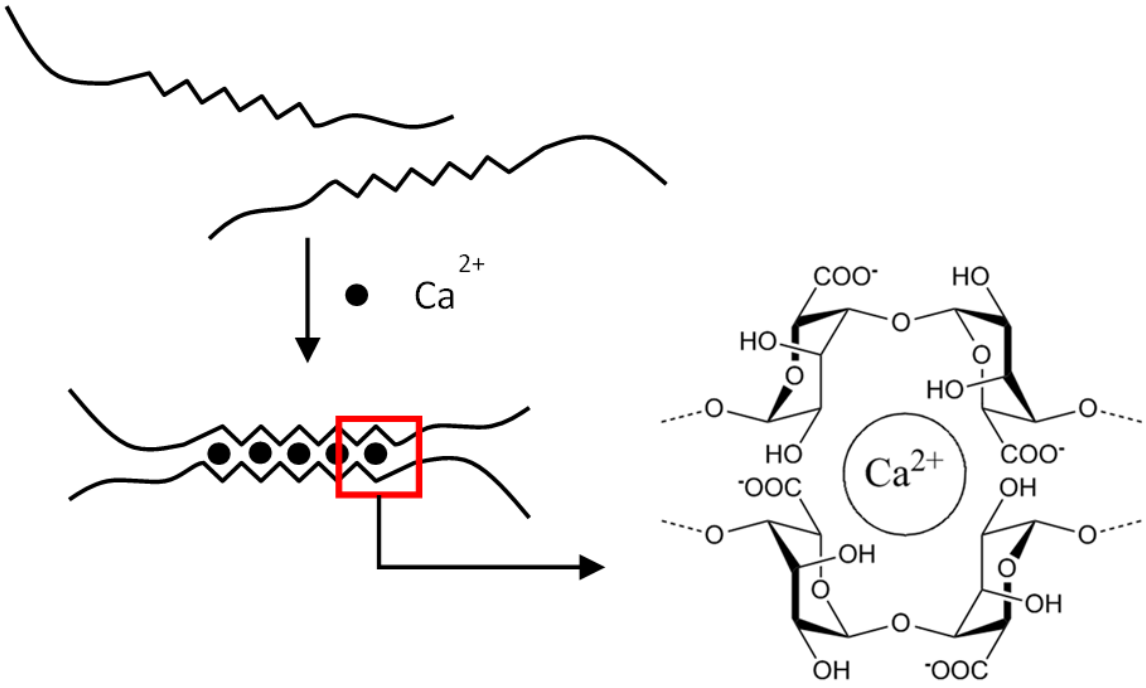


Figure 2.3. Calcium alginate gel formation. Four G residues of sodium alginate cross link with calcium cations forming an 'egg box' porous and strong gel matrix. Carboxylate functional groups of the G residues are exposed and serve as potential metal binding sites (Paques *et al.*, 2014).

Several studies have incorporated nanotechnology for the sorption of heavy metals. Hao, Man and Hu (2010) synthesized a magnetic nanoadsorbent for the adsorption of Cu from polluted river and tap water. The nanoadsorbent was prepared by covalently binding 1,6 hexadiamine to the surface of Fe_3O_4 nanoparticles. The maximum adsorption capacity of Cu was 25.77 mg/g and 98 % of Cu was removed from the contaminated waters. A study conducted by Qu *et al.* (2014) developed ZnS nanocrystal (NC) sorbent by coating ZnS NC onto the surface of α-Al_2O_3 nanoparticles for the removal of mercury (Hg) from solutions. Approximately 2000 mg/g Hg was the saturated adsorption capacity of the ZnS NCs and 99.9 % of Hg was removed by the ZnS NC sorbent from aqueous solutions. In a separate study, Neyaz and Siddiqui (2015) developed a nanosorbent composed of a silica-coated magnetite nanocomposite functionalized with ethylenediaminetetraacetic acid (EDTA). The nanosorbent was used to remove Cu from aqueous solutions. The maximum capacity of the nansorbent was 0.099 mole/g and 90-95 % of the Cu ions was removed. These studies indicate the potential use of nanotechnology for treatment of heavy metal contaminated water.

2.6 Problem statement and research gap

The water resources of Gauteng are constantly being polluted with elevated concentrations of heavy metals arising from mining and industrial effluent (McCarthy, 2011). As a result, the natural water quality and quantity in Gauteng has deteriorated. The local communities in the surrounding areas utilize the metal contaminated water resources for personal and agricultural purposes (McCarthy, 2011). Upon ingestion of toxic metal concentrations, these communities are exposed to detrimental health hazards (Duruibe, Ogwuegbu and Egwurugwu, 2007). The reclamation of AMD wastewater may potentially serve as an alternative source of water for both domestic and agricultural purposes. It therefore becomes necessary to investigate and develop treatment strategies that are eco-friendly and effective in removing heavy metals from the water resources.

2.7 Present study

The dead biomass of *M. luteus* is highly efficient in the biosorption of relatively high concentrations of metals and could therefore be exploited as a biosorbent (Jackson *et*

al., 2009; Wang and Chen, 2009; Rani *et al.*, 2010; Puyen *et al.*, 2012). One particular study performed by Leung *et al.* (2000) found that immobilizing *M. luteus* on calcium alginate improved metal removal abilities. Some studies have also exploited *Enterobacter* sp. for the removal of heavy metals from effluents (Lu *et al.*, 2006; Panda and Sarkar, 2012). Sinha and Khare (2012) found that immobilizing *Enterobacter* sp. in calcium alginate enabled reuse of the cells for multiple cycles in the removal of Hg from aqueous solutions.

The present study focuses on treating synthetic wastewater using metal biosorbents composed of the dead biomass of *E. xiangfangensis* Pb204 and *M. luteus* which were isolated from acid mine decant on the West Rand, Johannesburg, South Africa (26°06'26.8"S 27°43'20.2"E). Studies performed by Vallabh (2014, 2017) had shown *E. xiangfangensis* Pb204 and *M. luteus* to be highly tolerant of heavy metals. When used as a consortium, the biosorption of Pb, Ni, Co, Mn and Zn from multi-metal systems was enhanced thereby indicating their potential for application as a commercial biosorbent (Vallabh, 2014, 2017). Due to the properties of CANPs in relation to metal sorption, the present study aimed at improving the biosorptive ability of *E. xiangfangensis* Pb204 and *M. luteus* biosorbents by immobilization onto CANPs as nanobiosorbents.

The biosorption treatment strategy investigated in this study shows potential in re-use of AMD wastewater as an alternative source of water. Rural communities that struggle to find access to potable water may potentially use the biosorbent or nanobiosorbent treated AMD wastewater as a water source for domestic and agricultural purposes.

2.8 Research hypothesis

Immobilization of bacteria is important to maintain extended use as well as to control the presence of bacteria (even if dead) in water (Ahalya, Ramachandra and Kanamadi, 2003). Finding a suitable immobilization matrix is a challenge that could be answered by utilizing CANPs. CANPs could protect and enhance the metal binding properties of the biosorbent. A mixture of *M. luteus* and *E. xiangfangensis* Pb204 as a biosorbent is known to successfully biosorb high concentrations of metals from sampled wastewaters. Therefore, the study hypothesizes that further immobilizing the bacterial

mixture on CANPs would enhance the metal biosorption abilities of the bacterial biosorbent.

Water scarcity and access to water for domestic and agricultural purposes in rural communities is limited (Ochieng, Seanego and Nkwonta, 2010). Subsequently, heavy metal contamination affects agriculture and the food chain particularly when metals are taken up by crops irrigated with heavy metal contaminated water (Durand, 2012). The study additionally seeks to verify the suitability of synthetic wastewater treated with the nanobiosorbent for agricultural use. This aspect was investigated in tomato plants as they are widely grown by subsistence farmers in local communities. Furthermore, local tomato production contributes 22 % to the total amount of vegetables produced in South Africa making it a high value product (DAFF, 2018). The study hypothesizes that the concentration of metals taken up by the tomato plants watered with treated synthetic AMD wastewater would be within acceptable limits and that the treatment would provide a short-term solution for water reuse in subsistence/home-grown farming.

Chapter 3: Biosorption of heavy metals from neutralized synthetic AMD wastewater

3.1 Introduction

Heavy metal contamination of water resources in Gauteng has resulted from urban industrialization. In particular, the mining industry generates high levels of toxic heavy metal contaminated wastewater in the form of AMD (Ochieng, Seanego and Nkwonta, 2010). The heavy metals in AMD seeps into the environment where they accumulate in animals and plants. Heavy metals also accumulate in humans via the food chain (Duruibe, Ogwuegbu and Egwurugwu, 2007). At elevated concentrations, heavy metals pose chronic environmental health risks to ecosystems, agriculture and humans (Duruibe, Ogwuegbu and Egwurugwu, 2007). Physicochemical treatment strategies such as chemical precipitation using limestone or membrane filtration are currently being exploited for the remediation of toxic heavy metals from wastewaters (Fu and Wang, 2011). This treatment strategy is highly efficient however, it is expensive and generates large amounts of toxic sludge that is damaging to the environment. As such, low-cost biological alternatives are being developed for the efficient removal of heavy metals from wastewater in an eco-friendly manner (Fashola, Ngole-Jeme and Babalola, 2016).

Previous studies have shown that the biomass of dead bacteria may be exploited for the biosorption of heavy metals from wastewaters (Ayangbenro and Babalola, 2017). During the passive process of biosorption, external metal cations bind to the anionic functional groups present on the outer surface of dead bacterial cells (Gaur *et al.*, 2014). Binding of the metal cations to the surface of the cell occurs via various mechanisms including ion exchange, complexation and adsorption (Ahalya, Ramachandra and Kanamadi, 2003). The drawback of utilizing dead bacterial biomass includes poor strength, low density and mechanical instability (Gupta, Nayak and Agarwal, 2015). Immobilization of dead bacterial cells improves the stability, mechanical strength and porosity of the biosorbent. It also prevents the physical degradation of the chemical and microbial properties of the biosorbent (Zabochnicka-Świątek and Krzywonos, 2014). Moreover, immobilization using nanomaterials increases the surface area to volume ratio which subsequently increases the reactivity

and sorption properties of the biosorbent (San *et al.*, 2014). It further provides higher stability to the biosorbent (San *et al.*, 2014). Alginate, originating from brown algae, is an anionic polymer that is biodegradable and non-toxic (Paques *et al.*, 2014). A calcium alginate gel matrix exposing carboxylate functional groups forms upon crosslinking of alginate guluronic residues with calcium ions (Paques *et al.*, 2014). The calcium alginate gel matrix may be exploited for the removal of heavy metals from aqueous solutions as well as for the immobilization of the dead bacterial biomass (Rani *et al.*, 2010; Mishra, 2013).

A study performed by Vallabh (2017) reported on the use of *E. xiangfangensis* Pb204 and *M. luteus* as biosorbents for Pb in single and multi-metal systems. Maximal biosorption occurred at 25 °C, pH 7 with a biomass concentration of 2 g/L. Furthermore, the study showed that in addition to Pb, the biosorbents were able to take up Co, Mn, Ni and Zn when used individually or in combination. The present chapter describes the neutralization and treatment of synthetic AMD under the conditions described by Vallabh (2017) using free and immobilized *E. xiangfangensis* Pb204 and *M. luteus*.

3.2 Experimental procedure

Luria Bertani (LB) culture media, sodium alginate and metal salts (trace metal grade) were sourced from Sigma-Aldrich®, USA. Filter-sterilized stock solutions (0.5 M) of metal salts were used to prepare mine water with different metal concentrations as indicated in Table 3.1. The metal salts used included sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4.7H_2O$), manganese sulphate ($MnSO_4.H_2O$), copper sulphate ($CuSO_4.5H_2O$), cobalt chloride ($CoCl_2.6H_2O$), nickel chloride ($NiCl_2.6H_2O$), aluminium sulphate ($Al_2(SO_4)_3.18H_2O$), lead nitrate ($Pb(NO_3)_2$), zinc sulphate ($ZnSO_4.7H_2O$) and iron sulphate ($FeSO_4.7H_2O$). Each treatment was performed in triplicate.

3.2.1 Preparation of synthetic AMD

Three different AMD water samples were prepared based on metal concentrations reported for Gauteng wastewaters in 2017 (Table 3.1) with some modification using the prepared 0.5 M metal stocks. The first sample, AMD-1, was representative of metals in the Central Basin as reported by Humphries, McCarthy and Pillay (2017).

The second sample, AMD-2, and the third sample, AMD-3, represented mine water in the Witwatersrand Basin of Gauteng before and after treatment through a high density sludge (HDS) mine water treatment plant, respectively (Hobbs, 2017).

Table 3.1. Elemental composition of synthetic AMD representative of the Central and Witwatersrand Basin.

	AMD-1 (Humphries, McCarthy and Pillay, 2017)	AMD-2[1] (Hobbs, 2017)	AMD-3[2] (Hobbs, 2017)
Metal	Concentration (ppm)		
Na	170	22	22
K	20	2	2
Ca	280	54	54
Mg	172	33	33
Mn	47	86	16
Cu	0.33	0	0
Co	4.7	1.3	0.2
Ni	10.6	1.7	0.35
Al	122	1.04	0.15
Pb	0.003	0.009	0.009
Zn	9.12	0	0
Fe	40	712	21

[1] AMD from the Witwatersrand Basin before treatment

[2] AMD from the Witwatersrand Basin after treatment. Some of the metal concentrations were not reported after treatment and were therefore maintained as prior to treatment

The metal concentrations of all three AMD samples were quantified by ICP-OES in a Spectro ARCOS ICP-OES (Spectro).

3.2.2 Preparation of biosorbent

M. luteus and *E. xiangfangensis* Pb204 were grown to stationary phase at 37 °C for 24 hours in LB broth with shaking (200 rpm). The cells were harvested by centrifugation (Heraeus™ Multifuge™ X1R centrifuge, Thermo Scientific™) at 10000 x *g* for 10 minutes and heat-killed by autoclaving for 20 minutes at 121 °C. The cells were washed twice in physiological saline solution (9 g/L NaCl) by centrifuging at 5000 x *g* for 5 minutes in order to remove remnants of broth or contaminants from the

surface of the cells. After the final wash, the pelleted cells were stored at -20 °C until required for treatment. Prior to treatment, the cells were resuspended in sterile deionized water to give a final concentration of 10 g/L (Vallabh, 2017) unless stated otherwise.

3.2.3 Synthesis of CANPs

CANPs were synthesised according to a protocol kindly provided by Ms. Vidya Keshav at the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa. A 0.06 % (w/v) sodium alginate solution was dissolved at 70 °C and cooled to 25 °C with continuous stirring using the MSH10 hotplate magnetic stirrer (Labcon). Thereafter, a peristaltic pump (EP-1 Econo Pump, Biorad) was used to add 22 mM $CaCl_2$ dropwise at a flow rate of 0.5 mL/min to the sodium alginate solution while stirring to form the free CANPs. The nanoparticles were stirred for an additional hour for homogenization followed by three washes with deionized water (15000 x *g*, 10 minutes) to remove impurities and excess Ca^{2+} ions. The free CANPs were resuspended in deionized water to give a final concentration of 10 mg/mL and were sonicated thrice on ice using the Q125 sonicator (QSonica) for 30 seconds on and 30 seconds off with 50 % amplification to disperse aggregated nanoparticles.

3.2.4 Synthesis of nanobiosorbent

Nanobiosorbent was prepared using the same protocol as in section 3.2.3 with slight modification. A 0.06 % (w/v) sodium alginate solution was dissolved at 70 °C and cooled to 25 °C with stirring. Thereafter, 0.5% (v/v) glutaraldehyde was added to the solution and stirred for 30 minutes. Glutaraldehyde was used to efficiently crosslink sodium alginate in the presence of calcium chloride with the biosorbent. Biosorbent prepared as described in section 3.2.2 was added to give a final concentration of 10 mg/mL and was stirred for 30 minutes. Thereafter, 22 mM $CaCl_2$ was added as described in section 3.2.3 while stirring to form the nanobiosorbent. Following agitation for an hour, the nanobiosorbent was washed thrice with deionised water (15000 x *g*, 10 minutes) and dispersed by sonicating thrice on ice (30 seconds on, 30 seconds off, 25 % amplification) using the Q125 sonicator (QSonica).

3.2.5 Characterization and stability of the free CANPs and nanobiosorbent

The stability of the CANPs and nanobiosorbent was determined over a period of 2, 4 and 8 weeks when stored at both 4 °C and 25 °C. The surface morphology and characterisation of the CANPs and nanobiosorbent was performed using scanning electron microscopy (SEM) at the Microscopy and Microanalysis Unit (University of the Witwatersrand) (Nova Nanolab™ 600 SEM, FEI). The SEM samples were prepared by adding a 5 µl drop of sample onto carbon tape fixed to an aluminium stub. The sample was dried at 60 °C in the Optolab oven incubator (model 325) for an hour. Thereafter, the samples were coated with one coating of carbon and two coatings of gold palladium before analysis.

3.2.6 Neutralization and biological treatment of the synthetic AMD samples

Prior to treatment with biosorbent, nanobiosorbent or free CANPs, the AMD samples were neutralised either with ground limestone powder (Wonder B3059 Agricultural Lime, Efekto) or 1 M sodium hydroxide (NaOH). The neutralized samples were analysed by ICP-OES for metal composition before and after removal of any metal precipitation to determine the best neutralizing agent to use. Metal precipitates formed due to the neutralization process were removed by centrifuging at 24 000 x g for 10 minutes. Subsequent experimental work was performed with limestone-neutralized AMD water samples. The percentage reduction in metal concentration by the neutralizing agents was determined using Equation 1 (Ahmad *et al*., 2012):

$$\% \text{ Reduction} = 100 \times (C_b - C_a) / C_b \qquad [1]$$

Where C_b and C_a refers to the concentration of metal in solution (mg/L) before and after the removal of metal precipitate, respectively.

After removing any metal precipitates, AMD-1, AMD-2 and AMD-3 were treated for 24 hours with 2 g/L of either *M. luteus*, *E. xiangfangensis* Pb204 or a 2:1 ratio of *E. xiangfangensis* Pb204 to *M. luteus*. The AMD samples were incubated at 25 °C with shaking (150 rpm) to allow optimal interaction between the metal ions and biosorbent during treatment. Cell-bound metal ions were separated from unbound metal ions

through centrifugation (24 000 x g, 10 minutes). The supernatant of each sample was nitrified to a pH ≤ 2 in preparation for analysis of metal composition by either ICP-OES (Spectro ARCOS ICP-OES, Spectro) or ICP-MS (NexION 300 ICP-MS, PerkinElmer® Inc.). Furthermore, neutralized mine water samples were treated with 2 g/L of either *M. luteus*, free CANPs, nanobiosorbent prepared with only *M. luteus* or a mixture of *M. luteus* biosorbent and free CANPs in a 1:1 ratio. The untreated and treated samples were analysed using ICP-OES. ICP-MS analysis was performed on the same samples before and after treatment for the following metals: Al, Pb, Fe, Zn, Co, Cu and Ni. The percentage of metal biosorbed by the biosorbent was determined using Equation 2 (Ahluwalia and Goyal, 2005):

$$\% \text{ Biosorption} = 100 \times (C_i - C_f) / C_i \qquad [2]$$

Where Ci and C_f refers to the initial and final concentration of metal in solution (mg/L) before and after the biosorption treatment, respectively.

3.2.7 Statistical analysis of data

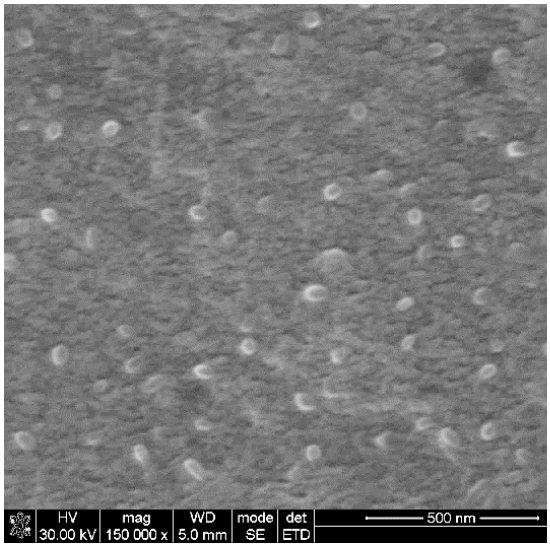
The statistical significance of the different treatments was analysed using IBM SPSS Statistics, version 25. A one-way ANOVA statistical test was used to determine the significance at a 95% confidence interval ($p < 0.05$) of the parameters tested between the neutralization or treatment groups.

3.3 Results

3.3.1 Characterization and stability of CANPs and nanobiosorbent

For the purpose of this study, the nanobiosorbent of choice was composed of *M. luteus* immobilized onto CANPs. The choice of bacterial species was based on findings discussed in section 3.3.2 from the different treatment options that were performed during the biosorption studies. SEM imaging showed that majority of the free CANPs formed monodisperse spherical structures that were approximately 55-85 nm in size (Figure 3.1a). Figure 3.1b shows the spherical CANPs coating the surface of *M. luteus* to form the nanobiosorbent.

a



b

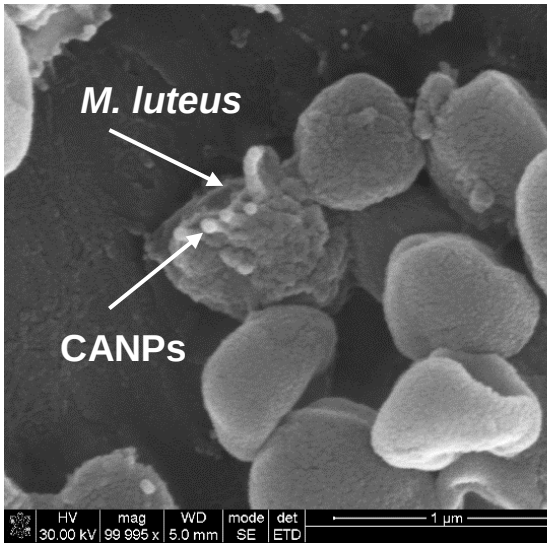
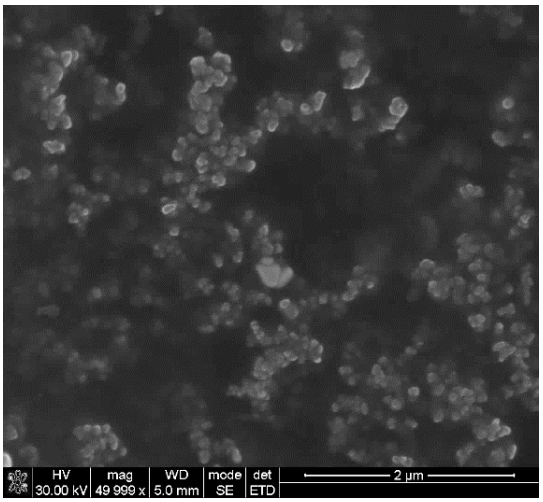


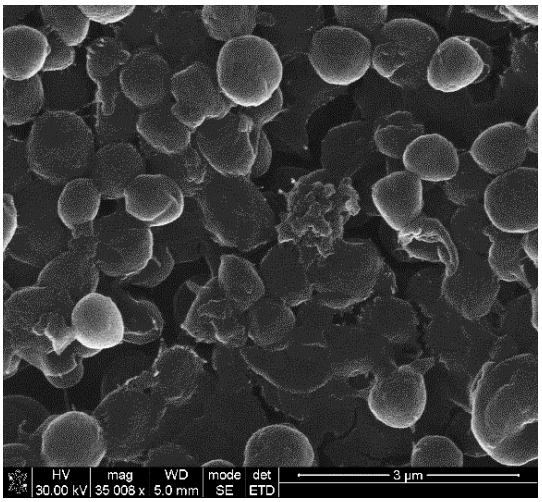
Figure 3.1. SEM images of free CANPs (a) and nanobiosorbent comprising *M. luteus* (b). Majority of the free CANPs were spherical in structure. *M. luteus* bacterial cells were coated with CANPs as indicated by the arrows.

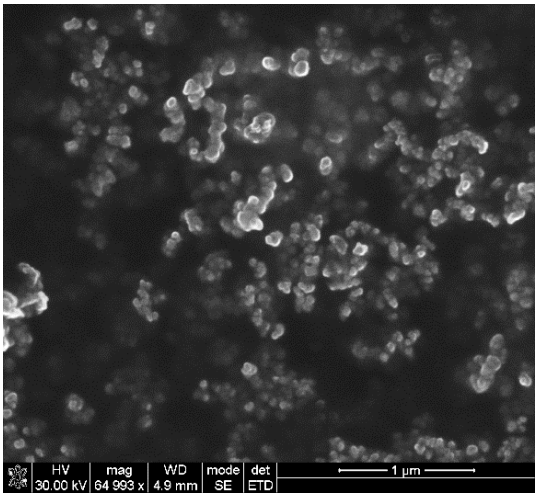
Both the free CANPs and nanobiosorbent displayed structural stability when stored up to 8 weeks at 4 °C and 25 °C (Figure 3.2). The cell walls of the bacteria in the nanobiosorbent remained intact and the spherical CANPs coating the surface of the bacterial cells maintained their association throughout the stored period at both temperatures.

a



b



c 

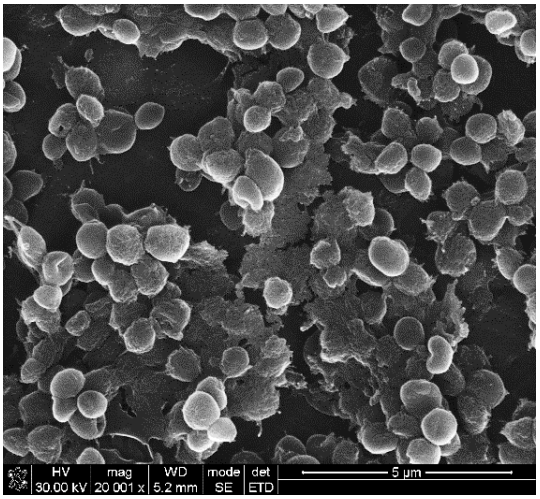
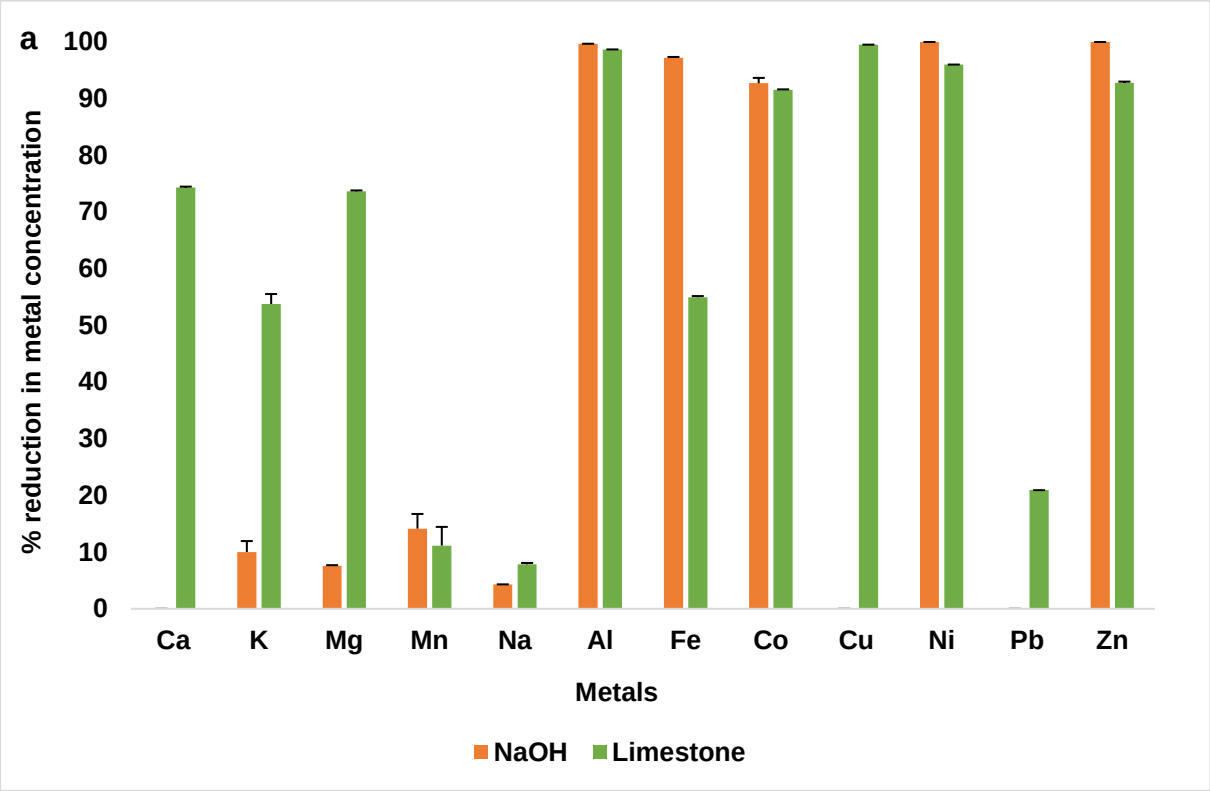
d 

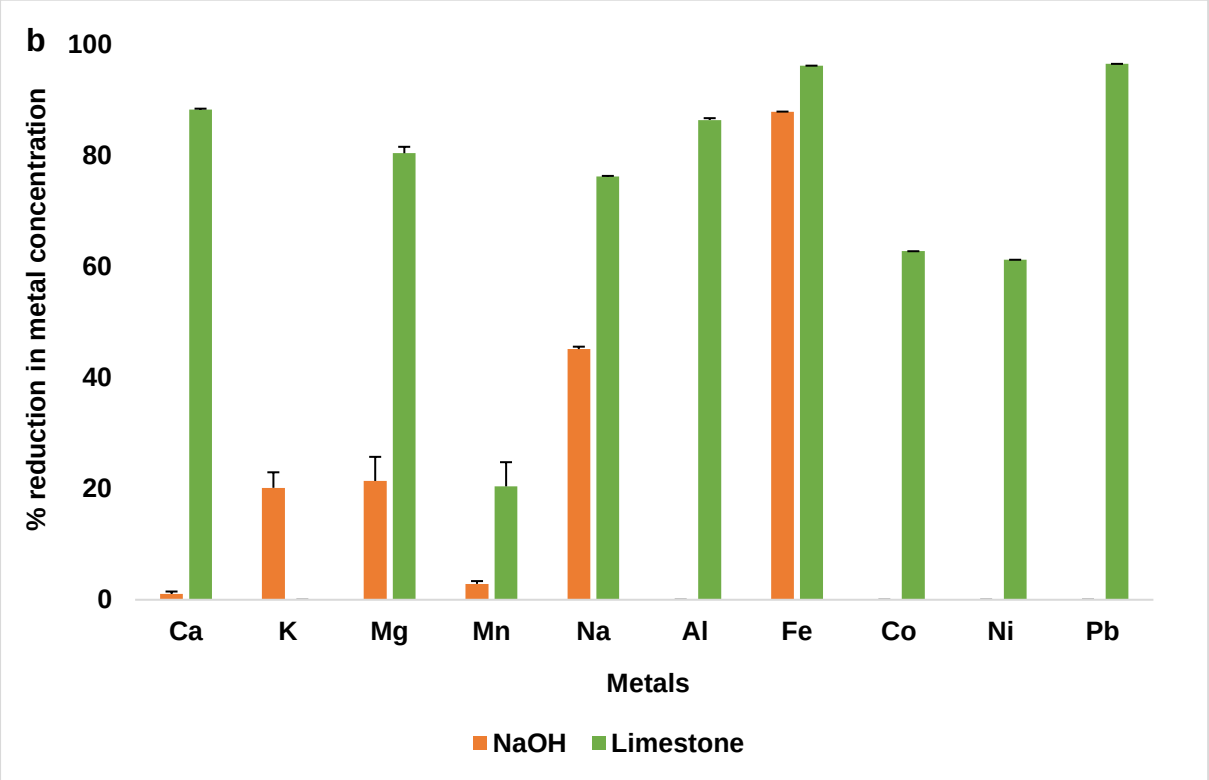
Figure 3.2. SEM images of free CANPs and nanobiosorbent that were stored for 8 weeks. CANPs at 4 °C (a); nanobiosorbent at 4 °C (b); CANPs at 25 °C (c) and nanobiosorbent at 25 °C (d).

3.3.2 Neutralization and treatment of AMD-1, AMD-2 and AMD-3

Application of either limestone or NaOH to neutralize the low pH of AMD-1 (pH 4), AMD-2 (pH 4) and AMD-3 (pH 5) resulted in the precipitation of metals. All 12 metals were reduced in concentration when AMD-1 was neutralized with limestone as a result of precipitation while neutralization with NaOH reduced the concentration of Ca, Zn, Ni, Al, Co, Fe, K, Mg, Mn and Na. A one-way ANOVA analysis indicated that all 12 metal concentrations were significantly different when comparing the two neutralization strategies (Table A.1 in Appendix A). For AMD-2, limestone neutralization resulted in the precipitation and reduction of Al, Fe, Ca, Mg, Mn, Na, Ni, Co and Pb as compared to the use of NaOH which only reduced Ca, Na, Mg, Mn, Fe and K. Of these metals, the reduction in concentration of Co, Mn, Ni, Ca, Fe, Mg and Na during limestone neutralization were found to be statistically significant (Table A.1 in Appendix A). Neutralization of AMD-3 (a treated version of AMD-2) with NaOH reduced the concentration of only Fe and K resulting from precipitation. Neutralization and precipitation with limestone resulted in the reduction of all metal concentrations with the exclusion of Cu and Zn. Statistical significance between neutralization with limestone and NaOH was observed for all metals except for Mn, Cu and Zn (Table A.1 in Appendix A). Figure 3.3 represents the concentration of metals in all three AMD samples before and after the metal precipitates were removed by centrifugation.

Based on these findings, all subsequent experimental work was performed on AMD samples neutralized with limestone.





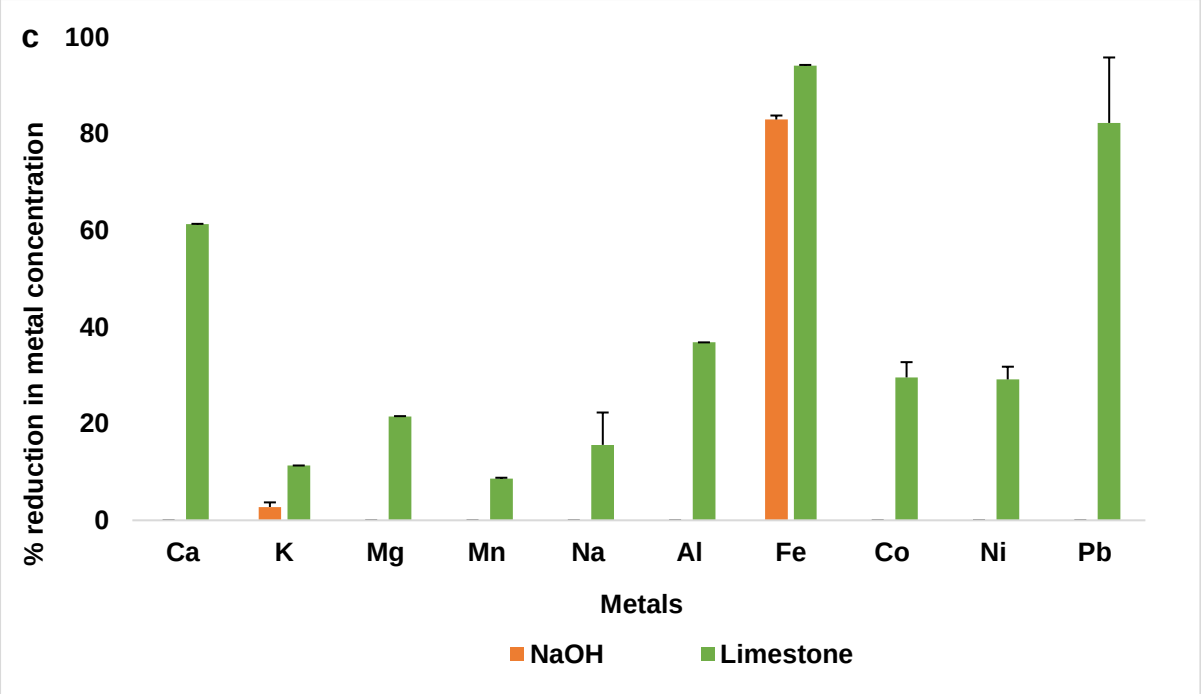


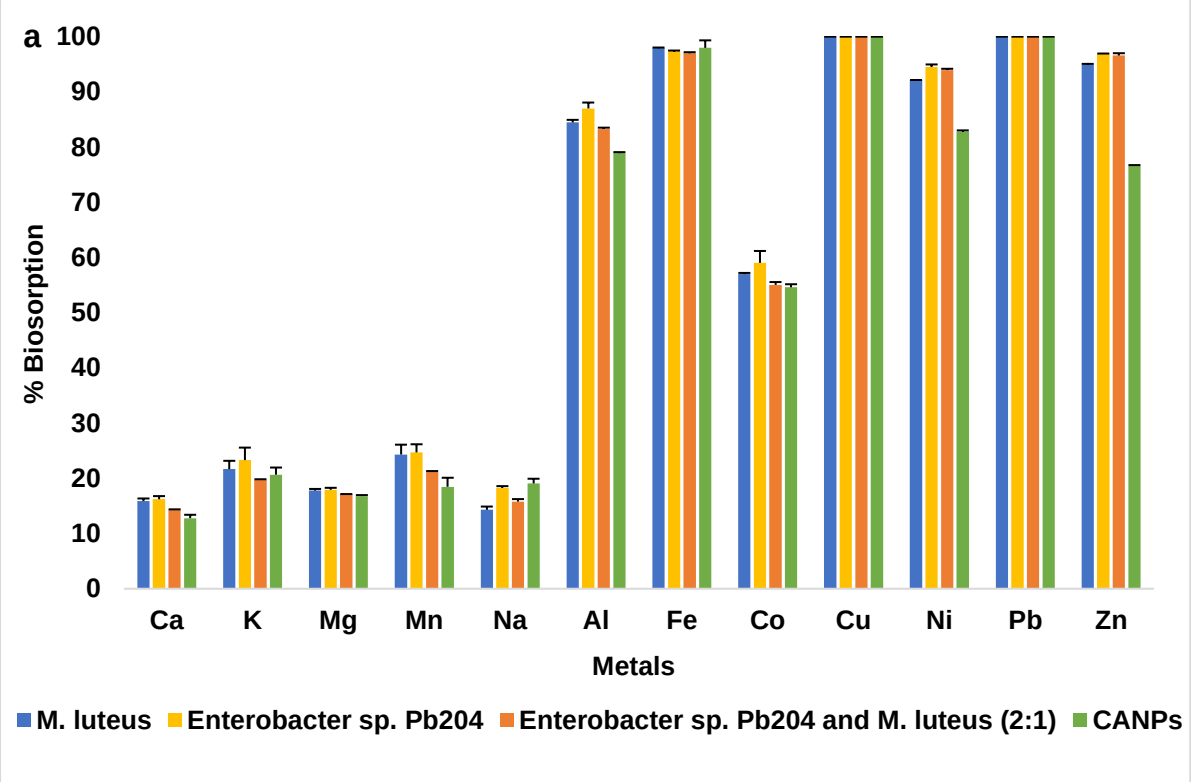
Figure 3.3. The percentage reduction in metal concentrations after neutralization of AMD-1 (a), AMD-2 (b) and AMD-3 (c) with NaOH or limestone.

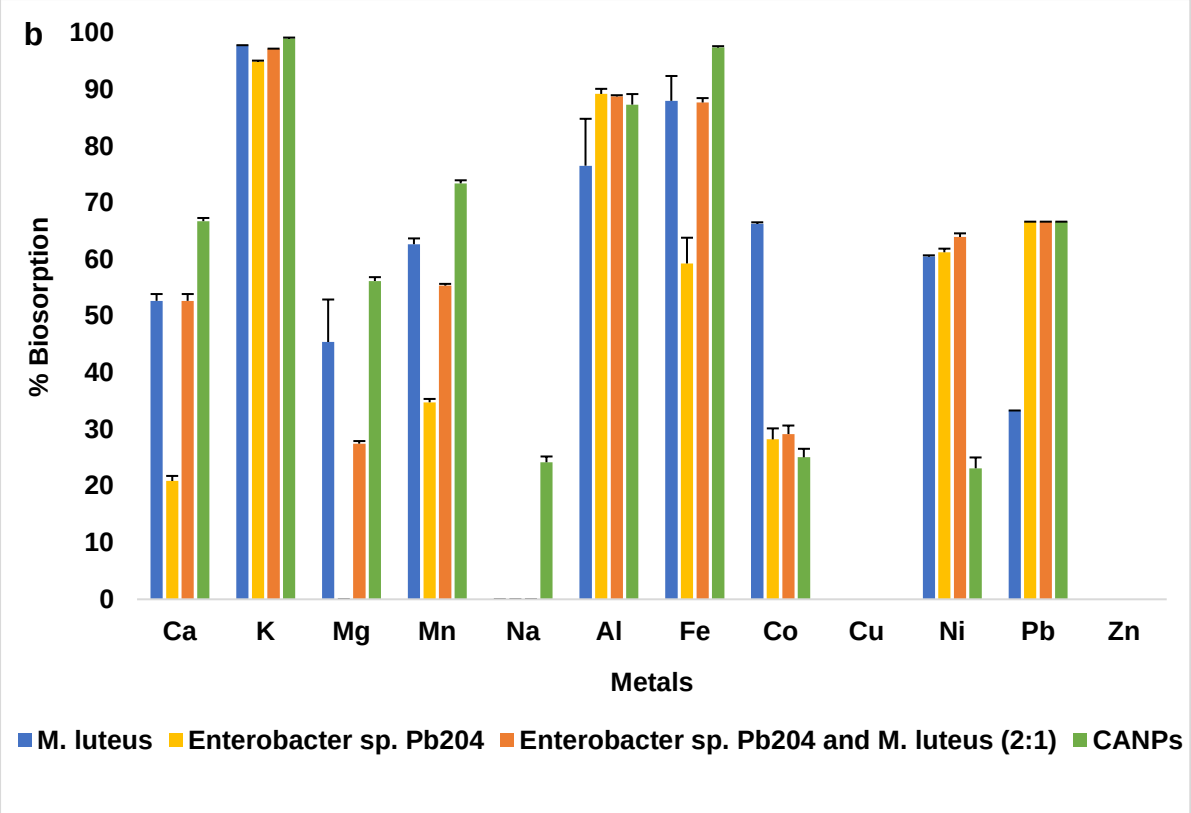
Post-neutralization with limestone, the concentration of Mn (43.4, 92.7 and 12.8 mg/L), Fe (1.3, 1.8 and 0.4 mg/L), Ni (0.242, 0.666 and 0.269 mg/L) and Al (2.98, 1.4 and 0.3 mg/L) remained above the acceptable agricultural levels of 10 mg/L, 0.3 mg/L, 0.02 mg/L and 0.225 mg/L (DWAF, 1996) in AMD-1, AMD-2 and AMD-3, respectively. Further treatment with a biosorbent or nanobiosorbent was anticipated to bring these levels within the stipulated limits.

Biological treatment of limestone neutralized AMD-1 with either *E. xiangfangensis* Pb204, *M. luteus* or a 2:1 combination of the two bacterial species resulted in further reduction of Ni to acceptable agricultural levels. However, Al and Mn remained above acceptable agricultural levels for all four biological treatments as shown in Figure 3.4a. In addition, all treatments reduced the Fe concentration to acceptable agricultural levels. Comparison of treatment strategies for AMD-1 using one-way ANOVA analysis indicated significant differences in Ni concentrations across all treatments (p = 0.001, Table A.2 in Appendix A) except between *E. xiangfangensis* Pb204 and the combination of biosorbents (p = 0.175, Table A.2 in Appendix A). Significant differences were also observed in the treatment of Fe when comparing *M. luteus* to the other three treatments (p < 0.05, Table A.2 in Appendix A).

For AMD-2, treatment with the individual and combined bacterial biosorbents and the free CANPs did not lower the concentration of Mn and Ni below agricultural limits. When used individually, both *E. xiangfangensis* Pb204 and *M. luteus* lowered the concentration of Fe by 59.3 % and Al by 76.5 %, respectively (Figure 3.4b); these levels were still above the acceptable limit of 0.3 mg/L for Fe and 0.225 mg/L for Al. However, when used in combination or with free CANPs, the concentration of Fe was reduced by 87.7 and 97.4 %, respectively and Al was reduced by 88.8 and 87.3 %, respectively. Individually, *E. xiangfangensis* Pb204 reduced the Al concentration by 89.2 % and *M. luteus* reduced the Fe concentration by 88 %. No significant differences are reported for Al concentrations for AMD-2 across all treatments (p = 0.515, Table A.3 in Appendix A). Significant differences were observed for the treatment of Fe between *M. luteus* and *E. xiangfangensis* Pb204 (p = 0.044, Table A.3 in Appendix A) and between the combination of biosorbents and free CANPs (p = 0.003, Table A.3 in Appendix A).

All metal concentrations except for Ni were reduced to acceptable agricultural levels in AMD-3 when using any of the four treatments (Figure 3.4c) with statistical significance reported for Ni across all treatment strategies (p = 0.001, Table A.4 in Appendix A).





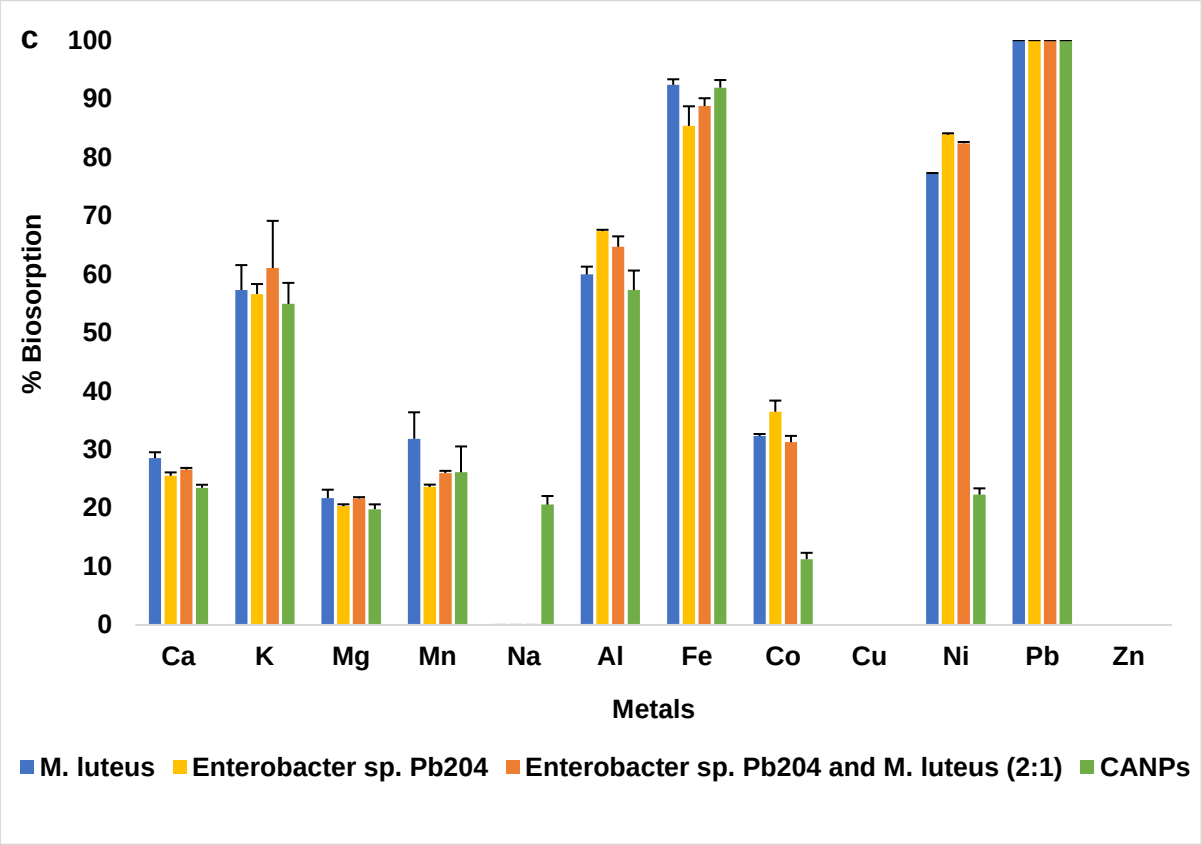


Figure 3.4. The percentage biosorption of metal from limestone neutralized AMD-1 (a), AMD-2 (b) and AMD-3 (c) treated with *M. luteus*, *E. xiangfangensis* Pb204, a combination of *E. xiangfangensis* Pb204 and *M. luteus* (2:1) and CANPs.

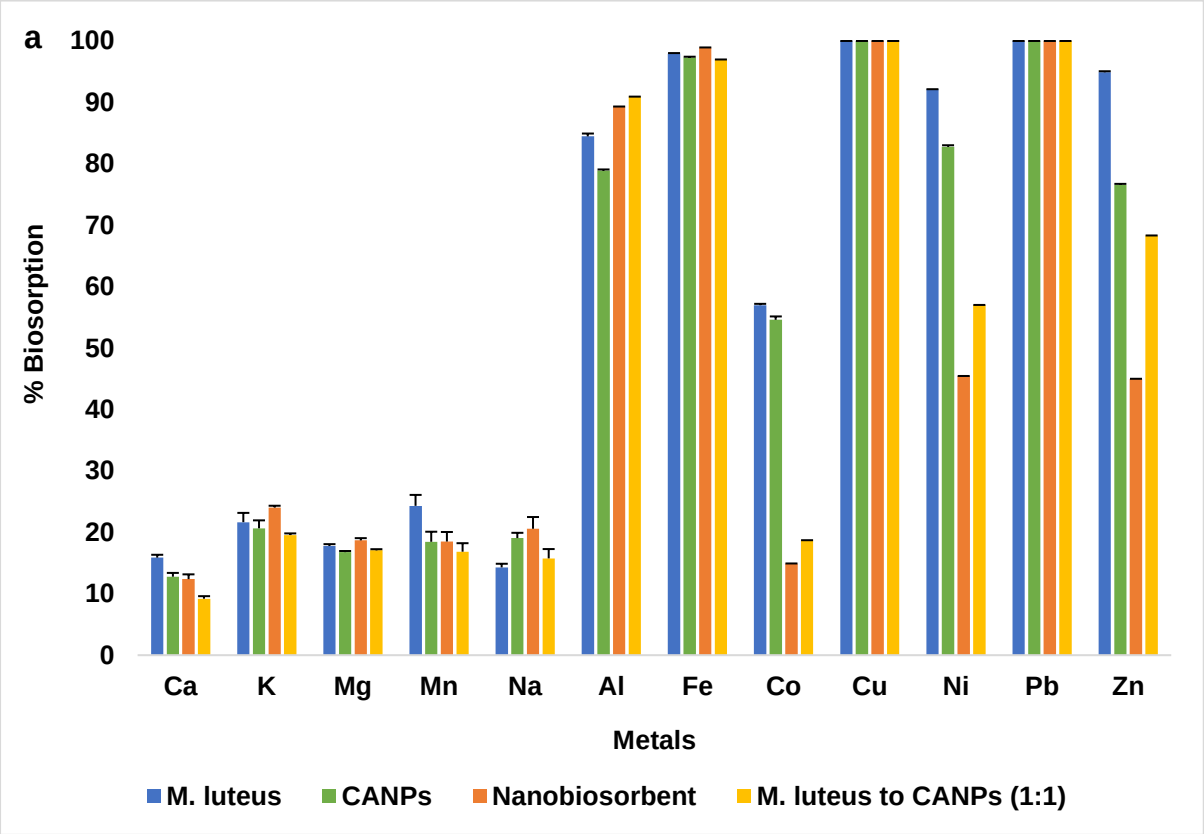
Overall, all four treatments were able to reduce the metal concentrations in each of the AMD samples. For each AMD sample, a different treatment was observed to reduce more metal. For example, *E. xiangfangensis* Pb204 reduced some of the metals more in AMD-1 compared to the other treatments while for AMD-3, treatment with *M. luteus* resulted in higher reduction. However, based on the statistical data (Table A.2-A.4 in Appendix A), treatment with *M. luteus* showed significant reduction ($p < 0.05$) in more metals from all three AMD samples and was selected for immobilization onto CANPs to prepare the nanobiosorbent. Treatment of the three AMD samples with the nanobiosorbent was then compared to treatment with a 1:1 mixture of *M. luteus* and free CANPs in addition to using the bacterial biosorbent or free CANPs on their own (Figure 3.5).

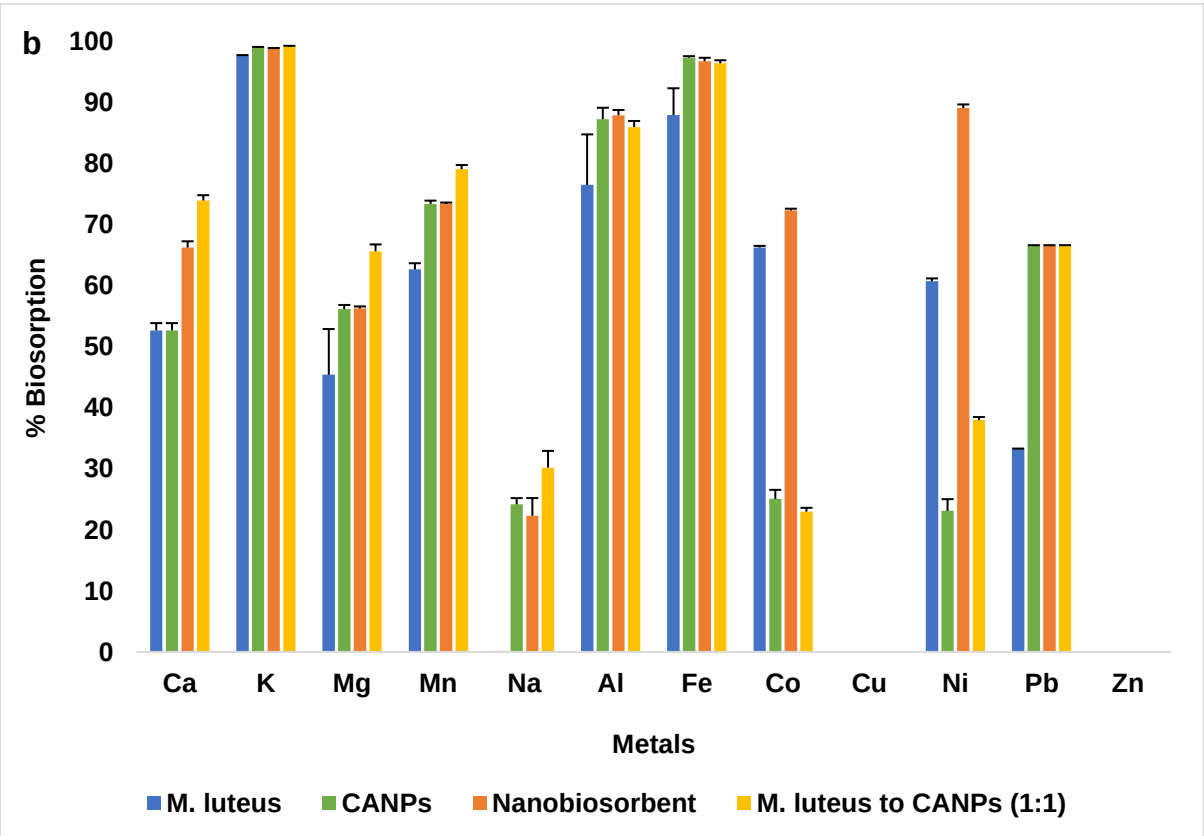
All four treatments of limestone neutralized AMD-1 resulted in some biosorption of metals. However, treatment with *M. luteus* yielded the most reduction in five of the metals (Ca, Co, Mn, Ni and Zn) while four of the metals (Fe, K, Mg and Na) were reduced the most by the nanobiosorbent. The difference in reduction between the free biosorbent and nanobiosorbent for these metals was significant ($p < 0.05$, Table A.5 in Appendix A). Aluminium was the only metal that was reduced slightly more than the other treatments when using a mixture of *M. luteus* and free CANPs; this was only significant when compared to *M. luteus* ($p = 0.001$, Table A.5 in Appendix A). Across all treatments, Al, Mn and Ni remained above the acceptable limits for agricultural use except when treated with *M. luteus*; the Ni concentration was reduced by 92.2 %.

For AMD-2, using a mixture of *M. luteus* with the free CANPs resulted in a significant reduction of the most number of metals present which included Ca, K, Mg, Mn, Na and Pb (Table A.6 in Appendix A). When comparing the reduction in the metals between the nanobiosorbent and free biosorbent, the nanobiosorbent performed better as seen in Figure 3.5b. Although most metals were reduced to below agricultural limits, Mn and Ni remained above 10 mg/L and 0.02mg/L, respectively for all four treatments.

Treatment of AMD-3 with the nanobiosorbent or free biosorbent each reduced four of the twelve metal concentrations more than the other treatments. These included K, Fe, Mn and Na for the nanobiosorbent and Al, Ca, Mg and Ni for the free biosorbent (Figure 3.5c). Of these metals, significant differences in concentration were obtained for Al, Fe, K, Na and Ni ($p = 0.001$, Table A.7 in Appendix A). Treatment of AMD-3

with *M. luteus* resulted in 77.3 % reduction of Ni however, the concentration exceeded the acceptable agricultural level for Ni.





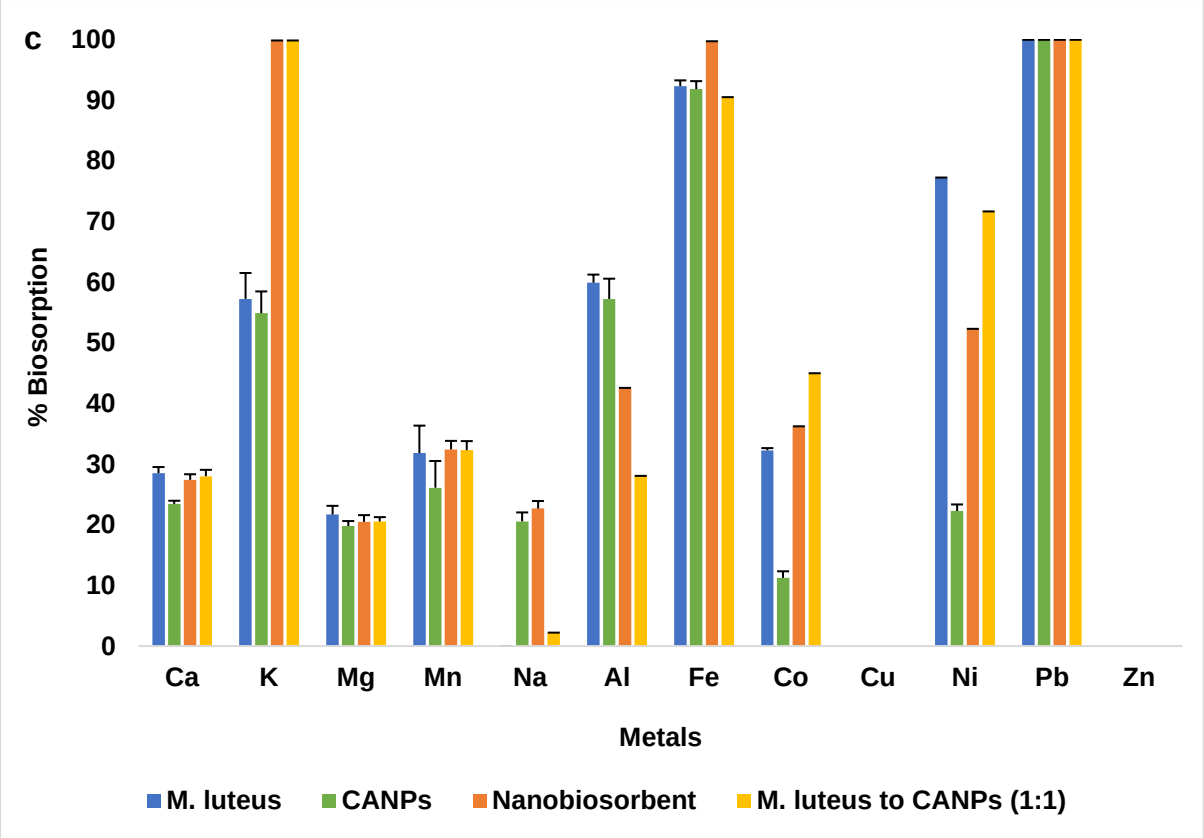


Figure 3.5. The percentage biosorption of metal from limestone neutralized AMD-1 (a), AMD-2 (b) and AMD-3 (c) treated with *M. luteus*, CANPs, nanobiosorbent and 1:1 mixture of free CANPs and *M. luteus*.

Overall, a conclusive finding on the single best treatment for the AMD water samples could not be reported. A summary of the treatments that resulted in the reduction of the concentration of the most metals to the least for each AMD sample is presented below.

AMD-1: *M. luteus*>Nanobiosorbent>Mixture>free CANPs

AMD-2: Mixture>Nanobiosorbent>free CANPs>*M. luteus*

AMD-3: Nanobiosorbent=*M. luteus*>Mixture>free CANPs

3.4 Discussion

Heavy metal contamination of water resources as a result of urban industrialization poses a prominent concern for availability of potable water and biotoxicity to human health (Duruibe, Ogwuegbu and Egwurugwu, 2007). Chemical precipitation, ion exchange and membrane filtration are currently being employed for the removal of

heavy metals however these methods are expensive and produce secondary toxic pollution (Fu and Wang, 2011). The biological removal of heavy metals using the dead biomass of bacteria through the passive process of biosorption has been widely studied (Ahalya, Ramachandra and Kanamadi, 2003; Ayangbenro and Babalola, 2017). These methods were found to be less expensive, eco-friendly and equally effective compared to their chemical counterparts. The present chapter focused on comparing the treatment of neutralized synthetic AMD wastewater using variations of heat-killed bacterial biosorbents. Furthermore, the Gram positive bacterial biosorbent, *M. luteus*, was immobilized onto CANPs as it was the best performing biosorbent for AMD-3 and the effect of immobilization on biosorption was evaluated.

3.4.1 Structural formation and stability of CANPs and nanobiosorbent

Majority of the CANPs had formed stable monodisperse spherical structures ranging between 55-85 nm in size (Figure 3.1). A study performed by Daemi and Barikani (2012) established that the concentration of $CaCl_2$ and sodium alginate used to synthesize the CANPs affects the size and distribution of the nanoparticles. Synthesis of CANPs with a $CaCl_2$ concentration of 18 mM or 36 mM and a sodium alginate concentration of 0.06 % (w/v) resulted in spherical CANPs ranging between 40-70 nm in dimension (Daemi and Barikani, 2012). The CANPs formed in this study are slightly larger compared to the dimensions reported by Daemi and Barikani (2012) when 22 mM $CaCl_2$ and a 0.06% (w/v) sodium alginate solution was used for synthesis. A study performed by Liu *et al.* (2006) confirmed that spherical structures commonly form as a result of minimizing the interfacial energy during synthesis of calcium alginate microparticles. The unaffected morphology and lack of degradation of the bacterial cell walls with evidence of the CANPs still attached (Figure 3.2) indicate that the nanobiosorbent was stable over a period of 8 weeks irrespective of the storage at 4 °C or room temperature.

3.4.2 Comparison of the neutralization strategies

The metal concentrations used to prepare AMD-1 as well as AMD-2 and AMD-3 were based on the most recent values reported for AMD originating in the Central Basin (Humphries, McCarthy and Pillay, 2017) and the Witwatersrand Basin (Hobbs, 2017), respectively. The composition of AMD-3 represents that of AMD-2 after being treated

through a HDS water treatment plant. It is this treated AMD that is released into the Tweelopie Spruit ending up in the Crocodile River (Hobbs, 2017). Synthetic mine water was used for the purpose of this study due to the need for a regular, fresh supply of water for treatment by biosorption and use in irrigation. Collecting large volumes of AMD from a mine on a daily basis was not feasible while the study was conducted. An acidic pH between 3 and 5 is characteristic of AMD water. However, Vallabh (2017) reported that the optimal pH at which the bacterial biosorbents selected for this study function is at a pH of 7. Therefore, prior to biological treatment of the AMD it was necessary to neutralize the pH.

Two chemicals, limestone and NaOH, were compared in the neutralization of all three AMD samples. Both chemical agents resulted in the precipitation of some of the metals. Limestone was able to significantly reduce majority of the metal concentrations compared to NaOH; majority of the *p* values were <0.05 (Table A.1 in Appendix A). Metal precipitation occurs upon binding of metal ions to the hydroxyl groups forming metal hydroxide precipitates. Limestone is composed of calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$) which ionizes to produce two hydroxyl groups (OH^-) upon interaction with water whereas NaOH ionizes to produce only one hydroxyl group which may account for the higher precipitation of metals by limestone (Baltpurvins, Burns and Lawrance, 1996). Currently, limestone is exploited as chemical precipitating agents to precipitate metal hydroxides out of toxic mining effluents as a result of increasing the pH of the effluent to neutral levels (Potgieter-Vermaak *et al*., 2006). The process commonly involves the addition of limestone as slurry or in a dry form to the AMD wastewater (Akcil and Koldas, 2006). Kapil and Bhattacharyya (2017) found limestone to be a more effective neutralizing agent compared to NaOH similar to the findings obtained in this study. Limestone is also much more cost-effective as opposed to NaOH (Kapil and Bhattacharyya, 2017) and therefore remains a better choice in the neutralization of large volumes of AMD. As a result, subsequent experiments in the study were performed with limestone neutralized AMD water. Although neutralization does precipitate many of the metals present in AMD, their concentrations tend to remain above the acceptable water quality limits for potable or agricultural use. This was true for Al, Fe, Mn and Ni in the present study. It was proposed that additional treatment with a bacterial biosorbent may result in further reduction in the concentration of these and other metals.

3.4.3 Comparison of the bacterial biosorbents

The bacterial biosorbents, *E. xiangfangensis* Pb204 and *M. luteus*, were particularly selected for this study as they were found to remove multiple metals from solution under optimised laboratory conditions as reported by Vallabh (2017). Maximum metal biosorption was obtained when these two bacterial biosorbents were used in a 2:1 combination. As such, the combination of the bacterial biosorbents were also used to treat the synthetic AMD wastewater in this study.

Treatment with *E. xiangfangensis* Pb204, *M. luteus* and a combination of the two bacterial biosorbents resulted in the further reduction of the metal concentrations from the lime neutralized AMD samples. Majority of the metals were reduced to below the acceptable agricultural limits with the exception of Mn in AMD-1 and AMD-2. The highest biosorption of Mn for AMD-1 was 24.7 % obtained using *E. xiangfangensis* Pb204 while *M. luteus* biosorbed 62.7 % Mn in AMD-2. The lowest concentration of Ni in AMD-2 and AMD-3 as well as that of Al in AMD-1 though further reduced after neutralization also remained above their acceptable limits. The findings obtained in this study are also supported by many studies that report on different biosorbents having varying affinities for metal ions (Nakajima and Tsuruta, 2004, Ghaed, Shirazi and Marandi, 2013, Vallabh, 2017). These differences may occur between species such as bacteria and algae and also within a species for example between different bacterial species and strains (Wang and Chen, 2009). The variation in biosorbent affinities for metals may also be attributed to the amount of available metal binding sites present in different biosorbents, initial concentration of metal ions, biomass concentration, temperature, presence of other metal ions and pH (Das, Vimala and Karthika, 2008).

The pH of a solution affects the nature of the functional binding groups available on the bacterial cell walls and the solution chemistry of the metals. Each metal ion has a different solubility curve. The bioavailability of metal ions increases as the pH levels decrease and all metals have different thresholds for solubility (Salomons, 1995). At neutral pH, the solubility of Mn, Ni and Al begins to decrease. Hence they were not completely bioavailable which resulted in the reduced biosorption reported in this study. Additionally, the initial metal concentrations influence biosorption whereby the metal binding sites on the surface of the biosorbent become saturated when ions in

solution are excessive (Fourest and Roux, 1992). Some metal binding sites have a higher affinity to some metal ions compared to others. When multiple metals are present in solution, the metal ions begin to compete with each other for binding sites on biosorbents (Zabochnicka-Świątek and Krzywonos, 2014). The higher concentrations and presence of multiple metals in the AMD samples may have affected the selectivity and biosorption affinity of some metals over others.

Overall, more metals were reduced in concentration when using an individual bacterial biosorbent compared to using them in combination. Although *E. xiangfangensis* Pb204 performed better than *M. luteus* for AMD-1, *M. luteus* biosorbed significantly more metals both in terms of the number of different metals as well as metal concentration for both AMD-2 and AMD-3. The metal uptake differences observed between the two bacterial biosorbents may be attributed to the amount of available metal binding sites present in each bacterial biosorbent. Gram positive bacteria such as *M. luteus* are known to have thicker cell walls made of peptidoglycans layers. Large amounts of teichoic acids and lipoteichoic acids are found in the peptidoglycans which are composed largely of carboxylate and phosphate anionic functional groups where metal binding occurs (Hansda and Kumar, 2015). Cross bridging between peptidoglycan layers via peptide bonds occurs commonly in Gram positive bacteria compared to Gram negative bacteria (Gupta, Nayak and Agarwal, 2015). The anionic functional groups such as hydroxyls, thiols and amines present in these structural components serve as potential metal binding sites. Gram negative bacteria such as *E. xiangfangensis* Pb204 have thinner peptidoglycans consisting of lipopolysaccharides, lipoproteins, phospholipids and glycoproteins (Hansda and Kumar, 2015). These structural components also contain multiple anionic functional groups however the quantity of metal binding sites available may vary between bacterial species (Wang and Chen, 2009). The biosorption performance by *M. luteus* in the removal of majority of the metals across all AMD samples may have been better compared to *E. xiangfangensis* Pb204 as a result of having a higher quantity of available metal binding sites. A biosorption study performed by Nwidi and Agunwamba (2015) explored the abilities of five different biosorbents for the removal of Zn, Cu and Mn. The study found that the Gram positive bacteria, *Staphylococcus xylosus* removed Zn and *Bacillus circulans* removed Cu and Mn best compared to the Gram negative bacteria, *Pseudomonas aeruginosa*. The study concluded that Gram positive bacteria may have

performed better compared to the Gram negative bacteria due to their thicker peptidoglycan layers present in the cell wall where metal binding occurs. As such, the Gram positive bacteria, *M. luteus*, was selected for immobilization onto CANPs for the subsequent treatments in this study.

3.4.4 Effect of immobilization

Bacterial biosorbents are small in size which enable high biosorption capacities. However, this property has limitations such as low density, poor mechanical strength and low rigidity and solid-liquid separation difficulties (Yun, Vijayaraghavan and Won, 2011). The benefits of immobilizing bacterial biosorbents includes the improved stability of biomass and separation from effluent as well as the utilization of the biosorbent for successive sorption and desorption cycles (Bai and Abraham, 2003; Yun, Vijayaraghavan and Won, 2011). CANPs are a suitable, eco-friendly polymeric matrix for immobilization of biosorbent due to the biodegradable properties of the alginate component (Paques *et al*., 2014). Many studies have used calcium alginate for immobilization of biosorbents for metal biosorption purposes (Bai and Abraham, 2003; Arica *et al*., 2004). On a nanoscale, the use of CANPs may further improve the surface area to volume ratio, reactivity and sorptive properties of the biosorbent (Qu, Alvarez and Li, 2013).

Many studies have exploited calcium alginate for the removal of metals from solution as they are capable of binding their ions (Ibáñez and Umetsu, 2002; Papageorgiou *et al*., 2006; Mata *et al*., 2009). Similarly, free CANPs were shown to reduce the metal concentrations from the neutralized AMD samples in this study. However, the bacterial biosorbent performed better in metal reduction when used separately; combining the biosorbent with free CANPs also resulted in greater metal reduction. Overall, immobilizing *M. luteus* onto CANPs maintained the biosorptive properties of the bacteria and resulted in better biosorption for majority of the metals compared to using the free CANPs alone (Figure 3.5). This was evident in the treatment of all three AMD samples. The difference in biosorption abilities between free CANPs and bacterial biosorbent may be attributed to the amount of available metal binding sites (Das, Vimala and Karthika, 2008). The calcium alginate component in CANPs contains only carboxylate anionic functional groups where metal binding occurs (Paques *et al*., 2014) whereas several different metal binding functional groups such as hydroxyls,

phosphoryl, carboxyl and amine groups are present in the bacterial biosorbent (Etesami, 2018). Calcium alginate nanoparticles are mostly reported to biosorb single metal elements from solution (Geetha *et al*., 2016; Khezri *et al*., 2016; Sadiq, Choubey and Bajpai, 2018). Metal ions exhibit variation in their affinities for carboxylate and other functional groups due to the ionic radii of the cations and bond energies between the cation and functional group. The difference in bond energies is a result of the various geometries of ionic co-ordination such as planar (e.g. Ni ions), tetrahedral (e.g. Ca ions) and off-centred (e.g. Pb ions) that have been shown to play an important role in cation binding especially with carboxyl groups (Bala *et al*., 2007). For this reason, metal binding to the CANPS may have been more restricted compared to that of the bacterial biosorbent which offers a variation of metal binding groups. This would also result in more competition for the binding sites on the CANPs.

The findings from the study provide no conclusive evidence on the further improvement of metal uptake using nanobiosorbent compared to biosorption using free bacterial biosorbent. However, immobilization of the bacterial biosorbent onto CANPs did not significantly affect the overall ability of the biosorbent to take up metals. These findings suggest that using CANPs is a suitable strategy for immobilization and can be implemented for recovery and reuse of the biosorbent. The findings further suggest that incorporating CANPs with bacterial biosorbent enhanced metal uptake compared to using the free CANPs alone. A study performed by Tuzen *et al*. (2008) reported on the improved stability of *P. aeruginosa* when immobilized onto multiwalled carbon nanotubes for the biosorption of Co, Cd, Pb, Mn, Cr and Ni. The biosorptive abilities of the biosorbent were maintained and showed reusability properties. In a separate study, Akhtar *et al*. (2009) successfully immobilized *Trichoderma harzianum* onto a calcium alginate support matrix which improved the stability of the biosorbent as well as the uranium biosorption capacity. Furthermore, regeneration and re-use of the immobilized biosorbent did not alter the biosorbent capacity significantly. Ozdemir, Ceyhan and Manav (2005) reported on the successful immobilization of *Chryseomonas* luteola TEM05 bacteria as well as the extracellular polysaccharide of the bacteria onto calcium alginate beads for the biosorption of Ni and Cu from solution. The study found that incorporating the bacterial components with calcium alginate improved the biosorption of Ni and Cu compared to using the calcium alginate beads alone.

Treatment with a mixture of *M. luteus* and free CANPs was performed to determine whether immobilization of the bacterial biosorbent onto CANPs by crosslinking with glutaraldehyde affected the biosorptive abilities of the CANPs or bacterial biosorbent. Although the mixture of the free CANPs and bacterial biosorbent were shown to take up metals especially for AMD-2, there was no conclusive evidence that crosslinking with glutaraldehyde interfered with the performance of the individual biosorbents. This can be surmised from the performance of the nanobiosorbent which performed better in terms of metal uptake for AMD-1 and AMD-3. Crosslinking the bacterial biosorbent to CANPs is preferred due to the benefits associated with immobilization such as long-term stability. This was evident in the present study where storing the nanobiosorbent at room temperature for 8 weeks did not result in any degradation in its structure (Figure 3.2). Furthermore, immobilizing the biosorbent is a better option compared to using a mixture of free bacterial biosorbent and CANPs due to the potential for recovery and reuse of the nanobiosorbent which is important for commercialization purposes and economic feasibility.

3.5 Conclusions

A combination of limestone neutralization and biological treatment with heat-killed *M. luteus* is effective in reducing the concentrations of majority of the heavy metals present in AMD water found in the Central and Witwatersrand Basins of Gauteng, South Africa to within acceptable agricultural limits. However, high concentrations of Mn and Ni are not reduced effectively. The inclusion of an additional biosorption step in the treatment process may improve the limitations of saturation which biosorbents reach rapidly in solutions with high metal concentrations. This was a proof of concept study however in a pilot study, biosorbent or nanobiosorbent would be fixed onto a removable membrane. Immobilization of *M. luteus* onto CANPs provided stability to the biosorbent while maintaining its biosorptive properties. It also enabled easy separation of the biosorbent with bound metal ions from the water as precipitate or sediment compared to the removal of free suspended bacterial biosorbent. Further studies would be to determine desorption abilities of the biosorbent and nanobiosorbent for reusability. This is important when considering a biosorbent for commercialization. These findings show the potential for the application of the nanobiosorbent described here in the treatment of heavy metal polluted wastewaters.

Chapter 4: Quantification of heavy metals in tomato plants watered with treated synthetic AMD wastewater

4.1 Introduction

Plants acquire essential nutrients from the environment however they are also able to take up high concentrations of unnecessary and toxic metals such as Pb from contaminated soils (Cobb *et al*., 2000). Urban industrialization and particularly the mining industry generates large volumes of heavy metal contaminated effluent that leaches into the environment accumulating in other water bodies, soils, crops, animals and eventually humans (Durand, 2012). Kamunda, Mathuthu and Madhuku (2016) found that heavy metals originating from the mines are taken up by plants when grown in contaminated soils. This phenomenon poses severe ecological and human health concerns. Although some metals are retained in the roots of the plants, many metals are transported via the xylem and phloem of the plants where they redistribute to other structures of the plants (Page and Feller, 2015). Home grown vegetables or agricultural crops that are exposed to elevated concentrations of heavy metals are likely to accumulate the toxic metals in the roots, leaves and fruits of the plants (Cobb *et al*., 2000). Ingestion of vegetables or fruits with high metal content poses chronic health risks to animals and humans such as cardiovascular diseases, nephrotoxicity and skin cancer (Zhou *et al*., 2016).

Tomatoes (*Solanum lycopersicum*) account for 22 % of the total amount of vegetables produced in South Africa making it a high value product (DAFF, 2018). They are usually grown for domestic use by homestead and subsistence farmers in local communities. However, such communities have limited access to clean water for domestic and agricultural purposes (Ochieng, Seanego and Nkwonta, 2010). In addition, South Africa is currently facing severe water scarcity making every water source critical for human and animal use. The water that is used to irrigate homestead and subsistence gardens originates from natural water sources that are likely contaminated with mining and industrial effluent. Consequently, the fruit produced from such plants are likely to accumulate heavy metals making them unsafe for human consumption.

The present chapter describes the growth of tomato plants irrigated with the biosorbent treated synthetic AMD water prepared in this study. Additionally, the concentration of individual heavy metals in the various plant structures was quantified to trace heavy metal transfer from the water and soil to the plant. It was hypothesized that the tomato plants watered with treated synthetic AMD wastewater would have acceptable amounts of metal in the edible parts of the plant for safe human consumption. The use of biosorbent treated synthetic wastewater for agricultural use may potentially provide a short-term solution for water reuse in subsistence/home-grown farming especially within local mining communities.

4.2 Experimental procedure

4.2.1 Tomato plant pot trial

Forty tomato seedlings of the Rodade tomato plant variety were kindly supplied by the Agricultural Research Council (ARC, Pretoria). Twenty seedlings were divided randomly into four groups of five plants each to determine the effect of using treated water on tomato production. Each group received a different water treatment as follows: (1) tap water (control), (2) untreated acidic AMD-3 mine water, (3) limestone neutralized AMD-3 mine water treated with *M. luteus* biosorbent or (4) limestone neutralized AMD-3 mine water treated with nanobiosorbent (described in Chapter 3). Each treatment consisted of 5 biological tomato plant replicates. The remaining twenty seedlings were divided and treated similarly to serve as a replicate trial. Due to space and time constraints, only 10 tomato plants in total could be grown per treatment in replicate. The replicate study was carried out within a short period of the first study commencing.

A single tomato seedling was sown per 23 cm pot (with drainage holes) that was filled with potting mix and fertilized with bone meal (Wonder K9716 Bonemeal, Efekto). Each tomato plant was fertilized with approximately 3 mL/L of liquid organic fertiliser (Margaret Roberts Organic Supercharger Fertilizer, Kirchhoff's) rich in nitrogen, phosphorus and potassium for every two weeks over the first month to promote healthy growth of the stems and leaves of the tomato plants (Uchida, 2000). The pots were placed indoors in an enclosed room as planting was carried out in July (winter). Overhead supplemental lighting (16 hours/day) was provided by LED grow lights and

the temperature maintained at 24 °C during the day and 20 °C at night as recommended by Schwarz, Thompson and Kläring, (2014). The seedlings were irrigated with ~75 mL of the respective water treatment twice a week. As the tomato plants matured and the water requirement increased, the plants were watered with ~150 mL thrice a week. When the plants reached the flowering stage after 10 weeks, 1 g/L of phosphorus rich fertilizer (Dr Fisher's Multifeed Flower Grow, Efekto) was used every two weeks to promote the growth of the tomato flowers and fruits. Additionally, the flower buds were sprayed every two weeks with a solution of 2.5 g/L $MgSO_4$ (Epsom Salt, Robertsons) to promote flowering and fruition (Stiles, 1999). Plant growth was monitored by measuring the stem height, leaf area and leaf count for each plant (Cornelissen *et al*., 2003). The tomato plants were uprooted and the roots, stems, leaves, flowers and fruits were harvested for quantification of heavy metals by ICP-OES and ICP-MS. All replicates were maintained and processed separately. The percentage of the metal concentrations of each experimental group higher or lower than the tap water control group was determined using Equation 3:

$$\% \text{ Higher/lower} = 100 \times C_{ex} / C_{tp} \quad [3]$$

Where C_{ex} and C_{tp} refers to the metal concentrations (mg/kg) obtained for the experimental group and tap water group, respectively, for each plant structure.

4.2.2 Sample preparation and heavy metal analysis

The roots, stems, leaves, flowers and fruits harvested per treatment and its replicate were dried individually in the Optolab oven incubator, model 325, at 80 °C for 48 hours. Thereafter, the samples were ground to powder using a pestle and mortar and 0.2 g of each sample was placed in acid-cleaned vessels and digested using a microwave digestion system (MARS 6™, CEM) for 10 minutes at 200 °C with 2 mL of concentrated nitric acid (HNO_3) and 1 mL of concentrated hydrogen peroxide (H_2O_2) (Nakano, Higashide and Ahn, 2017). The digested samples were diluted with extra pure water and the metal concentrations of Al, Cu, Co, Pb, Ni, Zn, Na, Ca, Mn, Mg and K were determined in a Spectro ARCOS ICP-OES (Spectro) and ICP-MS (NexION 300 ICP-MS, PerkinElmer® Inc.). One-way ANOVA (IBM SPSS Statistics, version 25) was used to compare metal concentrations between treatment groups and between each plant structure. The ability of the metals to translocate to the aerial parts

of the plants were determined by calculating the translocation factors (TF) of the metals for each treatment. The TF was obtained as the quotient of the metal concentration in the edible parts of the plant (fruits) and the metal concentration in the roots of the plants (Ng *et al*., 2016). A TF value greater than 1 would indicate that the metal was translocated from the roots to the aerial parts of the plants while a TF value less than 1 indicates that the metal had accumulated in roots in higher concentrations compared to the aerial parts of the plants.

Soil samples from each treatment were processed similarly to the plant material with slight modification. Soil samples were digested for 10 minutes at 175 °C using the microwave digestion system with 9 mL HNO_3 and 3 mL hydrochloric acid (HCl) as reported in Method 3015a by the Environmental Protection Agency (1999). The soil samples were then diluted and analysed for metal concentration using ICP-OES and ICP-MS. Water samples of each treatment were also analysed using ICP-OES and ICP-MS.

4.3 Results

4.3.1 Analysis of tomato plant growth

Rodade tomato seedlings were received from the ARC 8 weeks post-germination and transplanted individually into pots at an average height of 25 cm. The tomato plants across all water treatments grew over a period of 28 weeks to maturity. Figure 4.1-4.4 show the presence of healthy, green leaves in the four treatments over this period.

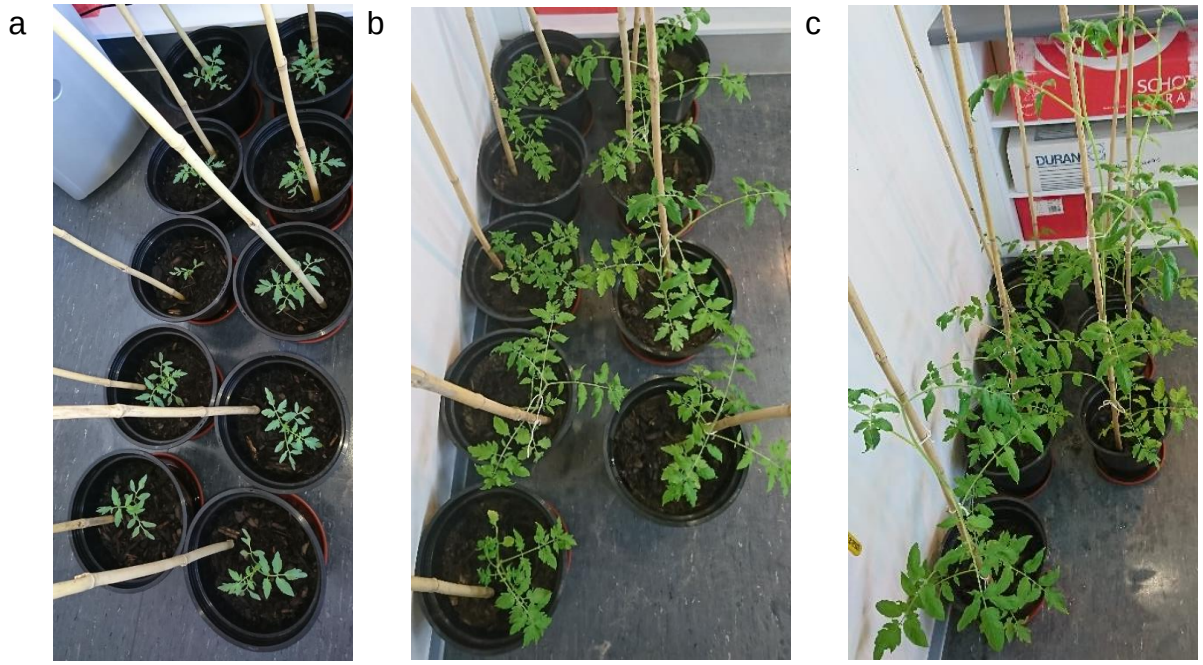


Figure 4.1. Tomato plants grown with tap water at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.

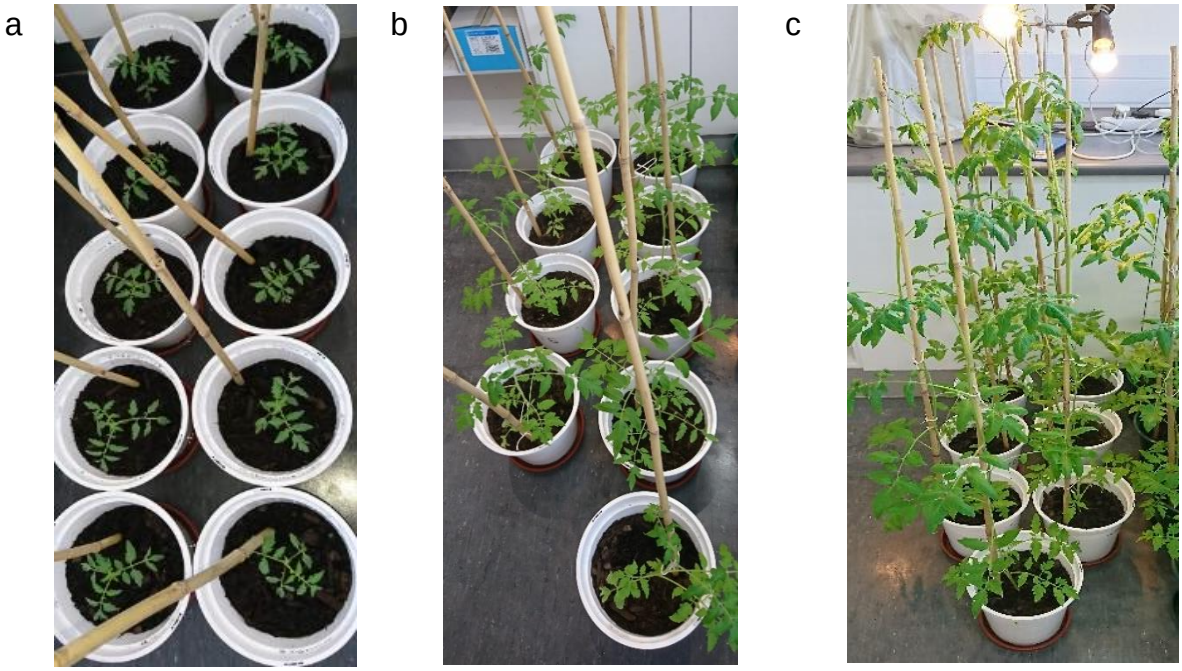


Figure 4.2. Tomato plants grown with limestone neutralized AMD-3 water treated with *M. luteus* biosorbent at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.

a b c

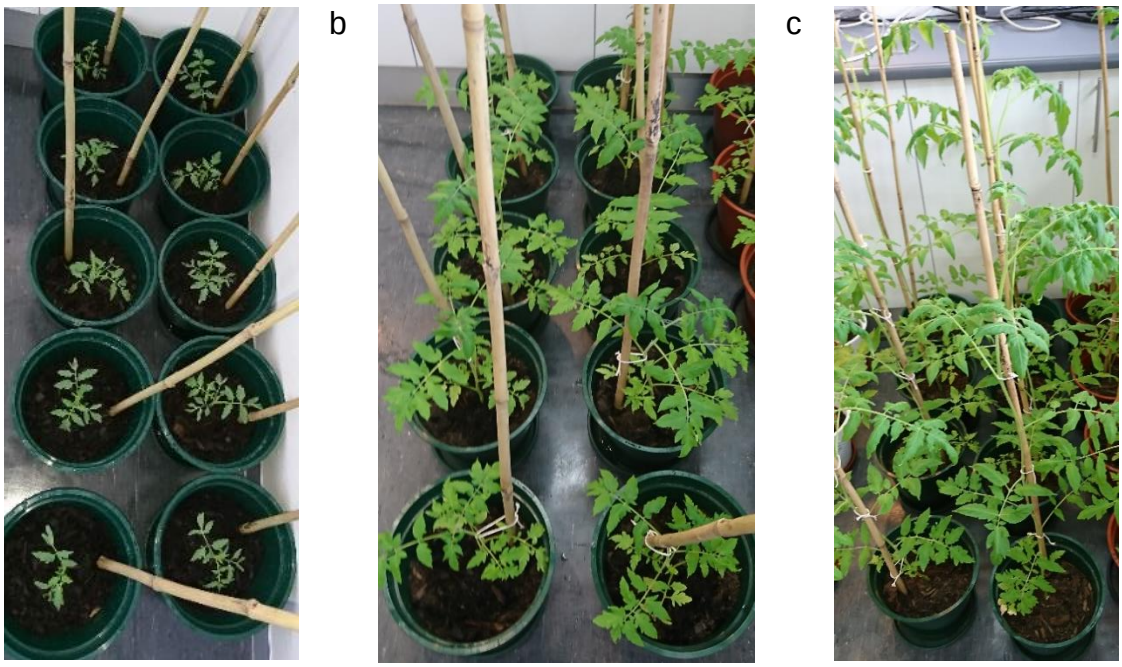


Figure 4.3. Tomato plants grown with limestone neutralized AMD-3 water treated with nanobiosorbent at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.

a b c

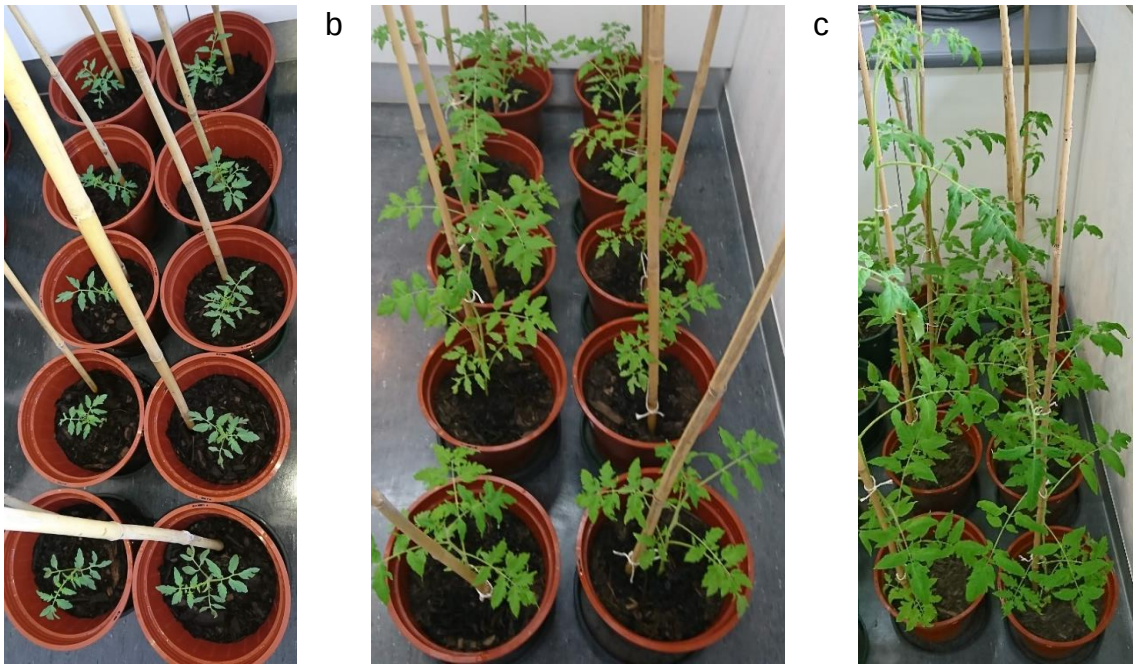
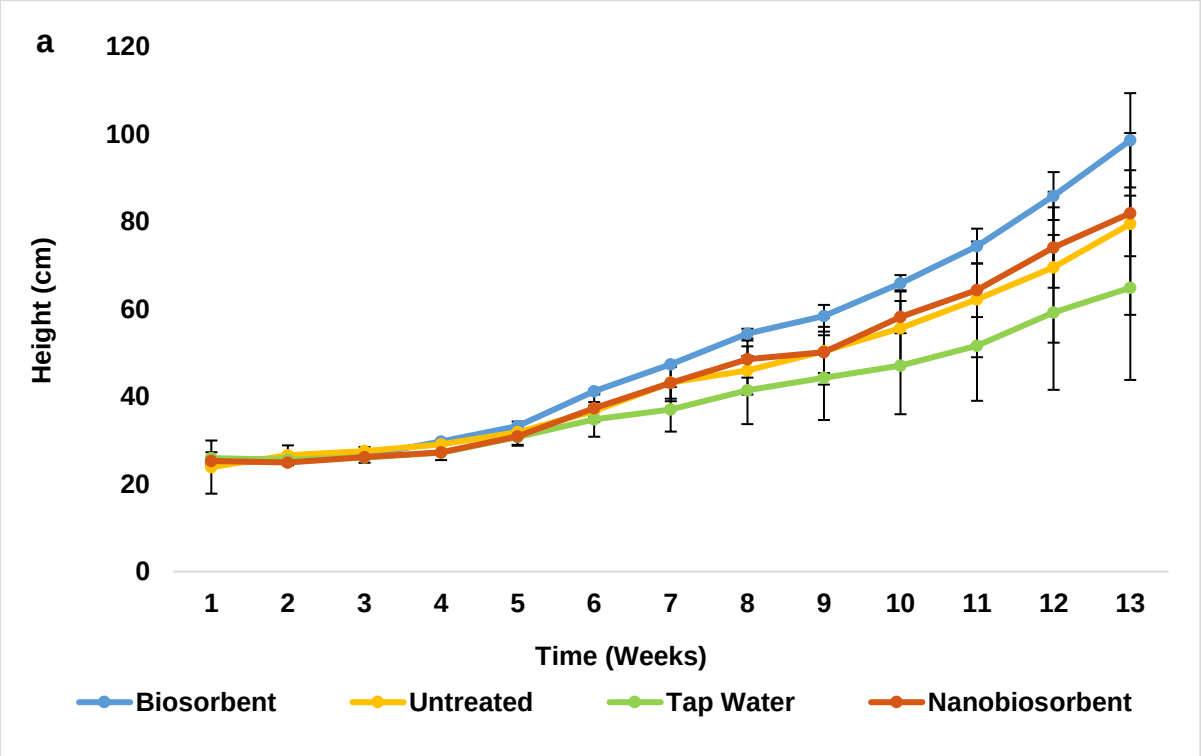
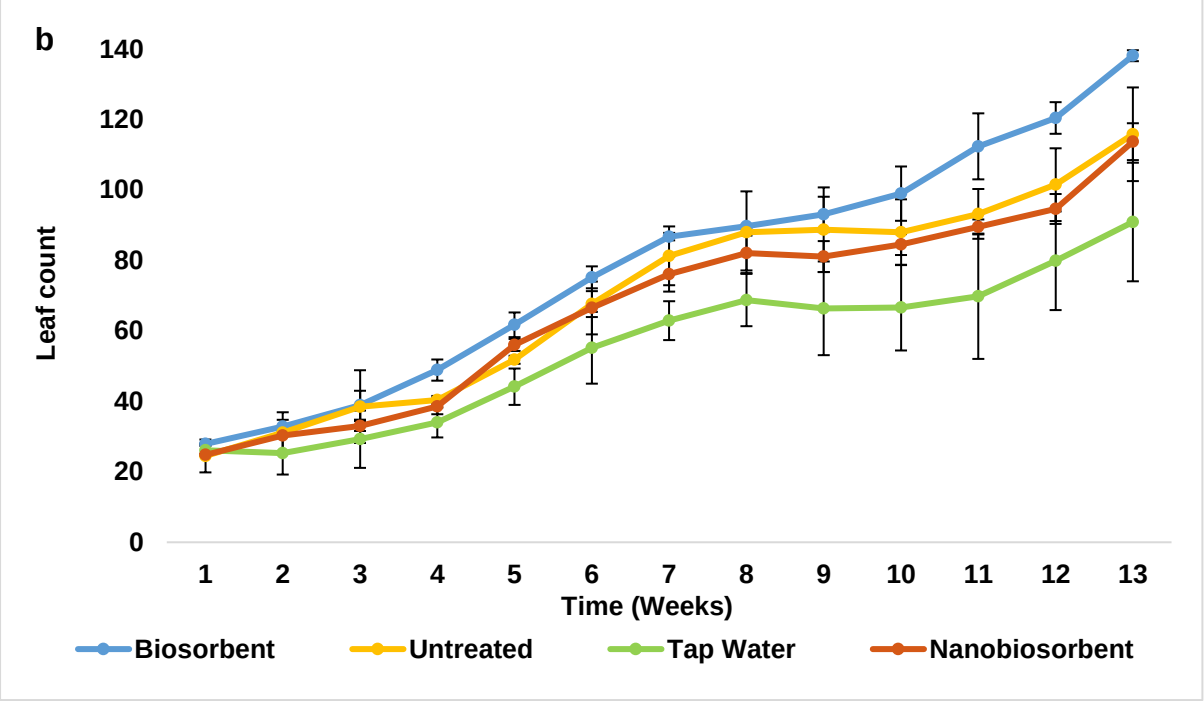


Figure 4.4. Tomato plants grown with untreated AMD-3 water at 1 week (a), 10 weeks (b) and 16 weeks (c), post-transplant showing healthy leaf formation and increased height over the growth period.

Tomato plants grown with the biosorbent treated limestone neutralized AMD-3 water grew more in terms of plant height, leaf count and leaf area compared to the plants grown with the other water treatments as shown in Figure 4.5. This was followed by tomato plants grown with the nanobiosorbent and untreated AMD-3 water. Plants irrigated with tap water grew the least in terms of all three parameters.





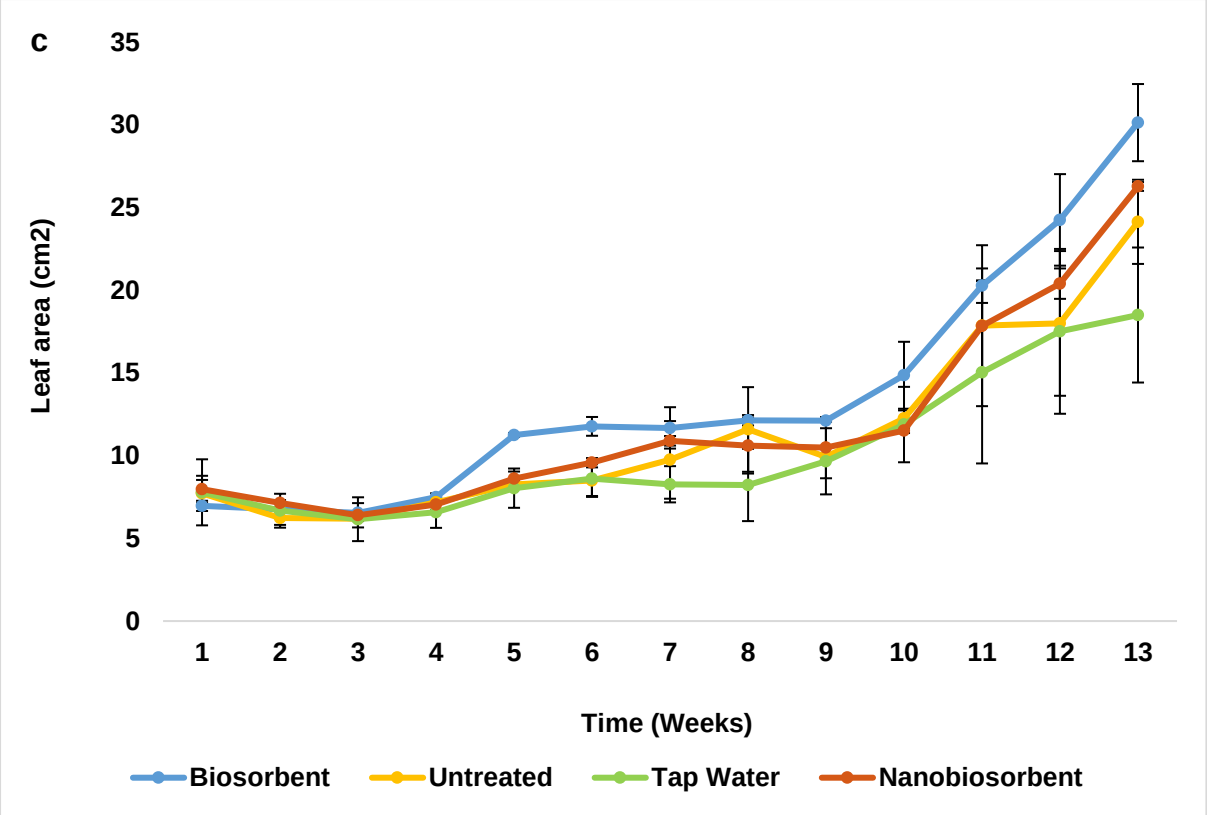


Figure 4.5. Plant height (a), leaf count (b) and leaf area (c) for each water treatment group over a period of 13 weeks. Data presented is based on an average of two replicate groups consisting of five plants each.

The onset of flowering in all tomato plants was slow and began in week 10 when flower buds were observed in tomato plants irrigated with tap water and limestone neutralized nanobiosorbent treated AMD-3 water. Flowers had appeared randomly on individual plants within each treatment group by week 19 but not all plants within a treatment were flowering. Fruiting was observed in the group treated with tap water (Figure 4.6a) by week 19 but flowers in the biosorbent and untreated groups were still developing even though the fruits appeared at the same time as the group treated with tap water. Tomato fruit started developing in the plants irrigated with biosorbent treated limestone neutralized AMD-3 water (Figure 4.6b) and untreated water (Figure 4.6d) at week 20 and 22, respectively. Fruit had not developed in the group irrigated with nanobiosorbent treated limestone neutralized AMD-3 water by week 28 when the study was terminated.

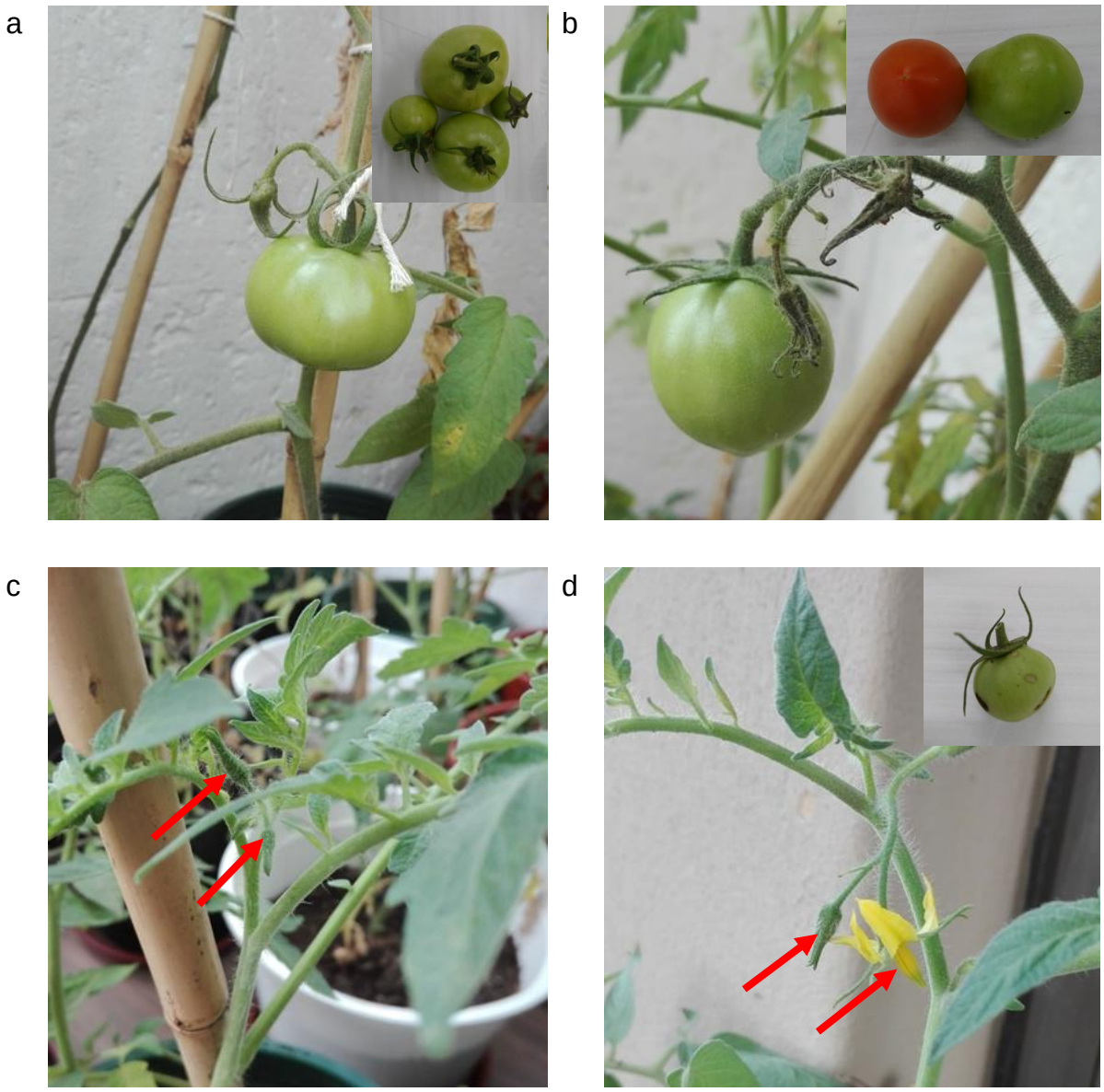


Figure 4.6. Development of tomato fruit in plants irrigated with tap water (a) and biosorbent treated limestone neutralized AMD-3 water (b) at 21 weeks. Flowers (indicated by arrows) with no visible fruit in plants irrigated with nanobiosorbent treated limestone neutralized AMD-3 water (c) and untreated AMD-3 water (d) at 21 weeks. Inset figures represent the final tomato fruits per treatment that were digested for heavy metal quantification.

4.3.2 Heavy metal accumulation in tomato plant structures

Majority of the metals taken up by the tomato plants accumulated in higher concentrations in each plant structure across all treatments compared to the tap water group (Figure 4.7a-e). High metal concentrations for majority of the metals (Al, Fe, Ca,

Mn, Mg, Na, Ni, K, Pb, Zn) accumulated within the plant structures in the following order:

Flowers>Roots>Stems>Leaves=Fruits

Across all treatments, significant differences for each metal was observed when comparing accumulation between plant structures (Table A.8-A.11 in Appendix A). Table 4.1 shows the translocation factors (TF) of the metals from the roots of the plants to the fruits of the tap water, biosorbent treated and untreated plants or flowers of the nanobiosorbent treated plants. The TF of K was greater than 1 for all four treatments indicating translocation of K to the aerial parts of the plants. The TF of Ca, Cu, K, Mg, Mn, Ni, Pb and Zn were greater than 1 indicating translocation of these metals to the aerial parts of the plants of the nanobiosorbent treatment group.

Table 4.1. Translocation factors of metals in tomato plants for all four treatments.

Element (TF)	Treatment			
	Tap water	Biosorbent treated	Nanobiosorbent treated	Untreated
Al	0.005	0.000	0.000	0.000
Ca	0.062	0.031	7.244	0.137
Co	0.004	0.008	0.000	0.006
Cu	0.059	0.145	1.072	0.128
Fe	0.002	0.003	0.112	0.004
K	1.534	1.752	19.115	1.639
Mg	0.121	0.133	12.079	0.169
Mn	0.059	0.056	8.600	0.136
Na	0.139	0.148	0.000	0.123
Ni	0.005	0.007	3.058	0.011
Pb	0.000	0.590	2.157	0.337
Zn	0.122	0.182	4.082	0.118

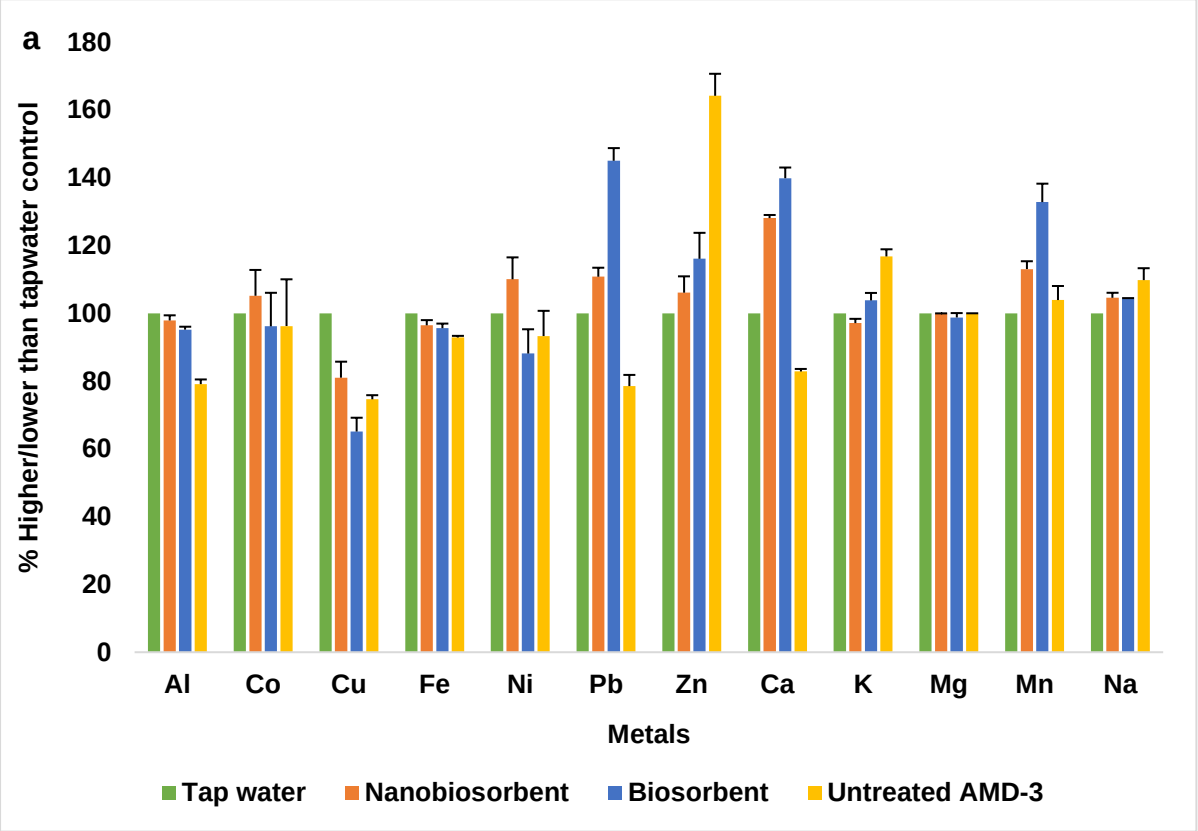
Majority of the metals that accumulated in the tomato fruits (as this is the structure consumed) were above the permissible levels for human consumption as shown in Table 4.2. The levels of Na and Ni in fruit from all three treatments remained within acceptable limits. The tomato fruits obtained from plants watered with limestone

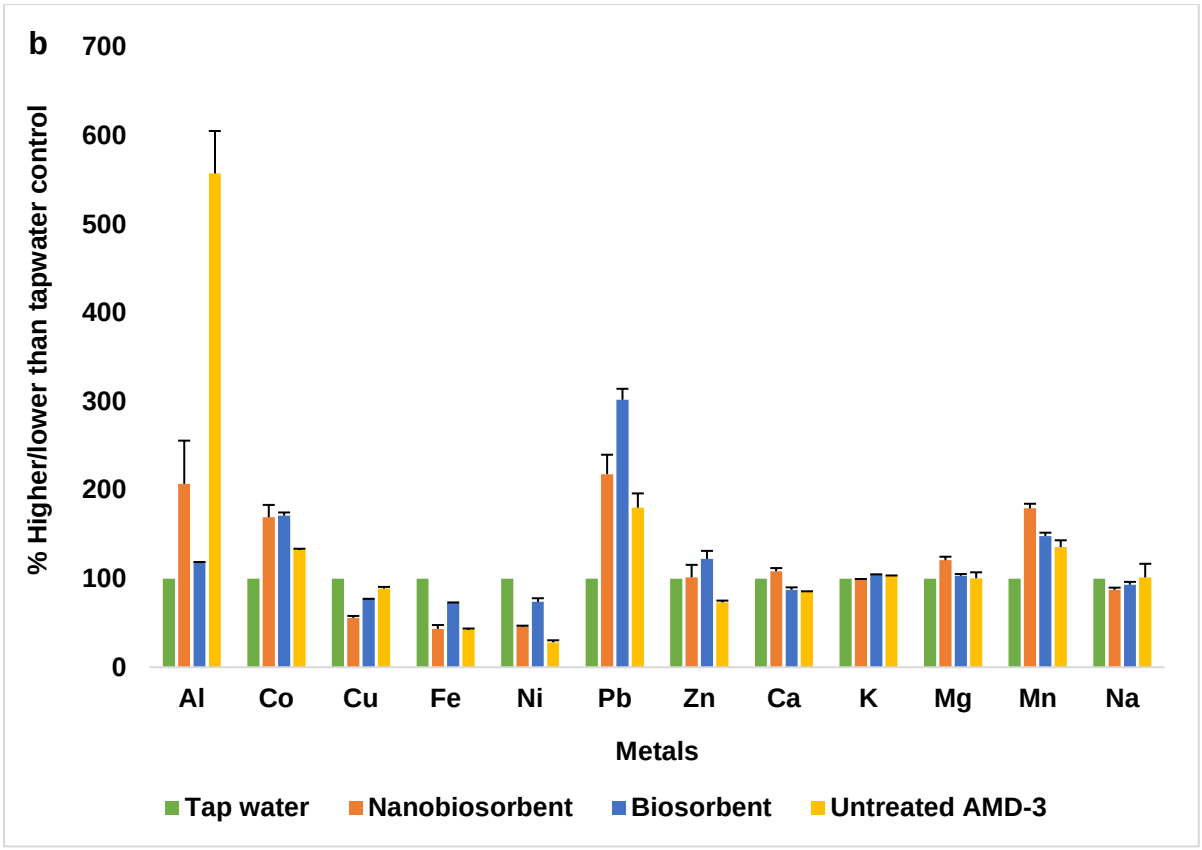
neutralized biosorbent treated AMD-3 water contained 1.93 mg/kg Pb while those from the untreated AMD-3 water only contained 0.75 mg/kg of the metal. Plants from the tap water treatment were the only ones to produce fruit without any Pb.

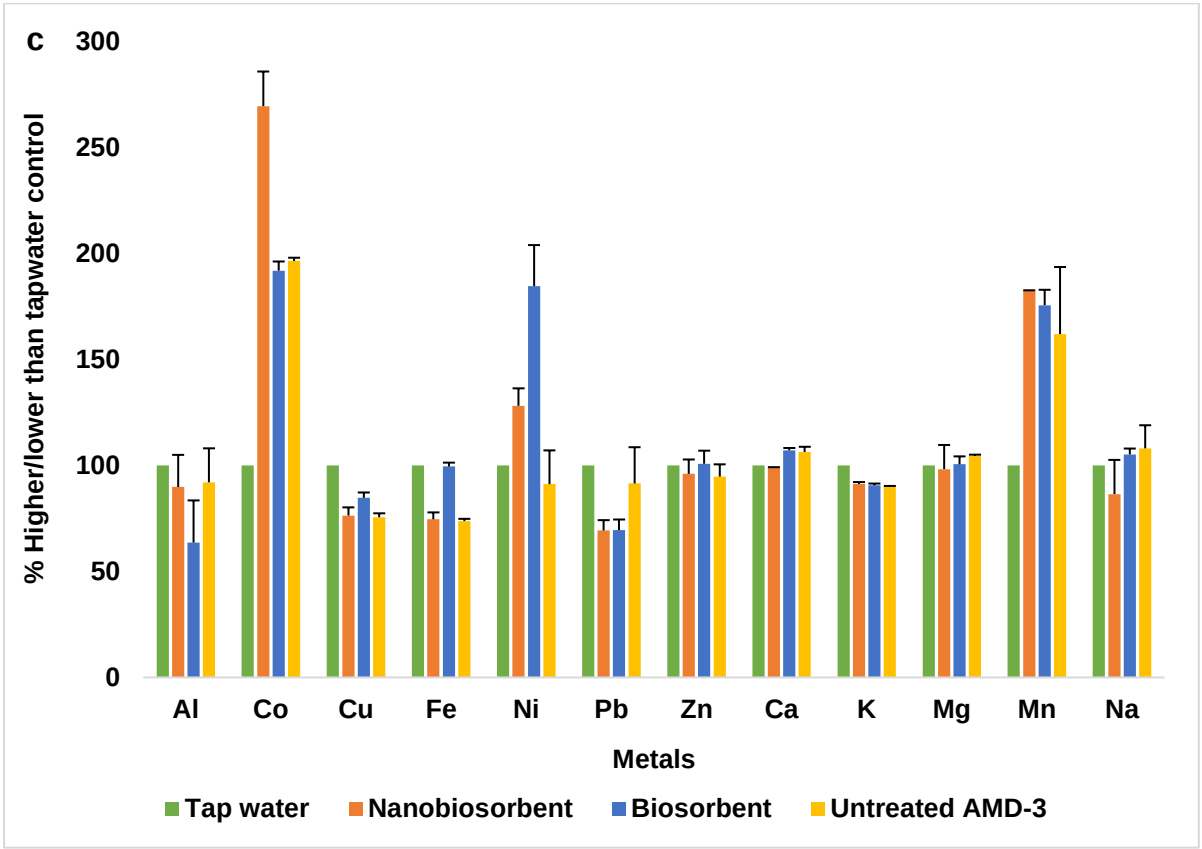
Table 4.2. Elemental composition of tomato fruits produced by plants from different treatment groups in the present study.

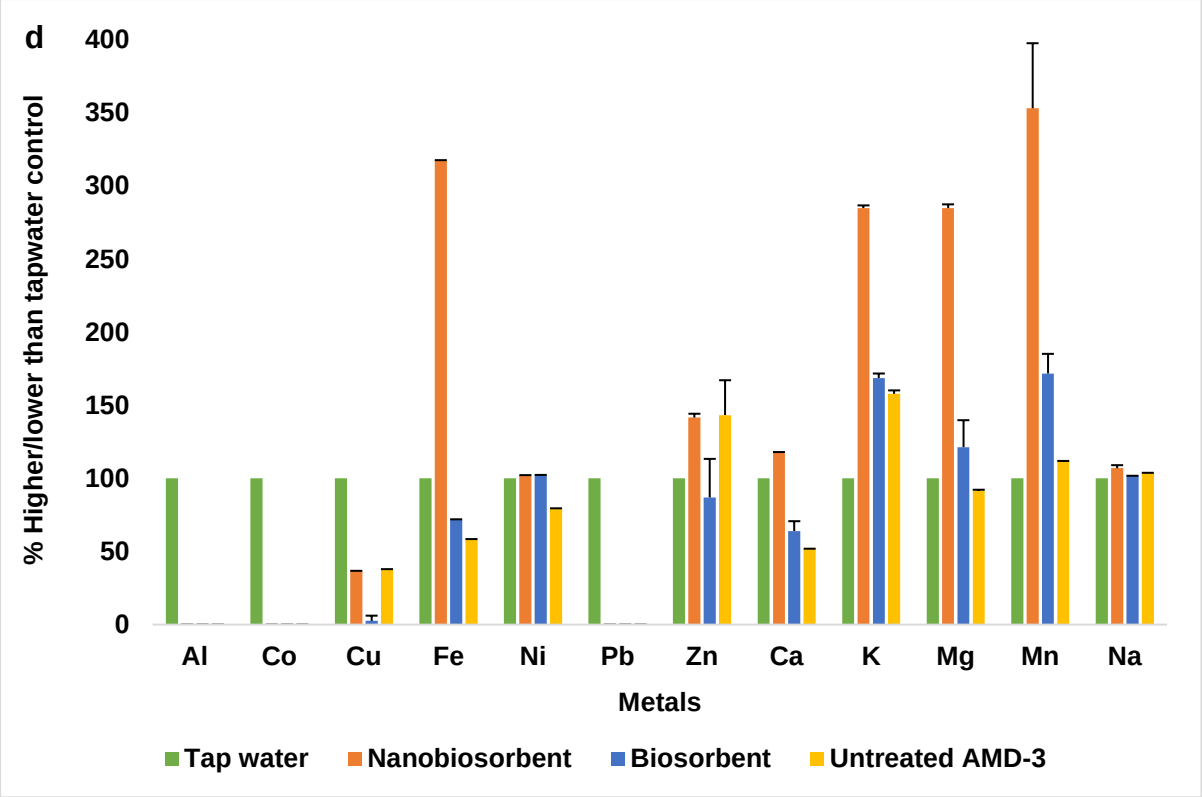
Element (average mg/kg)	Treatment			Limit/Maximum daily intake	Reference
	Tap water	Untreated	Biosorbent treated		
Al	72.708	3.590	2.020	-	
Ca	1116.663	2034.863	769.463	1000 mg	Lenntech (2019)
Co	0.083	0.105	0.152	0.05-0.1 mg/kg	FAO/WHO (2016)
Cu	5.878	9.516	9.398	2 mg	Lenntech (2019)
Fe	30.707	50.680	39.542	18 mg	Lenntech (2019)
K	43422.750	54159.525	51463.900	3500 mg	Lenntech (2019)
Mg	2145.825	2983.700	2324.200	400 mg	Lenntech (2019)
Mn	64.999	156.225	82.075	2 mg	Lenntech (2019)
Na	431.800	405.275	462.975	2400 mg	Lenntech (2019)
Ni	0.194	0.384	0.223	1.5 mg/kg	FAO/WHO (2016)
Pb	0.000	0.745	1.929	0.1 mg/kg	FAO/WHO (2016)
Zn	16.823	26.613	29.051	15 mg	Lenntech (2019)

Overall, fruit of the plants from the group watered with untreated AMD-3 accumulated the highest concentrations of seven (Ca, K, Mg, Mn, Fe, Cu and Ni) of the metals quantified in this study while those from the group watered with limestone neutralized biosorbent treated AMD-3 had the highest concentrations for Na, Co, Pb and Zn. Fruit from the tap watered group accumulated the highest concentration of Al (72.71 mg/kg) for which there is no reported limit. The concentrations reported here were significantly different between treatment groups ($p < 0.05$, Table A.12 in Appendix A).




b
% Higher/lower than tapwater control
700
600
500
400
300
200
100
0
Al
Co
Cu
Fe
Ni
Pb
Zn
Ca
K
Mg
Mn
Na
Metals
Tap water
Nanobiosorbent
Biosorbent
Untreated AMD-3


c
% Higher/lower than tapwater control
300
250
200
150
100
50
0
Al
Co
Cu
Fe
Ni
Pb
Zn
Ca
K
Mg
Mn
Na
Metals
Tap water
Nanobiosorbent
Biosorbent
Untreated AMD-3



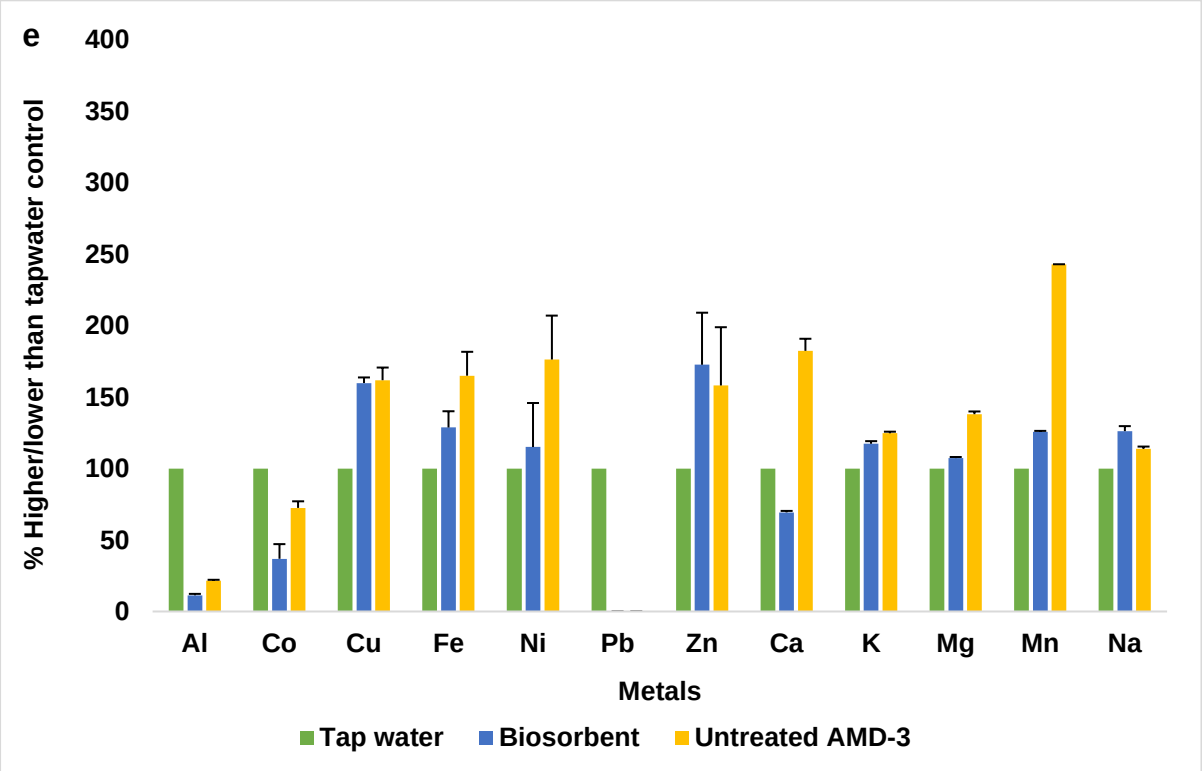


Figure 4.7. The graph represents the percentage of the metal concentrations of each experimental group relative to the tap water control group for the tomato plant roots (a), stems (b), leaves (c), flowers (d) and fruits (e). No fruits had developed in the nanobiosorbent treated plants by the end of the study.

4.3.3 Heavy metal quantification in water and soil samples

The concentration of metals in the tap water, untreated, biosorbent treated and nanobiosorbent treated AMD-3 water used to irrigate the tomato plants was determined by ICP-OES and ICP-MS. All the metals in the tap water were within the acceptable limits outlined by DWAF (1996), except for Pb (0.014 mg/L) which was slightly higher than the acceptable agricultural level of 0.010 mg/L. For all three variations of AMD-3 water, Mn and Fe were consistently higher than 10 mg/L Mn and 0.3 mg/L Fe (DWAF, 1996), respectively. Untreated AMD-3 water had the highest concentration of both metals (24.10 mg/L Mn and 21.35 mg/L Fe). The nanobiosorbent treated limestone neutralized AMD-3 water had a higher Mn content (18.18 mg/L) than biosorbent treated limestone neutralized AMD-3 water (17.98 mg/L) while the opposite was true for the Fe content. Both the untreated AMD-3 water and nanobiosorbent treated water had above acceptable concentrations of Ni, 0.522 mg/L and 0.254 mg/L, respectively. However, biosorbent treated limestone neutralized AMD-3 water had a Ni concentration of 0.018 mg/L. The Mn and Fe concentrations were significantly different (p = 0.001, Table A.13 in Appendix A) when comparing the untreated AMD-3 water to the other three treatment groups.

Analysis of the commercial potting soil used in the study prior to planting showed that although majority of the metals were within the acceptable limits for soil quality in South Africa (Molewa, 2014), Mn and Cu levels were higher. The acceptable limit for Mn is 740 mg/kg and that of Cu is 16 mg/kg; the potting soil contained 1327.92 mg/kg Mn and 17.11 mg/kg Cu. This profile was the same for the soil from all four treatments at the end of the study. The highest concentration of Mn at 1551.66 mg/kg and Cu at 25.08 mg/kg was obtained for the soil in the untreated AMD-3 treatment group. As expected, majority of the metal concentrations were lower in the potting soil compared to the other soil groups (Figure 4.8). Interestingly, from the graphs in Figure 4.8 and the statistical data reported in Table A.14 (Appendix A), the soil from the nanobiosorbent and biosorbent treated AMD-3 groups had significantly lower metal concentrations overall compared to the soil from the tap water group. This could be as a result of greater metal uptake by the tomato plants irrigated with either of the treated water which is reflected in the higher concentration of metals found in the different plant structures that were analysed. However, there was little difference between the

soil from the nanobiosorbent and biosorbent treated AMD-3 group. The only significant difference in concentration was obtained for Ca and Na (p = 0.001) where the biosorbent treated AMD-3 soil had a lower concentration for both metals.

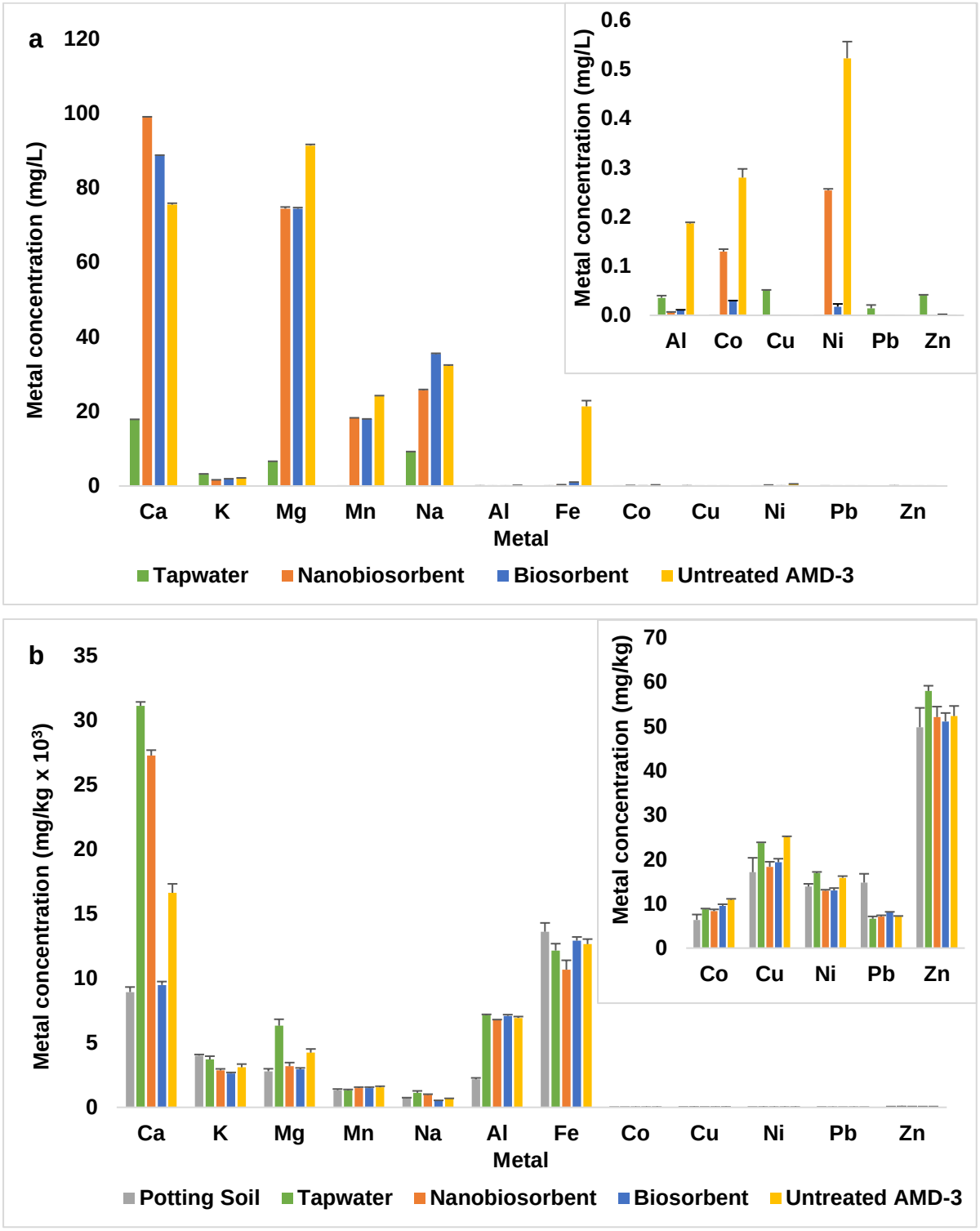


Figure 4.8. Metal concentrations of water (a) and soil (b) samples from each tomato plant treatment group. Inset graphs show the profile for metals with very low concentrations in the sample set.

4.4 Discussion

The quality of natural waters in Gauteng, South Africa has deteriorated as a result of heavy metal contamination originating from mining and industrial effluent (McCarthy, 2011). The discharge of AMD wastewater into the surrounding environment results in the accumulation of elevated metal concentrations in the soil and plants posing environmental health concerns as it induces biotoxicity (Duruibe, Ogwuegbu and Egwurugwu, 2007). Metal concentrations may be reduced from effluent using the dead biomass of bacteria through biosorption and this process may be stabilized through immobilization of the biomass with CANPs as shown in Chapter 3. The present chapter focused on the growth and development of tomato plants irrigated with tap water, limestone neutralized nanobiosorbent treated AMD-3 water, limestone neutralized biosorbent treated AMD-3 water and untreated AMD-3 water. Furthermore, the heavy metal accumulation in the tomato plant structures, soil and water per treatment group was evaluated and compared to the permissible consumable and agricultural levels, respectively.

4.4.1 Plant growth and development

Out of the three AMD variations treated as described in Chapter 3, AMD-3 was selected for the purpose of irrigation of the tomato plants in the pot trial. According to Hobbs (2017), AMD-3, which originates in the Witwatersrand Basin and is treated by an HDS process for the removal of trace metals, is released into the Tweelopie Spruit where it eventually flows into the Crocodile River. Human settlements around the Crocodile River are therefore exposed to these particular metal concentrations when they consume the water for domestic or farming purposes.

The general maturity period of a tomato plant is approximately 90-100 days. In the present study, plants from the tap water, biosorbent and untreated plant groups only reached the fruiting stage after 131, 140 and 154 days, respectively, while the nanobiosorbent group remained at the flowering stage for the duration of the pot trial (Figure 4.6c). These findings suggest that plant growth and development was slightly stunted likely as a result of the conditions the plants grew under.

Tomato plants prefer to grow in summer or under warm conditions with exposure to 8 hours of sunlight (ARC-VOPI, 2013). However, due to the lack of access to a

greenhouse and seedlings only becoming available in July (Winter), plant pot trials were performed indoors with regulated temperature and light to allow the study to progress. Several factors have been reported to affect plant growth and development. The photosynthesis and carbohydrate production are affected by carbon dioxide (CO_2) concentration (optimum at 200-1500 µmol mol^{-1}) and daily light integral whereas temperature (optimum at 10-35 °C) and relative humidity (optimum between 30-90 %) affect the rate at which the plant develops (Schwarz, Thompson and Kläring, 2014). Previous studies have reported the daily light integral of 13.0–17.3mol m^{-2} d^{-1} photosynthetic active radiation (PAR) was essential for growth and production of carbohydrates (Schwarz, Thompson and Kläring, 2014). The light intensity provided by the LED lights and grow lights may have been insufficient for optimal photosynthesis as compared to natural sunlight when plants are grown outside. Regulating the CO_2 concentrations and humidity may have further improved the growth and development of the plants to the fruiting stage. Overall, the plants may have grown optimally outdoors during summer season.

The tap watered plants fruited earlier and were bigger in size compared to the other treatment groups. The untreated group fruited the slowest and was smallest in size while the nanobiosorbent group yielded no fruit (Figure 4.6). The differences in the fruiting periods and size between treatment groups may have been as a result of the quality of the water used for irrigation. As expected, majority of the metal concentrations were found in higher concentrations in the fruit of the untreated group compared to the tap water and biosorbent groups. Particularly, K, Mn and Fe were found in higher concentrations in the flowers of the nanobiosorbent group and the fruits of the untreated group compared to the tap water group (Figure 4.7d-e). More metals were also shown to translocate to the aerial parts of the nanobiosorbent treated plants compared to the other treatment groups (Table 4.1). Previous studies have found that an excess of K, Mn or Fe induces an imbalance in the uptake of other nutrients and also has a negative impact on the fruit yield of tomato plants (Sainju, Dris and Singh, 2003; Asri and Sonmez, 2012; Kleiber, 2015). Therefore, the high concentrations of these metals reported in this study may have contributed to the lack of and poor fruit development in the nanobiosorbent and untreated groups, respectively.

All four plant treatment groups were fertilized uniformly during the seedling stage and during the flowering stage to supply metal nutrients such as K, Ca, Mg, Fe, Mn, Zn, Ni and Cu that are essential for plant growth (Cobb *et al*., 2000; Sainju, Dris and Singh, 2003). The plants irrigated with biosorbent treated limestone neutralized AMD-3 water grew the most in terms of plant height, leaf count and leaf area. The nanobiosorbent treated AMD-3 watered plants also grew better compared to the tap watered plants during the initial phase (Figure 4.5). Both the biosorbent and nanobiosorbent treated limestone neutralized AMD-3 water contained higher amounts of Ca and Mg originating from the limestone which could have accounted for the better growth in these treatment groups. Calcium is an essential macronutrient responsible for not only maintaining the structure and functioning of the cell walls and membranes in fruiting and vegetable plants (Poovaiah, 1986) but also for regulating the growth and development of plants (Hepler, 2005). It also has an important role in the nutrition and physiology of the plant (Kirkby and Pilbeam, 1984). Magnesium forms part of the chlorophyll molecules that are essential for photosynthesis to occur and is also a key co-factor in other physiological processes (Huber and Jones, 2012).

Surprisingly, the plants watered with untreated AMD-3 also initially developed better than the tap water control group in terms of plant height and leaf count. This may be explained by the increased bioavailability of most metal ions due to the acidity of the untreated AMD-3 water (pH 5) compared to the neutral pH of tap water. The solubility of metals increases as the pH levels decrease therefore slight changes in pH levels can affect the bioavailability of metals (Salomons, 1995).

These results suggest that the metal ions from the limestone and the AMD water itself can become available as supplementary nutrients leading to improved growth and development of the plants. However, uptake of excess metal concentrations over time in the plants affects the growth and development of the fruits as seen in the treatment groups. Plants that are exposed to heavy metals exhibit a decrease in growth rate (Yaqvob *et al*., 2011). A study performed by Oancea, Foca and Airinei (2005) found that mercury, Cd, Zn and thallium contaminated water used for the irrigation of tomato plants affected the growth of the plants and resulted in a decline in the functioning of the physiological and biochemical processes within the plants.

4.4.2 Metal accumulation in tomato plants

Most of the metal concentrations had accumulated in the tomato plant structures in the following order: flowers>roots>stems>leaves=fruits. Metals are translocated to the aerial parts of the plant as indicated by a TF>1 while TF<1 indicates that the metal was mostly located in the roots of the plants and did not translocate to the aerial parts of the plants (Ng *et al.*, 2016). Potassium was the only metal shown to translocate to the aerial parts of the plants for all four treatment groups as indicated by a TF>1. Within tomato plants, K is reported to be highly mobilized whereby the metal is transported through the xylem and phloem to translocate in all structures and organs of the plant (Nzanza, 2006). As mentioned in section 4.4.1, high concentrations of K may have affected the rate at which the other metals were taken up by the plants.

Majority of the metal concentrations found in the fruits from the tap water, untreated AMD-3 and biosorbent treated AMD-3 groups exceeded either the FAO limits for consumable tomatoes or the maximum daily intake for an adult except for Na and Ni. Both the biosorbent and untreated AMD-3 groups produced tomatoes with above acceptable concentrations of Pb. A study performed by Parashar and Prasad (2013) similarly reported that the irrigation of tomato plants with treated and untreated wastewater resulted in the accumulation of Pb and Cd in tomato fruits at unsafe levels. The ingestion of high amounts of Pb can lead to possible health implications such as disorders of the nervous system, abdominal pains and encephalopathy (Järup, 2003).

Both Ca and Mg are essential for growth and development of tomato plants and small amounts are generally present in the fruits. However, human exposure to high doses of Ca causes hypercalcemia which affects kidney and bowel function (Peacock, 2010) while too much Mg leads to nausea, coma, cardiac defects and paralysis (De Baaij, Hoenderop and Bindels, 2015). Potassium is another macronutrient important for plant growth; tomatoes are considered to be rich in K. On average, a medium-sized tomato contributes between 6-8% of the recommended daily intake of K (3500 mg). On the other hand, when consumed in high amounts such as the reported K concentrations in the tomatoes from the present study, it may result in bradycardia, renal failure, hyperchloremic metabolic acidosis and paraesthesia (Weisberg, 2008).

Manganese, Cu, Zn, Fe and Co are essential trace elements that are used by the enzymes as co-factors in normal cellular metabolism but at elevated levels they may become hazardous. When Mn is ingested at high concentrations it becomes toxic resulting in neurological disorders upon ingestion of toxic concentrations (Santamaria, 2008). The high concentrations of Mn in the tomatoes likely originated from both the water and potting soil as evident from Figure 4.8. Another element that accumulated in the tomatoes from the soil was Cu. Copper toxicity in humans induces neurological disorders such as Alzheimer's disease as well as liver diseases (Uriu-Adams and Keen, 2005). High exposure to Zn may induce apoptosis particularly in the brain (Plum, Rink and Haase, 2010) while Fe is reported to contribute to the onset of neurodegenerative diseases and brain aging (Agrawal *et al*., 2017). A study performed by Jensen and Tuchsen (1990) reported on the carcinogenic effects of high Co exposure in humans and mice. Overall, the ingestion and exposure to toxic concentrations of the elements discussed here have negative implications on human health. Consequently, none of the treatments in this study, including the tap water group, produced tomatoes safe enough for human consumption.

As the plants in the nanobiosorbent treated AMD-3 group did not fruit, there is no data to suggest whether the use of this water in the irrigation of tomato plants is suitable. Since the flowers of the tomato plant develop into fruit, it may be suggested that the level of metal accumulation in the flowers would be an indication of levels that could accumulate in the fruit. In this study, the metal accumulation in the flowers exceeded all the other plant structures yet the fruit from the other three treatment groups had accumulated the lowest amount of metal. Therefore, the metal concentration in the flowers cannot be used to predict whether the fruit from the nanobiosorbent treated group would have (or not) contained metals within acceptable limits for consumption. However, a general observation from the findings reported here suggest that the nanobiosorbent and biosorbent treated groups yielded similar results in metal accumulation in the other plant structures and therefore the fruit from the nanobiosorbent treated group may have exhibited a similar metal profile to that of the biosorbent treated group.

The metals that accumulated in the different structures of the plants are likely to have originated from several sources. These include the water, potting soil and fertilizers

that were used during the study. The tap water was of a good quality as expected except for the concentration of Pb (0.014 mg/L) which was slightly over the acceptable agricultural level of 0.010 mg/L. Lead is easily taken up by plants and is known to accumulate in the aerial parts of the plant (Sharma and Dubey, 2005; Salem, Albanna and Awwad, 2016). However, the Pb levels were still within the permissible levels in the fruit of the tap water group. Water from the other three groups had high concentrations of Mn and Fe that could have contributed to the accumulation of these metals in the tomato plants. In addition, high levels of Mn were found in the potting soil prior to planting and in the different treatment groups. Together these sources are likely to have resulted in high concentrations of Mn exposure to the plants. Manganese is soluble under neutral and acidic conditions which increases the bioavailability of the metal (Hem, 1963). Although the nanobiosorbent treated and untreated AMD-3 water had above acceptable limits of Ni, the fruit from the untreated AMD-3 did not. In addition to Mn, the soil from each treatment group had high concentrations of Cu which likely contributed to the levels obtained in the fruit from all treatments as AMD-3 did not contain Cu. Lead, Cu and Zn contaminated soils are also known to cause stunted growth in plants (Sainju, Dris and Singh, 2003; Sharma and Dubey, 2005) as was observed in this study.

The high levels of the other metals found in the fruits and other plant structures of the tap water, untreated AMD-3, biosorbent and nanobiosorbent treated AMD-3 groups can be attributed to the accumulation of the metals in the soil over time from regular watering. During the period of study more metal would have become available for the tomato plants to take up. This was evident from the lower metal composition of the initial potting soil compared to the soil across all treatments at the end of the study where majority of the metal concentrations were higher. The use of pots to grow the tomato plants as opposed to planting in the ground therefore serves as a limitation of the study. This is because irrigation of tomato plants grown in the ground would have likely resulted in the dispersion of the metals deeper into the soil or they may have drained into the ground water. This may have subsequently resulted in the exposure of the plants to lower metal concentrations. Thus, the tomato plant structures and fruits would have possibly accumulated lower concentrations of metals over time. In the present study, metals originating from the water or fertilizers may have accumulated in high concentrations in the potting soil over time which likely impacted the amount of

metals take up by the plants during the potting trial. The reason for performing a pot trial was to avoid contributing towards soil contamination as a result of the heavy metals accumulating in the ground which would subsequently deteriorate the ground water quality and surface water quality through accumulation in downstream water sources (Issaka and Ashraf, 2017). New trials could be performed in land designated solely for this purpose and that is distant from any water source in order to verify this hypothesis. The findings from this study suggest that tap water and the biologically (both biosorbent and nanobiosorbent) treated AMD was not suitable for agricultural purposes.

4.5 Conclusions

The study concludes that using nanobiosorbent or biosorbent treated limestone neutralized AMD-3 water for the irrigation of tomato plants resulted in the production of plants that incurred stunted growth with fruits that were not safe for human consumption. The same was observed in the tap watered plants suggesting that the conditions the plants grew under and the quality of the soil also affected the overall growth of the plants and quality of the fruits produced.

Chapter 5: Conclusions and future recommendations

The natural waters in Gauteng, South Africa are contaminated with heavy metals originating from industrial effluent. Toxic metals accumulate in the water and food chain posing various health risks to the local communities that are exposed to the toxic metals. The development of eco-friendly metal removal treatment strategies is necessary for the reclamation of AMD water. The treated water may potentially be reused for agricultural purposes. This study aimed to determine if AMD water treated with bacterial biosorbent or nanobiosorbent was suitable for agricultural purposes.

The study concludes that using limestone is a suitable strategy for the neutralization of AMD water as it precipitated majority of the metals. Limestone is currently being used in South Africa during the wastewater treatment process and is a cheaper option compared to NaOH. A single biosorbent that can effectively take up metals from different variations of AMD could not be described. This implies that the types and concentrations of the metals that constitute a particular wastewater sample influence their biosorption likely due to competition for binding sites on the bacterial cell wall. Nevertheless, a biosorbent comprised of *M. luteus* was determined to result in the reduction of most metal concentrations found in AMD water typical of the Central and Witwatersrand Basins of South Africa. Furthermore, immobilization of *M. luteus*, with CANPs maintained its structural and biosorptive properties. This was evident from the stability of the nanobiosorbent over extended periods at 4 and 25 ° C. Furthermore, the resultant quality of water produced after treatment with the nanobiosorbent was similar to that of biosorbent treated AMD. Regardless of which treatment was used, the concentration of Mn and Ni remained above acceptable agricultural limits. Although this made the treated AMD water generally unsuitable for agricultural purposes, it was hypothesised that these metals would accumulate in the roots of tomato plants without affecting the edible fruit if the water was used for irrigation purposes.

For the purpose of this study, biologically treated AMD-3 was selected for irrigating tomato plants. According to Hobbs (2017) this is the quality of AMD that is released into the Tweelopie Spruit eventually reaching Crocodile River where local communities get their water from. Overall, plant growth occurred to maturity across all treatments

except in the group irrigated with nanobiosorbent treated AMD-3 water which failed to reach the fruiting stage by the time the study was terminated. However, development was stunted even in the tap water group which served as a positive control. This may have resulted from a combination of factors which include the controlled conditions in which the plants were grown in, the quality of the water used for irrigation, the quality of the initial potting soil and the design of the growth trial which eventually resulted in the high accumulation of metals in all plant structures. Overall, the concentration of most metals in the fruits produced by tomato plants irrigated with tap water, untreated AMD-3 and biosorbent treated AMD-3 exceeded the permissible levels for safe consumption. The study therefore concludes that AMD water treated with the *M. luteus* biosorbent or nanobiosorbent may not be suitable for agricultural purposes.

This study focused on the importance of developing biological alternatives for the treatment of heavy metal contaminated wastewater due to the health implications of heavy metal exposure. Furthermore, it sought to determine if recycled AMD can be used as an alternative source of water for agricultural purposes especially in these times of water scarcity. It is therefore recommended that another plant trial is performed on farming land to establish whether metal accumulation in the tomatoes could have occurred due to its containment in the soil within the pots regardless of the water quality. In addition, the inclusion of a second biosorption step for further uptake of metals from the wastewater would be of value especially due to the saturation effect experienced when biosorbents are exposed to very high metal concentrations. Alternatively, other novel, eco-friendly biosorbents from extreme environments can be explored as options for the reclamation of AMD water as well as the treatment of metal contaminated soils.

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Appendices

Appendix A

Tables showing the *p* values for each neutralization or treatment group per metal after performing one-way ANOVA statistical tests. A *p* value < 0.05 indicates a significant difference at a confidence interval of 95%.

Table A.1. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations after neutralization of AMD-1, AMD-2 and AMD-3 with NaOH or limestone.

Metal	AMD-1	AMD-2	AMD-3
		p values	
Al	0.001	1.000	0.001
Ca	0.001	0.001	0.001
Co	0.004	0.001	0.002
Cu	0.001	-	-
Fe	0.001	0.001	0.001
K	0.001	0.061	0.001
Mg	0.014	0.005	0.002
Mn	0.038	0.004	0.055
Na	0.002	0.006	0.008
Ni	0.001	0.001	0.001
Pb	0.001	1.000	0.028
Zn	0.010	-	-

Table A.2. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-1 after treatment with CANPs, *E. xiangfangensis* Pb204, *M. luteus* or a 2:1 combination of the two biosorbents.

Treatment	Metal											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.108	0.008	1.000	0.001	0.045	0.002	0.001	0.001	0.001	1.000	0.001
M vs E	0.298	0.916	0.517	1.000	0.019	0.702	0.913	0.988	0.001	0.001	1.000	0.001
M vs EM	0.085	0.224	0.034	1.000	0.001	0.092	0.089	0.097	0.174	0.001	1.000	0.001
M vs C	0.002	0.082	0.026	1.000	0.008	0.625	0.004	0.001	0.001	0.001	1.000	0.001
E vs EM	0.219	0.231	0.195	1.000	0.179	0.256	0.057	0.056	0.033	0.175	1.000	0.447
E vs C	0.098	0.071	0.160	1.000	0.993	0.409	0.004	0.001	0.546	0.001	1.000	0.001
EM vs C	0.001	0.313	0.756	1.000	0.182	0.405	0.390	0.102	0.002	0.001	1.000	0.001

M = *M. luteus*, E = *E. xiangfangensis* Pb 204, EM = 2:1 combination of biosorbents, C = CANPs

Table A.3. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-2 after treatment with CANPs, *E. xiangfangensis* Pb204, *M. luteus* or a 2:1 combination of the two biosorbents.

Treatment	Metal									
	Al	Ca	Co	Fe	K	Mg	Mn	Na	Ni	Pb
All	0.515	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.119
M vs E	0.480	0.004	0.001	0.044	0.001	0.112	0.006	0.008	0.932	0.414
M vs EM	0.495	0.104	0.001	0.999	0.032	0.326	0.088	0.311	0.141	0.144
M vs C	0.542	0.033	0.001	0.067	0.001	0.505	0.033	0.056	0.001	0.144
E vs EM	0.906	0.019	0.908	0.124	0.001	0.006	0.006	0.003	0.267	0.845
E vs C	0.665	0.002	0.157	0.097	0.001	0.001	0.001	0.011	0.001	0.845
EM vs C	0.751	0.005	0.097	0.003	0.001	0.002	0.005	0.017	0.001	1.000

M = *M. luteus*, E = *E. xiangfangensis* Pb 204, EM = 2:1 combination of biosorbents, C = CANPs

Table A.4. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-3 after treatment with CANPs, *E. xiangfangensis* Pb204, *M. luteus* or a 2:1 combination of the two biosorbents.

Treatment	Metal									
	Al	Ca	Co	Fe	K	Mg	Mn	Na	Ni	Pb
All	0.083	0.002	0.001	0.008	0.522	0.002	0.001	0.001	0.001	0.859
M vs E	0.137	0.017	0.014	0.011	0.998	0.211	0.024	0.001	0.001	0.892
M vs EM	0.238	0.077	0.737	0.191	0.792	1.000	0.085	0.001	0.001	1.000
M vs C	0.758	0.002	0.001	0.991	0.935	0.086	0.185	0.001	0.001	1.000
E vs EM	0.486	0.191	0.004	0.232	0.703	0.019	0.006	0.001	0.046	0.892
E vs C	0.263	0.037	0.001	0.016	0.974	0.567	0.554	0.001	0.001	0.892
EM vs C	0.306	0.007	0.001	0.281	0.475	0.008	1.000	0.001	0.001	1.000

M = *M. luteus*, E = *E. xiangfangensis* Pb 204, EM = 2:1 combination of biosorbents, C = CANPs

Table A.5. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-1 after treatment with CANPs, *M. luteus*, nanobiosorbent or a 1:1 mixture of *M. luteus* and free CANPs.

Treatment	Metal											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.016	0.001	1.000	0.001	0.001	0.001	0.001	0.001	0.001	1.000	0.001
M vs C	0.001	0.082	0.001	1.000	0.001	0.625	0.006	0.002	0.002	0.001	1.000	0.001
M vs NB	0.001	0.108	0.001	1.000	0.001	0.043	0.023	0.005	0.001	0.001	1.000	0.001
M vs MC	0.001	0.011	0.001	1.000	0.001	0.067	0.073	0.001	0.507	0.001	1.000	0.001
C vs NB	0.001	0.935	0.001	1.000	0.001	0.004	0.001	1.000	0.387	0.001	1.000	0.001
C vs MC	0.001	0.068	0.001	1.000	0.019	0.300	0.567	0.514	0.020	0.001	1.000	0.001
NB vs MC	0.001	0.133	0.001	1.000	0.001	0.011	0.002	0.606	0.004	0.001	1.000	0.001

M = *M. luteus*, C = CANPs, NB = Nanobiosorbent, MC = 1:1 Mixture of *M. luteus* and CANPs

Table A.6. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-2 after treatment with CANPs, *M. luteus*, nanobiosorbent or a 1:1 mixture of *M. luteus* and free CANPs.

Treatment	Metal									
	Al	Ca	Co	Fe	K	Mg	Mn	Na	Ni	Pb
All	0.409	0.001	0.001	0.002	0.001	0.016	0.001	0.003	0.001	0.119
M vs C	0.542	0.001	0.001	0.004	0.001	0.505	0.001	0.056	0.001	0.286
M vs NB	0.521	0.001	0.001	0.007	0.001	0.502	0.001	0.043	0.001	0.286
M vs MC	0.598	0.001	0.001	0.008	0.001	0.288	0.001	0.041	0.001	0.406
C vs NB	0.964	0.945	0.001	0.991	0.536	0.996	1.000	0.750	0.001	1.000
C vs MC	0.822	0.001	0.092	0.969	0.354	0.004	0.001	0.133	0.001	0.768
NB vs MC	0.386	0.001	0.001	0.998	0.064	0.006	0.001	0.086	0.001	0.768

M = *M. luteus*, C = CANPs, NB = Nanobiosorbent, MC = 1:1 Mixture of *M. luteus* and CANPs

Table A.7. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations of limestone neutralized AMD-3 after treatment with CANPs, *M. luteus*, nanobiosorbent or a 1:1 mixture of *M. luteus* and free CANPs.

Treatment	Metal									
	Al	Ca	Co	Fe	K	Mg	Mn	Na	Ni	Pb
All	0.001	0.001	0.001	0.001	0.001	0.066	0.096	0.001	0.001	1.000
M vs C	0.349	0.001	0.001	0.888	0.733	0.044	0.185	0.001	0.001	1.000
M vs NB	0.001	0.535	0.001	0.001	0.001	0.442	0.989	0.001	0.001	1.000
M vs MC	0.001	0.884	0.001	0.095	0.001	0.487	0.995	0.001	0.001	1.000
C vs NB	0.001	0.006	0.001	0.001	0.001	0.809	0.061	0.186	0.001	1.000
C vs MC	0.001	0.001	0.001	0.253	0.001	0.766	0.069	0.001	0.001	1.000
NB vs MC	0.001	0.888	0.001	0.001	1.000	1.000	0.999	0.001	0.001	1.000

M = *M. luteus*, C = CANPs, NB = Nanobiosorbent, MC = 1:1 Mixture of *M. luteus* and CANPs

Table A.8. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation in per tomato plant structure for the plants irrigated with tap water.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.002
Fl vs L	0.124	0.019	0.984	0.001	0.079	0.001	0.005	0.001	0.139	0.690	0.001	0.020
Fl vs S	0.524	0.002	0.974	0.001	0.060	0.002	0.014	0.011	0.013	0.223	0.001	0.109
Fl vs R	0.025	0.001	0.001	0.001	0.032	0.001	0.001	0.085	0.069	0.001	0.001	0.053
Fl vs Fr	0.008	0.002	1.000	0.039	0.089	0.439	0.001	0.001	0.001	1.000	0.001	0.184
L vs S	0.069	0.036	1.000	0.001	0.251	0.001	0.017	0.010	0.198	0.757	0.005	0.310
L vs R	0.025	0.400	0.001	0.001	0.032	0.001	0.014	0.982	0.371	0.001	0.001	0.087
L vs Fr	0.328	0.019	0.988	0.001	0.084	0.713	0.001	0.001	0.169	0.757	0.004	0.034
S vs R	0.025	0.001	0.001	0.001	0.032	0.001	0.018	0.152	0.244	0.001	0.001	0.157
S vs Fr	0.130	0.001	0.979	0.030	0.061	0.075	0.017	0.007	0.013	0.257	0.992	0.105
R vs Fr	0.025	0.001	0.001	0.001	0.032	1.000	0.001	0.090	0.080	0.001	0.001	0.030

Fl = Flower, L = Leaves, Fr = Fruit, S = Stem, R = Root

Table A.9. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation in per tomato plant structure for the plants irrigated with nanobiosorbent treated limestone neutralized AMD-3 water.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.003	0.049	0.001
Fl vs L	0.830	0.001	0.248	0.003	0.036	0.008	0.096	0.001	0.160	0.034	0.857	0.005
Fl vs S	0.972	0.028	0.460	0.164	0.084	0.001	0.027	0.024	0.024	0.003	0.121	0.001
Fl vs R	0.001	0.006	0.001	0.001	0.013	0.011	0.001	0.017	0.012	0.049	0.058	0.001
L vs S	0.971	0.001	0.911	0.013	0.015	0.001	0.801	0.019	0.196	0.124	0.255	0.034
L vs R	0.001	0.006	0.001	0.001	0.013	0.002	0.245	0.028	0.217	0.051	0.155	0.001
S vs R	0.001	0.033	0.001	0.001	0.013	0.001	0.054	0.013	0.975	0.051	0.831	0.010

Fl = Flower, L = Leaves, S = Stem, R = Root

Table A.10. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation in per tomato plant structure for the plants irrigated with biosorbent treated limestone neutralized AMD-3 water.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.006	0.006	0.006
Fl vs L	0.710	0.009	0.605	0.001	0.017	0.001	0.034	0.039	0.026	0.215	0.767	0.057
Fl vs S	0.979	0.029	0.580	0.021	0.100	0.001	0.017	0.025	0.034	0.037	0.926	0.069
Fl vs R	0.001	0.021	0.001	0.001	0.130	0.001	0.012	0.038	0.002	0.076	0.012	0.061
Fl vs Fr	1.000	0.001	0.999	0.019	0.086	0.001	0.001	0.001	0.001	0.717	0.017	0.193
L vs S	0.938	0.001	1.000	0.003	0.022	0.001	0.105	0.049	0.050	0.998	0.993	0.101
L vs R	0.001	0.031	0.001	0.001	0.013	0.001	0.056	0.091	0.062	0.068	0.032	0.067
L vs Fr	0.668	0.010	0.644	0.003	0.003	0.001	0.041	0.040	0.029	0.217	0.046	0.088
S vs R	0.001	0.008	0.001	0.001	0.013	0.001	0.003	0.052	0.415	0.081	0.023	0.138
S vs Fr	0.978	0.031	0.616	1.000	0.004	0.001	0.019	0.027	0.038	0.016	0.032	0.031
R vs Fr	0.001	0.022	0.001	0.001	0.013	0.001	0.012	0.040	0.001	0.076	0.993	0.027

Fl = Flower, L = Leaves, S = Stem, R = Root

Table A.11. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation in per tomato plant structure for the plants irrigated with untreated AMD-3 water.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Fl vs L	0.818	0.021	0.846	0.001	0.013	0.001	0.005	0.179	0.097	0.505	0.001	0.001
Fl vs S	0.658	0.001	0.928	0.001	0.026	0.002	0.063	0.052	0.143	0.958	0.970	0.001
Fl vs R	0.001	0.008	0.001	0.001	0.005	0.017	0.001	0.037	0.031	0.001	0.001	0.001
Fl vs Fr	1.000	0.001	1.000	0.001	0.098	0.008	0.001	0.001	0.001	0.998	0.001	0.022
L vs S	0.997	0.033	0.999	0.001	0.002	0.001	0.154	0.283	0.330	0.825	0.002	0.454
L vs R	0.001	0.034	0.001	0.001	0.005	0.028	0.009	0.482	0.172	0.001	0.001	0.001
L vs Fr	0.835	0.022	0.885	0.001	0.003	0.013	0.001	0.196	0.116	0.646	0.001	0.009
S vs R	0.001	0.025	0.001	0.001	0.005	0.001	0.077	0.026	0.857	0.001	0.001	0.001
S vs Fr	0.678	0.001	0.953	0.543	0.030	0.001	0.088	0.067	0.159	0.995	0.001	0.003
R vs Fr	0.001	0.003	0.001	0.001	0.005	0.003	0.001	0.042	0.034	0.001	0.010	0.001

Fl = Flower, L = Leaves, S = Stem, R = Root

Table A.12. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation per treatment in the fruits.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.650	0.012	0.103	0.001	0.001	0.001	0.001	0.065	0.002	0.275
TP vs TB	0.001	0.001	0.695	0.007	0.197	0.001	0.002	0.001	0.016	0.579	0.002	0.272
TP vs UT	0.001	0.001	1.000	0.062	0.112	0.001	0.001	0.001	0.033	0.138	0.007	0.405
TB vs UT	0.900	0.001	0.695	0.931	0.243	0.038	0.001	0.001	0.001	0.062	0.023	0.926

TP = Tap water, TN = Nanobiosorbent treated limestone neutralized AMD-3 water, TB = Biosorbent treated limestone neutralized AMD-3 water, UT = Untreated AMD-3 water

Table A.13. The *p*-values for the one-way ANOVA statistical comparison of metal concentrations in the water per treatment.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.013	0.001
TP vs TN	0.011	0.001	0.031	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.016	0.001
TP vs TB	0.022	0.001	0.001	0.001	0.061	0.001	0.003	0.001	0.001	0.750	0.022	0.001
TP vs UT	0.001	0.004	0.052	0.001	0.059	0.001	0.002	0.001	0.001	0.001	0.022	0.001
TN vs TB	0.118	0.002	0.040	1.000	0.092	0.001	1.000	0.124	0.001	0.001	1.000	0.008
TN vs UT	0.002	0.002	0.076	1.000	0.060	0.001	0.001	0.001	0.001	0.001	1.000	1.000
TB vs UT	0.006	0.013	0.058	1.000	0.061	0.001	0.001	0.001	0.001	0.001	1.000	0.008

TP = Tap water, TN = Nanobiosorbent treated limestone neutralized AMD-3 water, TB = Biosorbent treated limestone neutralized AMD-3 water, UT = Untreated AMD-3 water

Table A.14. The *p*-values for the one-way ANOVA statistical comparison of metal accumulation per treatment in the soil.

Treatment	Metals											
	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
All	0.039	0.001	0.005	0.019	0.228	0.001	0.001	0.024	0.001	0.004	0.001	0.173
TP vs TN	0.365	0.001	0.613	0.186	0.405	0.001	0.001	0.239	0.191	0.008	0.653	0.309
TP vs TB	0.997	0.001	0.170	0.157	0.566	0.001	0.001	0.251	0.001	0.008	0.118	0.179
TP vs UT	0.775	0.001	0.009	0.031	0.816	0.013	0.001	0.076	0.001	0.249	0.601	0.292
TP vs PS	0.001	0.001	0.450	0.419	0.391	0.562	0.001	1.000	0.001	0.335	0.051	0.416
TN vs TB	0.501	0.001	0.081	0.826	0.249	0.495	0.856	1.000	0.001	1.000	0.075	0.985
TN vs UT	0.892	0.001	0.009	0.150	0.273	0.477	0.012	0.888	0.015	0.067	1.000	0.999
TN vs PS	0.001	0.001	0.540	0.977	0.157	0.001	0.541	0.142	0.005	0.627	0.059	0.922
TB vs UT	0.911	0.001	0.035	0.121	0.913	0.045	0.003	0.872	0.167	0.026	0.010	0.941
TB vs PS	0.001	0.629	0.317	0.860	0.732	0.001	0.973	0.151	0.176	0.650	0.064	0.979

TP = Tap water, TN = Nanobiosorbent treated limestone neutralized AMD-3 water, TB = Biosorbent treated limestone neutralized AMD-3 water, UT = Untreated AMD-3 water, PS = Potting soil