The effect of cadmium on earthworms (*Eisenia andrei*) and their intestinal bacteria

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PREFACE

The experimental work done and discussed in this dissertation for the degree *Magister Scientiae* in Environmental Sciences was carried out in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. This study was conducted part-time during the period of January 2014 to November 2017, under the supervision of Prof. C.C. Bezuidenhout and Prof. M.S. Maboeta.

The research done and presented in this dissertation signifies original work undertaken by the author and has not been submitted for degree purposes to any other university before. Appropriate acknowledgements in the text have been made, where the use of work conducted by other researchers have been included.

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SUMMARY

Cadmium contamination, predominantly from anthropogenic activities such as mining, have a significant impact on soil organisms. It alters the abundance, diversity, community structure, ecological functions and the species present in the soil. The reproduction of earthworms are adversely affected by very low cadmium concentrations. Consequently, earthworms are recognised bioindicators of cadmium contamination in soil. Soil bacterial communities also change dramatically with cadmium contamination. As bacteria provide essential molecules through their metabolic processes, a disturbance in bacterial community structure and function does have significant implications on other organisms. Both earthworms and bacteria have methods of resisting cadmium toxicity. Some bacteria are known for their ability to bind and detoxify cadmium, not only in soil but also within organisms. In this manner, intestinal bacteria may contribute to the cadmium resistance observed in earthworms after long-term exposure.

This study aimed to determine if *Eisenia andrei* acquired cadmium resistance after long-term exposure in comparison to *E. andrei* that have not had long-term exposure. The study compared earthworm resistance in terms of; mortality, reproduction, cadmium body burden and, aerobically culturable bacteria present in their casts. Bacterial results were compared in relation to: bacterial levels, diversity of the morphologically distinct culturable bacteria colonies, the species present in the casts and the overall Gram-positive to Gram-negative ratios.

The OECD guidelines for testing the effects of chemicals on earthworms was utilised to compare the earthworm resistance over a range of 10 to 400 μg Cd^{2+} g^{-1} after four weeks. Thereafter, the bacteria present in the casts were cultured on soil and nutrient agar augmented with a range of 10 to 400 μg Cd^{2+} L^{-1}. The cocoons produced during the four weeks and the juveniles that emerged after an additional four weeks were counted to determine difference in reproductive output. Bacterial levels and morphologically distinct colony diversity was compared from the

cultured bacteria. The 16S rDNA from the cultured bacteria was amplified, sequenced and compared to GenBanks' identified sequences for species identification.

Earthworms under long-term exposure acquired significantly greater resistance to cadmium according to their reproductive output. Furthermore, they had significantly less cadmium body burden at the highest soil-cadmium concentration. The bacteria from the long-term exposure group required a greater concentration of cadmium to significantly reduce bacterial levels. There were no conclusive results about the difference in diversity of culturable bacteria from the earthworm casts. The highly cadmium resistant species isolated, are all known to be metal resistant. Different species were isolated at the highest cadmium concentration from the two groups. *Cellulomonas persica* and *Bacillus subtilis* were the only Gram-positive bacteria isolated at the highest cadmium concentration and both of these were isolated from the more resistant earthworm group. The implications are that earthworms that have acquired greater resistance to cadmium have a reduced cadmium body burden and since the overall cadmium resistance of the bacteria are greater and the community structure of resistant bacteria are different, it is concluded that intestinal bacteria may contribute to earthworm resistance.

Keywords: Earthworms, cadmium resistance, intestinal bacteria, long-term exposure, cadmium body burden, hormesis, resource allocation

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPases	adenosine triphosphatases
CDF	cation diffusion facilitator
cfu	colony forming units
DNA	deoxyribonucleic acid
HSD	honest significant difference
ICP-MS	inductively coupled plasma mass-spectrometer
LB	lysogeny broth
LC_{50}	lethal concentration 50
MIC	minimum inhibitory concentration
MT	metallothionein
n	sample size
OECD	Organisation for Economic Cooperation and Development
p	calculated probability
PC	phytochelatins
PCR	polymerase chain reaction
PCS	phytochelatin synthase
ppm	parts per million

RND	resistance-nodulation-cell division
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
WHC	water holding capacity
$Worms_{LTE}$	Earthworms with long-term exposure to cadmium
$Worms_{U}$	Earthworms with no previous exposure to cadmium

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

CHAPTER 1

INTRODUCTION

1.1 General introduction and problem statement

Soil is a non-renewable resource that is crucial to sustainable development (Acton & Gregorich, 1995). Assessing soil quality with bioindicators such as earthworms is the principal measure of sustainable land utilisation (Doran & Zeiss, 2000). Earthworms are good bioindicators because they are sensitive, abundant, important to the ecosystem and inexpensive to culture. (Doran & Zeiss, 2000). Anthropogenic activities such as mining and agriculture can have a negative effect on soil (Sandrin & Maier, 2003; Galunin *et al.*, 2014) especially if they alter the heavy metal concentrations (Autier & White, 2004). Earthworms generally exhibit avoidance behaviour in the presence of toxins (Wentsel & Guelta, 1988; Yeardley *et al.*, 1996). One such toxin is the heavy metal cadmium in its divalent cation form (Cd^{2+}).

Earthworms are known to develop resistant to Cd^{2+} when exposed to sub-lethal concentrations for prolonged periods (Reinecke *et al.*, 1999; Spurgeon & Hopkin, 2000; Piearce *et al.*, 2002; Reid & Watson, 2005). Their mechanisms of Cd^{2+} resistance are not well understood. Apart from avoiding contaminants, it is known that earthworms produce metallothionein (MT) that has been implicated in Cd^{2+} detoxification by sequestration (Stürzenbaum *et al.*, 2001). Upon exposure to Cd^{2+}, MT gene transcription is increased leading to reduced oxidative stress and reduced Cd^{2+} accumulation (Liang *et al.*, 2011).

Earthworms have a large and diverse amount of bacteria in their intestines, which are pivotal to their digestion (Konig, 2006). They may harbour indigenous bacteria as indicated by Jolly *et al.* (1993) and Toyota & Kimura (2000). Many bacteria are capable of biosorption of metals (Ansari & Malik, 2007) for example some *Cupriavidus* spp. and *Pseudomonas* spp. It has been observed

that bacteria can reduce the bioavailability of Cd^{2+} in soil (Siripornadulsil & Siripornadulsil, 2013) and could contribute to metal resistance through microbial processes within organisms (Monachese *et al.*, 2012).

Since earthworms have displayed differentiation in Cd^{2+} resistance after long-term exposure (Fourie *et al.*, 2007), the question arises if their intestinal bacteria may play a role in their acquired resistance. If it does, it would be expected that their intestinal bacteria differ in terms of bacterial levels, diversity and species present. If Cd^{2+} resistant earthworms contain bacteria that have greater resistance to Cd^{2+}, or different species that are able to sequestrate or detoxify Cd^{2+} to a greater extent, such bacteria could have application in remediation of Cd^{2+} contaminated land.

1.2 Research aim and objectives

The aim of this study was to investigate the effects of different Cd^{2+} concentrations (0, 10, 40, 160 and 400 μg g^{-1} soil) on earthworms, *Eisenia andrei,* in terms of mortality, reproduction, metal body burden and aerobically culturable bacteria present in their gut. In addition, this study compares these results to *E. andrei* that have had long-term exposure to Cd^{2+}. The objectives of this study were to:

- Test the null hypothesis that the long-term exposure earthworms ($Worms_{LTE}$) has no significant different ($p<0.05$) responses in terms of: mortality, weight change, cocoon production and juveniles produced per cocoon, compared to earthworms with no previous exposure to Cd^{2+} ($Worms_{U}$). The alternative hypothesis being that there is significant differences.
- Test the null hypothesis that there are no differences in Cd^{2+} bioaccumulation between $Worms_{LTE}$ and $Worms_{U}$.
- Test the null hypothesis that aerobically cultured cast bacteria are not significantly different ($p<0.05$) in terms of bacterial levels and the diversity of the morphologically distinct

bacterial species. In addition, conclude if the Cd^{2+} resistant species present and the Gram-positive to Gram-negative ratios are different.

1.3 Outline of the chapters

In Chapter 2, the relevant literature is discussed and includes the importance of soil as a natural resource, mining and the exposure of Cd to the soil surface and minimum standards for remediation. The nature and consequences of Cd exposure is indicated as well as including how earthworms and bacteria resist the toxic effects of Cd. Lastly, the method and standards that has been utilised in similar research was critically evaluated. Chapter 3 the materials and methods used during the study is discussed. The experimental design is outlined and the methods selected and any modifications thereto elaborated upon. Noteworthy formulas and statistical methods are explained and their results attached in annexures. The results are tabulated, graphically presented and comprehensively discussed in chapter 4. Chapter 5 contains conclusions to the aims and provides recommendations for future studies. Finally, a combined list of references for all chapters is provided.

CHAPTER 2

LITERATURE REVIEW

2.1 The soil ecosystem

Soil is a combination of degraded rock, minerals and organic matter that develops over centuries (Ashman & Puri, 2008). The soil layer that covers most of the earth is paramount to the majority of terrestrial fauna and flora. Bacteria, fungi, protozoa, plants, invertebrates and vertebrates inhabit this layer and their interactions influence the biogeochemical cycling of essential elements (Prescott *et al.*, 2008). Soil is dynamic and its health is defined by its ability to sustain organisms and maintain or enhance water and air quality (Doran & Zeiss, 2000). Assessing the health and quality of soil and its change over time is the principal measure of sustainable land utilisation (Doran & Zeiss, 2000). Soil organisms can be used as indicators of soil health (Van Bruggen & Semenov, 2000; Schloter *et al.,* 2003; Zhang *et al.,* 2008; Park *et al.*, 2011).

The uneven distribution of naturally occurring elements in soil affords for differences in the abundance and diversity of the soil ecosystem (Begon *et al.*, 2006). Heavy metals are known to accumulate in the soil surface layer (Galunin *et al.*, 2014). Its presence reduces the abundance, diversity and stability of soil organisms (Malik *et al.*, 2008; Wahl *et al.*, 2012; Jubileus *et al.*, 2013). Anthropogenic activities affect soil by imposing physical, biological and chemical stresses on soil organisms. This makes anthropogenic activities a major ecological concern (Sandrin & Maier, 2003; Galunin *et al.*, 2014). Activities that increases heavy metal concentrations include the use of pesticides and fertilisers, electroplating, the manufacture of plastic, textile and paint and mining (Autier & White, 2004).

Most minerals and metals are found in abundance in South Africa making it an important mining country. Mining contributed 8% to South Africa's gross domestic product in 2015 (Zwane, 2015).

South Africa is a water scarce country and pollution from mining seriously impact soil (Claassens *et al.*, 2008; Jubileus *et al.*, 2013) and water quality (Durand, 2012). This places heavy metal pollution at the forefront of environmental complications (Ochieng *et al.*, 2010). South Africa has been defined as a resource cursed country because its mining has led to historic and present unmanaged pollution (Elbra, 2013). It had 1600 legal mines registered by 2010 (Eijsackers *et al.*, 2014). The spread of waste from mine dumps in South Africa reaches some human settlements by wind and water flow (Van Rensburg *et al.*, 2009). If not prevented, the pollutants are consumed when drinking water and eating vegetation cultivated in contaminated areas (Boussen *et al.*, 2013). The Witwatersrand district in South Africa (Johannesburg) has been impacted severely by mining with the focus being short-term profits as opposed to sustainability (Durand, 2012). Animals are also exposed to heavy metals through direct ingestion, inhalation and dermal uptake as well as the consumption of plants and animals that have bioaccumulated heavy metals (Winde & Van Der Walt, 2004; Li *et al.*, 2006; Yang *et al.*, 2006; Cunha *et al.*, 2008; Boussen *et al.*, 2013; Eijsackers *et al.*, 2014).

The Department of Environmental Affairs of South Africa published the National norms and standards for the remediation of contaminated land in May 2013 (Government notice no. 467 of 2013). The norms and standards are applicable to land owners and those who undertake and assess remediation of contaminated land. Its purpose is to provide an unambiguous, uniform approach by affording the minimum standards when assessing pollution and its remediation. It provides a list of screening values for the metals; arsenic (As), Cd, cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), Pb, vanadium (V) and zinc (Zn) and the minimum standard necessary for the protection of the environment and remediation measures (Table 1). The National Environmental Management Waste Act (59 of 2008) governs adherence to these standards. The act endeavours to protect human and environmental health by preventing pollution and ecological degradation and, makes special mention of waste generated by mining

(Act 59 of 2008). The soil screening values specifies the minimum standards for all land uses and land protective of water resources, as well as residential and industrial areas. Informal residential and standard residential areas are renowned for subsistence cultivation of vegetables (Dinham, 2003; Van Averbeke, 2009), a form of land use. Some heavy metals for example cadmium (Cd) are highly toxic at low concentrations (Newman & Clements, 2008).

Table 1: Soil screening values for metals (µg g^{-1} soil). The minimum standard values to which contamination and remediation is measured as indicated by the South African Department of Environmental Affairs (2013).

Parameter	All land uses protective of the water resource	Informal residential	Standards residential	Commercial /industrial
Arsenic	5.8	23	48	150
Cadmium	7.5	15	32	260
Chromium (III)	46 000	46 000	96 000	790 000
Chromium (VI)	6.5	6.5	13	40
Cobalt	300	300	630	5 000
Copper	16	1 100	23 600	19 000
Lead	230	110	230	1 900
Manganese	740	740	1 500	12 000
Mercury	0.93	0.93	1.0	6.5
Nickel	91	620	1200	10 000
Vanadium	150	150	320	2 600
Zinc	240	9 200	1 900	150 000

2.2 Cadmium

Heavy metals have a density greater than 5 g per cm^3. There are 53 heavy metals, some of which are essential (Nies, 1999). Essential metals are more abundant in soil and are lighter than non-essential metals although they have similar molecular binding characteristic (Newman & Clements, 2008). Cadmium is not biologically essential (Martelli *et al.*, 2006) except for the marine diatom *Thalassiosira weissflogii* (Lane *et al.*, 2005). It is a known carcinogen of the lung, mammary glands, pancreas, kidney and urinary bladder in humans (Martelli *et al.*, 2006; Huff *et al.*, 2007). It is closely associated with gold (Ag), Zn, Cu and Pb ores and mining waste (Martelli *et al.*, 2006; Galunin *et al.*, 2014). The divalent cations of Zn (Zn^{2+}) and Cd (Cd^{2+}) have highly

concentrated charges making them strong Lewis acids with great affinity for sulphur, nitrogen and oxygen containing ligands. The Pearson's Hard and Soft Acids and Bases theory (Pearson, 1963) indicates that large Lewis acids (soft acids) prefer large Lewis bases (soft bases). The atomic radii of Cd^{2+} is slightly larger than Zn^{2+} thus being a slightly softer acid and displacing Zn^{2+} in bonds with softer anions (Martelli *et al.*, 2006) with possible toxic effects (Bruins *et al.*, 2000). Because Zn^{2+} plays a crucial role in many proteins, especially enzymes that have low substrate specificity and attack small molecules such as hydrolytic enzymes, Cd^{2+} is an environmental concern (Newman & Clements, 2008). Their strong bonds to sulfhydryl groups attributes to their toxicity once inside a cell. The bonding could cause deformation and consequently interference with the functions of sensitive enzymes. There is a correlation between the dissociation constant of metal sulphides and the inhibitory concentration they have on bacteria. Thus, Cd^{2+} toxicity is related to its bioavailability to enzymes (Nies, 1999; Sandrin & Maier, 2003). Cadmium's bioavailability correlates with environmental conditions such as pH, temperature and dissolved ligands (Cunha *et al.*, 2008; Hu *et al.*, 2013; Park *et al.*, 2011).

Many alloys contain Cd; it improves thermal properties of electronics and soldering and reduces mechanical friction. It has been electroplated as coatings to reduce corrosion of other metals, a barrier to control nuclear fission, anodes for Ni-Cd batteries, as well as pigment in plastics, paints and ink. Cadmium is a widespread and persistent heavy metal being released at a very high rate from human activities (Cunha *et al.*, 2008). Cadmium is transported by wind mainly as salts, which easily dissolves in water and makes it available to organisms. It is known to swiftly cross pulmonary surfaces and the gut, gaining access to the blood stream (Martelli *et al.*, 2006). It enters cells by adsorption, passive diffusion, active transport, facilitated diffusion and endocytosis (Martelli *et al.*, 2006). After uptake it can undergo biotransformation that can enhance elimination, detoxification, sequestration, redistribution or alternatively, enhance its toxic effect (Newman & Clements, 2008).

Acute and chronically lethal effects have been observed for organisms exposed to Cd (Li *et al.*, 2006; Martelli *et al.*, 2006; Huff *et al.*, 2007) . High concentrations cause autolysis and apoptosis of animal cells (Martelli *et al.*, 2006; Prozialeck *et al.*, 2006). Low concentrations have sublethal effects that include changes in physiological processes, growth, behaviour and development. These changes could reduce fecundity and lead to ecological demise (Newman & Clements, 2008). Cadmium is also genotoxic (Takiguchi *et al.*, 2003; Fourie *et al.*, 2007; Liang *et al.*, 2011; Voua Otomo & Reinecke, 2010). Some DNA damage can undergo DNA-repair but others results in the destruction of cells. Cadmium is highly mutagenic, carcinogenic and can sensitise cells to other genotoxic agents by inhibiting DNA-repair (Fourie *et al.*, 2007). Furthermore, it deregulates DNA transcription by disturbing oxidation-reduction homeostasis (Martelli *et al.*, 2006).

Bioaccumulation occurs in many organisms and depends on the characteristics of Cd, the organism and the environment (Hobbelen *et al.*, 2006). Bioaccumulation by primary consumers can result in biomagnification in the food chain (Newman & Clements, 2008). Some bacteria bioaccumulate Cd (Costa & Duta, 2001; Limcharoensuk *et al.*, 2015). The soil-plant-animal pathway contributes substantially to the exposure to animals (Millis *et al.*, 2004; Cunha *et al.*, 2008; Hu *et al.*, 2013). Plants tend to accumulate Cd in the softer edible parts leading to greater biomagnification (Li *et al.*, 2006; Cunha *et al.*, 2008).

Many organisms show resistance to Cd through a range of mechanisms (Nies, 1999). Eukaryotes typically regulate intracellular metal ions by the expression of a metal binding protein called metallothionein (MT) (Valls & de Lorenzo, 2002). Mammalian cells have four known varieties of MT which has a relatively low molecular weight consisting of approximately 60 amino acids in animal systems (Haq *et al.*, 2003). A single MT molecule can bind up to seven Cd and Zn or 12 Cu divalent cations (Valls & de Lorenzo, 2002; Haq *et al.*, 2003). The main purpose of MT is believed to be the distribution and regulation of Zn and Cu. Eukaryotes with low levels of MT have been shown to be more sensitive to the effects of Cd (Martelli *et al.*, 2006).

2.3 Earthworms

Earthworms predominantly feed on decaying plant matter but also consume nematodes, fungi, bacteria and soil (Parle, 1963a; Edwards & Fletcher, 1988). According to Parle (1963a) the value of earthworms is its contribution to the degradation of substances such as cellulose in soil. They are bioindicators of soil health, instrumental to the food web and can assist in the remediation of contaminated land (Edwards & Bater, 1992; Johnson, 2017). Earthworms are monoecious and continuous breeders in warm, damp and dark environments. A cocoon is formed in which sperm and ovum are deposited for fertilisation. Adults are recognised by clearly developed clitella (Hickman, 2006). To function as respiratory organ, the earthworm integument contains a dense network of capillaries. It needs to remain damp to afford for diffusion and osmoregulation (Schmidt-Nielsen, 1997). These characteristics place earthworms in direct contact with the soil pore water and substances dissolved in it. As such, earthworms are indicators of soil quality (Eijsackers *et al.*, 2014).

It has been shown that earthworms exposed to low levels of Cd^{2+} (≤ 500 µg Cd^{2+} g^{-1} soil) for short periods (56 days weeks) are not terminally affected and do not display significant weight change but, do produce significantly less cocoons (Spurgeon *et al.*, 1994). Low concentration of Cd^{2+} has been observed to produce a hormetic effect on growth (Stebbing, 1981; Zhang *et al.*, 2009). Cadmium affects earthworm immunity, synthesis and release of hormones, osmoregulation and fecundity (Venables *et al.*, 1992). Non-lethal amounts of Cd^{2+} have been observed to cause DNA damage (Fourie *et al.*, 2007).

Earthworms avoid certain toxins by assembling as opposed to distributing throughout the substrate or, if possible, they move away from toxins (Wentsel & Guelta, 1988; Yeardley *et al.*, 1996). Lukkari and Haimi (2005) observed that various earthworm species avoid soil spiked with Cu and Zn at low concentrations. Earthworms are however able to acclimatise or resist toxic

heavy metals, such as Cd^{2+}, present at sublethal concentrations (Reinecke *et al.*, 1999; Spurgeon & Hopkin, 2000; Piearce *et al.*, 2002; Reid & Watson, 2005). They are present at highly contaminated sites (Stürzenbaum *et al.*, 2001) though the mechanisms of Cd^{2+} resistance are not implicit. Voua Otomo and Reinecke (2010) provided biomarker related evidence of cell metabolic activity and DNA damage resistance by *Eisenia fetida* that have had long-term exposure to sublethal concentrations of Cd^{2+}.

Earthworms bioaccumulate Cd (Spurgeon & Hopkin, 2000) to amounts beyond 1mg g^{-1} of total dry body weight (Stürzenbaum *et al.*, 2001). Accumulation develops particularly in tissue surrounding the digestive tract and inside the nephridia. This could render them toxic to their predators (Fourie *et al.*, 2007). Two MT isoforms (wMT-1 and wMT-2) are produced by earthworms with wMT-2 implicated in Cd^{2+} detoxification by sequestration (Stürzenbaum *et al.*, 2001). Protein folding by wMT-2 is better in the presence of Cd^{2+} than Zn^{2+} (Kowald *et al.*, 2016). Upon exposure to Cd^{2+}, wMT-2 gene transcription is increased leading to reduced oxidative stress and Cd^{2+} accumulation (Liang *et al.*, 2011).

Earthworms harbour large amount of diverse microbes in their intestines that are pivotal to the digestion of their food (Konig, 2006). Bacteria present in earthworm gut are also present in soil but at different ratios (Furlong *et al.*, 2002; Singleton *et al.*, 2003). The hindgut contains a 100-fold-higher culturable aerobic bacteria than the foregut (Karsten & Drake, 1995). Dempsey *et al.*(2011) found that earthworms change the ratio of bacteria to fungi. The breakdown of cellulose and to a lesser extent chitin by the earthworm might be aided by microorganisms within the hindgut (Parle, 1963a). Many invertebrates have microorganisms indigenous to the species for example grasshoppers, millipedes cockroaches, termites and fly larva (Barton & Northup, 2011). From a scanning electron microscopy study performed it appeared that indigenous bacteria are present in the earthworm intestine (Jolly *et al.*, 1993). Toyota and Kimura (2000) indicated *Aeromonas hydrophila* as Gram-negative bacteria indigenous to earthworm *Eisenia fetida*. Many

bacteria are capable of biosorption (Ansari & Malik, 2007). *Cupriavidus taiwanensis* and *Pseudomonas aeruginosa*, present in a Cd contaminated rice field, reduce Cd^{2+} uptake by the rice (Siripornadulsil & Siripornadulsil, 2013). This raises the question if bacteria present in Cd^{2+} resistant earthworm intestines may contribute to their resistance.

2.4 Earthworms as bioindicators

Earthworms are easily cultured in large quantities, mature in a short period and exhibit high reproductive rates and, are sensitive to contaminants and physical soil parameters (Peakall, 1992). Their use as bioindicators of soil health is well documented (Reinecke, 1992). The earthworm reproductive test is a sublethal indication of toxicity (OECD, 2004). Organisms struggle to maintain homeostasis when exposed to toxins. They may change their behaviour, resist toxins and/or adapt. They may become exhausted and fail to compensate for the effect of the contaminants and die. Lower concentrations might have sublethal effects that include changes in physiological processes, growth, behaviour and development. Such changes could reduce fecundity and lead to local extinction (Newman, 2010). Earthworm reproduction capacity is the result of four factors namely; fertilisation rate, timing of mating, reproductive lifespan and, the viability of the cocoons produced. Environmental pollution affects reproductive rates before affecting mortality (Peakall, 1992). Some species are less sensitive to certain contaminants than others. *Eisenia fetida* and *E. andrei* are the earthworm species used in most earthworm bioindicator studies and several international standard toxicity tests. Other species used include *Lumbricus rubellus, L. castaneus* and *L. terrestrisand* (Nahmani *et al.*, 2007). The Organisation of Economic Co-operation and Development (OECD) have adopted a ring- tested guideline for the testing of chemicals using the earthworms' *E. fetida* and *E. andrei.* The tests include both acute and chronic effects of amendments to artificial soil by way of measuring mortality, growth and reproductive output. The test periods coincide with the earthworm's reproductive cycle and specific statistical methods are suggested for hypothesis testing (OECD, 2004).

2.5 Bacteria

Bacteria are abundant in soil. Its diverse metabolic pathways provide crucial products to other organisms (Allison & Martiny, 2008). Soil bacteria provide fundamental ecological services such as biogeochemical cycling, suppression of pathogens, degradation of pollutants and litter, stabilisation of soil aggregates and, improvement of water retention and soil porosity. These ecological services are reduced by metal contamination (Hassen *et al.*, 1998; Sandrin & Maier, 2003; Park *et al.*, 2011). Metal reduces the functional diversity of bacteria although the total soil respiration does not necessarily change (Stefanowicz, 2006).

Resistance to metal contamination is likely to have evolved soon after bacterial life began because the environment has always had fluctuating concentrations of metals (Bruins *et al.*, 2000). Resistance of bacterial communities refers to the communities' ability to remain structurally and functionally similar when exposed to a disturbance such as metal contamination (Allison & Martiny, 2008). Many bacterial groups have acquired an array of responses to resist non-essential and, elevated levels of essential metals (Valls & de Lorenzo, 2002; Silver & Phung, 2009). Resistance occurs through active transport, intracellular sequestration, enzymatic detoxification, exclusion by semi-permeable barriers, extracellular sequestration and a reduction of metal sensitivity (Bruins *et al.*, 2000). Genes that bring about resistance are present in plasmid and chromosomal DNA (Silver, 1996; Bruins *et al.*, 2000; Liu *et al.*, 2008). Bacterial cell walls are remarkably different in the ability and manner that they resist metals (Bruins *et al.*, 2000). Gram-negative bacteria are less sensitive to metal ions than the Gram-positive bacteria (Morozzi *et al.*, 1986). Gram-negative bacteria exhibited 20% less biosorption of Cd^{2+} than Gram-positive bacteria. In both Gram-negative and Gram-positive bacteria, biosorption is mainly passive although Gram-positive bacteria use metabolic uptake to a greater extent (Gourdon *et al.*, 1990).

The manner in which some bacteria cope with high concentration of Cd^{2+}, such as sequestration and transformation, is also to the benefit of surrounding organisms. *Cupriavidus* spp. tolerate high concentrations of Cd^{2+}. The presence of *C. taiwanensis* appear to reduce the uptake of Cd^{2+} by rice when present in the same soil (Siripornadulsil & Siripornadulsil, 2013). It is known that *Pseudomonas* spp. accumulate Cd^{2+} in the periplasm and intracellularly (Minz *et al.*, 1996; Ahemad & Malik, 2012). *P. aeruginosa* produces thiol-rich compounds that may reduce toxic $CdCl_2$ to less toxic cadmium sulphide (Siripornadulsil & Siripornadulsil, 2013). Aeromonads have been reported as being resistant to metals (Akinbowale *et al.*, 2007) by ion eflux (Najiah *et al.*, 2009). Toyota & Kimura (2000) suggested that *Aeromonas hydrophila* might be indigenous to *E. fetida*. Its presence in metal resistant earthworms at high Cd^{2+} concentartions should be assessed.

2.5.1 Active transport

Non-essential Cd^{2+} enter bacterial cells through nutrient transport systems along with essential divalent cations such as Mg and Zn (Silver & Phung, 2005). Because the size of Cd^{2+} and Zn^{2+} are similar, there is little discrimination between there uptake and transport. When driven by the chemiosmotic gradient of divalent cations, a high concentration of a non-essential ions such as Cd^{2+} does not stop its transport if the total ionic concentration is still low. In addition, the transport enzyme is expressed continuously regardless of the physiological demand. The resulting accumulation of Cd^{2+} causes toxicity. Mutations with reduced expression of these chemiosmotic enzymes may have greater resistance to Cd^{2+}. They are however less vigorous than the wild type due to their reduced substrate uptake and are therefore supplanted in the absence of Cd^{2+}. Transport systems that are substrate specific are slower and uses adenosine triphosphate (ATP) hydrolysis for energy. These expensive uptake systems are expressed only when required and are inducible (Nies, 1999).

Resistant bacteria can accumulate up to 15 times less Cd than non-resistant bacteria (Bruins *et al.*, 2000). Resistance mainly results from membrane bound transport proteins that expel Cd^{2+}. Such transport proteins are referred to as efflux systems or pumps and are chromosomal or plasmid-encoded (Bruins *et al.*, 2000; Silver & Phung, 2009). There are seven types of efflux pumps. Two are adenosine triphosphatases (ATPases) antiporters and five are chemiosmotic cation antiporters. Efflux pump types may have evolved separately for Gram-positive and Gram-negative bacteria. Gram-positive bacteria such as *Staphylococcus, Bacillus* and *Listeria* spp. use ATPases to remove Cd^{2+} whereas Gram-negative bacteria use chemiosmotic cation antiporters (Silver, 1996; Silver & Phung, 1996). There are three known efflux systems relative to bacterial resistance of Cd^{2+} namely, the *CzcD* membrane-integrated protein, the *CzcCBA* transport system and the *CadA* P-type ATPases (Silver & Phung, 2009)

ATPases are enzymes that transport ions across cell membranes using ATP hydrolysis (Hoffman, 2007). The P-type ATPase are polypeptides that are set in the cell membrane and consists of numerous protein domains. (Silver & Phung, 2009). When a gamma-phosphate from ATP attaches to an ATPase membrane protein, structural changes occur. The changes allows the protein to move ions against the electro-chemical gradient. The staphylococcal resistance plasmid pI258 contains the genes for the *Cad* operon. The *Cad* operon encodes for *CadA* that is transcribed and translated to P-type ATPases. In addition, the *Cad* operon affords for its repressor (*CadC)* that binds to the operon promoter, inhibiting transcription. When Cd^{2+} is present it binds to *CadC* and releases it from the operon promoter, affording for transcription. Thus, resistance by the costly P-type ATPases is transcription regulated (Silver & Phung, 1996; Busenlehner *et al.*, 2003).

The chemiosmotic cation antiporters relevant to Cd^{2+} are *CzcD* and *CzcCBA.* The *CzcD* efflux pump is from the cation diffusion facilitator (CDF) group and was first observed in the *Cupriavidus metallidurans* bacterium. It is a single membrane polypeptide chemiosmotic efflux pump. Archaea,

yeast, plants and animals express CDF homologues. The acronym *Czc*, is derived from the ions Cd^{2+}, Zn^{2+} and Co^{2+} to which the gene product provides resistance. *CzcD* genes are present in the plasmids of *C. metallidurans* which also may contain encoding for numerous other metal resistance determinants (Silver & Phung, 1996; Paulsen & Saier Jr., 1997). The resistance-nodulation-cell division (RND) group of chemiosmotic antiporters consist of three polypeptides. It is also known as the CBA family referring to its three polypeptides in the order that the genes appear on the operon. The C protein is situated on the outer membrane and the A protein on the inner membrane. The B protein connects the A and C proteins forming a continuous channel through which cations Cd^{2+}, Zn^{2+} and Co^{2+} are conveyed. The *CzcCBA* expels cations that are obtained from both the endoplasm and the periplasm (Silver & Phung, 2005; Silver & Phung, 2009).

2.5.2 Intracellular sequestration

For bacteria, resistance by intracellular sequestration is the exception and not the rule. Bacteria rather employ resistance mechanisms such as efflux systems (Silver & Phung, 1996; Valls & de Lorenzo, 2002). Resistance by intracellular sequestration occurs when metals are bound to proteins within the cytoplasm (Bruins *et al.*, 2000; Sandrin & Maier, 2003). Bacteria such as *Synechococcus* sp. and *Pseudomonas* sp. (Bruins *et al.*, 2000), as well as eukaryotes, are known to produce cysteine-rich metal-binding proteins called metallothionein (MT) (Blindauer *et al.*, 2002; Haq *et al.*, 2003; Silver & Phung, 2005). *Synechococcus* contains the *smtA* gene that affords for a 56 amino acid-long polypeptide MT. Different from eukaryotic MT, it prefers binding to Zn^{2+} as opposed to Cd^{2+} (Silver & Phung, 1996). Sequestration supports homeostasis and protects organisms from oxidative injury (Haq *et al.*, 2003; Sandrin & Maier, 2003). Cells with lower amounts of MT have lower resistance to Cd^{2+} (Silver & Phung, 1996). Metallothionein containing less cysteine residuals can also bind less Cd^{2+}. Structural analysis of *Synechococcus*

MT indicate only four potential Cd^{2+} binding sites thus being less effective than MT produced by animals (Bruins *et al.*, 2000).

Regulation of MT expression occurs mainly at transcription by way of the repressor *SmtB* protein (Silver & Phung, 1996). Evidence suggests that some post-transcriptional mRNA moderating occurs. In addition to basal expression, metals such as Zn, Cd, Hg, Cu, BI, Ni and Co promote increased MT expression. The amount of metal required to induce transcription is unique to the metal. Organic signalling agents such as; cytokines, corticosteroids, vitamin D_3 and other redox active species also increases MT production (Haq *et al.*, 2003; Sauge-Merle *et al.*, 2012). In addition, gradual increases of Cd^{2+} have been observed to increase the amount of *SmtA* genes (Silver & Phung, 1996). A strain of *P. putida*, that demonstrates intracellular Cd sequestration, produces three low-molecular-weight cysteine-rich proteins that may be related to metallothionein (Bruins *et al.*, 2000).

Phytochelatins (PC) is a type of metal-binding polypeptide found in the yeast *Saccharomyces cerevisiae* and *Candida glabrata*. Phytochelatins form complexes with Cd^{2+}, Cu^{2+}, Ag^{2+} and As^{2+}. Their enhancement of Cd^{2+} resistance correlates with the accumulation of Cd^{2+}, suggesting cytosolic binding. Phytochelatins may be relevant to bacteria because phytochelatin synthase genes has been identified in in Cyanobacteria and Proteobacteria and they contain distantly related proteins (Clemens, 2006).

2.5.3 Exclusion by semipermeable barriers and extracellular sequestration

The outer envelope of Gram-positive and Gram-negative bacteria may contain metal binding functional groups (Johnson *et al.*, 2006). This may prevent metals from entering the cell and so doing provide resistance. Exopolysaccharide coatings provide binding sites for metal cations, as observed with *Klebsiella aerogenes*, *P. putida*, and *Arthrobacter viscosus* (Bruins *et al.*, 2000). When *A. viscosus*¸ was exposed to 100 mg L^{-1} Cd^{2+} the Cd^{2+} load was 30 mg Cd g^{-1} (Scott &

Palmer, 1988). Binding was pH dependent and at its optimum between a pH of four and nine. The separated and dried polysaccaride by itself does not bind the Cd^{2+} as extensively as when intact with the living organism (Scott & Palmer, 1988). Conformational changes to the cell membrane of some strains of *Staphylococcus aureus* are brought about by penicillinase plasmids. The changes restrict the entry of Cd^{2+} and other metals thus providing resistance. It is, however, only applicable to low-levels of Cd^{2+} (McEntee *et al.*, 1986). According to Aiking *et al.*, (1982) strain S45 of *K. aerogenes* ceased growth with the addition Cd^{2+} and then resumed growth after five hours. Once growth was steadily increasing, augmentation of Cd^{2+} had a reduced effect on growth relative to the control group. It was suggested that *K. aerogenes* removes Cd^{2+} ions from the substrate by excreting sulphur that limits the metal influx by external precipitation.

2.5.4 Reduced metal sensitivity

If a genetic mutation that results in a physiological change of a cellular component that leads to decreased sensitivity to metals, but does not disrupt the basic function of the cellular component, then the mutation can be considered a form of metal resistance. This includes DNA repair mechanisms and alterative pathways that bypass sensitive components (Bruins *et al.*, 2000). Such adaptations have been observed in *E. coli* cultured in media with high concentrations of Cd^{2+}. DNA damage was reduced in subcultures where initial DNA damage was considerable (McEntee *et al.*, 1986). In addition, the lag phase duration was reduced. Generating DNA repair mechanisms may be the cause of the initial extended lag phases (Bruins *et al.*, 2000).

2.5.5. Transfer of resistance

Qing *et al.*(2007) indicated that metal-resistant bacteria survive in soil with high levels of Cd^{2+} because they can acquire tolerance from genetic material in the environment. Horizontal transfer of genetic material afford bacteria the ability to adapt to changing environments. It can occur through conjugation, transformation and transduction (Ochman *et al.*, 2000). Conjugative plasmid transfer is the most prevalent mechanism for horizontal gene transfer by soil bacteria and the

main reason for its resistance to metals (Anjum *et al.*, 2011). The process of conjugation protects the DNA being transferred from direct exposure to metals that would degrade it (Mazodier & Davies, 1991). Plasmids can be transferred to the same or different species during conjugation (Ochman *et al.*, 2000). It can also be transferred between bacteria and yeast (Heinemann, 1991) as well as bacteria and archaea (Nelson *et al.*, 1999). In this manner, conjugation may afford for the transfer of resistance mechanisms between Gram-positive and Gram-negative bacteria (Courvalin, 1994; Martinez *et al.*, 2006). Transformation is the uptake of unprotected DNA from the cells environment. This form of gene transfer can only occur between distantly related organisms. Transferring genetic material via transduction requires a bacteriophage that can contain a very limited amount of DNA. Furthermore, transfer of the DNA from the phage is limited by the organism-phage receptor recognition (Ochman *et al.*, 2000).

Microbes that survive and remove or immobilise Cd^{2+} are of great interest to bioremediation (Gadd, 2004), however, by promoting metal resistance, we may promote antibiotic resistance. Antibiotic resistance appear to be more prevalent in freshwater microcosms that are exposed to higher concentrations of Cd^{2+} (Stepanauskas *et al.*, 2006). The same mechanisms used for survival; sequestration, detoxification and efflux of metals afford for antibiotic resistance (Hassen *et al.*, 1998; Seiler & Berendonk, 2012). Anthropogenic activities that exposes metal to soil, such as farming and mining, promote the spread of antibiotic resistance to soil bacteria (Seiler & Berendonk, 2012). It may serve as a selective pressure promoting the proliferation and evolution of antibiotic resistant bacteria (Seiler & Berendonk, 2012).

2.6 Isolation and identification of cadmium resistant bacteria

Bacteria harboured by soil invertebrates play a large role in their digestion (Breznak & Brune, 1994; Singleton *et al.*, 2003; König, 2006; Byzov *et al.*, 2007). The structure of bacterial communities change in the presence of Cd^{2+} in terms of the bacterial levels, species diversity,

species present and Gram-positive to Gram-negative ratios. (Qing *et al.*, 2007; Zhang *et al.*, 2008). The processes of bacterial communities may provide resistance to their hosts (Daane *et al.*, 1996) against Cd^{2+}. Comparing the intestinal bacterial communities of resistant and ordinary earthworm groups may indicate that bacteria play some role in earthworm resistance. Hence, bacteria from the earthworm intestines would have to be counted, isolated and identified.

2.6.1 Enumeration and isolation bacteria

For the enumeration of intestinal bacteria for culturing, earthworms are placed in sterile water for 24 hours. The cast containing water can then be cultured on nutrient agar by the dilution plate method as was done by Toyota & Kimura (2000). Placing the earthworms in sterile water has the risk of contamination by epidermal and soil bacteria. Prolonged exposure to sterile water may also destroy many of the culturable bacterial cells. In addition, supplementing the agar with soil extract may allow a greater diversity of culturable bacteria. In a study by Hamaki *et al.*(2005) it was establish that soil-extract agar afforded for several Actinobacteria species not observed in standard media. Furlong *et al.*(2002) and Byzov *et al.*(2007) first rinsed the earthworms in sterile water and then placed them in sterile petri plates, removing casts every two to three hours. The casts were vortexed for 30 seconds in saline solution and serially diluted in two types of media. The media contained soil extract. To obtain pure cultures the highest dilutions that had growth after two weeks were streaked on solid media containing soil extract (Furlong et al., 2002; Byzov *et al*., (2007). Furlong *et al.* (2002) reduced the risk of contamination by epidermal and soil bacteria by rinsing the earthworms in water. Placing them in sterile petri dishes and periodically removing the casts may preserve more bacteria than the prolonged exposure to sterile water however; bacteria may be eliminated by desiccation. Contamination would also not be completely eliminated. Dissecting earthworms and removing the gut content aseptically may eliminate contamination but would be too time consuming to obtain large samples.

There has been no discernible studies linking earthworm intestinal bacteria to earthworm Cd^{2+} resistance. Isolation and counting of Cd^{2+} resistant soil bacterial colonies, on ten percent nutrient agar, was performed by Kanazawa & Mori (1996). They observed that more Cd^{2+} resistant bacteria was present in soil polluted with Cd^{2+} than non-polluted soil. Culture-dependent methods are uncomplicated and cost effective but criticised for selecting only bacteria that can be cultured (Malik *et al.*, 2008). Culturability of bacteria are determined by the availability of nutrients and the physical properties of the media. Bacteria in the media will compete for nutrients and interfere with each other resulting in some species having limited or no growth. In addition, only viable cells will be cultured. The lag, log and death phases of species are not synchronised. Some species might be entering the log phase whilst others are already in the death phase (Barton & Northup, 2011). For this reason, a substantial period should be provided so that most species are present before isolating bacteria.

Culture-dependent methods are not a true reflection of the total microbial community and diversity because most species cannot be cultured (Hori *et al.*, 2006; Malik *et al.*, 2008). Culture-independent analysis of microorganisms may answer essential questions of microbiomes (Riesenfeld *et al.*, 2004; Zhang *et al.*, 2009). Metagenomics, for example, have been used to investigate single genes, pathways, organisms and communities by cloning DNA straight from the environment. It elucidates phylogenetic and genetic diversity in environments (Riesenfeld *et al.*, 2004) without having to culture microbes successfully. Nevertheless, culture-dependent methods are frequently used and can effectively indicate metal tolerant bacteria (Olsen & Bakken, 1987; Kanazawa & Mori, 1996; Hassen *et al.*, 1998; Ansari & Malik, 2007).

Cadmium resistant bacteria can be isolated by dissolving Cd salt in distilled water and adding it to solid media during preparation (Kanazawa & Mori, 1996; Xu *et al.*, 2012). To determine the minimum inhibitory concentration (MIC) of bacterial growth, a range of dilutions as referred to by Ansari & Malik (2007) would have to be applied. The range should correspond to that applied to

the earthworm reproductive test. It has to be considered that Cd^{2+} will interact with the other components of the media. This gives an inaccurate representation of Cd^{2+} toxicity as the interaction will reduce Cd^{2+} bioavailability (Hassen *et al.*, 1998). Thus, absence of bacteria at a specific concentration in agar does not equate to absence of bacteria in earthworm gut. Fungal growth may increase when Cd^{2+} is added to the growth medium (Stefanowicz, 2006). It can obstruct bacterial growth and prevent accurate counting and identification of bacteria. To exclude fungal growth, cycloheximide could be added to the media to interfere with fungal respiration, and eliminate its presence (Hamaki *et al.*, 2005).

Qing *et al.*(2007) morphologically distinguished colonies formed on solid media based on colour, shape, diameter, surface and edge. Morphologically distinct types can be isolated by picking them from spread or streak plates and creating pure cultures (Orndorff & Colwell, 1980; Olsen & Bakken, 1987; Hassen *et al.*, 1998). Considering the dilution factor, the amount of bacteria per morphological type can be expressed as colony forming units per gram of soil (cfu g^{-1} soil). Nutrient and soil extract agar have been utilised successfully to indicate the cfu in soil based biomes (Olsen & Bakken, 1987). Species can be identified with biochemical (MacFaddin, 1980) or molecular techniques (Furlong *et al.*, 2002).

2.6.2 Molecular techniques for identifying bacteria

Toyota & Kimura, (2000) extracted DNA from cultured bacteria and amplified the 16S ribosomal DNA (rDNA) by polymerase chain reaction (PCR). After sequencing the PCR products, they were able to identify species indigenous to *E. fetida.* Furlong *et al.*(2002) used the PCR product of 16S rDNA to establish if there was a difference in the bacteria of earthworm casts and the surrounding soil. Bacterial species identification by amplified 16S rDNA is well established (Singleton *et al.*, 2003; Vullo *et al.*, 2008). Rapid and high-yielding DNA extraction processes, such as that described by Neumann *et al.*(1992), Liu (2009), and Demeke & Jenkins, (2010) may be utilised. The microwave method as described by Carstens *et al.* (2014) could provide an economic means

of acquiring 16S rDNA template for PCR. Electrophoresis could confirm if the 16S rDNA extractions and amplification was successful. Amplification primers 27f and 1378r were utilised by Toyota & Kimura, (2000) whereas Furlong *et al.* (2002) use 27f and 1392r. More recently the primers 27f and 1492r have been utilised (Brodie *et al.*, 2006; Carstens *et al.*, 2014) as it is able to amplify most bacterial 16S rDNAs (Weisburg *et al.*, 1991). The pure culture amplicons can be sequenced and gene libraries such as that of the National Centre for Biotechnology Information (NCBI) searched for sequence similarities to identify the bacteria. Bacterial staining and optical microscopy could be used as confirmation of gene library searches.

2.6.3 Analysis of bacteria results

It is expected that cfu would be reduced with increased levels of Cd^{2+} (Oliveira & Pampulha, 2006; Wang *et al.*, 2010) and that resistance would be indicated by higher amounts of cfu at the same concentration (Qing *et al.*, 2007). The ratio of observable Gram-positive to Gram-negative bacteria may change (Bruins *et al.*, 2000; Gomes *et al.*, 2010) and the species present may differ (Toyota & Kimura, 2000; Lorenz *et al.*, 2006; Byzov *et al.*, 2007). Qing *et al.* (2007) indicated that morphological examinations need to be made on three replicate samples of each concentration to indicate statistically significant differences in cfu and diversity. Triplicate samples were also used by Martinez *et al.* (2006) as a result of the heterogeneous nature of the samples. All results are however depend on human abilities to visually distinguish between morphological types as well as the bacteria's culturability.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental design

The earthworm reproduction assay for testing chemicals, as described by the Organisation for Economic Co-operation and Developments (OECD), was used as a guideline (OECD, 2004). The assay was selected because it reduces the impacts that soil type, temperature, humidity, pH and feed type have on the results. It also affords for reproducibility of the results. Once resistance was confirmed, the Cd^{2+} body burdens of the earthworm groups could be compared for statistical significant differences ($p<0.05$). From the earthworm casts collected, the total colony forming units (cfu) could be counted and the morphologically distinct bacterial colonies isolated. The isolated species could then be identified through 16S rRNA gene sequencing. From comparing the sequencing results to known species sequences, the Gram-positive and Gram-negative ratios, alpha diversity and culturable morphologically distinct species present could be identified.

3.2 Earthworm reproduction test

3.2.1 Test organisms

The earthworm reproduction test (OECD, 2004) was used to test for statistically significant differences between two earthworm groups. The first groups ($Worms_{LTE}$) had long-term exposure to Cd^{2+} (Voua Otomo & Reinecke, 2010) while the second group ($Worms_{U}$) had no previous exposure. Both groups were from the species *Eisenia andrei* (Voua Otomo *et al.*, 2013). The worms were obtained from the North-West University laboratories in Potchefstroom South Africa. The $Worms_{LTE}$ stock, which have been used in numerous studies (Reinecke *et al.*, 1999; Voua Otomo & Reinecke, 2010), was sourced from the Stellenbosch University South Africa. These

worms have been cultured in manure that contained 0.01% $CdSO_4$. Studies by Voua Otomo & Reinecke (2010) have indicated a LC_{50} at 4000 µg Cd^{2+} g^{-1} of substrate.

3.2.2 Test substrate preparation

Artificial soil was prepared according to the OECD (2004) guidelines and consisted of:

1. 10% sphagnum peat which was finely ground and dried
2. 20% kaolin clay
3. 70% dry quartz sand with particles between 50 and 200 microns
4. Calcium carbonate to the value of 0.8% was added to obtain a pH of 6.0 ± 0.5.

The soil was sieved through a 2 mm-mesh to remove larger particles. Thirty chemically inert vessels were each filled with 500 g of the OECD soil. Each vessel's lid had four 1 mm holes to allow gaseous exchange. The vessels were placed in an incubator set at 20 ± 0.2 ^oC for eight days before starting the assay (OECD, 2004).

Water holding capacity (WHC) of the soil was calculated by collecting five of the OECD soil samples and placing it into tubes. The bottoms of the tubes were covered with filter paper that was held in place with rubber bands. The tubes were filled with water through capillary action by placing them in a water bath containing deionised water. They were gradually submerged over a period of three hours until the water level inside the tubes were above the soil level. Thereafter, the tubes were removed and left upright in a bed of wet fine quartz sand in a covered beaker. This was done for two hours to ensure that the excess water was completely drained from the soil. After draining, the wet soil was weighed whilst being heat-dried at 105 °C with a moisture analyser (Model MA 35) until the soil had a constant mass. The WHC was calculated by:

Equation 1: Water holding capacity

$$WHC = \frac{\text{mass of water saturated soil} - \text{mass of dry soil}}{\text{mass of dry soil}} \times 100$$

The mean of the five samples was taken as the 100% WHC and 60% of this (99 mL for 500 g soil) was added to each soil filled vessel after the test substance was dissolved in it (Annexure A).

3.2.3 Test substance

Cadmium sulphate octahydrate ($3CdSO_4.8H_2O$) was used as a source of Cd^{2+} to provide 0, 10, 40, 160 and 400 µg Cd^{2+} g^{-1} of soil (Annexure B). Because the test chemical is water-soluble, it was first dissolved in deionised water before adding it to the soil. It was dissolved by slowly heating and stirring the solution until no salt crystals could be observed. Once added to the soil it was thoroughly mixed to ensure its equal distribution throughout the vessels. Three replicates for each concentration group, for both the earthworm groups, thirty vessels, were prepared in this manner.

3.2.4 Test conditions and measurements

Test conditions were based on that described by the OECD earthworm reproduction test (OECD, 2004). The earthworms were cultured as indicated in Annexure 4 of the OECD guidelines for testing earthworm reproduction (OECD, 2004), apart from $Worms_{LTE}$ being exposed to 0.01% $CdSO_4$ before the test started. The earthworms were acclimatised by culturing them in large containers with damp horse manure. The containers were placed in a climate room set at 20 ± 2 ^oC with 90% humidity. From both the $Worms_{LTE}$ and $Worms_U$ groups the adult worms, of which the clitella were clearly visible, were randomly selected. The earthworms were removed from their substrate and left in petri dishes to depurate for twelve hours. Thereafter, they were individually rinsed in deionised water, placed on filter paper to remove access water and weighed individually. Ten worms were added to each vessel so that two groups, $Worms_{LTE}$ and $Worms_U$ each consisted of three replicates of 0, 10, 40, 160 and 400 µg g^{-1} soil. To each vessel containing, the soil, deionised water, Cd^{2+} and ten adult earthworms, a further five grams of damp horse manure were added. The vessels were then weighed and the weight documented.

During the test period, the vessels were incubated at 20 ± 2 ^{o}C. The weight of the vessels were verified weekly and the lost in weight, assumed to be water evaporation, would be replaced with deionised water. Once a week, for the initial 28 days of the incubation, an additional five grams of damp horse manure would be added to each vessel and the new weight documented. After 28 days the adult worms were removed. All the earthworms of each replicate were rinsed and placed in sterile petri plates on sterile damp filter paper (Furlong *et al.*, 2002; Byzov *et al.*, 2007). After twelve hours, the casts produced in the petri plates were used for bacterial culturing. The worms were rinsed and weighed and their weights noted. Three worms per replicate were individually frozen in Eppendorf tubes for the Cd^{2+} body burden analysis. Adult worm behaviour and substrate consistency were documented. The cocoons and juveniles were placed back into the vessels with an additional five grams of damp horse manure. After a further 28 days the juveniles and cocoons were counted by hand sorting and the results documented (OECD, 2004).

3.3 Earthworm Cadmium body-burden assessment

The individually weighed and frozen earthworms from the earthworm reproductive test, nine from each concentration, were used to determine the mean Cd^{2+} body burden for each concentration. Once thawed earthworms were digested with a method modified from that described by Blust *et al.* (1988). Each earthworm was transferred from its Eppendorf tube into a separate digestion tube. The Eppendorf tubes were rinsed twice with 1 mL 65% HNO_3 and decanted into the digestion tube to ensure that all of the sample has been removed. To each digestion tube an additional 5 mL of 65% HNO_3 and 1 mL of 30% H_2O_2 was added. The digestion tubes were placed into an Ethos Easy microwave where digestion took place at 180 ^{o}C. After cooling, the digestion tube contents were decanted into volumetric flasks and each flask filled to 50 mL (dilution factor) with 1% HNO_3. Each digested product was vacuum filtered through a 0.45 µm membrane filter. The filtrate was stored at 3 ^{o}C in 50 mL polypropylene tubes. Body burdens were analysed from a 10 mL sample of each digested product. This was done using an inductively coupled plasma

mass-spectrometer (ICP-MS) Agilent Model 7500 CE (Coleman *et al.*, 2010). The results (Annexure G) were calculated in parts per million (ppm) by:

Equation 2: Cadmium body burden parts per million (ppm)

$$Cd\ body\ burden = \frac{ppm\ \times dilution\ factor}{Sample\ weight}$$

3.4 Enumeration, isolation and identification of cadmium resistant bacteria

3.4.1 Resistant bacteria enumeration and isolation

Nutrient and soil agar petri plates were prepared to contain the same ratio of Cd^{2+} to substrate than what the earthworms were exposed to in the test containers (1^{-6}:1), thus 0, 10, 40, 160 and 400 mg Cd^{2+} kg^{1-} agar (Annexure B). To reduce chelation between Cd^{2+} and agar, Cd^{2+} had to be added to the agar after it had cooled down from the auto-clave sterilisation. In addition, 40 mg L^{-1} of cycloheximide had to be added to exclude fungal growth. To achieve this, the $3CdSO_4.8H_2O$ was first dissolved in deionised water by slowly heating and stirring until no Cd salt was visible. The cycloheximide was dissolved in the same manner. Both the Cd^{2+} and cycloheximide was added to the agar through sterilisation filters and mixed before poring the agar into petri plates. (Angle & Chaney, 1989; Hassen *et al.*, 1998; Harley & Prescott, 2001).

From the earthworm reproduction test, ten earthworms from each replicate (n=3) were rinsed in sterile water and placed in sterile petri plates to collect their casts for a 12 h period (Furlong *et al.*, 2002; Byzov *et al.*, 2007). The casts were aseptically removed and weighed in Eppendorf tubes. To each of the tubes 1.5 mL of phosphate buffered solution was added. The tubes were vortexed for 30 seconds to liberate and suspend the bacteria in the solution. The suspension was serially diluted, and 0.1 mL of each dilution plated onto soil and nutrient agar to a dilution factor of 1^{-9}. This was done using the standard spread plate technique (Harley & Prescott, 2001). Agar plates were incubated at 21 ^{o}C for four days.

After four days of incubation, the total cfu g^{-1} of casts were determined using the standard plate count method. A colony of each unique combination of: form, elevation, margin, dull or shiny appearance, optical properties, pigment and texture, were isolated by aseptically picking it from the agar. The amount of cfu of each specific type was counted and its dilution factor noted for statistical analysis. Unique colony types were streaked on soil or nutrient agar, depending on which agar they originated from. Their Cd concentration and dilution factor was noted on the bottom of each streak plate. Because identification of the culturable species would be done by molecular methods; DNA extraction, its amplification by polymerase chain reaction (PCR) and gene sequencing would be required. The colonies were streaked to ensure that they are pure cultures. The streak plates were incubated for a further three days at 21 ^{o}C. Single colonies from the streak plates were used to inoculate lysogeny broth (LB) (Harley & Prescott, 2001). The inoculated LB were incubated under agitation for one day at 37 ^{o}C before DNA extraction started. Because some of the samples did not have sufficient or any growth after this period, a further two days were afforded for incubation.

3.4.2 DNA extraction

Bacteria from the streak plate colonies and LB were Gram stained and observed under a microscope to ensure that they were pure cultures (Harley & Prescott, 2001). The Gram staining results were recorded to confirm the molecular identification results. DNA was extracted from the LB samples as soon as it appeared mildly turbid from growth. For the samples that did not grow in broth, the colonies were picked from the streak plates and its DNA extracted. A microwave method, modified from that which Carstens *et al.* (2014) refers to, was used to extract DNA from the LB and picked colonies.

The bacteria was separated from the LB by pipetting 1.5 mL broth into Eppendorf tubes and centrifuging it at 14,000 rpm for two minutes so that a bacteria cell pellet would form at the bottom of the tube. For samples that contained very little bacteria, the supernatant had to be decanted

and the process repeated several times until there was a visible pellet. If a visible pellet could not be obtained from the LB a colony from its streak plate would be inserted at the bottom of the tube. The cells were suspended in 20 µL of nuclease free water by vortex. The tubes were sealed and microwaved for two, and for some species three, minutes at 700 W for cell lysis to occur. Thereafter, the DNA was separated from the lipids and proteins by centrifugation at 14,000 rpm for one minute. The tubes were carefully placed on ice to prevent the resuspension of cellular debris and the interaction between molecules. The supernatant served as the template for DNA amplification.

3.4.3 DNA amplification and sequence confirmation

A polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene of bacteria extracted from the LB or picked colonies. Eubacterial primers 27f (5' - AGA GTT TGA TCM TGG CTC AG- 3') and 1492r (5' - CGG TTA CCT TGT TAC GAC TT- 3') (Brodie *et al.*, 2006) and double concentrated master mix from Thermo Scientific™, were selected for the PCR. The master mix consisted of 0.4 nM of dNTP, 0.05 units/µL of *Taq* DNA polymerase, and 4 mM of Mg_2Cl. One µL extracted DNA template was added to a suspension containing: 1 µL of equal proportioned forward and reverse primers, 12.5 µL of double concentrated PCR Master Mix and 10.5 µL of Nuclease-free water. A thermo cycler (Bio-Rad, UK) programmed for 300 seconds of denaturation at 95 °C, for followed by 35 cycles of; melting at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 60 seconds, was used for the PCR (Lane, 1991). Successful amplification of 16S rDNA was confirmed by agarose gel electrophoresis. Three µL of PCR products, a 1 kb DNA Ladder and a control containing no DNA was mixed with 2 µL of 6X Orange DNA Loading Dye (Thermo Scientific™) and loaded into the one percent agarose gel. Electrophoreses was done for 45 minutes at 80 V. Images of the agarose gel were taken by a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and stored for reference (Annexure E).

3.4.4 Gene sequencing and species identification

After confirming the presence of 16S rDNA, the remaining PCR product was frozen and sent to Inqaba Biotechnical Industries (Pty) Ltd for gene sequencing. The amplicons were purified by column purification and sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems® 3130xl genetic analyser. The resulting chromatograms of partial gene sequences were examined using Chromas trace viewer to estimate sequencing quality. The closest matches to the obtained sequences were indicated using BLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST) from the GenBank® database.

3.5 Statistical analysis

Statistical analysis was performed on IBM SPSS Statistics version 24.0 for all earthworm weight, reproduction and body burden results (Annexure C), as well as bacterial cfu and diversity results. Levene's test confirm homogeneity of variance of all earthworm weight and reproduction analysis. Shapiro-Wilk test confirmed homoscedasticity across different levels of independent variables. Independent samples t-test indicated initial weight differences. Dunnett's test compared tests group results to their specific control groups (Dunnets, 1964). One-way analysis of variance indicated if there were statistically significant different between the results of test and control groups. Post Hoc Tukey's honest significant difference test indicated if there were significant differences between the earthworm groups when subjected to the same concentration of Cd^{2+}. Where means were compared, the standard error of the mean (SEM) were calculated and indicated. Where individual results were compered the standard deviation (SD) was calculated and indicated. Microsoft Excel was used for graphical representation. Diversity was calculated by the Simson's index of diversity (Hunter & Gaston, 1988).

Equation 3: Simpson's diversity index

$$l = \frac{\sum_{i=1}^{R} n_{i(n_i-1)}}{N(N-1)}$$

Where n_i represents the number of colonies having the same colony morphology and N is the total number of colonies on the agar plate.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Earthworms

Table 2: Percentage weight change, cocoons produced per adult, juveniles per cocoon and cadmium body burden after 28 days of exposure.

	Concentration (µg Cd^{2+} g^{-1} soil**)**				
	0	10	40	160	400
Percentage weight change (±SEM) (n=3)					
$Worms_U$	-6.4±1.2 $^{A(a)}$	-2.3±1.6$^{A(a)}$	2.4 ±2.5 $^{A(b)}$	5.0±3.2$^{A(b)}$	-7.3±1.3$^{A(a)}$
$Worms_{LTE}$	-3.8±1.6$^{A(a)}$	-8.6±1.5$^{A(a)}$	13.9±2.1$^{B(a)}$	-9.0±3.93$^{B(a)}$	-7.5±4.3$^{A(a)}$
Cocoons/adult (±SEM) (n=3)					
$Worms_U$	0.67±0.1$^{A(a)}$	0.40±0.1$^{A(b)}$	0.23±0.1$^{A(b)}$	0.1±0.0$^{A(b)}$	0.0±0.0$^{A(b)}$
$Worms_{LTE}$	0.63±0.0$^{A(a)}$	0.67±0.0$^{B(a)}$	0.43±0.1$^{A(a)}$	0.47±0.1$^{B(a)}$	0.27±0.1$^{B(b)}$
Juveniles/cocoon (±SEM) (n=3)					
$Worms_U$	5.9±0.5$^{A(a)}$	5.8±0.2$^{A(a)}$	4.1±0.2$^{A(a)}$	2.0±0.6$^{A(b)}$	0.0±0.0$^{A(b)}$
$Worms_{LTE}$	6.6±0.4$^{A(a)}$	5.6±0.3$^{A(a)}$	5.8±0.6$^{A(a)}$	3.6±0.3$^{A(b)}$	3.1±0.4$^{B(b)}$
Body burden (µg g^{-1}) (±SD) (n=9)					
$Worms_U$	0.8±0.4$^{A(a)}$	32.0±8.6$^{A(a)}$	89.3±27.9$^{A(b)}$	191.6±15.4$^{A(b)}$	236.3±54.2$^{A(b)}$
$Worms_{LTE}$	17.7±10.2$^{A(a)}$	59.5±17.9$^{A(b)}$	112.5±26.2$^{A(b)}$	151.7±43.1$^{A(b)}$	176.3±43.0$^{B(b)}$

[1] Standard error of the mean (±SEM) was calculated as the mean of three vessels of the same treatment, each containing 10 earthworms.

[2] Standard deviation (±SD) was calculated by the mean of nine earthworms, three from each vessel per treatment.

[3] Different upper-case letters indicate statistically significant differences ($p<0.05$) between groups for each concentration, whereas different lower-case letters in parentheses indicate significant ($p<0.05$) differences between the control group (0) and the test group.

4.1.1 Weight change

Levene's Test confirmed the homogeneity of variance of the initial earthworm weighs within the previously unexposed earthworms ($Worms_U$) and the long-term exposure earthworms ($Worms_{LTE}$). Apart from $CdSO_4$ at 0.01% of the feed weight (Voua Otomo & Reinecke, 2010) $Worms_U$ experienced the same environmental factors as $Worms_{LTE}$ prior to the experiment. Independent samples t-test however, indicated that the initial weight of $Worms_U$ were significantly ($p<0.05$) larger (0.332 g ± SD 0.068 g) than $Worms_{LTE}$ (0.298 g ± SD 0.066 g). The long-term exposure resulted in the difference of the initial weight (Swain *et al.*, 2004)

There was no earthworm mortalities form either group at any of the Cd^{2+} concentrations. This confirms results by Spurgeon *et al.* (1994) at a similar range of Cd^{2+} concentrations. The Shapiro–Wilk test confirmed homoscedasticity of weight change across the different concentrations (0, 10, 40, 160 and 400 µg Cd^{2+} g^{-1} soil). Analysis of variance (ANOVA) indicated statistically significant differences in the percentage weight change of $Worms_U$ ($p=0.007$) but not for $Worms_{LTE}$ ($p=0.259$). For $Worms_U$, when comparing the 10, 40, 160 and 400 µg Cd^{2+} g^{-1} soil (test groups) to the 0 µg (control group), Dunnett's test indicated a significant increases in weight at 40 and 160 µg Cd^{2+} (Table 2). The increase in weight may be a hormetic effect since low concentrations of a Cd^{2+} exposure had an increase in growth. Such observation have been observed for *E. fetida* exposed Cd^{2+} by Zhang *et al.* (2009). Alternatively, the weight increase does coincide with a statistically significant decrease in cocoon production thus, possibly the result of a change in resource allocation from reproduction to growth. A similar suggestion have been made by Aira *et al.* (2007) who subjected *E. fetida* to physical stress by frequently handling them with forceps. For the $Worms_{LTE}$ test groups, the weight change was not significantly different from its control group.

4.1.2 Reproduction

4.1.2.1 Cocoon production

Levene's test confirmed the homogeneity of variance for cocoon production and juveniles produced per cocoon. Analysis of variance indicated statistically significant differences between the cocoons produced for both earthworm groups. The test groups of $Worms_U$ had significantly less cocoons than its respective control group (Table 2). Spurgeon *et al.* (1994) observed similar results with a comparable Cd^{2+} range.

Reduction of cocoons started at 10 µg Cd^{2+} g^{-1} soil for $Worms_U$. According to the national norms and standards for the remediation of contaminated land (Notice 467 of 2013 by the Department of Environmental Affairs of South Africa), 15 µg g^{-1} soil is an acceptable Cd level for informal residential settlements (Table 1). Informal residential settlements in South Africa frequently utilises vegetable gardens for subsistence (Baiphethi & Jacobs, 2009). As earthworms provide crucial ecological services (Johnson, 2017), long terms studies of the effects of low level contamination on earthworms, and possibly the revision of the national norms and standards for the remediation of contaminated land, may be required.

$Worms_U$ produced no cocoons at the 400 µg Cd^{2+} g^{-1} soil. $Worms_{LTE}$ did have cocoons at the same concentration, however, it was significantly less than its control group. A post-hoc Tukey's HSD test indicated that cocoon production by $Worms_U$ at 10, 160 and 400 µg was significantly less than that produced by $Worms_{LTE}$ at the same concentration. Therefore, in terms of cocoon production, the Cd^{2+} applications affected $Worms_{LTE}$ less than $Worms_U$. As cocoons were counted at the end of the first four weeks, when the adults were removed, some cocoons may have been unrecognisable or decomposed.

4.1.2.2 Juveniles per cocoon

The 10 and 40 μg Cd^{2+} g^{-1} soil test groups did not have significantly less juveniles per cocoon compared to their control groups. In addition, they were not significantly different from each other. The 160 μg tests groups had significantly less juveniles per cocoon compared to their control groups but were also not different from each other. Because juveniles were left in the soil for four weeks after the adults were removed, the reduction observed may be the result of cocoon viability or juvenile mortality. Spurgeon *et al.* (1994), who removed the cocoons from the substrate before they hatched, concluded no statistical difference in juveniles emerged per cocoon. With the reduction observed at 160 μg Cd^{2+} g^{-1} soil, juvenile mortality may be inferred but requires further testing. Where cocoons were produced, the two earthworm groups did not have statistically significant differences in juveniles per cocoon. As no cocoons were observed for $Worms_U$ at 400 μg Cd^{2+} g^{-1} soil, there were also no juveniles. $Worms_{LTE}$ did have cocoons at 400 μg Cd^{2+} g^{-1} soil but the juveniles produced per cocoon were similar to the ratio of juveniles produces for the same group at 160 μg Cd^{2+} g^{-1} soil. This supports the inference that juvenile mortality did not occur.

4.1.3 Cadmium body burden

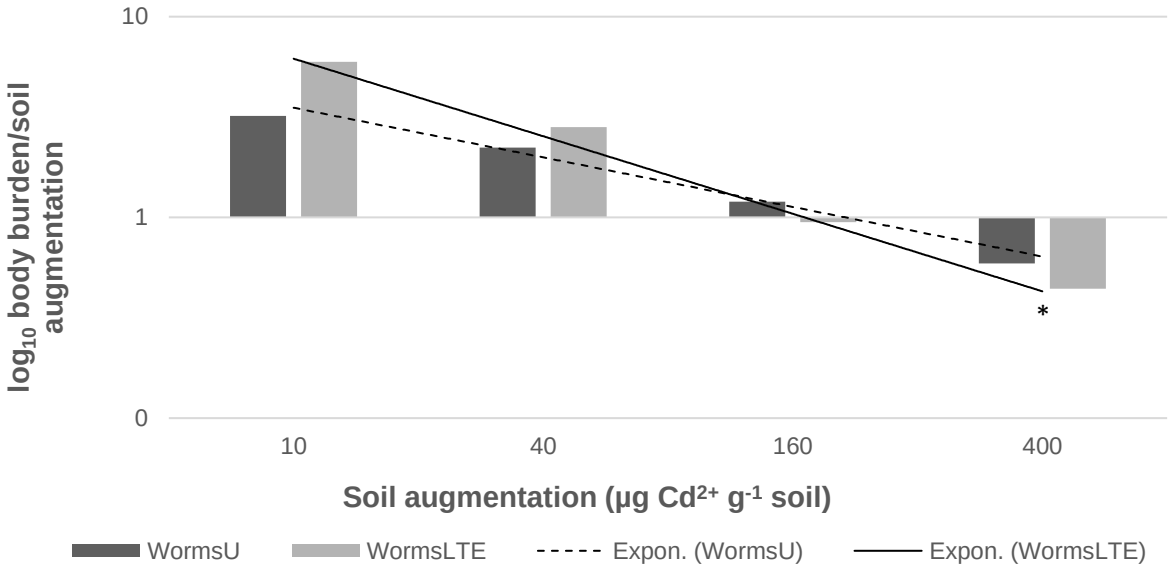

Figure 1: Earthworm mean cadmium body burden after, 28 days of exposure, as a fraction of the cadmium augmentation to the soil on a logarithmic scale. Asterisks (*) indicates statistically significant differences.

The body burdens of the two earthworm groups were not assessed before the experiment. $Worms_{LTE}$ did have prior exposure to Cd^{2+} (Voua Otomo & Reinecke, 2010) and earthworms are known to bioaccumulate Cd^{2+} (Spurgeon & Hopkin, 2000; Fourie *et al.*, 2007). The $Worms_{LTE}$ control group, which had no exposure to Cd^{2+} for the four-week test period, had a greater body burden than the $Worms_{U}$ control group (Table 2). Although the difference between the averages are big, they are not statistically different ($p<0.05$) due to large differences of body burden between individual earthworms within each control group. The low body burden of the $Worms_{LTE}$ control group, even though they had Cd^{2+} exposure prior to the experiment, may be explained by the depuration of Cd^{2+} together with faeces and water whilst in the control soil, as indicated by Veltman *et al.* (2007).

$Worms_{U}$ did not have a significantly larger body burden at 10 µg Cd^{2+} compared to its control group but $Worms_{LTE}$ at the same Cd^{2+} level did. The higher body burden of $Worms_{LTE}$ at 10 µg Cd^{2+} could be because of the combination of prior exposure and the test exposure at 10 µg Cd^{2+}.

At 40, 160 and 400 µg both earthworms groups had significantly greater body burdens compared to their control groups. The only statistically different body burdens between the groups was at 400 µg Cd^{2+} g^{-1} soil.

Comparing the trends in body burden accumulation, Figure 1 indicates that Cd^{2+} body burden accumulated differently for the groups. A logarithmic scale, of the earthworm Cd^{2+} body burdens divided by the Cd^{2+} soil augmentations, shows that although $Worms_{LTE}$ started off with a greater, yet not statistically different body burden, as the Cd^{2+} soil levels were increased $Worms_{LTE}$ body burden decreased relative to $Worms_{U}$ (Figure 1). The 10 and 40 µg test groups contained more than twice the augmented amount. At 160 µg Cd^{2+} the body burden of $Worms_{LTE}$ was less than $Worms_{U}$ and less than the substrate augmentation. At 400 µg Cd^{2+} both earthworm groups had less body burden than the soil augmentation and $Worms_{U}$ had a significantly greater ($p<0.00$) body burden than $Worms_{LTE}$. Thus, at higher concentrations, $Worms_{LTE}$ did not accumulate Cd^{2+} to the same extent as $Worms_{U}$. The reduced body burden may account for $Worms_{LTE}$s' ability to produce cocoons at higher Cd^{2+} concentrations (Table 2).

4.1.4 Qualitative results

In addition to the quantitative results, it was observed that at 400 µg Cd^{2+} g^{-1} soil, earthworms from $Worms_{U}$ had a yellow discolouration of their posterior segments. The discolouration is a characteristic of poisoning (Reinecke *et al.*, 1999). $Worms_{LTE}$ did not exhibit this discolouration. $Worms_{U}$ clustered at the centre of their vessels and the soil appeared to be less aerated and was odorous. Clustering may have been an avoidance technique of $Worms_{U}$ (Wentsel & Guelta, 1988; Yeardley *et al.*, 1996). Alternatively, the toxic effect could be causing them to move around less. This behaviour in itself may reduce reproduction and therefore further investigation at a molecular

level needs to be investigated. $Worms_{LTE}$ did not cluster together in the same manner. The consistency of the control soil was similar to that of the 400 µg Cd^{2+} g^{-1} soil test groups.

4.2 Bacteria

4.2.1 Bacterial levels

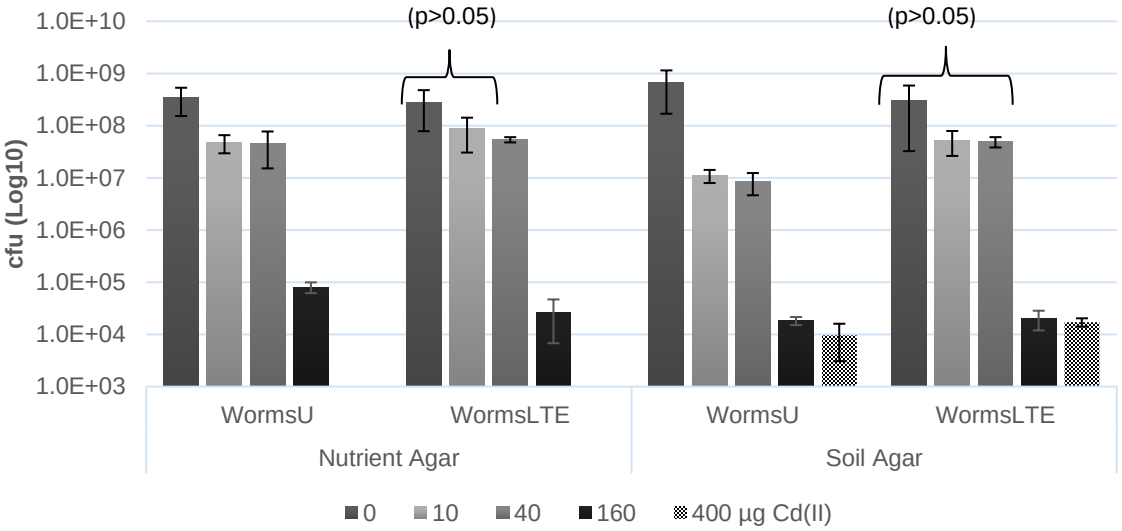

Figure 2: Colony forming units (cfu) of cadmium resistant isolates per gram of earthworm casts. Error bars indicate SD (n = 3). Braced concentrations in a groups indicate cfu that are not statistically different from 0 µg Cd2+ ($p>0.05$).

Earthworm casts were analysed for differences in the bacterial levels using colony forming units (cfu) per gram of casts. Tukey's HSD test of cfu indicated no statistically significant differences between $Worms_{LTE}$ and $Worms_{U}$ at any specific concentration. Comparing the test groups to their respective control groups, Dunnett's test, less prone to type II errors (Dunnets, 1964), indicated that augmenting both growth media with 10, 40, 160 and 400 µg Cd^{2+} L^{-1}, reduced colony forming units (cfu) significantly for $Worms_{U}$ bacteria (Figure 2). Conversely, at 10 µg in nutrient agar and, 10 and 40 µg in soil agar, cfu for $Worms_{LTE}$ were not statistically different to its control group. This result indicates that the amount of Cd^{2+} needed to reduce bacterial growth for $Worms_{LTE}$ was greater at the lower concentrations than that needed to reduce growth for $Worms_{U}$. On the nutrient agar, at 400 µg Cd^{2+} L^{-1}, both earthworm groups had no bacterial growth but on soil agar there were colonies. Chelation of nitrogen compounds between soil agar and Cd^{2+} may have made

Cd^{2+} less available to bacteria (Hassen, *et al.,* 1998). Chelation of Cd^{2+} in the soil would also afford for the intestinal microbiome experiencing a lower concentrations of Cd^{2+} than that introduced to the soil. For this reason, the lower Cd^{2+} concentrations may give a better indication of what the earthworm microbiome experienced.

Should the species present in the gut of $Worms_{LTE}$ and $Worms_{U}$ be the same, $Worms_{LTE}$s' bacteria may have undergone an overall upturn in their resistance mechanisms because of their the long-term exposure to Cd^{2+} (Silver, 1996; Nies, 1999). If different species are present, the ecological functions of the bacteria, on which the earthworms may be dependent (Barton & Northup, 2011), might not be occurring at the same rate. Loss of ecological function of its intestinal microbiome could have adverse repercussions such as mortality and reduced reproduction as well as a change in their behaviour due to a lack of nutrition (Larsen *et al.*, 2016). Alternatively, even though some of the bacterial species may be excluded from the community, those bacteria may be functionally redundant because other species that are Cd^{2+} resistant could perform the same ecological function at the same rate in their absence (Allison & Martiny, 2008).

4.2.2 Diversity of morphologically distinct colonies

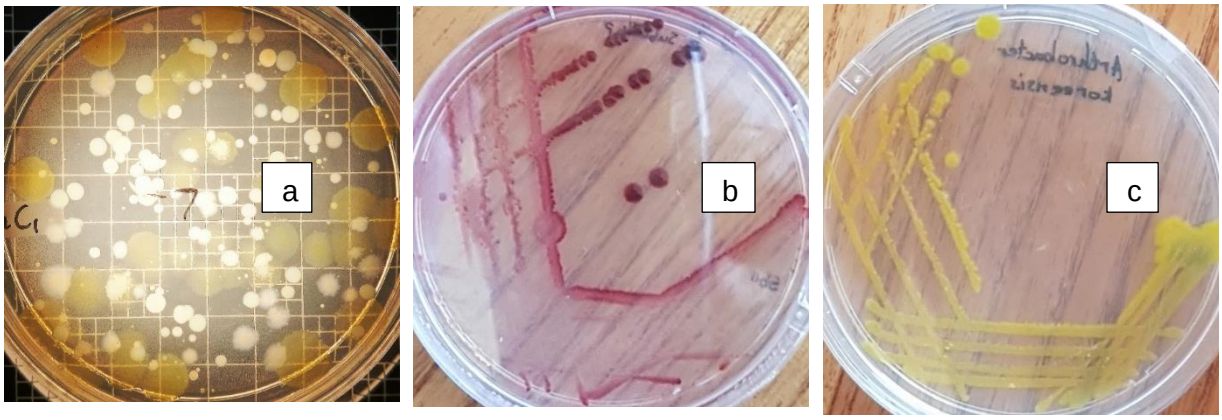

Figure 3: (a) An example of a control group spread plate at 1^{-7} dilution with clearly visible morphologically distinct species from which colony counts and species isolation were done. Distinct species were isolated on streak plates (b and C) and, identified by DNA sequencing.

Subtle differences between colony characteristics made their counting challenging. Some of the unique colonies selected were discovered to be of the same species after DNA sequencing. In those cases the cfu of species were added together in further analysis. Many colonies may have appeared to be the same and not isolated and identified. Such colonies could have been different species. Bacteria that were cultured on nutrient agar were easier to count and morphologically identify than on soil agar (Figure 3). This is because the nutrient agar was translucent and the soil agar opaque. Moreover, a specific bacteria, later identified as *Flavobacterium denitrificans*, with only a few distinct semi-translucent yellow colonies, would overwhelm the agar plates, possibly reducing the growth of other species. *F. denitrificans* is frequently observed from earthworm gut cultures and is known for its contribution to green house gasses as it produces nitrous oxide (Horn *et al.*, 2005).

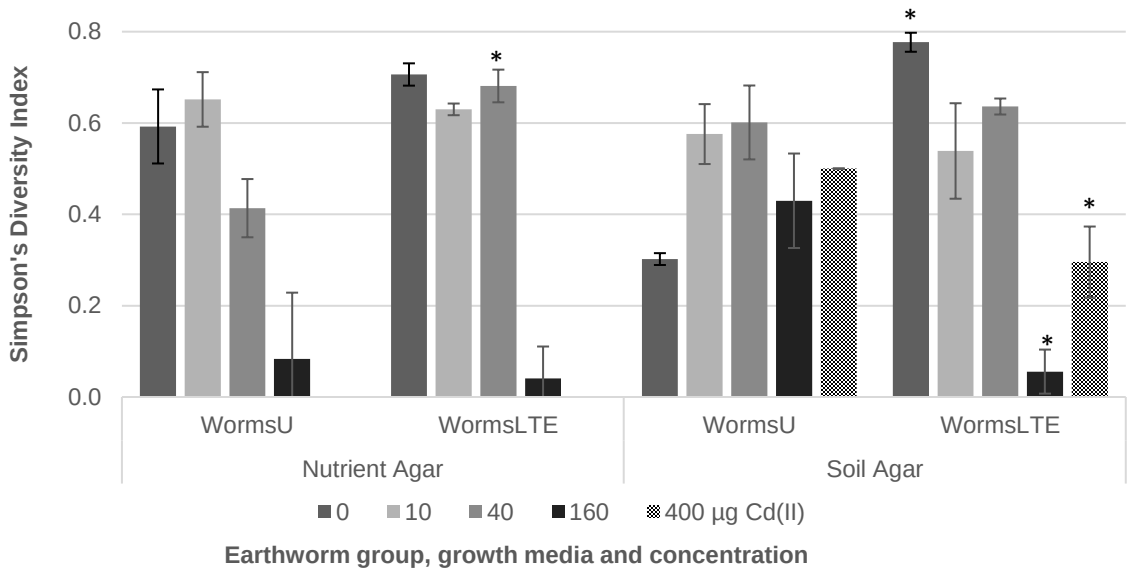

Figure 4: Alpha diversity estimates calculated with Simpson's diversity index based on observed morphologically distinct species. Asterisks (*) indicate significant differences ($p<0.05$) between $Worms_U$ and $Worms_{LTE}$ at a specific concentration for each growth medium.

Simpson's diversity index was used to compare the richness and evenness of morphologically distinct colonies. On nutrient agar, the diversity markedly decreased from ≥40 µg Cd^{2+} for $Worms_U$ but for $Worms_{LTE}$ it only decreased from ≥160 µg (Figure 4). An indication that, in terms of Cd^{2+} eliminating some species, $Worms_{LTE}$ may be more resistant than $Worms_{U.}$ Tukey's HSD test confirmed that the diversity at 40 µg was significantly greater for $Worms_{LTE}$.

On soil agar, the diversity at 0 µg Cd^{2+} was significantly greater for $Worms_U$ compared to $Worms_{LTE}$. The lack of diversity in $Worms_{LTE}$ was largely contributed to the extensive number of *Pseudomonas entomophila* observed at this concentration. Furthermore, in opposition to the results on nutrient agar, on soil agar $Worms_U$ had a greater diversity ≥160 µg Cd^{2+}. Tukey's HSD confirmed that $Worms_{LTE}$ had significantly less diversity at 160 and 400 µg Cd^{2+} compared to $Worms_U$. As cocoon production of $Worms_{LTE}$ was significantly greater at these concentrations, it can be inferred that the reduction in diversity had no harmful effect on the earthworms.

4.2.3 Species isolated

Table 3: Species identification on GenBank, based on morphologically distinct colonies from earthworm casts

Nucleotides used	GenBank ID	Locus	% Similarity	E value
677bp	*Achromobacter denitrificans* strain CCUG 407	NR_018398	99	0.0
918bp	*Achromobacter mucicolens* strain R-46658	NR_117613	99	0.0
760bp	*Aeromonas hydrophila* strain ATCC 7966	NR_074841	98	0.0
814bp	*Agrococcus citreus* strain IAM 15145	NR_041542	99	0.0
634bp	*Arthrobacter koreensis* strain CA15-8	NR_025665	99	0.0
726bp	*Bacillus cereus* strain JCM 2152	NR_113266	99	0.0
849bp	*Bacillus eiseniae* strain A1-2	NR_108906	97	0.0
553bp	*Bacillus safensis* strain NBRC 100820	NR_113945	99	0.0
596bp	*Bacillus subtilis* strain JCM 1465	NR_113265	99	0.0
628bp	*Cellulomonas persica* strain JCM 18111	NR_114320	99	0.0
822bp	*Cellulosimicrobium cellulans* DMS 43879	NR_119095	99	0.0
839bp	*Cupriavidus campinensis* strain WS2	NR_025137	99	0.0
767bp	*Flavobacterium denitrificans* strain ED5	NR_042088	96	0.0
762bp	*Kaistia adipata strain* Chj404	NR_042723	99	0.0
834bp	*Pseudomonas alcaligenes* strain ATCC 14909	NR_114472	98	0.0
835bp	*Pseudomonas entomophila* strain L48	NR_102854	98	0.0
796bp	*Pseudomonas nitroreducens* strain DSM 14399	NR_114975	99	0.0
797bp	*Pseudomonas plecoglossicida* strain FPC 951	NR_024662	98	0.0

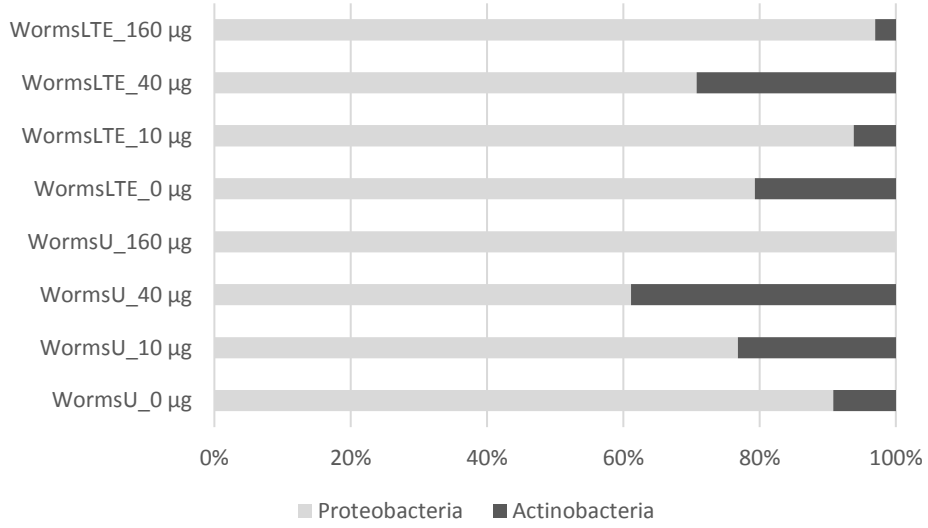

Figure 5: Phyla isolated from nutrient agar.

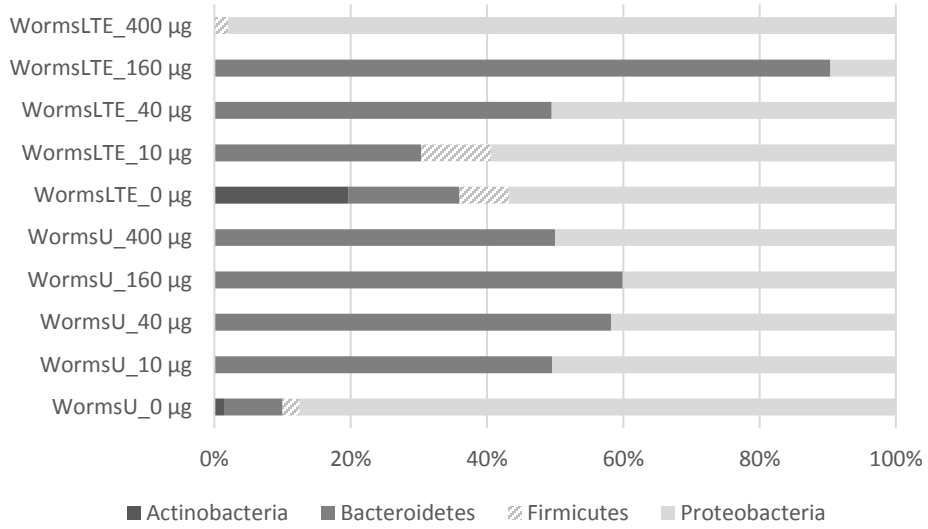

Figure 6: Phyla isolated from soil agar.

Table 4: Bacterial species observed per cadmium concentration.

	Concentration (µg Cd^{2+} g^{-1} growth media**)**				
$Worms_U$	0	10	40	160	400
Nutrient agar	*Achromobacter denitrificans*	*Achromobacter denitrificans*	*Arthrobacter koreensis*	*Cupriavidus campinensis*	
	Aeromonas hydrophila	*Aeromonas hydrophila*	*Pseudomonas entomophila*	*Pseudomonas plecoglossicida*	
	Arthrobacter koreensis	*Arthrobacter koreensis*	*Pseudomonas nitroreducens*		
	Pseudomonas nitroreducens	*Pseudomonas nitroreducens*			
Soil agar	*Aeromonas hydrophila*	*Flavobacterium denitrificans*	*Achromobacter denitrificans*	*Flavobacterium denitrificans*	*Achromobacter mucicolens*
	Agrococcus citreus	*Kaistia adipata*	*Flavobacterium denitrificans*	*Pseudomonas nitroreducens*	*Flavobacterium denitrificans*
	Bacillus cereus	*Pseudomonas alcaligenes*	*Kaistia adipata*		
	Flavobacterium denitrificans	*Pseudomonas entomophila*	*Pseudomonas entomophila*		
	Kaistia adipata		*Pseudomonas nitroreducens*		
	Pseudomonas alcaligenes				
	Pseudomonas entomophila				
$Worms_{LTE}$					
Nutrient agar	*Achromobacter denitrificans*	*Achromobacter denitrificans*	*Achromobacter denitrificans*	*Cellulomonas persica*	
	Aeromonas hydrophila	*Arthrobacter koreensis*	*Arthrobacter koreensis*	*Cupriavidus campinensis*	
	Arthrobacter koreensis	*Pseudomonas entomophila*	*Pseudomonas entomophila*	*Pseudomonas entomophila*	
	Pseudomonas entomophila	*Pseudomonas nitroreducens*	*Pseudomonas nitroreducens*		
	Pseudomonas nitroreducens				
Soil agar	*Bacillus eiseniae*	*Bacillus eiseniae*	*Flavobacterium denitrificans*	*Flavobacterium denitrificans*	*Achromobacter denitrificans*
	Bacillus safensis	*Flavobacterium denitrificans*	*Kaistia adipata*	*Pseudomonas alcaligenes*	*Bacillus subtilis*
	Cellulomonas persica	*Pseudomonas alcaligenes*	*Pseudomonas alcaligenes*	*Pseudomonas entomophila*	*Pseudomonas nitroreducens*
	Cellulosimicrobium cellulans	*Pseudomonas entomophila*	*Pseudomonas entomophila*		
	Flavobacterium denitrificans				
	Pseudomonas alcaligenes				
	Pseudomonas entomophila				

The gut bacteria of soil invertebrates are predominantly from the Gram-negative phyla of Proteobacteria and, the *Bacteroides* and *Flavobacterium* genera of Bacteroidetes. The Gram-positive bacteria are from the Actinobacteria and Firmicutes phyla (König, 2006). Eighteen species were identified from earthworm casts (Table 4), based on their colony morphology (Annexure E). Bacteria isolated from nutrient agar were from the Actinobacteria and Proteobacteria phyla (Figure 5), whereas soil agar also included species from Bacteroidetes and Firmicutes (Figure 6). These results are similar to that compiled in a review by König (2006). Identification of uncultured bacteria by Furlong *et al.* (2002) and Singleton *et al.* (2003) have identified up to nine bacterial phyla from earthworm casts.

4.2.3.1 Nutrient agar

On nutrient agar at 160 µg Cd^{2+} L^{-1}, $Worms_U$ contained no observable colonies of Actinobacteria (Figure 4) whereas $Worms_{LTE}$ did. Only one species of Actinobacteria was morphologically unique and identified as *Cellulomonas persica* (Table 4). *C. persica* was also observed in the control group of $Worms_{LTE}$ but at no other Cd^{2+} concentration (Table 5). Although insufficient literature exists on the cadmium resistance of *Cellulomonas* it has been isolated from cadmium contaminated rhizospheres and is recognised for its contribution to soil fertility (Dell' Amico *et al.*, 2005). *C. persica* degrades cellulose (Elberson *et al.*, 2000) and cellulose degradation is pertinent to earthworm survival and soil fertility. Parle (1963b) indicated that enzymes excreted by earthworms themselves, and not by microorganisms, resulted in the degradation of cellulose inside earthworm intestines. *C. persica* being observed as Cd^{2+} resistant inside $Worms_{LTE}$ and not in $Worms_U$ provides merit for further studies in its interactions with earthworms. It may not contribute to cellulose degradation inside earthworms but its proliferation inside and distribution by earthworms still contribute to soil fertilisation by cellulose degradation. *Cellulomonas* has been utilised for soil bioremediation (Banjoko, 2016). Thus, the introduction of resistant earthworms harbouring Cd^{2+} resistant cellulose degrading bacteria may improve soil fertility in Cd^{2+}

contaminated soil because earthworms can act as vectors for the dispersal of resistant bacteria (Daane *et al.*, 1996).

Cupriavidus campinensis was isolated in both $Worms_{LTE}$ and $Worms_{U}$ at 160 µg Cd^{2+}. *Cupriavidus* spp. are well known for its metal resistance (Siripornadulsil & Siripornadulsil, 2013; Maynaud *et al.*, 2014; Limcharoensuk *et al.*, 2015). It uses the membrane-bound CzcCBA complex which is believed to transport cations such as Cd^{2+} and Zn^{2+} from the periplasms through outer membrane proteins (Silver & Phung, 2009). It has been shown to immobilise (Liu *et al.*, 2012) and bioaccumulate Cd^{2+} (Limcharoensuk *et al.*, 2015) reducing its availability to plants (Siripornadulsil & Siripornadulsil, 2013). In literature, the species mainly identified for its resistance is *Cupriavidus metallidurans*. In a study by Reith *et al.* (2009), gold biomineralisation by *C. metallidurans* has been suggested not only as a means of bioremediation but also for bioexploration and bioprocessing of metals. As Cd^{2+} resistant *C. campinensis* was isolated from both earthworm groups its presence could not have contributed to resistance observed from $Worms_{LTE}$.

4.2.3.2 Soil agar

On soil agar, Bacteroidetes and specifically *F. denitrificans*, was isolated from all concentrations of both groups apart from $Worms_{LTE}$ exposed to 400 µg Cd^{2+} L^{-1} (Figure 5 and Table 5). The *F. denitrificans* is capable of dissimilative nitrate reduction, denitrifying nitric oxide (NO) to nitrous oxide (N_2O) for energy (Horn *et al.*, 2005). *Flavobacterium* spp. have been recognised as being Cd^{2+} resistant, having been isolated from Cd^{2+} contaminated soil (Kanazawa & Mori, 1996). A study by Zhang *et al.* (2008), using cad1 and cad2 PCR primers, identified *CadA* genes within *Flavobacterium* spp. They suggested that *Flavobacterium* might have acquired *CadA* from *Bacillus* spp. The *CadA* gene is known to provide resistance to *Bacillus subtilis* when expressed (Tsai *et al.*, 1992).

Instead of *F. denitrificans*, $Worms_{LTE}$ exposed to 400 µg Cd^{2+} had two other denitrifying species from the Proteobacteria phylum namely *Achromobacter denitrificans* and *Pseudomonas nitroreducens*. *A. denitrificans* was not identified in distinct colonies on $Worms_U$ at 400 µg although it was observed at lower concentration for the group (Table 5). The presence of *A. denitrificans* confirms the results of a study done by Abyar *et al.* (2012). It was illustrated in their study that *A. denitrificans* has a higher resistance to Cd^{2+} than *P. putida*. Its biosorption increases with substrate Cd^{2+} content to the point where its growth decreases and, it absorbs a considerable amount of Cd^{2+} having application in bioremediation (Abyar *et al.*, 2012).

4.2.3.3 Gammaproteobacteria species isolated

In a study by Malik & Jaiswal (2000) it was found that more than 70% of the 45 *Pseudomonas* spp. isolated from soil, were Cd^{2+} resistant. *Pseudomonas* spp. accumulate Cd in the periplasm and intracellularly (Minz *et al.*, 1996; Ahemad & Malik, 2012). In this study, nearly 50% of species isolated form agar containing high concentrations of Cd^{2+} (≥160 µg), were *Pseudomonas* spp. Apart from $Worms_U$ on nutrient agar at 160 µg Cd^{2+} (Table 5), *Pseudomonas* spp. was isolated on both media at all Cd^{2+} concentrations. At high concentrations of Cd^{2+}, *P, alcaligenes P. entomophila, P. nitroreducens* and *P, plecoglossicida* were isolated. *Aeromonas hydrophila*, suggested as being indigenous to *E. fetida* (Toyota & Kimura, 2000), and by extension *E. andrei* (Voua Otomo *et al.*, 2013), was observed in both earthworm groups but not at high Cd^{2+} concentrations.

For $Worms_U$ at 160 µg Cd^{2+} on nutrient agar, distinct colonies of *P. plecoglossicida* were isolated that were not observed on $Worms_{LTE}$. Conversely, $Worms_{LTE}$ contained colonies of *P. entomophila.* Both these *Pseudomonas* spp. are close relatives to *P. putida* (Anzai *et al.*, 2000; Vodovar *et al.*, 2006). Ingestion of *P. entomophila* is lethal to Dipteran *Drosophila melanogaster* (Matthijs *et al.*, 2009). A study by (Nikolouli & Mossialos, 2016) indicated that *P. entomophila* is highly resistant to antibiotics. Genetic analysis indicated encoding for a RND TtgABC efflux pump,

similar to that found in *P. putida*, which afford for its resistance. Its antibiotic resistance may indicate cross resistance to Cd^{2+} (Stepanauskas *et al.*, 2006). This is potentially due to efflux pumps occurring. The *P. plecoglossicida* is an opportunistic pathogen that, amongst others, causes haemorrhagic ascites disease in fish (Park *et al.*, 2000). Its resistance to copper and its possible utilisation in bioremediation has been indicated (Andreazza *et al.*, 2010). As both these species have displayed resistance to Cd^{2+}, its possible contribution to the Cd^{2+} resistance observed in $Worms_{LTE}$ is uncertain.

Pseudomonas alcaligenes and *Pseudomonas nitroreducens* have both been placed in the *Pseudomonas aeruginosa* group of the Gammaroteobacteria subclass due to their 16S rRNA indicating phylogenetic affiliation (Anzai, *et al.*, 2000). The *P. aeruginosa* produces thiol-rich compounds which detoxifies $CdCl_2$ (Siripornadulsil & Siripornadulsil, 2013). Both *P. alcaligenes* and *P. nitroreducens* are denitrifying bacteria and in general, most denitrifying bacteria are from the phylum Proteobacteria. Ten to 15% of all the bacteria in soil are denitrifying and they have extensive diversity. Soil with a higher diversity of microbes have a higher diversity of nitrifying bacteria. There are twice as many nitrifying bacteria in earthworm intestines than in soil (König, 2006). Their population shifts, redundancy and responses to environmental changes are not well understood (Hallin *et al.*, 2007). Most of the earthworm intestinal bacteria are from the gamma subclass of Proteobacteria (König, 2006). The *P. alcaligenes* is known for its metal resistance and has been utilised in bioremediation (O'Mahony *et al.*, 2006). $Worms_{LTE}$ had significantly less diversity than $Worms_U$ at 400 µg Cd^{2+} (Figure 3) because *P. nitroreducens* cfu dominating the $Worms_{LTE}$ agar plates. It did however contain three observably distinct denitrifying cfu types where $Worms_U$ only contained two (Table 5).

4.2.3.4 Gram-positive and. Gram-negative ratios

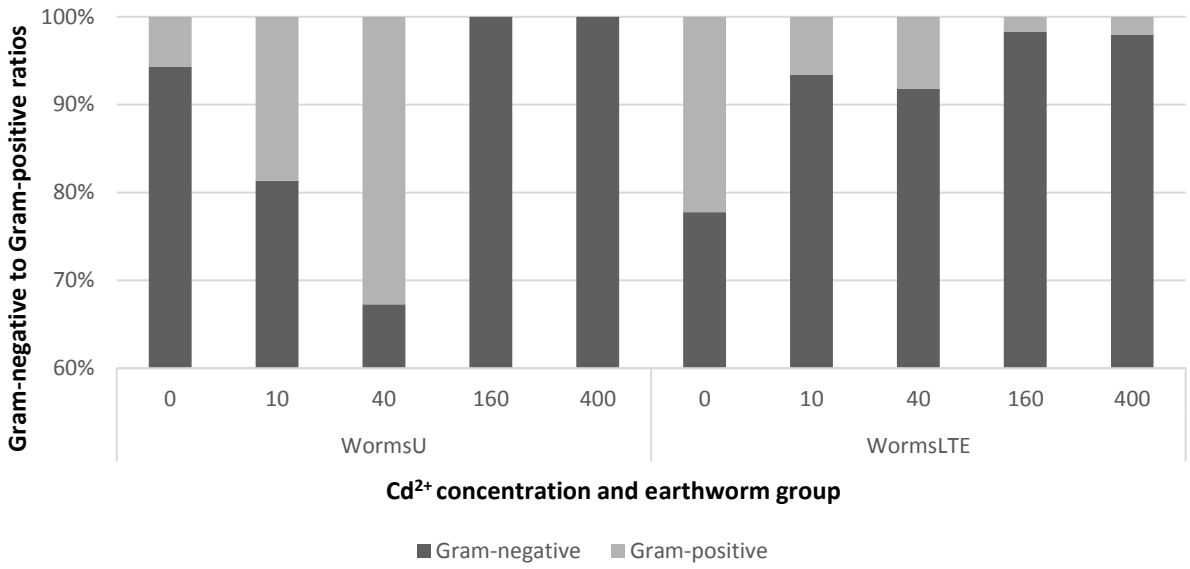

Figure 7: Gram-positive and Gram-negative bacteria per concentration for both media as identified from distinct colony forming units.

The morphologically distinct colonies from each media was counted and the Gram-stained to determine if they were Gram-positive or Gram-negative. Gene sequencing and species identification later confirmed the results. Most species isolated were Gram-negative (Figure 7). Almost 50% of species were from the Proteobacteria phylum (Table 5) and more than 70% of *Pseudomonas* spp. are regarded as resistant to cadmium (Malik & Jaiswal, 2000). The results concurs with that of Morozzi *et al.* (1986), Bruins (2000) and König (2006). $Worms_U$ had no Gram-positive bacteria at ≥160 µg Cd^{2+} whereas $Worms_{LTE}$ did. This may be an indication of greater tolerance to Cd^{2+} by Gram-positive earthworm intestinal bacteria. Cadmium resistance by the two species *B. subtillis* and *C. persica* is well established (Boyanov *et al.*, 2003; Banjoko, 2016). Their resistance being the result of costly ATPases (Silver & Phung, 2005; Palmgren & Nissen, 2011).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Earthworms

In terms of earthworm mortality, the null hypothesis that long-term Cd^{2+} exposure earthworms ($Worms_{LTE}$) were not significantly ($p<0.05$) different from previously unexposed earthworms ($Worms_U$), when exposed to 10, 40, 160 and 400 µg Cd^{2+} g^{-1} soil, could not be rejected. A difference could not be determined since none of the earthworms died from Cd^{2+} exposure.

The null hypothesis that $Worms_{LTE}$ were not different from $Worms_U$ in relation to weight change was rejected for low Cd^{2+} levels (40 and 160 µg g^{-1} soil). There were statistically significant increases in weight observed for $Worms_U$ that was not observed in $Worms_{LTE}$. However, the null hypothesis could not be rejected for the highest Cd^{2+} concentration (400 µg g^{-1} soil) which displayed no significant statistical difference between the two groups.

With regards to reproduction, the null hypothesis that $Worms_{LTE}$ are not different form $Worms_U$ was rejected. Even at the lowest concentration (10 µg Cd^{2+} g^{-1-} soil) $Worms_U$ had produced significantly less cocoons than its control group and $Worms_{LTE}$ at equal concentrations. The concentration at which reproduction of $Worms_U$ were significantly ($p<0.05$) reduced, was less than the minimum standards for Cd^{2+} for informal settlements as set out by the national norms and standards for the remediation of contaminated land. At the highest Cd^{2+} concentration, $Worms_U$ had no cocoons where $Worms_{LTE}$ did. It is therefore concluded that long-term exposure to Cd^{2+} created selective pressure on *E. andrei* that resulted in Cd^{2+} resistance.

On assessing whether there were significant ($p<0.05$) differences in Cd^{2+} body burden accumulation in the test period, the null hypothesis was rejected. The trend indicated that, as the

Cd^{2+} levels were increase in the soil, the body burden of $Worms_{LTE}$ was decreased in relation to the body burden of $Worms_{U}$ at the same Cd^{2+} levels. At 400 μg Cd^{2+} g^{-1} soil, the body burden of $Worms_{LTE}$ was significantly ($p<0.05$) less than $Worms_{U}$. Thus, $Worms_{LTE}$ were more resistant to accumulating Cd^{2+}.

5.2 Bacteria

Aerobically culturable bacterial levels present in earthworm casts were significantly ($p<0.05$) reduced at the lower Cd^{2+} levels for $Worms_{U}$ in relation to its control group. Low Cd^{2+} concentrations did however not reduce bacteria levels for $Worms_{LTE}$ compared to its control. The aerobically culturable bacterial levels of $Worms_{LTE}$ were therefore more resistant to Cd^{2+} and the null hypothesis that bacterial levels between the two groups would be the same is rejected.

On nutrient agar, the diversity of the visibly distinct bacterial colonies of $Worms_{U}$, decreased to a greater extent with increased Cd^{2+} levels when compared to $Worms_{LTE}$. At 160 μg Cd^{2+} L^{1-} nutrient agar the diversity was significantly greater for the $Worms_{LTE}$. In contrast to this result, diversity on soil agar was greater for $Worms_{U}$. The null hypothesis of equal diversity can therefore not be rejected.

Gram staining indicated that most species were Gram-negative with the majority of species isolated being from the *Pseudomonas* genus. The highest Cd^{2+} levels for $Worms_{U}$ bacteria on soil agar did not contain any observable *Pseudomonas* species whereas $Worms_{LTE}$ did. Casts from $Worms_{LTE}$ also contained highly Cd^{2+} resistant Gram-positive bacteria where none was isolated from $Worms_{U}$. It is therefore concluded that differences exist in the aerobically culturable morphologically distinct bacterial species present in $Worms_{U}$ and $Worms_{LTE}$ casts. These differences may contribute to earthworm resistance.

It can therefore concluded that long-term exposure to Cd^{2+} afford *E. andrei* with Cd^{2+} resistance and that the bacteria present in the intestinal tract may contribute to this resistance.

5.3 Recommendations

Significant increases in weight were observed for $Worms_U$ when exposed to low Cd^{2+} levels. This increase in weight correlated with a decrease in cocoon production. It is recommended that the cause for weight increase and cocoon production decrease be investigated at a molecular level. Such research would shed light on the hormetic or metabolic shift effects observed.

After four weeks, reproduction was significantly reduced by exposure to 10 μg Cd^{2+} g^{-1} soil. Because earthworms are bioindicators of soil health and sustainable land utilisation, it is advisable to study the body burden and bioaccumulation of low-level Cd^{2+} exposure on earthworms and other soil organisms in the long-term. The national norms and standards for the remediation of contaminated land indicates that 15 μg Cd^{2+} g^{-1} soil are acceptable for informal residential settlement, however, people in these settlements do use the land for subsistence. If long-term studies show significant Cd^{2+} bioaccumulation, the norms and standards for the remediation of contaminated land may have to be revised.

In this study, the cocoons were left in the contaminated soil for the test period and the juveniles hatched only counted thereafter, as recommended by the OECD guidelines. Some juveniles may have perished after hatching because of the Cd^{2+} toxicity. Other studies have focused more on cocoon viability by removing the cocoons from the contaminated soil after they were produced. Because the juvenile stage is the most sensitive, it is advised to study the effects of Cd^{2+} specifically on juveniles. Although there was no mortality for adults in this study, mortality may have occurred in juveniles.

Along with the reproduction difference observed between the earthworm groups, differences were observed in bacterial levels present in the earthworm casts. Intestinal bacteria may therefore contributed to elevated Cd^{2+} resistance although proteins produced by the earthworms, such as metallothionein, may be the main resistance determinant. Proteomic and genomics studies are recommended to confirm if there is a difference in the metallothionein production in Cd^{2+} resistant earthworms. Differences in bacteria present in casts could be the results of resistance obtained from their long-term exposure or by proteins produced by their resistant hosts. It is further recommended to compare the amount of Cd^{2+} present in the casts as well the amount of Cd^{2+} sequestered by the bacteria in the casts.

Only a fraction of the bacteria present in casts are culturable. Culture-based methods are not as accurate as non-culture based methods for observing bacterial levels, its diversity and the species present in a microbiome. It is therefore suggested that metagenomics is used to present and compare the intestinal microbiomes (Frank & Pace, 2008). The results of such studies could further be compared to community level physiological profiles (Akmal *et al.*, 2005) in order to have a better understanding of how the intestinal microbiome could contribute to resistance.

Further studies on the mechanisms that confer Cd^{2+} resistance to bacteria inside earthworm intestines are needed. Especially if the resistance arises from the sequestration of metal, thus reducing its bioavailability. Exploiting this ability may prove valuable for the bioremediation of Cd^{2+} contaminated land.

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ANNEXURE A

Water holding capacity calculation of the of OECD soil.

100% WHC (in % of dry mass) = $\frac{S-D}{D} \times 100$

Where:

- S = mass of water-saturated soil
- D = mass of dry soil

Sample 1: S = 1.913 g D = 1.250 g ∴ $\frac{1.913\ g - 1.250\ g}{1.250\ g} = 0.347$

Sample 2: S = 2.470 g D = 1.656 g ∴ $\frac{2.470\ g - 1.656\ g}{1.656\ g} = 0.330$

Sample 3: S = 2.371 g D = 1.580 g ∴ $\frac{2.371\ g - 1.580\ g}{1.580\ g} = 0.334$

Sample 4: S = 3.258 g D = 2.295 g ∴ $\frac{3.258\ g - 2.295\ g}{2.295\ g} = 0.307$

Sample 5: S = 1.316 g D = 0.878 g ∴ $\frac{1.316\ g - 0.878\ g}{0.878\ g} = 0.333$

$$\therefore\ 60\ \%\ WHC\ = \left(\frac{60}{100}\right) \times \left(\frac{0.347 + 0.330 + 0.334 + 0.307 + 0.333}{5}\right) \times 500\ g\ soil$$

$$= \mathbf{99\ mL\ water / 500\ g\ Soil}$$

ANNEXURE B

Amount of $3CdSO_4.8H_2O$ to be added to 500 g soil

Calculating the molar mass of $3CdSO_4.8H_2O$

Cd = 337.233
S = 96.198
O = 318.988
H = 16.1264
$\therefore 3CdSO_4.8H_2O$ = 769.5454 g/mol

Calculation of $3CdSO_4.8H_2O$ g to be added to 99 mL water (WHC) to supply 0, 10, 40, 160 and 400 µg Cd^{2+} to soil.

$$\therefore 0\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times 500\ g\ soil$$

$$= \mathbf{0.0000\ g\ 3CdSO4.8H2O}$$

$$\therefore 10\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times 500\ g\ soil$$

$$= \mathbf{0.0114\ g\ 3CdSO4.8H2O}$$

$$\therefore 40\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times 500\ g\ soil$$

$$= \mathbf{0.0456\ g\ 3CdSO4.8H2O}$$

$$\therefore 160\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times 500\ g\ soil$$

$$= \mathbf{0.1826\ g\ 3CdSO4.8H2O}$$

$$\therefore 400\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times 500\ g\ soil = \mathbf{0.4564\ g\ 3CdSO4.8H2O}$$

Amount of $3CdSO_4.8H_2O$ to be added to 1 L agar

Calculation the amount of $3CdSO_4.8H_2O$ g to be added to 1 L agar to supply 0, 10, 40, 160 and 400 µg Cd^{2+} agar.

$$\therefore 0\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times \left(\frac{1000\ g}{1\ kg}\ \text{agar}\right)$$

$$= \mathbf{0.000\ g\ 3CdSO4.8H2O}$$

$$\therefore 10\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times \left(\frac{1000\ g}{1\ kg}\ \text{agar}\right)$$

$$= \mathbf{0.023\ g\ 3CdSO4.8H2O}$$

$$\therefore 40\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times \left(\frac{1000\ g}{1\ kg}\ \text{agar}\right)$$

$$= \mathbf{0.091\ g\ 3CdSO4.8H2O}$$

$$\therefore 160\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times \left(\frac{1000\ g}{1\ kg}\ \text{agar}\right)$$

$$= \mathbf{0.365\ g\ 3CdSO4.8H2O}$$

$$\therefore 400\ \mu g\ Cd(II) \times \left(\frac{1\ g}{1000000\ \mu g}\right) \times \left(\frac{769.54543\ g\ 3CdSO4.8H2O}{337.233\ g\ Cd2+}\right) \times \left(\frac{1000\ g}{1\ kg}\ \text{agar}\right)$$

$$= \mathbf{0.913\ g\ 3CdSO4.8H2O}$$

ANNEXURE C

Earthworm results and analysis

Initial earthworm weight

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Weight	Equal variances assumed	1.129	.289	-4.379	298	**.000**
	Equal variances not assumed			-4.379	297.854	**.000**

Change in weight

Discriptives

weight_changeW_U

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
1	3	-6.435630205000001	2.019802771000000	1.166133674000000	-11.453098440000000	-1.418161971000000
2	3	-2.281868967000000	2.801818701000000	1.617630781000000	-9.241972463000000	4.678234529000000
3	3	2.398609095000000	4.277409458000000	2.469563502000000	-8.227065048000000	13.024283240000000
4	3	5.033354734000000	5.606886910000000	3.237137667000000	-8.894924486000000	18.961633950000000
5	3	-7.302465200000000	2.245145572000000	1.296235400000000	-12.879715980000000	-1.725214416000000
Total	15	-1.717600109000000	5.869231208000000	1.515428981000000	-4.967872014000000	1.532671797000000

weight_changeW_LTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
6	3	-3.763802334000000	2.726412416000000	1.574094942000000	-10.536586230000001	3.008981565000000
7	3	-8.551574839000000	2.544935912000000	1.469319434000000	-14.873546110000000	-2.229603567000000
8	3	-13.932013110000000	3.579516811000000	2.066634994000000	-22.824025810000002	-5.040000409000000
9	3	-9.044927163000000	6.810511605000000	3.932050708000000	-25.963175880000000	7.873321552000000
10	3	-7.476816234000000	7.428406347000000	4.288792404000001	-25.930000580000000	10.976368110000001
Total	15	-8.553826736000001	5.453606819000000	1.408115226000000	-11.573933530000000	-5.533719944000000

ANOVAs

ANOVA

weight_changeW_U

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	348.862	4	87.216	6.538	.007
Within Groups	133.408	10	13.341		
Total	482.270	14			

ANOVA

weight_changeW_LTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	159.811	4	39.953	1.557	.259
Within Groups	256.575	10	25.657		
Total	416.386	14			

Multiple Comparisons

Dependent Variable: Worms_U
Dunnett t (2-sided)[a]

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
10	0	4.153761238000000	2.982256023000000	.474	-4.466390780000000
40	0	8.834239300000000*	2.982256023000000	**.044**	.214087283000000
160	0	11.468984940000000*	2.982256023000000	**.011**	2.848832921000000
400	0	-.866834995000000	2.982256023000000	.995	-9.486987013000000

Based on observed means.
The error term is Mean Square(Error) = 13.341.
*. The mean difference is significant at the .05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable: Worms_LTE
Dunnett t (2-sided)[a]

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
10	0	-4.787772505000000	4.135815167000001	.618	-16.742264400000000
40	0	-10.168210780000000	4.135815167000001	.101	-22.122702670000000
160	0	-5.281124828000000	4.135815167000001	.543	-17.235616720000000
400	0	-3.713013899000000	4.135815167000001	.782	-15.667505790000000

Based on observed means.
The error term is Mean Square(Error) = 25.657.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Reproduction results and statistical analysis

Cocoons per Adult

Descriptive

Dependent Variable	CdConcentration	Mean	Std. Error	95% Confidence Interval Lower Bound	Upper Bound
CocoonsPerAdultWormsU	0	.667	.049	.557	.777
	10	.400	.049	.290	.510
	40	.233	.049	.123	.343
	160	.100	.049	-.010	.210
	400	-2.706E-16	.049	-.110	.110
CocoonsPerAdultWormsLTE	0	.633	.056	.509	.758
	10	.667	.056	.542	.791
	40	.433	.056	.309	.558
	160	.467	.056	.342	.591
	400	.267	.056	.142	.391

Univariate analysis of variance

ANOVA

CocoonsPerAdult**WormsU**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.537	3	.179	19.515	.000
Within Groups	.073	8	.009		
Total	.610	11			

ANOVA

CocoonsPerAdult**WormsLTE**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.316	4	.079	8.464	.003
Within Groups	.093	10	.009		
Total	.409	14			

Compare to Control

Multiple Comparisons

Dependent Variable: CocoonsPerAdultWormsU

Dunnett t (2-sided)[a]

(I) CdConcentration	(J) CdConcentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
10	0	-.267*	.0699	.011	-.469	-.065
40	0	-.433*	.0699	.000	-.635	-.231
160	0	-.567*	.0699	.000	-.769	-.365
400	0	-.667*	.0699	.000	-.869	-.465

Based on observed means.
The error term is Mean Square(Error) = .007.
*. The mean difference is significant at the .05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable: CocoonsPerAdultWormsLTE

Dunnett t (2-sided)[a]

(I) CdConcentration	(J) CdConcentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
10	0	.033	.0789	.978	-.195	.261
40	0	-.200	.0789	.089	-.428	.028
160	0	-.167	.0789	.173	-.395	.061
400	0	-.367*	.0789	.003	-.595	-.139

Based on observed means.
The error term is Mean Square(Error) = .009.
*. The mean difference is significant at the .05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Juveniles per Cocoon

Descriptive

Dependent Variable	CdConcentration	Mean	Std. Error	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
JuvenilesPerCocoonWormsU	0	5.900	.352	5.116	6.684
	10	5.767	.352	4.983	6.551
	40	4.067	.352	3.283	4.851
	160	2.000	.352	1.216	2.784
	400	-8.882E-16	.352	-.784	.784
uvenilesPerCocoonWormsLTE	0	6.600	.401	5.706	7.494
	10	5.567	.401	4.673	6.460
	40	5.767	.401	4.873	6.660
	160	3.567	.401	2.673	4.460
	400	3.133	.401	2.240	4.027

Univariate analysis of variance

ANOVA

JuvenilesPerCocoonWormsU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29.953	3	9.984	21.510	.000
Within Groups	3.713	8	.464		
Total	33.667	11			

ANOVA

JuvenilesPerCocoonWormsLTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.943	4	6.736	13.955	.000
Within Groups	4.827	10	.483		
Total	31.769	14			

Compare to Control

Multiple Comparisons

Dependent Variable: JuvenilesPerCocoonWormsU

Dunnett t (2-sided)[a]

(I) CdConcentration	(J) CdConcentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
10	0	-.133	.4975	.996	-1.571	1.305
40	0	-1.833*	.4975	.014	-3.271	-.395
160	0	-3.900*	.4975	.000	-5.338	-2.462
400	0	-5.900*	.4975	.000	-7.338	-4.462

Based on observed means.
The error term is Mean Square(Error) = .371.
*. The mean difference is significant at the .05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable: JuvenilesPerCocoonWormsLTE

Dunnett t (2-sided)[a]

(I) CdConcentration	(J) CdConcentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
10	0	-1.033	.5673	.267	-2.673	.606
40	0	-.833	.5673	.431	-2.473	.806
160	0	-3.033*	.5673	.001	-4.673	-1.394
400	0	-3.467*	.5673	.000	-5.106	-1.827

Based on observed means.
The error term is Mean Square(Error) = .483.
*. The mean difference is significant at the .05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Body burden

Soil augmentation g^{-1}	WormsLTE	WormsU	Soil augmentation g^{1}	WormsLTE	WormsU
0 µg	2.73	0.85	160 µg	161.76	185.25
0 µg	1.60	1.41	160 µg	96.87	204.14
0 µg	25.36	1.22	160 µg	107.57	191.13
0 µg	32.16	0.52	160 µg	168.01	185.08
0 µg	19.60	0.43	160 µg	133.85	207.07
0 µg	22.63	0.38	160 µg	175.29	160.77
0 µg	21.43	0.67	160 µg	118.25	154.98
0 µg	4.68	1.24	160 µg	237.15	192.34
0 µg	12.71	0.65	160 µg	166.78	206.82
10 µg	41.91	34.80	400 µg	127.45	286.98
10 µg	59.97	46.15	400 µg	139.28	240.85
10 µg	51.67	32.54	400 µg	143.82	233.96
10 µg	78.72	111.78	400 µg	352.89	281.79
10 µg	72.63	63.29	400 µg	247.79	316.96
10 µg	76.46	95.16	400 µg	208.71	173.16
10 µg	64.38	21.09	400 µg	216.20	174.91
10 µg	66.00	31.95	400 µg	168.42	248.06
10 µg	23.51	25.63	400 µg	158.93	169.68
40 µg	88.55	62.18			
40 µg	115.88	52.63			
40 µg	93.14	75.83			
40 µg	127.19	75.87			
40 µg	101.63	83.15			
40 µg	94.58	89.71			
40 µg	169.63	142.15			
40 µg	93.54	112.33			
40 µg	128.16	110.01			

Multiple Comparisons

Dependent Variable: WormsU_Samples

Dunnett t (2-sided)[a]

(I) WormsU_Dunnets	(J) WormsU_Dunnets	Mean Difference (I-J)	Std. Error	Sig.
10	0	31.20443	15.65593	.167
40	0	88.49788*	14.00309	.000
160	0	190.75391*	14.43405	.000
400	0	235.44076*	14.00309	.000

Dependent Variable: WormsLTE_Samples

(I) WormsLTE_Dunnets	(J) WormsLTE_Dunnets	Mean Difference (I-J)	Std. Error	Sig.
10	0	41.80948*	15.06908	.029
40	0	94.81531*	15.06908	.000
160	0	134.06170*	15.06908	.000
400	0	158.66309*	15.50596	.000

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

ANNEXURE D

Examples of agarose-gel electrophoresis results

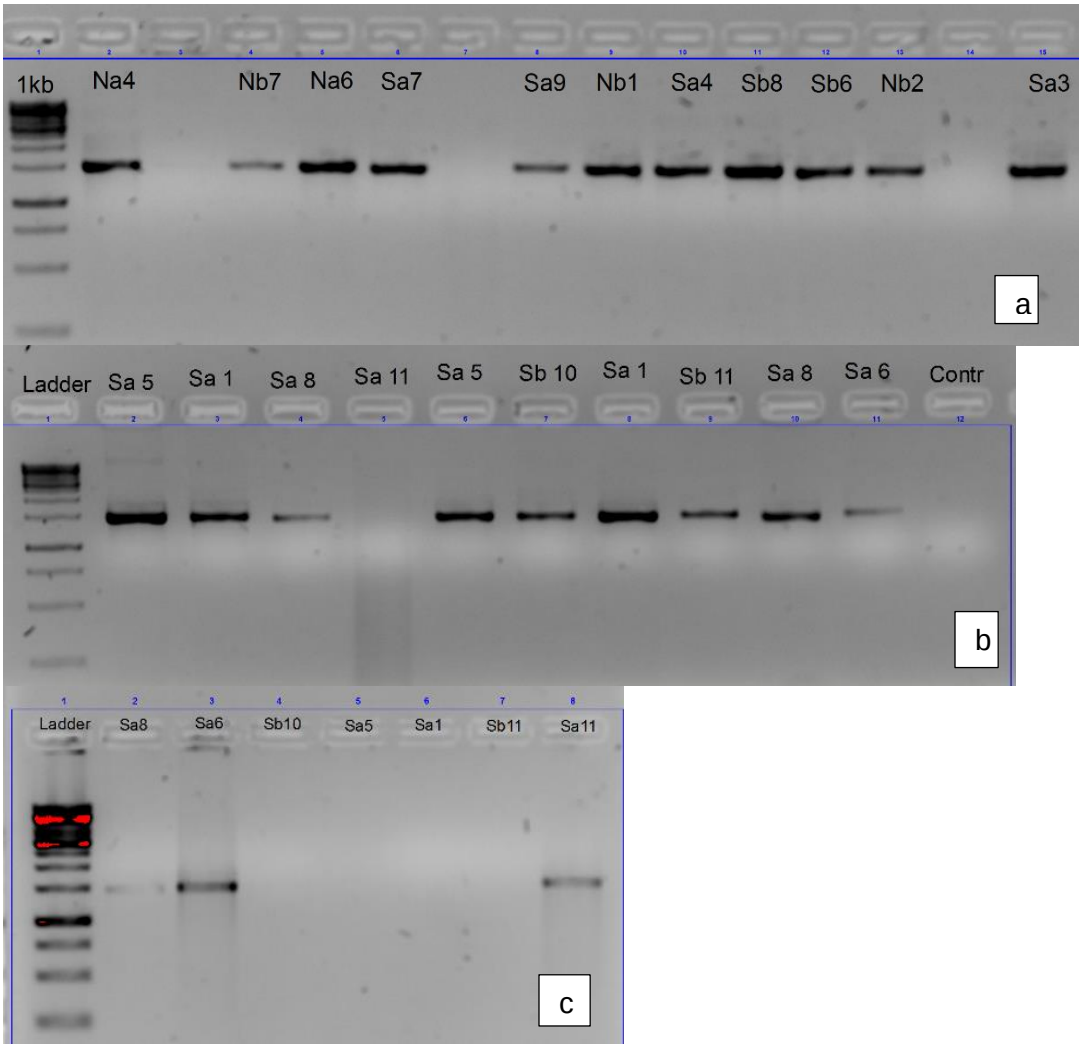

Figure 4: Examples of agarose-gel electrophoresis results as viewed with Gene Genius Bio Imaging System (Syngene, Synoptics, UK). Tracks; Sa6, Sa8 and Sa11 (from b) are examples where little or no growth was observed in LB broth and DNA had to be extracted directly from streak plate colonies (c).

ANNEXURE E

Colony morphology

Each unique colony type was given an identity code based on its morphology. Similar codes were revised. Once distinct colonies were confirmed it would be streaked on agar to ensure that it is a pure culture.

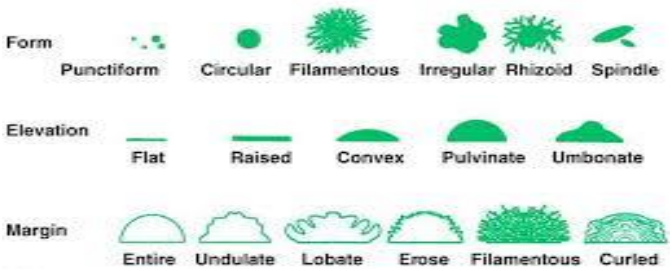

Figure 5: Colony morphology as indicated by Harley & Prescott (2001)

Table 5: Unique identifying codes given to morphologically distinct types

Characteristic	a	b	c	d	e	f	g	h
Form	Punctiform	Circular	Filamentous	Irregular	Rhizoid	Spindle		
Elivation	Flat	Raised	Convex	Pulvinate	Umbonate			
Margin	Entire	Undulate	Lobate	Erose	Filamentous	Curled		
Appearance	Shiny	Dull						
Optical properties	Opaque	Translucent	Transparent					
Pigmentation	Purple	Red	Yello	Cream	Tan	White	blue	green
Texture	Rough	Smooth						

Colony counts of distinct colony forming units (cfu) per genus

After the distinct colonies were identified by their 16S rDNA sequences, the colony counts were added and tables per genus.

Table 6: Colony counts of district colony forming units per genus on nutrient agar (±SD).

		Concentration (µg Cd(II) g^{-1} nutrient agar**)**			
Group	Genus	0	10	40	160
	Aeromonas				
$Worms_U$		1.7E8 ±6.5E7	2.3E7 ±7.6E6	0.0E0 ±0.0E0	0.0E0 ±0.0E0
$Worms_{LTE}$		1.2E7 ±5.4E6	0.0E0 ±0.0E0	0.0E0 ±0.0E0	0.0E0 ±0.0E0
	Achromobacter				
$Worms_U$		9.1E7 ±5.1E7	7.4E6 ±2.3E6	0.0E0 ±0.0E0	0.0E0 ±0.0E0
$Worms_{LTE}$		4.9E7 ±2.8E7	1.9E7 ±2.4E6	9.6E6 ±8.4E5	0.0E0 ±0.0E0
	Pseudomonas				
$Worms_U$		5.3E7 ±1.9E7	6.6E6 ±3.1E6	2.8E6 ±1.8E6	7.4E4 ±1.4E4
$Worms_{LTE}$		1.7E8 ±6.6E7	6.5E7 ±2.9E7	3.6E7 ±1.4E6	2.6E4 ±1.1E4
	Arthrobacter				
$Worms_U$		3.1E7 ±1.8E7	1.1E7 ±1.9E6	1.8E7 ±6.7E6	0.0E0 ±0.0E0
$Worms_{LTE}$		4.6E7 ±2.2E7	2.5E6 ±1.3E3	8.5E6 ±2.7E6	0.0E0 ±0.0E0
	Cupriavidus				
$Worms_U$		0.0E0	0.0E0	0.0E0	2.9E3 ±2.9E3
$Worms_{LTE}$		0.0E0	0.0E0	0.0E0	5.7E2 ±5.7E2
	Cellulomonas				
$Worms_U$		0.0E0 ±0.0E0	0.0E0 ±0.0E	0.0E0 ±0.0E0	0.0E0 ±0.0E0
$Worms_{LTE}$		0.0E0 ±0.0E0	0.0E0 ±0.0E0	0.0E0 ±0.0E0	2.9E2 ±2.9E2

Table 7: Colony counts of district colony forming units per genus on soil agar (±SD).

		Concentration groups (µg Cd(II) g^{-1} soil agar**)**				
Earthworm Group	Genus	0	10	40	160	400
	Aeromonas					
$Worms_U$		2.3E7 ±1.5E7	0.0E0	0.0E0	0.0E0	0.0E0
$Worms_{LTE}$		0.0E0	0.0E0	0.0E0	0.0E0	0.0E0
	Achromobacter					
$Worms_U$		0.0E0	0.0E0	1.2E6±3.3E5	0.0E0	4.8E3±1.9E3
$Worms_{LTE}$		0.0E0	0.0E0	0.0E0	0.0E0	2.4E3±1.3E3
	Pseudomonas					
$Worms_U$		5.6E8±2.4E8	5.3E6±1.6E6	1.6E6±3.6E5	7.3E3 ±1.8E3	0.0E0
$Worms_{LTE}$		1.6E8±8.4E7	3.4E7 ±9.3E6	2.3E7±3.8E6	6.9E2 ±3.6E2	1.5E4 ±5.5E2
	Cellulomonas					
$Worms_U$		0.0E0	0.0E0	0.0E0	0.0E0	0.0E0
$Worms_{LTE}$		3.7E7±2.4E7	0.0E0	0.0E0	0.0E0	0.0E0
	Flavobacterium					
$Worms_U$		5.8E7±2.7E7	5.7E6±7.4E5	5.1E6±1.8E6	1.1E4±3.4E3	4.8E3±1.9E3
$Worms_{LTE}$		5.7E7±3.4E7	1.7E7±6.7E6	2.4E7±2.9E6	2.0E4±4.4E3	0.0E0
	Bacillus					
$Worms_U$		1.1E7 ±5.6E6	0.0E0	0.0E0	0.0E0	0.0E0
$Worms_{LTE}$		2.3E7±1.1E7	1.9E6±1.9E6	0.0E0±0.0E0	0.0E0±0.0E0	2.4E2±1.3E1
	Agrococcus					
$Worms_U$		6.4E6±5.5E6	0.0E0	0.0E0	0.0E0	0.0E0
$Worms_{LTE}$		0.0E0	0.0E0	0.0E0	0.0E0	0.0E0
	Kaistia					
$Worms_U$		0.0E0	1.6E5±1.6E5	6.0E5±1.8E4	0.0E0	0.0E0
$Worms_{LTE}$		0.0E0	0.0E0	2.0E6±1.9E5	0.0E0	0.0E0
	Cellulosimicrobium					
$Worms_U$		0.0E0	0.0E0	0.0E0	0.0E0	0.0E0
$Worms_{LTE}$		3.2E7±1.4E7	0.0E0	0.0E0	0.0E0	0.0E0