

**ASSESSMENT OF PLATINUM MINE TAILINGS STORAGE FACILITIES:
AN ECOTOXICOLOGICAL PERSPECTIVE**

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DECLARATION

The experimental work conducted and discussed in this dissertation was carried out at the School of Environmental Sciences and Development, Zoology, North-West University, Potchefstroom Campus. This study was conducted from February 2007 to November 2008 under the supervision of Prof. Mark. S. Maboeta, co-supervision of Prof. Pieter D. Theron and assistant supervision of Dr. Sarina Claassens.

The study represents original work undertaken by the author and has not been previously submitted for degree purpose to any other university. Appropriate acknowledgements have been made in the text where the use of work conducted by other researchers has been included.



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SUMMARY

South Africa is one of the most important mining countries in the world, hosting the world's largest reserves of platinum group metals (PGMs). Even though mining is clearly an important activity in South Africa, contributing approximately US\$ 7.4 billion annually to the countries' gross domestic product (GDP), the costs to the environment are not insignificant. One of the most severe environmental aspects associated with mining is the storage of mineral waste on tailings storage facilities due to their impacts on air quality, ground water quality, aesthetics and land use. It is also unknown whether the environmental effects of tailings storage facilities increase or decrease over time. The aim of this study was to determine the ecotoxicity of platinum tailings storage facilities of different ages by means of soil physical and chemical analysis, earthworm ecotoxicological studies, dehydrogenase activity and soil mesofauna studies. Samples were obtained from three platinum tailings storage facilities of different ages of which two were already rehabilitated while the third was still operational at the time this study was performed. The latter was used as a negative control for the purpose of the study. Soil samples were physically and chemically analysed. Earthworm ecotoxicological studies were conducted to determine changes in biomass, reproduction, mortality, neutral red retention times and tissue metal concentrations. Dehydrogenase activity was determined before the introduction of earthworms and manure, after introductions of manure and after introductions of earthworms and manure. Soil mesofauna were extracted and identified in order to determine species richness, diversity, abundance and functional grouping. Soil chemical analysis indicated that concentrations of certain heavy metals, especially chrome (Cr), present in platinum tailings materials could have a potential effect on microorganisms, microbial processes and earthworms. Earthworm ecotoxicological results indicated that earthworms that bioaccumulated higher levels of heavy metals showed poor hatchability of cocoons. Dehydrogenase activity indicated that earthworms play a significant role in increasing the number and biomass of soil microbes because significant increases in dehydrogenase activity were noticed after the addition of earthworms to platinum tailings materials. Results from the earthworm ecotoxicological studies, dehydrogenase activity, and soil mesofauna composition indicated that environmental impacts of tailings storage facilities did not increase with age, but is more likely to be an indication of the rehabilitation measures administered to the different tailings storage facilities.

Keywords: *earthworms, bioassays, dehydrogenase activity, soil mesofauna, metals, biomarkers*

OPSOMMING

Suid-Afrika is een van die belangrikste mynboulande met, sover bekend, die grootste reserwes van die platinumgroep-metale (PGM) in die wêreld. Alhoewel mynbou duidelik 'n belangrike aktiwiteit in Suid-Afrika is, wat 'n bydrae van ongeveer US\$ 7.4 biljoen jaarliks tot die land se bruto binnelandse produk (BBP) lewer, is die impak van mynbou op die omgewing baie groot. Een van die meer ernstige omgewingsaspekte geassosieer met mynbou is die berging van mynslik, te wyte aan die impak daarvan op lugkwaliteit, grondwaterkwaliteit, visuele impak en landelike gebruik. Dit is onbekend of die omgewingseffekte van slikdamme verhoog of verlaag oor tyd. Die doel van hierdie studie was om die toksisiteit van platinum slikdamme van verskillende ouderdomme aan die hand van fisiese- en chemiese analyses van die substraat, asook deur erdwurm-toksisiteit, dehidrogenase-aktiwiteit en grondmesofauna-studies te bepaal. Grondmonsters was verkry van drie slikdamme van verskillende ouderdomme waarvan twee reeds gerehabiliteer was, en die derde steeds 'n operasionele slikdam was ten tye van hierdie studie. Die laasgenoemde slikdam was as 'n negatiewe kontrole vir hierdie studie gebruik. Grondmonsters was fisies en chemies geanaliseer. Erdwurm-toksisiteit was gedoen om enige veranderings in biologiese massa (biomassa), reprodusering, sterfte, neutraalrooi-retensietyd en weefsel-metaalkonsentrasies te bepaal. Dehidrogenase-aktiwiteit was bepaal voor die invoeg van erdwurms en beesmis, na die invoeg van beesmis en na die invoeg van erdwurms en beesmis. Grondmesofauna was geëkstraheer en geïdentifiseer om spesierikheid, diversiteit en funksionele groepe te bepaal. Chemiese analyses van grondmonsters het aangetoon dat konsentrasies van sekere swaar metale, veral chroom (Cr), se teenwoordigheid in platinum slikdamme die potensiaal het om mikroorganismes en mikrobiële prosesse asook erdwurms te affekteer. Biologiese ontledings van erdwurms dui aan dat erdwurms met hoër vlakke van swaar metale in hul weefsels, 'n swak uitbroedingsyfer van kokonne toon. Dehidrogenase-aktiwiteite dui aan dat erdwurms 'n belangrike rol speel in die getalstoename en biomassa van grondmikrobes omdat groot toename in dehidrogenase-aktiwiteit voorgekom het na die byvoeging van erdwurms tot platinum slikdam-materiaal. Resultate van die biomassa-studie van erdwurms, dehidrogenase-aktiwiteit en grondmesofauna-samestelling dui aan dat omgewingsimpakte van slikdamme nie verhoog het met verloop van tyd nie. Hierdie bevindinge kan heelwaarskynlik dien as aanduiding van die tipe rehabilitasiemaatreëls wat op elk van die verskillende slikdamme aangewend was.

Sleutelwoorde: erdwurms, bio-analises, dehidrogenase-aktiwiteit, grondmesofauna, metale, biomerkers

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CHAPTER 1

1. Introduction

1.1 Mining and associated problems

Africa is the producer of some of the world's most important minerals and metals including gold, platinum group metals (PGMs), diamonds, uranium, manganese, chromium, nickel, bauxite and cobalt (Mbendi Information Services, 2008). The continent also hosts approximately 30% of the world's mineral reserves including 40% of the world's gold, 60% of the world's cobalt and 90% of the world's PGM reserves. South Africa is one of the most important mining countries in Africa and in the world, hosting the world's largest reserves of chrome, gold, vanadium, manganese and PGMs, while also being the largest producer and supplier of PGMs in the world, supplying 56.7% of the total world supply of PGMs in 2005 (DME, 2007; Mbendi Information Services, 2008). South Africa also has a known reserve base of 87.7% of the world reserve of PGMs (DME, 2007). Platinum group metals include platinum (Pt), palladium (Pd), iridium (Ir), osmium (Os), rhodium (Rh) and ruthenium (Ru) and have a variety of uses e.g. in the manufacturing of jewellery, in industrial applications like high technology and in the electrical, chemical and petroleum refining industries (DME, 2007). The main consumer of PGMs is the automotive industry where platinum and palladium are used as oxidation catalysts in catalytic converters. Anglo American Platinum (Anglo Platinum) is the largest producer of platinum and palladium in South Africa, followed by Impala Platinum (Implats), Lonmin Platinum (Lonplats), Northam Platinum and Aquarius Platinum (Mbendi Information Services, 2008). Even though mining is clearly an important activity in South Africa, contributing approximately US\$ 7.4 billion annually to the country's gross domestic product (GDP) (Mbendi Information Services, 2008), the costs to the environment are not insignificant. These include environmental impacts such as water pollution, air pollution, soil pollution and generation of domestic and hazardous wastes. These impacts are caused by activities such as de-watering of aquifers due to shaft and opencast mining, dust from roads, milling activities, blow-off from tailings storage facilities, sulphur dioxide and other gaseous emissions during processing of the metals, hydrocarbon spills, soil erosion and soil quality depletion caused by removal and storage of top soils.

One of the most severe environmental aspects associated with mining is the storage of mineral waste and the impacts it may have on air quality, ground water quality, aesthetics and land use at tailings storage facilities. Tailings is a mineral waste stream that is derived from the concentration of ore bearing minerals into a concentrated product. It is transported to the tailings storage facility in the form of slurry, thus depositing a proportional amount of water in addition to the mineral waste. It is unknown whether the environmental effects of tailings storage facilities increase or decrease

over time. This is especially relevant when determining the financial liabilities of closure of tailings storage facilities on mining companies in South Africa. In order to assess the possible environmental and human risks of tailings storage facilities over time, as well as the risk of ecological receptors associated with contaminated soils, an ecological risk assessment is required. This process usually consists of a tiered framework whereby sites are firstly screened for contaminants where after pathways to humans or wildlife are identified (Hankard *et al.*, 2005). Ecological risk assessments have been used in the United States (Stavola & Craven, 1992), the U.K. (Greig-Smith, 1992), and the Federal Republic of Germany (Kokta & Rothert, 1992) to determine the effects of pesticides on the environment. The first phase of the ecological risk assessment consisted of screening potential contaminants by means of soil physical and chemical analyses. Soil chemical analyses have been used in traditional risk assessment of contaminated soils and are used to calculate potential ecological risks. However this method does not take biological availability into account. Biological availability of elements is not only dependent on the availability of elements in the soil solution, but on the entry, action mode, metabolism, storage and excretion of elements by organisms (Landis & Yu, 1995). Biological availability of chemical compounds is a major factor in the toxicity of environmental samples (Umbreit *et al.*, 1988). The bioavailable fraction of a chemical compound is a more relevant parameter than the chemically extractable fraction from an environmental point of view (Verstraete & Mertens, 2004).

For the latter part of the ecological risk assessment, ecotoxicological analyses are recommended (Stephenson *et al.*, 2002; Lobiner *et al.*, 2003). Butler (1978) described ecotoxicology as the study of toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems including transfer pathways of those agents and their interactions with the environment. In the U.S, soil contamination studies have increasingly relied upon biological tests for developing site-specific human health and ecological risk assessments (Callahan & Linder, 1992). These sites usually contain a mixture of contaminants and require a toxicity based approach instead of a chemically based approach in order to determine the potential environmental impact related with a mixture of contaminants (Callahan & Linder, 1992). Chemically based approaches are dependent on extensive chemical analysis of environmental samples, whereas toxicity based approaches integrate biological information into the assessment process (Pankhurst *et al.*, 1995). There are a variety of methodologies that can be used to conduct an ecotoxicological study, for example, the use of vegetation as an indicator of toxicological stress, the use of predatory birds as indicators of the effects of bioaccumulation of contaminants, and a variety of field based studies.

The second phase of the risk assessment for this study, includes an acute toxicity test using bioassays, which is the most relevant methodology used for testing in the laboratory (Kula, 1998). This methodology can be used instead of field based studies, firstly because acute toxicity tests and bioassays determine the level of concentration of a xenobiotic which produces harmful responses on the test organism during continuous short term exposure (Reinecke, 1992). Secondly, field studies are time-consuming and habitats in South Africa are not always suitable for these tests because of exposure of organisms to harsh weather conditions. Laboratory evaluations tend to correspond with field test results for mortality and uptake of contaminants (Callahan & Linder, 1992), therefore it is not necessary to carry out field tests to obtain objective results. Thirdly, bioassays are more sensitive than chemical analysis as they determine the contaminant bioavailability as well as the associated adverse biological and toxic effects and they integrate the effects of the total concentrations of contaminants and chemical mixtures (Callahan & Linder, 1992; Fernández *et al.*, 2005). Lastly, they can be directly related to toxic effects of contamination for soil fauna (Callahan & Linder, 1992). Biological effects such as reduced growth, reduced reproduction, morbidity and death are associated with exposure to a mixture of contaminants (Callahan & Linder, 1992). Sub-lethal endpoints are selected according to the level of exposure of organisms to the impacts of pollutants. This exposure occurs at different levels of functional complexity when organisms are exposed to toxic stress and this becomes apparent at the molecular, cellular and physiological levels (Moore & Halton, 1977; Moore, 1985). This then leads to effects on survival, growth, and reproduction, which all have an effect on the population level (Crommentuijn *et al.*, 1995).

1.2 Soil physical and chemical properties and their use in ecotoxicological studies

Based on a previous study (Wahl, 2007), the contaminant most likely to be present in the tailings material is heavy metals as tailings material consists of concentrated mineral waste. Heavy metals are those elements with a molecular weight of more than 55 on the periodic table such as manganese (Mn) and iron (Fe) (Coyne, 1999). However those that are of environmental concern are chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), technetium (Tc), cadmium, (Cd), mercury (Hg), and lead (Pb). It should be taken into account that although Zn, Cu, Mo and Fe are micro-elements required for plant growth, quantities in excess of these trace amounts can lead to toxicity. Heavy metals known to be persistent in platinum tailings materials are Cr, Ni, Cu, Al, Zn, Pb, Mn and Fe (Wahl, 2007).

The bioavailability of contaminants is dependent on soil physical and chemical properties such as pH, organic matter content and texture (Fent, 2004). Soil chemical analysis can provide an indication of the concentrations of heavy metals in the tailings substrate as well as their potential

levels of toxicity. According to van Breemen (2004), pH is one of the most important soil properties for plants. This is because the solubility of many elements (both toxic and non-toxic) depends on pH, e.g. the solubility of many metal ions like Al increases with decreasing pH resulting in toxicity of this element at a pH of less than 3.5. Metal deficiencies can occur in alkaline soils, e.g. Fe and Zn. Molybdenum, on the other hand, increases with pH (van Breemen, 2004). Studies conducted on the toxicity of Cu have determined that the bioavailability of Cu is not dependent on pH, but on soil properties like soil organic matter content (Jäggy & Streit, 1982; Bengtsson *et al.*, 1986; van Gestel, 1992) and cation exchange capacity (CEC) (van Gestel, 1992). However studies conducted by Ma (1982) indicated that pH may have an effect on the bioavailability of Cu when it is the only variable. Bioavailability of Cd, Pb and Zn are dependent on soil pH and CEC, which influences the uptake of these metals by earthworms (Ma, 1982; Bengtsson *et al.*, 1986). The uptake of Pb is also dependent on soil organic matter (van Gestel, 1992). In aerobic soils at a low pH, metal levels will be high in pore water, whereas in anaerobic soils at high pH, metals will be sorbed or precipitated (Peijnenburg, 2004). The latter soils will, however, pose no threat to organisms exposed to pore water. Studies conducted by Fordham & Wilber (1992) on sewerage sludge indicated that mortality of earthworms was significantly higher in soil with lower pH and the bioavailability of metals was also higher in this soil type. Metal mobility and bioavailability increase inversely with pH and thus toxicity also increases (Fordham & Wilber, 1992).

Soil texture is an important factor when assessing the bioavailability of metals, because it determines the percentage of clay minerals available for forming colloidal particles (Ashman & Puri, 2002). Colloidal particles are made up of a variety of minerals, however, clay minerals are the most important mineral colloids found in soils world wide. Colloidal particles play an important role in the exchanging of elements by forming charges which attract elements of opposite charge. Soils with high concentrations of colloidal particles have the capacity to replace cations in the soil solution when they are removed from the soil, this is known as the buffer capacity of the soil (Ashman & Puri, 2002). Well-buffered soils maintain the concentration of nutrients in soil solution and thus maintain the bioavailable fraction. Therefore, soil texture is important when assessing bioavailability of metals and the ability of soils to hold and replace metals in solution. Clay and silt are known to hold higher percentages of heavy metals than sand soils due to the ability to adsorb a higher amount of elements.

In metal contaminated soils, direct chemical methods provide data by measuring the total and soluble-fraction concentrations (Morgan *et al.*, 1992). Toxicity of pesticides is determined by the soil-pore-water concentration (van Gestel & Ma, 1990). This information can, however, not provide accurate estimates of the bioavailability of heavy metal components (Morgan *et al.*, 1992). Total

heavy metal concentrations, measured in the soil substrate by means of chemical analysis, indicate the sum of the exchangeable and the soluble portion of the heavy metals on the exchange sites of soil colloids and in the soil pore water, respectively. The soluble metal concentrations measured during soil chemical analysis indicates the metals available in solution for uptake by plants and organisms in the soil. The soluble metal concentrations measured in soil do not necessarily indicate the concentrations that will be accumulated by organisms. Chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicological consequences in biota (Fent, 2004). It is thus important that ecotoxicological tests are conducted on biota in order to determine this.

1.3 Earthworms and their use in ecotoxicological studies

Earthworms belong to the phylum Annelida, subphylum Clitellata and class Oligochaeta. They are divided into general categories, based on basic features such as burrowing abilities, food preferences and body colour, shape and size (Bouché, 1977; Lee, 1985; Curry, 1994). Earthworms are the most commonly used test organism for toxicity tests conducted on soil contaminants. This is because they are cosmopolitan, common in many soils, are vulnerable to impacts on soil and are good indicators of land use and soil fertility (Reinecke, 1992; Paoletti, 1999). Earthworms are therefore the obvious choice for ecotoxicological testing in terrestrial habitats (Becker, 1992). Earthworms also play a beneficial role in promoting soil fertility, are a source of food for other organisms, are convenient representatives for soil fauna, and are important due to their relevance in soil structure and nutrient cycling (Greig-Smith, 1991). Their functions include incorporation of decaying matter into the soil, improving aeration, drainage and moisture holding capacity (Reinecke, 1992). They are also recognised as the most important soil faunal component, not only because they are involved in the regulation of organic matter decomposition and nutrient cycling, but also because they change the diversity and structure of soil microbiota (Enami *et al.*, 2001). This is due to their relatively large size, their behaviour and high biomass. They are also more sensitive than other soil fauna and will thus provide an indication of safety for other fauna (Greig-Smith, 1991). They are also suitable for laboratory testing as they are easy to handle, suitable for captive breeding, readily available and can be used as a bioindicator and test organism for chemicals in the soil environment (Greig-Smith, 1991; Reinecke, 1992).

Biological indicators of stress should be sensitive, stable, show variability in response to different pollutants, and be consistent in response to non-pollutant environmental changes (Venables *et al.*, 1992). Test organisms must be well suited to laboratory use, easily cultured in the laboratory and provide a reliable prediction of the hazard (Bouché, 1992). It is for this reason that *Eisenia fetida* is the standard species used for ecotoxicological testing with earthworms even though they are not the most sensitive earthworm species (Edwards & Coulson, 1992; Spurgeon & Hopkin, 1996; Kula

& Larink, 1997). *Eisenia fetida* belong to the Epigéal group of earthworms which are surface-active, pigmented, non-burrowing, dwell in litter and have a short generation time and have several hatchlings per cocoon (Bouché, 1977; Lee, 1985; Curry, 1994). Bouché (1992) recommended that *Eisenia fetida* remain the standard laboratory species for initial laboratory screening, i.e. in Organization for Economic Co-operation and Development (OECD) guideline 207 (1984). *Eisenia fetida* are suitable laboratory test species because of their high reproductive potential, ease of captive breeding, convenience for testing and are easily available (Greig-Smith, 1991; Kokta & Rothert, 1992; Reinecke *et al.*, 2001). Substantial toxicity tests have been conducted on *Eisenia fetida* resulting in an assortment of data available for comparison (Bouché, 1992; Reinecke *et al.*, 2001). *Eisenia fetida* are the least sensitive earthworm species when compared with that of other earthworm species and can thus endure high levels of contaminants, which is a property that must be taken into consideration when using them for environmental toxicity studies (Bouché, 1992; Kula & Larink, 1997; Paoletti, 1999).

Mortality is the main endpoint in acute toxicity tests using *Eisenia fetida* as a test organism (OECD, 1984). A good correlation exists between laboratory data on mortality of *Eisenia fetida* and effects in the field (Kokta, 1992). Growth is a sub-lethal endpoint of an acute toxicity test, however it is not the most sensitive parameter compared to reproduction (Neuhauser & Callahan, 1990). Growth of worms provides information about the sub-lethal sensitivities of the worms (Reinecke, 1992). Reproduction and growth disturbances are the sub-lethal effects that are most likely to best mediate population effects and are also sensitive endpoints that have been identified in earthworm ecotoxicology (Kokta, 1992; Maboeta *et al.*, 1999). Reproduction is also an important factor of ecotoxicological assessment due to its influence on population dynamics (Moriarty, 1983; Spurgeon *et al.*, 1994). Cocoon production, hatching rate and survival of juveniles are parameters that can be investigated in order to indicate levels of stress on *Eisenia fetida* (Kokta, 1992) (Figure 1).

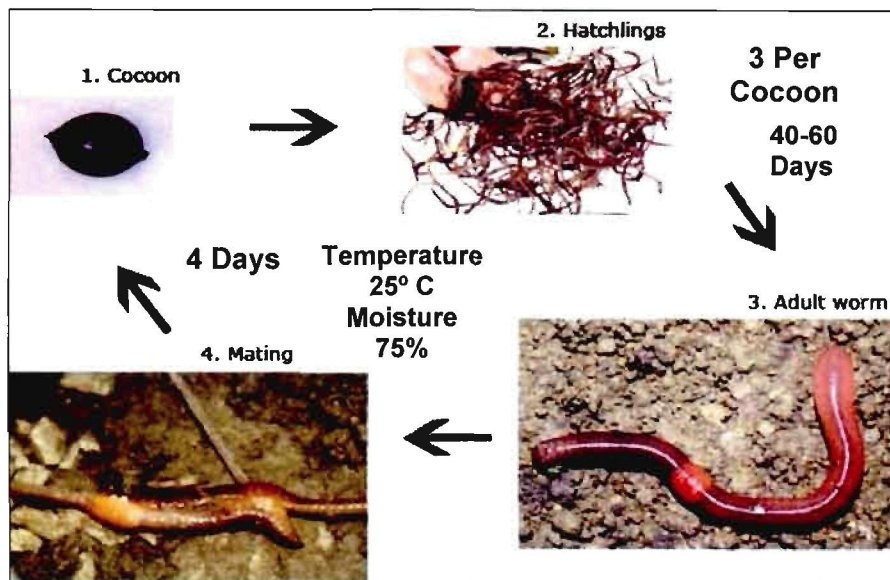


Figure 1. Life cycle of *Eisenia fetida* (Adapted from Venter & Reinecke, 1988; University of Central Lancashire, 2008)

The use of biomarkers in ecological surveys is one way to verify the biological availability and presence of relevant concentrations in biota (Bucheli & Fent, 1996). Biomarkers are used as an early warning system for the detection of effects in organisms and are related to a group of contaminants as well as single compounds (Peekall, 1992). They are indicators of toxic effects as they reflect either an individual biological reaction or reactions on the cellular, molecular or genetic level (Cortet *et al.*, 1999). Biomarkers are the potential solution to the limitations of the classical environmental toxicological approach in which the residue of a chemical was measured either in the organism or the environment and relating that to the acute effects (Peekall, 1994). Van Gestel & Van Brummelen (1996) recommended that the use of biomarkers deserves a place in modern ecotoxicology.

Earthworms play an important role as *in situ* indicators for assessing biological risks from toxic components in terrestrial environments (Morgan & Morgan, 1988; Weeks & Svendsen, 1996). Lysosomal membrane stability is one biomarker that was amended for earthworms (Weeks & Svendsen, 1996). It is an integrative, nonspecific biomarker and will thus be affected by a combination of pollutants present (Huggett *et al.*, 1992). When organisms are exposed to xenobiotics, one of the characteristic pathological alterations is the decreased integrity of the lysosomal membrane (Moore, 1988). This is also true when organisms are exposed to nonspecific abiotic and biotic stress (Moore, 1985). Although the mechanisms compromising the integrity of membrane stability is unknown, Mayer *et al.* (1992) has suggested that it may have something to do with the direct effects of chemicals on the membrane or increased frequency of secondary

lysosomes in toxicant-stressed cells. The lysosomal membrane stability measures the effect of toxic substances on the membrane stability of lysosomes *in vitro*, by means of the use of a neutral red dye, which penetrates the cell membranes by passive diffusion, accumulating intracellularly in the lysosomes (Koenig, 1962; Allison & Young, 1969; Nemes *et al.*, 1979). Lysosomes in healthy cells retain the cationic dye after the initial uptake while lysosomes in unhealthy cells in which the integrity of the lysosomal membrane has been impaired, will leak neutral red dye from the cell (Svendsen *et al.*, 2004). Neutral red dye diffuses out of the lysosomes and stains the cytosol at a time period relating to the degree of damage (Moore, 1985). When the cell is exposed to increasing lysosomal membrane damage, decreasing times for reddening of the cell cytosol will be measured (Svendsen *et al.*, 2004).

Two techniques were traditionally used prior to the development of the neutral red retention assay. These were the cytochemical technique (Moore, 1976) and the biochemical technique (Baccino & Zurretti, 1975). These techniques were based on the measurement of the amount of lysosomal enzyme that leaks through the lysosomal membrane after pH or hypo-osmotic shock was administered (Svendsen *et al.*, 2004). The neutral red retention assay is much simpler than both of these techniques (Svendsen *et al.*, 2004) and was developed as an *in vitro* cytotoxicity assay, which was based on the use of mammalian cells in culture (Babich & Borenfreund, 1990). This methodology was further developed by Lowe *et al.* (1992), while working with cells isolated from organisms *in vivo*. An assay was further developed that quantified the process of leakage of the dye by lysosomes under *in vitro* conditions (Lowe & Pipe, 1994). Further modifications implemented by Svendsen & Weeks (1995) and Weeks & Svendsen (1996), allowed the neutral red retention assay to be used with freshwater and terrestrial invertebrates. This technique has minimum treatment stress on cells due to the manner in which sample preparation and observation is completed under physiologically relevant *in vitro* conditions. It also has an increased time resolution and therefore has a higher sensitivity (Svendsen & Weeks, 1995; Weeks & Svendsen, 1996).

The neutral red retention time assay (NRR-T) measures changes in fragility of earthworm coelomocyte lysosome membranes following exposure to stress (Moore, 1988). NRR-T is a general assay in that it is indicative of the effects of more than one detrimental factor (Reinecke & Reinecke, 2007). NRR-T has already been established as pollutant responsive in earthworms exposed to metals and certain organic contaminants in a laboratory study (Hankard *et al.*, 2004). The use of the NRR-T assay has proven to be reliable, dose-related and practical (Svendsen *et al.*, 1996) to use in marine (Lowe *et al.*, 1992; Lowe & Pipe, 1994), freshwater (Svendsen & Weeks, 1995) and terrestrial systems (Weeks & Williams, 1996). The major benefit of using the NRR-T assay is that the results obtained reflect the effects of the toxicants experienced by the biota, thus

avoiding problems associated with the assessment of bioavailability (Svendsen *et al.*, 1996). Another benefit of the NRR-T assay is its responsiveness to a wide range of chemicals (Svendsen *et al.*, 2004). NRR-T is a widely used assay and has been used in a variety of toxicity tests.

This assay however doesn't go without criticism. Dimethyl sulfoxide (DMSO) and neutral-red are chemicals used to make up the base solution for the NRR-T tests. These chemicals are potentially cytotoxic compounds, however, the concentrations used in this assay have been successfully used in a variety of studies, without causing damage to lysosomal membranes (Svendsen *et al.*, 1996). Another drawback of this assay is that several types of coelomocytes are found in the coelomic fluid of earthworms (Stein *et al.*, 1977). Therefore, only the coelomocytes with a pronounced complex of pseudopodia are likely to fix themselves on the microscope slide thus having a better chance to be observed (Svendsen *et al.*, 1996). Coelomocytes with a poor complex of pseudopodia may not adhere to the slide and may possibly be missed during the observation. These coelomocytes also constitute the majority of the coelomocytes in the coelomic fluid and thus provide biased results as only those with profound pseudopodia are possibly counted (Stein *et al.*, 1977). The possible acclimation of earthworms as a result of long-term exposure and the possible selection of tolerant populations due to environmental pollutants exerting selective pressure on natural populations could bias the results from the NRR-T assays (Van Straalen, 1993; Reinecke *et al.*, 1999).

In order for the abovementioned studies to provide an early warning about the risk of ecological effects occurring on a long term basis, clear links of ecotoxicological significance between biomarkers and ecological endpoints like lethality, reproductivity and growth impairment need to be established (Svendsen & Weeks, 1997). These results can be used to extrapolate data from laboratory tests and microcosm studies to field situations (Reinecke, 1992). Determination of the metals bioaccumulated in the tissues of earthworms will also provide an indication of the cause of the lethal and sub-lethal effects experienced. Tissue metal analysis of organisms exposed to contaminants is another parameter that can produce vital information during an ecotoxicological study. Contaminants like persistent organic pollutants (POPs), heavy metals and pesticides can be accumulated via food uptake (van den Brink, 2004). Earthworms and other soil organisms accumulate organic contaminants which possibly results in elevated levels within the body (Ma *et al.*, 1998). Heavy metals have a high affinity for organs with high blood flow, like the kidneys, hair in mammals or bird feathers and bird eggs have been used as indicators in heavy metal studies (Denneman & Douben, 1993; Ma, 1994; Nolet, 1994).

Accumulated concentrations of metals in tissues of suitable monitoring organisms is an additional method in determining the relative degrees of metal pollution (Morgan *et al.*, 1992). Earthworms are used for such studies because they are efficient accumulators of soil metals and a proportion of the total soil metals are accumulated by them depending on a number of edaphic factors such as moisture content, pH and availability of nutrients (Morgan *et al.*, 1992). Earthworms are valuable indicators for investigating ecological effects of contaminants because they are sensitive to many chemicals, easily maintained test organisms and can be used to investigate potential food web effects due to their ability to bioaccumulate contaminants (Fordham & Wilber, 1992). Earthworms are exposed to a variety of pollutants, either singly or in combination, in polluted soil environments, through bodily contact and by ingestion of contaminated soil or food (Kokta & Rothert, 1992; Reinecke & Reinecke, 2003). These pollutants are absorbed and accumulated at different rates due to the different pathways of metabolism, excretion, and detoxification (Morgan & Morgan, 1989a&b). Earthworms tend to concentrate heavy metals in their body tissue and even though they may not be harmed by these heavy metals, it may affect organisms higher up in the food chain (Carter *et al.*, 1983; Reinecke, 1992). Animals feeding on invertebrates can thus be contaminated by these metals. From 1979 -1981, several thousand wildfowl, waders and gulls died from alkyl lead poisoning after consuming contaminated invertebrates (Bull *et al.*, 1983).

Toxicity of chemicals on organisms is determined by the critical concentration in their tissue (van Gestel, 1992). The persistence and toxicity of contaminants is determined by the bioavailability and bioaccessibility of the toxicants (Peijnenburg, 2004). Bioavailability is "the fraction of the total amount of a chemical present in soil that, within a given time span, is either available or can be made available for uptake by microorganisms or plants, from either the organisms' direct surrounding or the plant, or by ingestion of food" (Peijnenburg, 2004). This author continues by saying that the bioaccessible fraction "is the fraction of the total amount of a chemical present in ingested soil particles that, at maximum, can be released during digestion". A factor that affects bioaccessibility is pH in the different compartments of the gastrointestinal tract. In larger animals, the bioaccessibility of ionizable contaminants and metals present in ingested soil is more than in soil-dwelling invertebrates (such as earthworms) who regulate gut pH at neutral (Peijnenburg, 2004). Uptake of metals is driven by fugacity difference between water and the organism. The free metal ion is capable of passing through biological membranes and speciation of metals determines the free metal ion activity. The free metal ion concentration provides a better indication of availability and toxicity than total or dissolved concentrations (Sunda & Guillard, 1976; DiToro *et al.*, 2001).

1.4 Microbial analysis and its use in ecotoxicological studies

Enzyme activities are used as indicators of ecosystem stress as they integrate information from microbial status and from soil physico-chemical conditions (Aon *et al.*, 2001). They are mainly produced by soil microorganisms and plants and drive the physiology and metabolism of soil microorganisms as well as create microbial habitats in soil (Coyne, 1999; Aon & Colaneri, 2001). High levels of microbial activity are fundamental in maintaining soil quality and enzyme activity is a major factor contributing to overall soil microbial activity (Visser & Parkinson, 1992; Garcia *et al.*, 2002). Environmental research into enzymes plays a role in understanding the state and behaviour of cellular and extracellular enzymes in the environment and applies this knowledge in environmental and agricultural studies (Coyne, 1999). Soil management practices like additions of organic matter increases the microbial community. This can be explained by the feeding habits of soil microbes and their ability to biochemically degrade organic matter (Aira *et al.*, 2002). Earthworms drive this process by conditioning the substrate and altering biological activity (Aira *et al.*, 2002). Earthworms are thus key in increasing microbial activity in soils which in turn increases the enzymatic activity.

Enzymes found in soils are either extracellular or cellular (Skujins, 1976; Burns, 1982; Nannipieri *et al.*, 1983; Dick, 1994; Sinsabaugh, 1994). Cellular enzymes are associated with proliferating cells and extracellular enzymes are those that are released into the environment by secretion or lysis, as well as active enzymes from dead cells and other non-living soil components (Dick, 1994; Sinsabaugh, 1994). Table 1 indicates enzymes found in soils as well as their functionality as indicators of soil health. Enzymes play a role as organic protein catalysts that transform inorganic and organic substances without being changed (Coyne, 1999). Enzyme activity is also a measure of functional diversity of soil microbes and can be used to determine the diversity of enzyme patterns in soil extracts (Verstraete & Mertens, 2004).

Enzymes such as dehydrogenase are used as indices for the effects of contaminants on microbial activity (Hattori, 1992; Chander & Brookes, 1993; Dick, 1994). It is an intracellular enzyme that is correlated with microbial biomass and this activity is an indirect parameter of microbial biomass measurement (von Mersi & Schinner, 1991). Dehydrogenase activity measures intracellular catalysis and is correlated with the activity of extant cells (Dick, 1997). It is also considered to be an accurate measure of microbial oxidative activity of the soil and should thus have a direct relationship to total viable microorganisms (Taylor *et al.*, 2002).

Table 1. Soil enzymes as indicators of soil health (Verstraete & Mertens, 2004).

Soil Enzyme	Enzyme Reaction	Indicator of
Dehydrogenase	Electrol transport system	Microbial activity
Beta-glucosidase	Cellobiose hydrolysis	C-cycling
Cellulase	Cellulose hydrolysis	C-cycling
Phenol oxidase	Lignin hydrolysis	C-cycling
Urease	Urea hydrolysis	N-cycling
Amidase	N-mineralization	N- cycling
Phosphatase	Release of PO_4^{3-}	P-cycling
Arylsulphatase	Release of SO_4^{2-}	S-cycling

The enzyme reaction of dehydrogenase takes place in the electrol transport system and the main localization of this enzyme is in the plasma membrane of bacteria or in mitochondrial membranes of fungi (Dick *et al.*, 1996; Aon & Colaneri, 2001).

1.5 Soil mesofauna and their use in ecotoxicological studies

In the past, soil ecotoxicology focused on the adverse effects of soil contaminants on single individuals or populations. However, this discipline now focuses on the assessment of contaminants on the level of community structure and ecosystem functioning (Baird *et al.*, 2001). Soil can be defined as a living system in which plants (roots), microorganisms and soil fauna interact with each other and with the abiotic environment (Verhoef, 2004). Soil quality should be linked to sustained functioning of soil communities and processes and should include equilibrium and stability of these systems (Verhoef, 2004). Equilibrium includes the balance between synthesis and breakdown of organic matter, enrichment of soil organic matter or its depletion and the balance between increase and decrease of species to conserve diversity and ecological functions and stability which is the recovery of soil communities and processes after disturbance (Verhoef, 2004).

The upper ten centimeters of soil is called the living system due to most of the biological activity occurring in this layer. This occurs due to the heterogeneous nature of the soil, both vertically and horizontally, the variety in habitat scale and environmental conditions as well as the difference in ecological feeding strategies of the soil mesofauna, which include saprophagous (consume dead organic matter), phytophagous (consuming living plant and root material), predators (consuming living animals) and scavengers (consuming dead plant and animal matter) (Hickman *et al.*, 1997; Eijssackers, 2004). Predation amongst soil fauna affects soil processes, for example, predation on microbial-feeding nematodes effects mineralization and primary productivity. Microorganisms play a crucial role in recycling nutrients from plant and animal residues back into the soil where they are

available for plant uptake (Ashman & Puri, 2002). Therefore, if these microbes are preyed upon by microbial-feeding nematodes, this process is affected. Predation on Collembola results in an increase in fungal biomass, a reduction in litter mass loss and enhanced C-mineralization (Hedlund & Öhrn, 2000; Lawrence & Wise, 2000; Verhoef *et al.*, in press). This is because Collembola feed on fungi, organic matter and bacteria involved in C-mineralization. Predation on fungal feeding and detritivorous mesofauna has no effect on plant growth (Laakso & Setälä, 1999).

Soils have been found to function, in terms of providing sufficient nutrients to sustain vegetation, even though they have poor soil organism diversity. This can be explained by the redundancy of most soil organisms in species diverse soils. Type of soil, vegetation, moisture, presence of competitors, organic matter content of soil, soil texture and soil acidity are parameters determining species abundance in habitats (Anderson, 1977; David *et al.*, 1999; Ponge *et al.*, 2003). Studies conducted by Andrés & Mateos (2006) confirm that the richness of taxa is indicative of the maturity of the vegetal community. Although species abundance is an important indicator, species diversity plays an important role in the sustainable functioning of faunal communities. Soil mesofaunal diversity is also responsible for above ground ecosystem function due to their catalyzing role in cycling of essential soil elements, vegetation diversity and succession (van Straalen, 2004). Soil faunal biodiversity is also essential to sustain functionality in the community during catastrophic events e.g. during drought periods. Species performing similar functions in the community are essential and should one species diminish, another species performing a similar function in the same community will continue to carry out the function in that community. After a catastrophic event, recovery of the invertebrate community takes a minimum of fifteen years while it can take as long as eighty to one-hundred-and-two years for the recovery of the forest Collembolan community (Neumann, 1991; Webb, 1994; Addison *et al.*, 2003). The reason why restoration of the soil mesofaunal communities takes so long is based on the fact that the surrounding soil mesofaunal communities has to immigrate to the disturbed site. Collembola and Trombididae (Prostigmatic mites) establish within the first fifteen months (Wanner & Dunger, 2002). The maximal mesofaunal density is reached over a period of two to three years, where after a reduction in density levels occurs for the next ten years (Koehler, 1998).

Biodiversity is such an ecosystem parameter which is characterized by indicators of structural and functional diversity (Eijsackers, 2004). The *Shannon-Weaver index* (Shannon & Weaver, 1949) was the first index to express the diversity of a community. This index takes into account the species richness and the relative contribution of each species to the total number of organisms present (Verstraete & Mertens, 2004). The exclusion approach has been used to evaluate the importance of specific groups for a specific soil process. Studies conducted by Faber & Verhoef (1991)

determined that Collembola played the most important role in N-mineralization out of the entire fauna group. Species identity has shown to be more important over species abundance (Mikola *et al.*, 2002), i.e. the type and diversity of species is more important over the abundance of species found in soil. When a diversity of species is available in the soil, there is also a diversity of functions performed by them, whereas when there is a large number of one type of species that only performs one function in the soil, there is a void of other functions performed and the community structure can fail. Wardle (2002) proposed that the main biotic controls of soil ecosystem functioning are most likely the key traits of dominant species, community composition of component organisms and nature of biotic interactions among organisms.

Soil fauna influence decomposition by modifying the biomass, composition and activity of soil microbial communities or by transforming organic matter (Verhoef, 2004). Protists, nematodes and enchytraeids are the process drivers of N-mineralization and therefore contribute most of the 35% to 37% of the total net N-mineralization attributed to soil fauna. Apart from their important roles in the soil faunal community, soil micro-arthropods can also be used as indicators of human impact on ecosystems and their reactions to increased pollutant concentrations have been used to determine critical levels of soil pollution in the laboratory (Hopkin 1989; Denneman & van Straalen 1991; Zaitsev & van Straalen, 2001). Effects of soil contamination can be determined in the field by means of species composition, relative abundance and biodiversity of soil invertebrate communities (Zaitsev & van Straalen, 2001). In contaminated soils, the biodiversity of a community is altered due to the toxicological processes. These processes include the uptake of the toxicant, sequestration, biotransformation and excretion. Community changes occur due to differential sensitivities of species to contaminants and priorities given to vital life processes by organisms such as feeding above reproduction (van Straalen, 2004).

According to Verhoef (2004), the most abundant organisms in European soils are protozoa (1×10^9 individuals per thousand cm^3), followed by nematodes (3×10^4 individuals per thousand cm^3), mites (Acarina) (2×10^3 per thousand cm^3), springtails (Collembola) (1×10^3 per thousand cm^3), rotifers (5×10^2 per thousand cm^3), arthropods (insects, millipedes and spiders) (1×10^2 per thousand cm^3), potworms (50 per thousand cm^3) and earthworms (2 per thousand cm^3). Soil mesofauna constitute soil faunal organisms between 0.2 and 10mm in size (Ashman & Puri, 2002). This group includes the nematodes, rotifers, springtails, mites and other small arthropods. Therefore the most abundant group of fauna found in soil is the soil mesofauna. Soil mesofauna numbers are dependent on the time of the year, the nutrient content of microbial resources, the number of grazers and the stage of degradation of litter (Hanlon & Anderson, 1979; Hanlon, 1981; Teuben, 1991; van Wensem, *et al.*, 1993). According to studies conducted by Wahl (2007), the most prominent groups of soil

mesofauna found in platinum tailings materials were Prostigmatic mites and Collembola, which belong to the phylum Arthropoda.

Van Straalen (2004) suggested that arthropods are by far the most species-rich group of invertebrates and that species richness of microarthropods are one of the best bioindicators used to date, along with ecological qualification of earthworms. Magurran (1988) indicated that the degree of disturbance in soil microarthropod communities can be measured by means of total density, species diversity, constancy of certain species and biodiversity indices and abundance distributions. Soil arthropods are considered to be efficient indicators of ecosystem maturity and have been used for rehabilitation monitoring on rehabilitated opencast pits (Andrés & Mateos, 2006). Soil arthropods are useful bioindicators as they have short generation times, large population sizes and the ability to occupy a wide range of ecosystems, microhabitats and niches (Kremen *et al.*, 1993; Longcore, 2003).

Insects (especially Collembola), and mites are the most abundant (Kaczmarek, 1977) and widespread soil arthropods (Peterson & Luxton, 1982). Insects belong to the phylum Arthropoda, subphylum Uniramia and class Insecta (Hickman *et al.*, 1997). The typical soil living species are usually less than 2mm long and, with the exception of the Collembola, contribute little to biomass. They eat bacteria, fungi and spores, decomposing organic matter, faeces, living plants, and animals and are active in fragmenting litter (Coyne, 1999). Mites belong to the phylum Arthropoda, subphylum Chelicerata, class Arachnida and subclass Acari. Mites of the suborders Mesostigmata (parasitic and free-living predatory mites), Prostigmata (trombidiform mites), Astigmata (including storage, scabies, mange and itch mites) and Cryptostigmata (oribatid or beetle mites) are often found in soils and are classified by feeding habits. Species from these orders are expected to be the most abundant organisms in the tailing materials and it is thus important to understand their distribution patterns and factors that affect their abundance (Wahl, 2007). Species distribution of insects is determined by soil and vegetation cover while species distribution of mites is mostly determined by moisture (Blackith & Blackith, 1975; Anderson, 1977). It is well known that Prostigmatic mites are the dominant taxon in the dry, sandier soils of the Southern African subregion and have been known to be significantly stimulated by drought (Holmstrup *et al.*, 2007).

1.6 Aim of the study

The aim of this study was to determine the ecotoxicity of platinum tailings storage facilities of different ages in an attempt to determine the sustainability of rehabilitation practices on these facilities and to determine whether their effects on the environment will increase with age. Specific objectives to achieve this were:

- Total metal analysis, soluble metal analysis and pH analysis of soil samples taken from three tailings storage facilities of different ages to determine the concentrations of heavy metals available in the tailings materials and the potential for these metals to pose a threat to earthworms and soil microorganisms.
- Soil physical analyses in terms of organic matter content and gravel, sand, silt and clay percentages were conducted in order to determine the effects of these parameters on the quality of tailings material as a growth medium and their effects on the bioavailability of heavy metals.
- Earthworm bioassays using *Eisenia fetida* to determine sub-lethal endpoints in the form of growth, reproductive success and mortality as well as the use of biomarkers in the form of the neutral red retention assay.
- Earthworm tissue metal analysis to determine the concentration of heavy metals accumulated by earthworms.
- Dehydrogenase assays in order to determine the effect of earthworms on microbial activity as well as to provide an indication of the effects of the contaminants on microbial activity, prior to the introduction of earthworms. Such information may be useful to indicate the potential toxicity of platinum tailings storage facilities on biota and can thus provide important information on the environmental impacts of platinum storage facilities over time.
- Soil mesofaunal diversity to determine the effects of heavy metals on the diversity, abundance and species richness of soil mesofauna in tailings materials from three different platinum tailings storage facilities of different ages.

CHAPTER 2

2. Materials and Methods

2.1 Site description

As discussed in the introductory chapter, tailings is a mineral waste stream derived from the concentrating process of raw ore into a concentrated product. Platinum group metals (PGMs) consist of six elements, namely: platinum (Pt), palladium (Pd), iridium (Ir), osmium (Os), rhodium (Rh) and ruthenium (Ru) with copper (Cu), nickel (Ni) and chrome (Cr) as byproducts. In South Africa, PGMs are found in the Bushveld Igneous Complex, where they are recovered from the tabular Merensky reef, the underground reef number two (UG2) and the Platreef (Mbendi Information Services, 2008). The UG2 reef is situated between two layers of chromite and thus contains a higher Cr percentage than that of the Merensky reef, resulting in tailings material of a darker colour. Platinum tailings material produced from all of the abovementioned reefs contain approximately 0.5g/t to 1.5g/t of PGMs as well as traces of Ni, Cu and Cr. These metals as well as chemicals from the floatation section (copper sulphate, xanthate, flocculant and depressant) may contribute to a mixture of contaminants found on platinum tailings storage facilities.

Three sites (from now on referred to as sites 1, 2 & 3) were selected on a platinum mine near Rustenburg for the purpose of this study (Figures 2 & 3). All sites are located in the savanna biome of South Africa and receive an annual average precipitation of between 600-800 mm (Tainton, 1999). See Table 2 for monthly rainfall averages for the Rustenburg area from January to December. The oldest tailings storage facility on the property, site 1 was decommissioned in 1999, but has been operational since the late 1970's (Figure 4). Its age is estimated at approximately 30 years old. Rehabilitation commenced in late 2004 and terminated early 2005. Most of the tailings disposed on site 1 was derived from Merensky ore, hence its light colour compared with that of sites 2 and 3 (Figure 5). Site 2 was decommissioned in 2004 and rehabilitated viz. early 2005. This tailings storage facility is 20 years old. Site 3 (Figures 5 & 8) is still an operational tailings storage facility and was used for the purpose of this study as a negative control i.e. "raw" tailings. Site 3 is currently 6 years old. The materials disposed on sites 2 and 3 are very similar in content as they are both disposed from the same two concentrator plants on the property, the only difference being site 2 is rehabilitated and site 3 is operational. Most of the tailings disposed on these two tailings storage facilities are derived from UG2 ore, therefore they have high Cr content, compared with that of site 1.

Table 2. Monthly rainfall averages for the Rustenburg area, North-West Province South Africa (South African Rain Atlas, 2008).

	Jan	Feb	March	April	May	June	July	Aug	Sep	Oct	Nov	Dec
Mean rainfall (mm)	120.5	98	80.6	43.7	18.1	7.3	4.8	7.7	19.3	51.7	86.7	113.5

Site 1 was rehabilitated by means of placing Hutton topsoil over the surface of the tailings storage facility and seeding the area with a mixture of 17 indigenous grass species. Site 2 was grassed with additions of manure and a mixture of indigenous grass species by means of conventional seeding methods. These tailings storage facilities were rehabilitated more or less over the same period. The only differences between these facilities were the age of the disposal facilities, the methodology used for vegetation establishment and the different types of reef processed (i.e. Merensky and UG2 reef). Site 1 (Figure 4) has shown an immense amount of die back of vegetation compared with that of site 2 (Figure 5). The estimated crown cover of site 1 (Figure 6) is about 60% compared to an 85% crown cover estimated on site 2 (Figure 7). In general the sustainability of vegetative cover on tailings dams are poor despite the efforts of the current grassing techniques. Certain patches of tailings storage facilities' sidewalls are re-grassed approximately every two years, in order to maintain an aesthetic appearance and to prevent dust liberation from the tailings storage facilities. This is a very expensive practice due to the vast areas that have to be re-grassed. It is thus important to find a solution to more sustainable vegetative growth practices.



Figure 2. Map of South Africa indicating the location of Rustenburg in the North-West Province (Google Image, 2008).

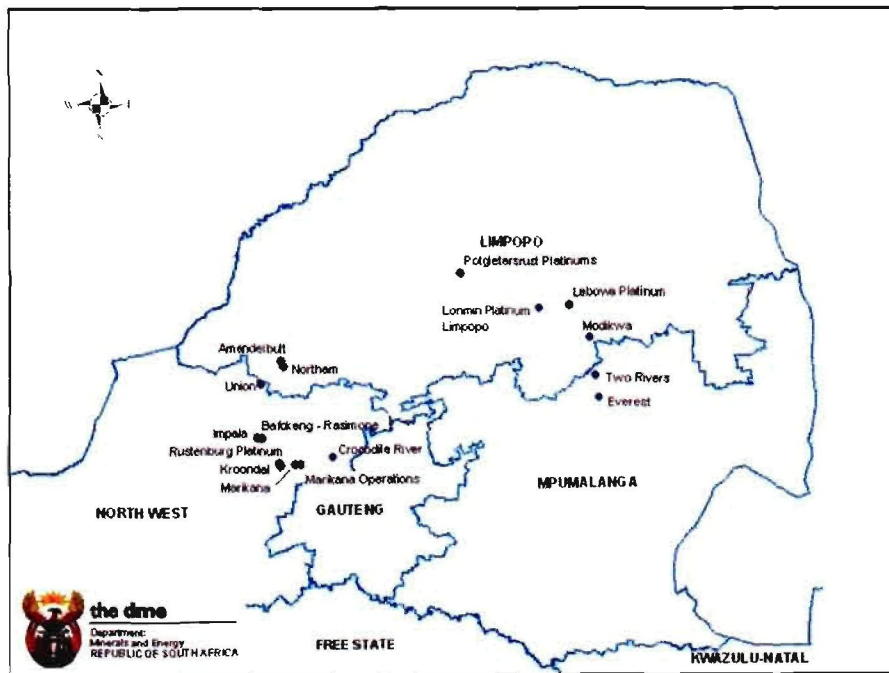


Figure 3. Map showing operational platinum-group metal mines in the Rustenburg area in the North-West Province, South Africa (DME, 2007)



Figure 4. Aerial photograph of site 1 (Google Earth, 2008). Light colours are attributed to the low chrome content of the Merensky reef.



Figure 5. Aerial photograph of site 2 and site 3, prior to rehabilitation of site 2 (Google Earth, 2008). The dark colours of the materials are attributed to the high chrome content of the UG2 reef.



Figure 6. Winter photograph of tailings site 1, facing North (dated July, 2007). Crown cover is estimated to be 60% on the top portion of this tailings disposal facility.



Figure 7. Winter photograph taken on the top of site 2, facing east (dated July 2007). Rehabilitation took place in 2005 and is showing signs of patchiness. The estimated crown cover on top of the tailings disposal facility is 85%.



Figure 8. Photograph of site 3 still in operation (dated July 2007).

2.2 Soil physical and chemical analysis

The sample plots on the tailings storage facilities consisted of one representative plot per disposal facility. At each site, six samples at 5m apart were obtained. Each sample weighed 3kg and was taken from the top 5cm of tailings material. These samples were placed in Ziploc bags, marked and sealed before they were transported to the Eco-Analytica Laboratory, Potchefstroom, where they were prepared for analysis. Total metal analysis, soluble metal analysis (1:2 water extract), pH (1:2 water extract) and physical analysis in the form of organic matter content and sand, silt and clay percentages were determined.

2.2.1 Gravel, sand, silt and clay

Gravel, sand, silt and clay percentages were determined in the Eco-Analytica Laboratory, Potchefstroom. Soil (50g) was placed in a 500ml glass beaker where after it was wetted with distilled water until saturated. Hydrogen peroxide (10mL) was added and the sample was left to stand for 10 minutes, where after the suspension was stirred well and placed on the stove to heat for 4 hours. Watch glasses were used to cover the beakers to prevent them from overflowing during heating. After 4 hours the beakers were removed from the heat and allowed to cool. Calgon (125mL) was added to each suspension and stirred well. A 53 μ m sieve was placed in a large funnel and the opening of the funnel was placed in a sedimentation cylinder. The suspension was poured over the 53 μ m sieve and the soil was washed with running tap water and a paint brush. Particles

less than 53µm were washed in the sedimentation cylinder and the fraction that stayed behind in the sieve was placed in a glass beaker and dried in the oven. The sedimentation cylinder was shaken well to collect all the particles in suspension and the first reading was measured with a hydrometer precisely after 40 seconds and recorded. Temperature was also noted. After precisely 7 hours without shaking the suspension, the second reading was observed. The oven dried fraction was sieved through a 53µm electrical sieve for 3 minutes, where after it was weighed and recorded. The last fraction that collected in a pan was sieved for 3 minutes through a 2000, 1000, 500, 250, 100µm and a 53µm sieve. The fraction collected on each sieve was weighed and recorded.

2.2.2 Organic matter content

The organic matter content of the tailings samples was determined in the Eco-Analytica Laboratory, Potchefstroom, by means of loss-on-ignition soil organic carbon methodology (Donkin, 1991). Samples were sieved through a 2mm screen, air-dried and bottled. Soil was taken (15g) from each sample and ignited in porcelain crucibles at a temperature of $450^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for a minimum of 6 hours in a muffle furnace (Towson & Mercer Model M8-1200). A temperature of $450^{\circ}\text{C} \pm 5^{\circ}\text{C}$ is the optimum temperature for loss-on-ignition analysis determined by Donkin (1991). The oven dry mass of the soil was determined.

2.2.3 Total metal analysis

The six samples from each tailings disposal facility were dried in an oven at a maximum temperature of 40°C . Samples were also protected from receiving direct sunlight. When the samples were dry, they were removed from the oven and sifted through a 2mm sieve.

Samples were further prepared in the Eco-Analytica Laboratory, Potchefstroom, for total metal analysis by using the United States Environmental Protection Agency (USEPA) method 3050 (Edgell, 1988). This method was used to determine the total bioavailable metals. Two grams of previously prepared soil sample per tailings storage facility was weighed, where after 15ml of nitric acid (HNO_3) was added to each sample. These samples were heated for a minimum of 1 hour and then cooled. Hydrogen peroxide (10mL) was added to each sample, followed by additions of 10mL of 3N HCl and heated for at least 1 hour. Samples were cooled and filtered and 50ml of deionised water was added to each sample. Samples were analysed by means of ICP/MS (Inductively Coupled Plasma Mass Spectrometry) in order to determine the total metal quantities.

2.2.4 Soluble metal analysis (1:2 water extract) and pH

Soluble metal analysis was determined by means of the 1:2 extraction methodology (Sonneveld & Van Den Ende, 1971). Deionised water (200mL) was poured into a plastic bottle and two to three drops of superflock (1%) was added to the deionised water which assisted the soil to settle. Soil (100mL) was added to the plastic bottle where after the samples were shaken for 30 minutes at 70 oscillations per minute. The suspension was decanted into low speed centrifugal containers and the samples were centrifuged for 10 minutes at 2000 rpm. Each sample suspension was decanted from the low speed centrifugal containers into high speed centrifugal containers per sample. The samples were further centrifuged for 12 minutes at 16500 rpm and were filtered into brown bottles where after the pH and the heavy metals were determined. pH was determined by means of a pH meter which is lowered into the solution to obtain a reading. Brown bottles were used to protect the samples from direct sunlight which could have broken down chemical compounds. This would have affected the results.

2.3 Earthworm ecotoxicological studies

2.3.1 Preparation of tailings materials

One sample plot per tailings storage facility was selected. At each site, six samples at 5m apart were obtained. Each sample consisted of one 3kg sample, taken from the top 5cm of tailings material. These samples were placed in Ziploc bags, marked, sealed and transported to the North-West University, Potchefstroom campus, where they were prepared.

Three randomly chosen samples from each disposal facility were mixed and dried in an oven at 40°C. The dried materials were then sifted through a 2mm sieve, in order to homogenise the samples and to exclude the bulk of undecomposed litter (Svendsen *et al.*, 1996). Each replicate was made up of 400g of tailings material and were moistened with 30ml of distilled water, providing a 7% moisture factor. The moisture factor was determined by adding distilled water until the medium appeared crumbly and moist. The substrates were inserted into glass jars (volume of 352ml). The lids of the glass jars were perforated for purposes of aeration and plastic discs were cut out from black plastic bags and placed under the lid to prevent the earthworms from escaping and to limit loss of moisture from the substrate. After the material was placed in the glass jars, the jars were stored in an environmental chamber set at 25°C for a period of seven days, during which the material stabilised.

A temperature of 25°C was chosen as this is the optimum temperature at which earthworms can reproduce and grow as determined in a variety of laboratory experiments (Jager *et al.*, 2006). A

temperature of 25°C was selected for the purpose of this experiment, as the ambient temperature in South Africa is normally higher than other countries, like the UK where most studies take place. Temperature is an important environmental factor governing physiological status in poikilothermic animals (Svendsen *et al.*, 2007). Temperature influences development time and reproductive rate in earthworms and increases in temperature initially result in faster growth and higher cocoon production (Reinecke & Kriel, 1981; Butt, 1997; Fayolle *et al.*, 1997). The environmental chamber was monitored to ensure that the temperature and humidity remained constant. The light in the chamber was also switched off to ensure that the earthworms were not exposed to light stress.

On the seventh day, food in the form of 4g (dry weight) of oven-dried horse manure was moistened with distilled water and added to each replicate. Food was replenished every seven days over the 28 day period, by means of wetting 4g oven dried horse manure with distilled water and mixing it in with the material. The provision of food during acute toxicity testing is important for the maintenance of high cocoon production rate in earthworms (Reinecke *et al.*, 1990). In addition to the replenishment of food, moisture in the form of distilled water was added to the replicates every week in order to maintain the original moisture percentage.

2.3.2 Preparation of artificial substrate (positive control)

Three replicates were made up of artificial material according to OECD guideline 207 (1984), which served as a positive control. Ten percent sphagnum peat (40g), 20% kaolin clay (80g was added with a kaolinite content above 30%) and 70% industrial sand (272g of industrial silica was obtained from a local silica sand mine) were mixed together. Calcium carbonate (8g) was added to adjust the pH to 6.0 ± 0.5 .

The dry constituents were added in the correct proportions and mixed thoroughly. Distilled water was added to provide the mixture with 35% moisture factor where after the medium was thoroughly mixed. The mixture was moistened in such a manner that it appeared to be wet and crumbly and not saturated to a point that water appeared when compressed. The contents of the mixture were then added to the glass jars and closed by means of a loosely placed plastic disc, followed by a perforated lid. The perforated lid allowed the jars to be aerated and the plastic disc prevented loss of moisture from the medium and prevented the earthworms from escaping from the medium.

These samples were also stored in the environmental chamber for a period of seven days during which the material settled. On the seventh day, the samples were removed and 4g (dry weight) of organic material in the form of oven dried horse manure were wetted with distilled water and added to each replicate.

2.3.3 Earthworm bioassays

Earthworms of the species *Eisenia fetida* were individually weighed. Ten worms were introduced into each glass jar (replicate) containing the tailings materials from site 1, 2, and 3 and the positive control, respectively. Weighing involved removing the earthworms from the substrate, washing them with distilled water and drying them on paper towels. They were then weighed by placing them in a Petri dish filled with distilled water. This prevented the worms from drying out. Earthworms were removed from the glass jars and weighed every seventh day, over a 28 day period. After the earthworms were weighed, 4g (dry weight) organic material in the form of oven dried horse manure were added to each replicate.

At the end of the 28 day period, the mature earthworms were removed from the glass jars, and the cocoons were left behind. The replicates, only containing cocoons, were returned to the environmental chamber where they remained for a period of 56 days. On the 56th day the hatched and unhatched cocoons (Figure 9), as well as the juveniles were counted and weighed, in order to determine the reproductive success (Hankard *et al.*, 2005).



Figure 9. Earthworm cocoons shown according to a 1:1mm scale.

Mortality was also noted in the replicates every seven days over the 28 day period. Unresponsive earthworms were poked with a syringe needle in the anterior side in order to determine their response. If the earthworm did not respond to the stimulus by pulling back or moving away from it, they were considered dead and were removed from the substrate. Missing earthworms were also considered to be dead.

2.3.4 Earthworm biomarkers

The neutral red retention time (NRR-T) assay was measured by using a modified version of the method described by Svendsen *et al.* (1996). This included making the following solutions, which were used to determine the NRR-T assay.

A base solution was made up by adding 10mg of neutral red dye (Toluylene Red; $C_{15}H_{17}N_4Cl$) to 0.5ml of DMSO (C_2H_6OS) in an Eppendorf container where after it was shaken to dissolve the contents.

Earthworm ringer solution was made up in a 500ml volumetric flask by adding 500ml of distilled water to 2.72g $NaCl_2$, 0.1775g KCl , 0.2088g $CaCl_2$, 0.1353g $MgSO_4$, 0.0272g KH_2PO_4 , 0.0213g Na_2HPO_4 , and 0.1764g $NaHCO_3$. The solution was shaken to dissolve the chemicals and the pH adjusted to ± 7.3 .

Ringer solution, which is used to keep the colomocytes alive whilst they are viewed under the microscope, was stored in the refrigerator during the seven day intervals between the NRR-T assay tests. On the testing days, the solution was removed from the refrigerator and allowed to warm up to room temperature before it was used for the NRR-T assay testing.

A working solution was made up by adding 10 μ l of base solution to 2.5ml of ringer solution in a polytop container. The polytop container was shaken to mix the contents. Working solution tended to crystallize every hour, therefore new solution had to be made up regularly during NRR-T assay tests.

Three earthworms from each replicate were evaluated every seventh day over a 28 day period by means of NRR-T assays. The earthworms were removed from the jars, cleaned with distilled water and dried on tissue paper. Each earthworm was held between the thumb and forefinger while coelomic fluid was extracted by means of a 10 μ l disposable syringe with a fine needle. Coelomic fluid samples were taken from the region posterior to the clitellum of the mature earthworms (Svendsen *et al.*, 1996). Equal volumes of coelomic fluid and ringer solution, making up a total of 20 μ l, were placed on a microscope slide. The solution was left for twenty seconds to adhere to the glass. Working solution (20 μ l) was added to the slide where after it was covered with a cover slip. The slide was immediately transferred to a light microscope where counting of the coloured and non-coloured cells commenced (Figure 10).

Cells were counted for 2 minutes at a time with intervals of 2 minutes between each counting session. After each counting session, the slide was placed in the moisture chamber consisting of a Petri dish with a paper towel moistened with distilled water in order to prevent the slide from drying out. When 50% of the total number of cells was stained, observation was stopped and the neutral red retention time was noted.

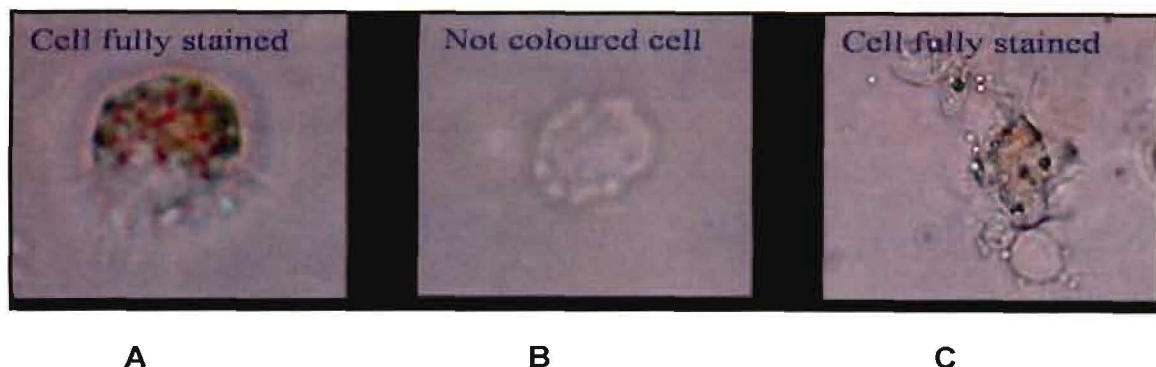


Figure 10. From left to right: A) a microscope slide showing a fully stained coelomocyte cell. Lysosomes are visibly stained; B) coelomocyte cell which has not been stained by the neutral red dye; C) a fully stained coelomocyte cell.

2.3.5 Earthworm tissue metal analysis

After the 28 day period, the adult earthworms were removed from the glass bottles and placed on wet filter paper in Petri dishes for a period of 24 hours in order for the earthworms to expel their gut contents. Earthworms were then placed into pill containers which were marked, sealed and then placed in the freezer until the earthworms were frozen.

Earthworm samples were then transported to the Eco-Analytica Laboratory, Potchefstroom, where the tissue metal content was determined. Earthworms were inserted in 2mL pure nitric acid (HNO_3) and left over night to dissolve. Solution (1mL) was extracted from each sample and 9mL of deionised water was added to obtain a ten times dilution. This solution was then injected through a 0.45 μm filter, where after it was analysed by means of the ICP/MS for heavy metals.

2.4 Microbial analysis

Dehydrogenase is an enzyme that is present in all microorganisms (Taylor *et al.*, 2002). Dehydrogenase activity is thus an indicator of microbial activity in soils. Dehydrogenase assays were performed before the introduction of manure, after the introduction of manure and after the introduction of manure and earthworms. These samples were assayed in order to determine the contribution of earthworms to dehydrogenase activity in soil samples.

2.4.1 Sampling and soil preparation

Samples were obtained from the same plots on each of the tailings storage facilities as that of the earthworm experiments. The top 0 to 10cm of tailings material was obtained by collecting 600g of material per sampling core. Samples were placed into Ziploc bags, marked and stored at 4°C until the time of analysis to preserve biological properties (Claassens *et al.*, 2006).

2.4.2 Determination of dehydrogenase activity

Samples were sifted through a 2mm sieve before assaying for dehydrogenase activity. The samples were kept at the soil's field capacity. Dehydrogenase activity was assayed according to the iodinitrotetrazolium chloride (INT) method (Von Mersi & Schinner, 1991; Alef & Nannipieri, 1995). The INT method was used because it is a rapid, precise and easily reproducible assay (Taylor *et al.*, 2002).

After Iodonitrotetrazolium chloride formazan (INF) (0.01g) was dissolved in 80ml of extractant and brought up to 100ml with the same extractant in a volumetric flask it was stored at 4°C. Extractant was made up of 100ml of N, N-dimethylformamide which was mixed with 100ml of ethanol. INF standard solution (0, 1, 2, 3, 4, 5 mL) was pipetted into test tubes, where after extractant solution (13.5mL) was added to each tube and mixed thoroughly. The optical density was determined at 464nm. Calibration concentrations were: 0, 100, 200, 300, 400 and 500 µg INF. A blank was made up and consisted of extractant solution only.

Chemicals and solutions were made up with 3M hydrochloric acid (HCl), Tris (hydroxymethyl)-aminomethane (THAM) buffer (1M, pH 7.0). The THAM (30.28g) was dissolved in 200ml of distilled water and the pH was adjusted to a pH of 7 with HCl (3M). Distilled water was used to fill the solution up to 250ml in a volumetric flask. The INT solution (9.88M) was made up of 0.5g of INT which was dissolved in 2mL of N,N-dimethylformamide and 50ml of distilled water. The solution was sonicated in an ultrasonic bath and was filled up to 100ml with distilled water in a volumetric flask. This solution was stored in the dark and only freshly prepared solution was used. The INF standard solution (100µg/ml) was made up of 0.01g of INF which was dissolved in 80ml extractant and was filled up to 100ml in a volumetric flask.

The INT procedure occurred as follows: 1g (wet weight) of soil was placed into a 100ml Schott bottle. The soil was mixed with 1.5ml THAM (1M, pH 7.0) buffer and 2ml INT solution. The Schott bottles were sealed and incubated at 40°C in the dark for 2 hours. After incubation, each sample was mixed with 10mL of extractant solution and kept in the dark for 1 hour. The samples were shaken every 20 minutes during this period. The soil suspension was filtered through a Whatman no. 2v filter paper. The absorbance of the filtrate was measured at 464nm.

Dehydrogenase activity was expressed as µg INF g⁻¹ dry weight (dwt) 2 h⁻¹ and was calculated by means of the following equation (Von Mersi & Schinner, 1991):

$$INF(\mu g g^{-1} dwt 2h^{-1}) = \frac{S_1 - S_0}{dwt}$$

where S_1 = INF (in μg) of the test
 S_0 = INF (in μg) of the control
 dwt = dry weight of 1g moist soil

This methodology was applied to the tailings materials and the artificial positive control material before the additions of earthworms and manure, after the additions of manure and after the additions of earthworms and manure.

2.5 Soil mesofauna

Soil samples were obtained from the same plots on the tailings storage facilities as that for the earthworm experiments, microbial activity and soil analyses. Six samples at 5m apart were obtained from each site. Each sample consisted of a 3kg sample, obtained from the top 10cm of tailings material. The samples were marked, sealed in Ziploc bags and stored in a cool, shady place to prevent contact with direct sunlight.

Samples were turned out onto a Berlese-Tulgren funnel (Woolley, 1982), where mesofaunal extraction commenced over a period of seven days. Soil mesofauna moved to the bottom of the soil column on account of the gradual drying out of the soil from above. Once the extraction process was complete, mites and Collembola were identified, recorded and sorted. Records were kept of species numbers per sample.

2.6 Statistical analysis

Data were analysed by means of the SigmaStat® computer program (SigmaStat for Windows Version 3.11, 2004 Systat Software Inc). Differences between treatment groups were determined by means of one-way analysis of variance (ANOVA). If treatment groups were significantly different ($p < 0.05$), the Student-Newman-Keuls test was used. All data were represented as the mean $\pm$ SD. The probability levels used to determine statistical significance were $p < 0.05$. Linear regressions were used to determine associations between dependent and independent variables.

Percentage increase of biomass was further determined by means of subtracting the mean masses recorded on day zero from that of day 28. The difference was then divided by the mean mass recorded on day zero and converted to a percentage to provide the mean percentage biomass increase experienced by the earthworms for each substrate. Mortality was further determined by

counting the number of survivors and determining the survival rate every seven days over the 28 day period. Soil mesofauna data were analysed by means of the Shannon-Weaver index for each site and were divided into functional groups as per methodology used by Wahl (2007). The Shannon-Weaver index was calculated by means of the following formula (Shannon & Weaver, 1949):

$$H = - \sum P_i \ln P_i$$

where: i = index number for each species present in the sample

$p_i = n_i/N$ = number of individuals within a species (n_i) divided by total number of individuals (N) present in the entire sample

$\ln$ = natural log

CHAPTER 3

3. Results

3.1 Soil physical and chemical analysis

3.1.1 Gravel, sand, silt, clay, organic carbon and pH

Gravel, sand, silt, clay, organic carbon and pH results are shown in Table 3. In most cases the control differed ($p < 0.05$) significantly from the test sites in gravel, sand, silt, clay and organic carbon percentages. There was, however, no significant difference ($p > 0.05$) in pH of the various test sites.

Table 3. Gravel, sand, silt, clay, organic carbon percentages and pH of control material and materials from sites 1, 2 and 3.

	Stones/Gravel >2 mm %	Sand 2-0.02 mm %	Silt 20-2 μ m %	Clay <2 μ m %	Organic Carbon %	pH
Control	1.93 $\pm$ 2.71 ^a	85.29 $\pm$ 0.36 ^a	4.35 $\pm$ 0.11 ^a	10.36 $\pm$ 0.26 ^{a,b,c}	1.12 $\pm$ 0.24 ^a	7.58 $\pm$ 0.05 ^a
Site 1	0.88 $\pm$ 1.27 ^{a,b}	71.99 $\pm$ 7.12 ^b	22.16 $\pm$ 5.75 ^a	5.85 $\pm$ 3.51 ^b	0.21 $\pm$ 0.13 ^b	7.69 $\pm$ 0.05 ^a
Site 2	0.16 $\pm$ 0.14 ^{a,b}	17.56 $\pm$ 5.92 ^c	61.83 $\pm$ 4.59 ^b	20.61 $\pm$ 3.38 ^c	1.08 $\pm$ 0.10 ^a	7.73 $\pm$ 0.05 ^a
Site 3	<0.01 $\pm$ <0.01 ^b	63.62 $\pm$ <0.01 ^b	33.12 $\pm$ <0.01 ^{a,b}	3.38 $\pm$ <0.01 ^b	0.06 $\pm$ <0.01 ^b	7.61 $\pm$ 0.10 ^a

a-c: Values with the same letter in superscript were not statistically different from each other ($p > 0.05$)

Gravel particle (>2mm) percentages in the control differed significantly ($p < 0.05$) from site 3. Sand particle (2-0.02mm) percentages in the control differed significantly from those in sites 1, 2 and 3. Silt particle (20-2 μ m) percentages in the control differed significantly from site 2, but not from sites 1 and 3. Clay particle (<2 μ m) percentages in the control did not differ significantly ($p > 0.05$) from those in sites 1, 2 and 3.

Organic carbon percentage in the control did not differ significantly from site 2, but differed significantly from sites 1 and 3. Organic carbon percentage indicates the following trend: Control > Site 2 > Site 1 > Site 3.

The pH did not differ significantly ($p > 0.05$) between the control and the three test sites. The pH trend indicates the following: Site 2 > Site 1 > Site 3 > Control.

3.1.2 Total and soluble metals

Table 4 shows the results of the total metal, soluble metal, earthworm tissue metal concentrations and the bioconcentration factor (BCF). Bioconcentration factor (BCF) is the concentration of the contaminant in the earthworm tissue/total soil concentration. The complete set of results obtained

from the chemical analysis can be seen in Table 20 in Appendix II. Total metal analysis conducted on the control material and platinum tailings material from sites 1, 2 and 3 were measured against toxicological benchmarks described in Efroymson *et al.* (1997). The Canadian Council of Ministers of the Environment (CCME) toxicity benchmarks represent levels considered protective of human health and the environment for specified uses of soil (in this case the most conservative use - agriculture). The Dutch National Institute of Public Health and Environmental Protection (RIVM) developed Ecotoxicological Intervention Values (EIV's). These values indicate concentrations in soil causing 50% of the species in the system to experience adverse effects. Earthworm benchmarks are derived from a number of acute toxicity tests that are used to benchmark concentrations of chemicals or heavy metals that are toxic to earthworms. Benchmark concentrations for toxicity of soil microorganisms and microbial processes were determined by effects on C-mineralization, N-transformation and enzyme activities (Efroymson *et al.*, 1997). These benchmarks were used since soil benchmarks do not exist for South Africa.

Total Na measured in site 3 (28.00 ± 0.00 ppm) differed significantly ($p < 0.05$) from that in the control (47.00 ± 8.66 ppm) and sites 1 (62.67 ± 13.20 ppm) and 2 (61.67 ± 8.02 ppm). Magnesium and K levels in the control and the three sites differed significantly from one another. The control differed significantly from the three sites in Al levels. The Al levels in site 1 (1433.33 ± 230.94 ppm), 2 (1300.00 ± 200.00 ppm) and 3 (660.00 ± 10.00 ppm) exceeded the toxicity levels of microorganisms and microbial processes (600.00 ppm). Chromium levels in the control and the three test sites differed significantly ($p < 0.05$) from one another. Total Cr at site 1 (7.97 ± 1.55 ppm) exceeded the earthworm toxicity benchmark (0.40 ppm). Total Cr at site 2 (46.67 ± 14.57 ppm) and 3 (11.00 ± 1.00 ppm) exceeded the earthworm toxicity benchmark (0.40 ppm) and the toxicity benchmark for microorganisms and microbial processes (10.00 ppm). Manganese levels in the control differed significantly from the Mn levels in the three test sites. Iron levels in the control differed significantly from the three test sites. Iron levels in site 1 (893.33 ± 526.24 ppm), site 2 (743.33 ± 141.54 ppm) and site 3 (270.00 ± 10.00 ppm), were above the toxicity benchmark for microorganisms and microbial processes (200.00 ppm). Nickel levels in the control and the three test sites differed significantly from one another. Copper levels in the control (0.16 ± 0.01 ppm), site 1 (4.50 ± 0.56 ppm), site 2 (3.40 ± 0.46 ppm) and site 3 (0.96 ± 0.02 ppm) differed significantly from one another. Zinc levels in the control (0.65 ± 0.02 ppm), site 1 (0.38 ± 0.14 ppm), site 2 (2.00 ± 0.98 ppm) and site 3 (0.19 ± 0.01 ppm) differed significantly from one another. Lead levels in the control differed significantly from all three test sites. Based on the total concentrations of metals the following trend could be determined: $Al > Fe > Mg > Na > K > Cr > Ni > Mn > Cu > Zn > Pb$. The following trend was determined for the sites: $Site\ 1 > Site\ 2 > Site\ 3 > Control$.

Sodium in solution at site 1 (1.17 ± 0.40 ppm) differed significantly ($p < 0.05$) from levels in solution of control (4.13 ± 0.06 ppm), site 2 (11.60 ± 7.49 ppm) and site 3 (7.63 ± 0.85 ppm). Aluminium in solution differed significantly between control (6.83 ± 0.85 ppm), site 1 (0.72 ± 0.42 ppm) and site 2 (2.07 ± 0.25 ppm), but there was no significant difference ($p > 0.05$) between the control (6.83 ± 0.85 ppm) and site 3 (5.57 ± 2.25 ppm). The control and all test sites differed significantly from one another in Mg, K, Cr, Cu, Zn and Pb concentrations in solution. Manganese in solution differed significantly between the control (0.56 ± 0.08 ppm) and site 1 ($0.01 \pm < 0.01$ ppm), there was no significant difference between the control and sites 2 ($0.06 \pm < 0.01$ ppm) and 3 (0.07 ± 0.02 ppm) and between site 1 and sites 2 and 3. Iron in solution differed significantly between the control and all three test sites and between site 1 (2.37 ± 0.35 ppm), the control (3.80 ± 0.26 ppm) and sites 2 (8.03 ± 0.85 ppm) and 3 (10.10 ± 3.39 ppm). Iron in solution at sites 2 and 3 did not differ significantly from one another. Nickel in solution differed significantly between site 1 ($0.03 \pm < 0.01$ ppm), the control (0.08 ± 0.02 ppm) and sites 2 (0.12 ± 0.01 ppm) and 3 (0.10 ± 0.03 ppm). Nickel in solution in the control did not differ significantly from site 3. Site 2 did not differ significantly from site 3. Based on the concentrations of metals in solution the following trend could be determined: $K > Na > Fe > Mg > Al > Cr > Mn > Zn > Cu > Ni > Pb$. The following trend was determined for the sites: Site 2 > Site 3 > Control > Site 1.

Linear regressions in Table 5a show a significant relationship between Al in soil and Al in solution ($r^2 = 0.95$; $p = 0.03$). Linear regressions show a strong relationship between Cr in soil and Cr in solution ($r^2 = 0.9$; $p = 0.07$). Linear regressions in Table 5b show significant relationships between Cr in *Eisenia fetida* and Cr in soil ($r^2 = 0.98$; $p = 0.012$), as well as Zn in *Eisenia fetida* and Zn in soil ($r^2 = 0.93$; $p = 0.04$). Linear regressions in Table 5c show a significant relationship between Mg in *Eisenia fetida* and Mg in solution ($r^2 = 0.91$; $p = 0.05$).

Linear regression relationships between pH and metal in soil (Table 6a) showed significant relationships between pH and Mg in soil ($r^2 = 0.90$; $p = 0.05$) and between pH and Mn in soil ($r^2 = 0.95$; $p = 0.03$). Strong relationships were shown between pH and Al ($r^2 = 0.86$; $p = 0.07$) and Fe ($r^2 = 0.85$; $p = 0.08$) respectively, in soil. There was a strong linear relationship between pH and Al in solution ($r^2 = 0.83$; $p = 0.09$) (Table 6b). There were no linear relationships between pH and metals in *Eisenia fetida* (Table 6c).

Significant ($p < 0.05$) relationships were determined between clay percentage and Zn in soil ($r^2 = 0.97$; $p = 0.01$), K in solution ($r^2 = 0.99$; $p = 0.01$) and Al in *Eisenia fetida* ($r^2 = 0.93$; $p = 0.04$) (Table 7). Strong relationships were determined between clay percentage and Na in *Eisenia fetida* ($r^2 = 0.88$; $p = 0.06$) and Zn in *Eisenia fetida* ($r^2 = 0.82$; $p = 0.09$). Linear regressions showed that clay percentage has a significant negative relationship with cocoon hatchability ($r^2 = 0.94$; $p = 0.03$) (Appendix I, Table

19). Linear regressions between organic carbon percentage (Appendix I, Table 16) and metals in soil, metals in solution and metals in *Eisenia fetida* did not show any significant relationships.

Table 4. Soil total metal analysis for control, site 1, 2 and 3 measured against the following benchmarks: CCME, the Canadian Council of Ministers of the Environment; RIVM (EIV's), the Dutch National Institute of Public Health and Environmental Protection, Ecotoxicological Intervention Values (EIV's); E/w, benchmarks for earthworm toxicity; SMO & MP, benchmark concentrations for toxicity to soil microorganisms and microbial processes, described by Efroymson *et al.* (1997). Earthworm tissue metal concentrations and bioconcentration factor (BCF) (i.e. Earthworm body concentration/total soil concentration) measured in ppm. Soluble metal analysis measured in ppm.

		Na	Mg	Al	K	Cr	Mn	Fe	Ni	Cu	Zn	Pb
CCME						750			150	150	600	375
RIVM						230			210	190	720	290
E/w						0.4			200	50	200	500
SMO & MP				600		10	100	200	90	100	100	900
Control	Soil	47.00±8.66 ^a	8.20±0.90 ^a	87.67±18.82 ^a	62.00±6.24 ^a	0.29±0.02 ^a	1.07±0.06 ^a	43.67±1.53 ^a	0.15±<0.01 ^a	0.16±0.01 ^a	0.65±0.02 ^a	0.11±0.01 ^a
	E/w	0.07±<0.01 ^a	<0.01±<0.01 ^a	0.01±<0.01 ^a	0.06±<0.01 ^a	<0.01±<0.01 ^a	0.03±<0.01 ^a	0.02±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a
	BCF	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	<0.01	0.01	<0.01	0.01	<0.01
	Soluble	4.13±0.06 ^a	3.50±0.20 ^a	6.83±0.85 ^a	6.00±0.69 ^a	0.02±<0.01 ^a	0.56±0.08 ^a	3.80±0.26 ^a	0.08±0.02 ^a	0.11±0.01 ^a	0.34±0.13 ^a	0.03±0.01 ^a
Site 1	Soil	62.67±13.20 ^a	88.00±19.16 ^b	1433.33±230.94 ^b	9.87±1.86 ^b	7.97±1.55 ^b	8.67±3.21 ^b	893.33±526.24 ^b	12.67±1.53 ^b	4.50±0.56 ^b	0.38±0.14 ^b	0.23±0.03 ^b
	E/w	0.07±<0.01 ^a	<0.01±<0.01 ^{a,c}	0.01±<0.01 ^a	0.06±<0.01 ^{a,c}	<0.01±<0.01 ^a	0.03±<0.01 ^a	0.03±<0.01 ^{a,c}	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a
	BCF	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
	Soluble	1.17±0.40 ^b	2.17±0.64 ^b	0.72±0.42 ^b	3.80±0.96 ^b	0.01±<0.01 ^b	0.01±<0.01 ^b	2.37±0.35 ^b	0.03±<0.01 ^b	0.02±<0.01 ^b	0.02±<0.01 ^b	<0.01±<0.01 ^b
Site 2	Soil	61.67±8.02 ^a	190.00±34.64 ^c	1300.00±200.00 ^b	13.67±0.58 ^c	46.67±14.57 ^c	8.73±0.51 ^b	743.33±141.54 ^b	9.43±0.98 ^c	3.40±0.46 ^c	2.00±0.98 ^c	0.23±0.03 ^b
	E/w	0.13±0.05 ^b	0.02±0.01 ^b	0.01±<0.01 ^b	0.08±0.01 ^b	<0.01±<0.01 ^b	0.04±<0.01 ^b	0.07±0.02 ^b	<0.01±<0.01 ^b	<0.01±<0.01 ^b	0.01±<0.01 ^b	<0.01±<0.01 ^b
	BCF	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Soluble	11.60±7.49 ^a	9.60±2.40 ^c	2.07±0.25 ^b	16.00±8.72 ^c	2.03±0.32 ^c	0.06±<0.01 ^{a,b}	8.03±0.85 ^c	0.12±0.01 ^c	0.16±0.02 ^c	0.05±0.01 ^c	0.01±<0.01 ^c
Site 3	Soil	28.00±<0.01 ^b	46.00±1.00 ^d	660.00±10.00 ^b	2.30±<0.01 ^d	11.00±1.00 ^d	2.80±0.10 ^b	270.00±10.00 ^b	3.97±0.06 ^d	0.96±0.02 ^d	0.19±0.01 ^d	0.04±0.00 ^c
	E/w	0.07±<0.01 ^a	0.01±<0.01 ^{b,c}	0.01±<0.01 ^a	0.06±<0.01 ^{b,c}	<0.01±<0.01 ^a	0.03±<0.01 ^a	0.04±<0.01 ^{b,c}	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a	<0.01±<0.01 ^a
	BCF	<0.01	<0.01	<0.01	0.03	<0.01	0.01	<0.01	<0.01	<0.01	0.02	<0.01
	Soluble	7.63±0.85 ^a	5.43±0.25 ^d	5.57±2.25 ^a	1.63±0.32 ^d	1.02±0.51 ^d	0.07±0.02 ^{a,b}	10.10±3.39 ^c	0.10±0.03 ^{a,a}	0.06±0.02 ^d	0.03±<0.01 ^d	<0.01±<0.01 ^d

a-d: Values with the same letter in superscript were not statistically different from each other (p>0.05)

Table 5. Simple linear regression equation ($y = b_0 + b_1x$) expressing relationships between (a) metal in solution (ppm) and in soil (ppm), (b) metal in soil (ppm) and in earthworm (ppm), and (c) metal in solution (ppm) and in earthworm (ppm) for *Eisenia fetida* cultured in control material and platinum tailings materials from sites 1, 2 and 3.

	(a) Metal in solution and metal in soil					(b) Metal in soil and metal in <i>Eisenia fetida</i>					(c) Metal in solution and metal in <i>Eisenia fetida</i>			
	b_0	b_1	r^2	p value		b_0	b_1	r^2	p value		b_0	b_1	r^2	p value
Na in soil	7.80	-0.03	0.01	0.88	Na in <i>Eisenia</i>	0.03	<0.01	0.25	0.50	Na in <i>Eisenia</i>	0.05	0.01	0.64	0.20
Mg in soil	2.60	0.03	0.57	0.25	Mg in <i>Eisenia</i>	<0.01	<0.01	0.84	0.09	Mg in <i>Eisenia</i>	<0.01	<0.01	0.91	0.05
Al in soil	7.70	-0.01	0.95	0.03	Al in <i>Eisenia</i>	0.01	<0.01	0.06	0.76	Al in <i>Eisenia</i>	0.01	<0.01	0.04	0.81
K in soil	6.60	0.01	<0.01	0.94	K in <i>Eisenia</i>	0.07	<0.01	0.13	0.64	K in <i>Eisenia</i>	0.06	<0.01	0.80	0.13
Cr in soil	0.06	0.04	0.86	0.07	Cr in <i>Eisenia</i>	<0.01	<0.01	0.98	0.01	Cr in <i>Eisenia</i>	<0.01	<0.01	0.86	0.07
Mn in soil	0.43	-0.05	0.56	0.25	Mn in <i>Eisenia</i>	0.03	<0.01	0.23	0.52	Mn in <i>Eisenia</i>	0.03	-0.01	0.08	0.72
Fe in soil	6.97	<0.01	0.04	0.80	Fe in <i>Eisenia</i>	0.03	<0.01	0.19	0.56	Fe in <i>Eisenia</i>	0.02	<0.01	0.29	0.46
Ni in soil	0.10	<0.01	0.11	0.66	Ni in <i>Eisenia</i>	<0.01	<0.01	0.06	0.75	Ni in <i>Eisenia</i>	<0.01	0.02	0.61	0.22
Cu in soil	0.10	-0.01	0.06	0.76	Cu in <i>Eisenia</i>	<0.01	<0.01	0.16	0.60	Cu in <i>Eisenia</i>	<0.01	0.01	0.54	0.27
Zn in soil	0.11	-0.01	<0.01	0.96	Zn in <i>Eisenia</i>	<0.01	<0.01	0.93	0.04	Zn in <i>Eisenia</i>	0.01	<0.01	0.08	0.71
Pb in soil	0.01	-0.03	0.05	0.77	Pb in <i>Eisenia</i>	<0.01	<0.01	0.02	0.85	Pb in <i>Eisenia</i>	<0.01	0.01	0.08	0.71

Table 6. Simple linear regression equation ($y = b_0 + b_1x$) expressing relationships between (a) pH in solution and metals in soil (ppm), (b) pH in solution and metals in solution (ppm), and (c) pH in solution and metals (ppm) in *Eisenia fetida* cultured in control material and platinum tailings materials from site number 1, 2 and 3.

(a) pH in solution and metal in soil					(b) pH in solution and metal in solution					(c) pH in solution and metal in <i>Eisenia fetida</i>				
	b_0	b_1	r^2	p value		b_0	b_1	r^2	p value		b_0	b_1	r^2	p value
Na in soil	-1288.60	174.90	0.56	0.25	Na in solution	-162.34	22.02	0.12	0.66	Na in <i>Eisenia</i>	-2.6	0.3	0.53	0.27
Mg in soil	-8098.38	1069.12	0.90	0.05	Mg in solution	-174.88	23.53	0.25	0.50	Mg in <i>Eisenia</i>	-0.5	0.01	0.33	0.43
Al in soil	-62619.90	8296.66	0.86	0.07	Al in solution	291.77	-37.63	0.83	0.09	Al in <i>Eisenia</i>	-0.05	0.01	0.33	0.43
K in soil	1694.45	-218.56	0.31	0.44	K in solution	-457.36	60.66	0.44	0.34	K in <i>Eisenia</i>	-0.81	0.12	0.49	0.30
Cr in soil	-1795.21	236.75	0.64	0.20	Cr in solution	-59.17	7.83	0.32	0.44	Cr in <i>Eisenia</i>	-0.09	0.01	0.51	0.29
Mn in soil	-420.53	55.65	0.95	0.03	Mn in solution	20.23	-2.62	0.50	0.29	Mn in <i>Eisenia</i>	-0.31	0.04	0.44	0.33
Fe in soil	-39938.50	5282.73	0.85	0.08	Fe in solution	2.60	0.46	<0.01	0.99	Fe in <i>Eisenia</i>	-1.67	0.22	0.55	0.26
Ni in soil	-536.19	70.92	0.78	0.12	Ni in solution	-0.26	0.04	0.01	0.92	Ni in <i>Eisenia</i>	-0.06	0.01	0.44	0.34
Cu in soil	-198.36	26.22	0.80	0.10	Cu in solution	-1.15	0.16	0.03	0.82	Cu in <i>Eisenia</i>	-0.05	0.01	0.59	0.23
Zn in soil	-59.76	7.91	0.45	0.33	Zn in solution	11.2	-1.44	0.43	0.34	Zn in <i>Eisenia</i>	-0.15	0.02	0.60	0.23
Pb in soil	-8.60	1.14	0.73	0.15	Pb in solution	0.65	-0.08	0.29	0.46	Pb in <i>Eisenia</i>	<0.01	<0.01	0.02	0.86

Table 7. Simple linear regression equation ($y = b_0 + b_1x$) expressing relationships between (a) Clay percentage in soil and metals in soil (ppm), (b) Clay percentage in soil and metals in solution (ppm), and (c) Clay percentage in soil and metals (ppm) in *Eisenia fetida* cultured in control material and platinum tailings materials from sites 1, 2 and 3.

	(a) Clay % and metal in soil					(b) Clay % and metal in solution					(c) Clay % and metal in <i>Eisenia fetida</i>			
	b_0	b_1	r^2	p value		b_0	b_1	r^2	p value		b_0	b_1	r^2	p value
Na in soil	37.26	1.25	0.34	0.41	Na in solution	2.24	0.39	0.43	0.35	Na in <i>Eisenia</i>	0.04	<0.01	0.88	0.06
Mg in soil	6.23	7.64	0.55	0.26	Mg in solution	1.90	0.33	0.59	0.23	Mg in <i>Eisenia</i>	<0.01	<0.01	0.67	0.18
Al in soil	677.43	19.19	0.06	0.77	Al in solution	4.74	-0.09	0.06	0.75	Al in <i>Eisenia</i>	0.01	<0.01	0.93	0.04
K in soil	15.65	0.63	0.03	0.82	K in solution	-1.45	0.83	0.99	0.01	K in <i>Eisenia</i>	0.05	<0.01	0.67	0.18
Cr in soil	-5.74	2.21	0.67	0.18	Cr in solution	-0.07	0.08	0.44	0.34	Cr in <i>Eisenia</i>	<0.01	<0.01	0.77	0.12
Mn in soil	3.15	0.22	0.17	0.58	Mn in solution	0.16	<0.01	<0.01	0.94	Mn in <i>Eisenia</i>	0.02	<0.01	0.79	0.11
Fe in soil	353.31	13.36	0.07	0.74	Fe in solution	5.61	0.05	0.01	0.90	Fe in <i>Eisenia</i>	0.02	<0.01	0.66	0.19
Ni in soil	5.39	0.12	0.03	0.84	Ni in solution	0.05	<0.01	0.30	0.45	Ni in <i>Eisenia</i>	<0.01	<0.01	0.74	0.14
Cu in soil	1.64	0.06	0.05	0.77	Cu in solution	0.01	0.01	0.78	0.12	Cu in <i>Eisenia</i>	<0.01	<0.01	0.79	0.11
Zn in soil	-0.26	0.11	0.97	0.01	Zn in solution	0.09	<0.01	0.01	0.88	Zn in <i>Eisenia</i>	<0.01	<0.01	0.82	0.09
Pb in soil	0.09	0.01	0.31	0.45	Pb in solution	0.01	<0.01	0.09	0.69	Pb in <i>Eisenia</i>	<0.01	<0.01	0.50	0.30

3.2 Earthworm ecotoxicological studies

3.2.1 Earthworm bioassays

3.2.1.1 Earthworm growth

The mean biomass of earthworms exposed to control material and tailings material is shown in Table 8. Earthworms exposed to tailings materials from sites 1, 2 and 3 showed an increase in biomass.

Earthworms exposed to the control medium showed a statistically significant ($p < 0.05$) increase in biomass from day one to day 14. From day one to day seven, there was no significant difference ($p > 0.05$) between the mean earthworm masses of the earthworms exposed to the tailings materials and the control. On day 14, there was a significant difference ($p < 0.05$) between the mean earthworm masses of the earthworms exposed to the control material and those exposed to the tailings materials. Mean earthworm masses of the earthworms exposed to the tailings material from site 1 differed significantly from the control and the other test sites. From day 21 to day 28 there was a significant difference ($p < 0.05$) in the earthworm masses of the earthworms exposed to the control material and those exposed to the tailings materials.

The mean biomass increase experienced by the earthworms in the three replicates of control medium was 90%. Earthworms exposed to tailings material from site 1 showed a statistically significant increase ($p < 0.05$) in biomass from day one to day seven with a mean biomass increase of 66%. The worms exposed to tailings material from site 2 showed a statistically significant increase ($p < 0.05$) in biomass from day one to day seven with a mean biomass increase of 58%. Those exposed to tailings material from site 3 showed a statistically significant increase ($p < 0.05$) in mean biomass from day one to day seven and a mean biomass increase of 63%.

All the test sites showed a statistically significant increase in mean biomass from day one to day seven, whereas the earthworms exposed to the control material showed a statistically significant ($p < 0.05$) increase in mean biomass from day one to day 14. Mean biomass percentage increase showed the following trend: Control (90%) > Site 1 (66%) > Site 3 (63%) > Site 2 (58%).

3.2.1.2 Earthworm mortality

Percentage mortality experienced by earthworms exposed to control material, tailings material from sites 1, 2 and 3 is shown in Table 8.

The earthworms exposed to the control material showed 0% mortality over 28 days. Earthworms exposed to tailings material from site 2 suffered 3% mortality over the 28 day period. Earthworm mortality occurred between day 21 and day 28. Earthworms exposed to tailings material from site 3 suffered a 10% mortality rate over the 28 day period, with 3% mortality occurring between day 14 and 21 and a further 7% occurring between day 21 and day 28. Earthworms exposed to tailings material from site 1 suffered a 21% mortality rate over the 28 day period. Ten percent mortality occurred between days seven and 14, a further 7% between days 14 and 21, and a further 4% between day 21 and 28. There was no statistical significant difference ($p>0.05$) between the mortality rates of the earthworms exposed to different substrates (i.e. control material, site 1, site 2 and site 3). The percentage mortality showed the following trend: Control < Site 2 < Site 3 < Site 1.

Table 8. Mean ($\pm$ SD) growth (g) and neutral red retention times (min) of *Eisenia fetida* over the 28 day period. *Eisenia fetida* percentage mortality every seventh day over the 28 day period.

	Growth (g)	NRR-T (min)	Mortality %
Day 1			
Control	0.27 $\pm$ 0.06 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 1	0.23 $\pm$ 0.06 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 2	0.23 $\pm$ 0.04 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 3	0.25 $\pm$ 0.05 ^a	37.89 $\pm$ 22.70 ^a	0 %
Day 7			
Control	0.39 $\pm$ 0.08 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 1	0.40 $\pm$ 0.09 ^a	11.00 $\pm$ 5.16 ^a	0 %
Site 2	0.37 $\pm$ 0.08 ^a	37.00 $\pm$ 9.80 ^a	0 %
Site 3	0.38 $\pm$ 0.07 ^a	21.00 $\pm$ 16.97 ^a	0 %
Day 14			
Control	0.51 $\pm$ 0.09 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 1	0.36 $\pm$ 0.11 ^b	5.00 $\pm$ 9.80 ^b	10 %
Site 2	0.45 $\pm$ 0.09 ^c	13.00 $\pm$ 10.12 ^{a,b}	0 %
Site 3	0.42 $\pm$ 0.10 ^c	8.33 $\pm$ 10.56 ^b	0 %
Day 21			
Control	0.55 $\pm$ 0.09 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 1	0.38 $\pm$ 0.11 ^b	14.33 $\pm$ 9.59 ^b	7 %
Site 2	0.44 $\pm$ 0.08 ^b	9.00 $\pm$ 8.49 ^b	0 %
Site 3	0.43 $\pm$ 0.13 ^b	2.78 $\pm$ 2.11 ^c	3 %
Day 28			
Control	0.51 $\pm$ 0.09 ^a	37.89 $\pm$ 22.70 ^a	0 %
Site 1	0.39 $\pm$ 0.09 ^b	8.11 $\pm$ 7.94 ^b	4 %
Site 2	0.37 $\pm$ 0.10 ^b	10.00 $\pm$ 10.42 ^{a,b}	3 %
Site 3	0.41 $\pm$ 0.12 ^b	8.00 $\pm$ 11.66 ^b	7 %
Total After 28 Days	Biomass increase		Total
Control	90 %	37.89 $\pm$ 22.70 ^a	0 %
Site 1	66 %	8.11 $\pm$ 7.94 ^b	21 %
Site 2	58 %	10.00 $\pm$ 10.42 ^{a,b}	3 %
Site 3	63 %	8.00 $\pm$ 11.66 ^b	10 %

a-c: Values with the same letter in superscript were not statistically different from each other ($p > 0.05$).

3.2.1.3 Earthworm reproduction

Earthworm reproduction results are shown in Table 9. Number of cocoons and hatchability percentage did not show significant differences ($p>0.05$) between the test sites and the control. There was, however, a significant difference in the number of juveniles that hatched per cocoon and the biomass of juveniles between the control and the test sites.

Table 9. Reproduction results obtained from earthworms exposed to control material and tailings material from sites 1, 2 and 3.

	Number of Cocoons	Hatchability %	Juveniles hatched per cocoon	Biomass of Juveniles (g)
Control	21.67±5.86 ^a	90.56±10.05 ^a	2.83±0.54 ^a	0.01±0.01 ^a
Site 1	18.33±5.51 ^a	92.68±6.87 ^a	1.06±0.15 ^b	<0.01±<0.01 ^b
Site 2	17.00±4.00 ^a	80.45±6.75 ^a	1.83±0.27 ^c	0.01±<0.01 ^b
Site 3	21.00±2.00 ^a	93.45±3.45 ^a	0.88±0.39 ^b	0.01±<0.01 ^b

a-c: Values with the same letter in superscript were not statistically different from each other ($p>0.05$)

There were no statistically significant differences ($p>0.05$) between the mean number of cocoons produced and the hatchability of the cocoons in the control and the test sites. The number of juveniles that hatched per cocoon in the control differed significantly from the mean number of juveniles that hatched per cocoon in tailings material from sites 1, 2 and 3. Those from site 1 and 3 did not differ significantly from the control, while the ones from site 2 differed significantly from the control and the other test sites. The mean biomass of juveniles exposed to the control material differed significantly from the juveniles exposed to tailings materials from sites 1, 2 and 3. Based on the number of juveniles that hatched per cocoon the following ranking can be identified: Control > Site 2 > Site 1 > Site 3

3.2.2 Earthworm biomarkers

At the start of the experiment there were no statistically significant differences ($p>0.05$) between the mean NRR-T of the earthworms exposed to the control material and those exposed to the tailings material (Table 8). On day seven, there were no statistically significant differences ($p>0.05$) between the control and tailings materials from sites 1, 2 and 3. On day 14, there was a statistically significant difference ($p<0.05$) between the NRR-T of the earthworms exposed to the control material and those exposed to tailings material from sites 1 and 3. On day 21, there were a

statistically significant differences ($p<0.05$) between the NRR-T of the earthworms exposed to the control material and the tailings materials from sites 1, 2 and 3. Neutral red retention times of earthworms exposed to tailings material from site 3 differed significantly ($p<0.05$) from those exposed to the control material and those exposed to tailings materials from sites 1 and 2. On day 28, there were a statistically significant differences ($p<0.05$) between the NRR-T of the earthworms exposed to control material and those exposed to tailings materials from sites 1 and 3. NRR-T assays resulted in the following trend ranked from highest neutral red times to lowest neutral red times: Control > Site 2 > Site 1 > Site 3

3.2.3 *Earthworm tissue metal analysis*

Results from earthworm tissue metal analysis are shown in Table 4. In general earthworms did not bioconcentrate large concentrations of heavy metals. Earthworms exposed to tailings material from site 3 bioaccumulated higher concentrations of heavy metals, followed by those exposed to the control material, then those exposed to tailings materials from site 1 and finally site 2.

Bioconcentration factors indicated that negligible amounts ($BCF=<0.01$) of Na, Mg, Al, Cr, Fe, Cu and Pb were bioaccumulated by the earthworms exposed to control and tailings materials from sites 1, 2 and 3. Earthworms exposed to tailings material from site 3 ($BCF=0.03$) bioaccumulated more K than those exposed to tailings materials from sites 1, 2 ($BCF=0.01$) and the control ($BCF=<0.01$). Manganese bioaccumulated the most in the earthworms exposed to the control material ($BCF=0.02$), followed by those exposed to tailings material from site 3 ($BCF=0.01$), then by those exposed to tailings materials from site 1 and 2 ($BCF=<0.01$). Nickel also bioaccumulated the most in the earthworms exposed to the control material ($BCF=0.01$), followed by those exposed to tailings materials from sites 1, 2 and 3 ($BCF=<0.01$). Zinc bioaccumulated the most in earthworms exposed to tailings material from site 3 ($BCF=0.02$), followed by those exposed to control material and tailings material from site 1 ($BCF=0.01$), then by those exposed to tailings material from site 2 ($BCF=<0.01$). Bioconcentration factors showed the following trend: Zn > K > Mn > Pb > Na > Ni > Cu > Cr > Fe > Mg > Al.

3.2.4 *Linear regressions*

Table 10 shows linear regressions between earthworm masses on day 28 and NRR-T, metals in soil, metals in solution and dehydrogenase activity after additions of earthworms and manure. The complete table which also includes other bioassay results, soil physical, soil chemical and dehydrogenase can be seen in Table 17 in Appendix I.

Linear regressions indicated that there are strong positive relationships between earthworm masses and neutral red retention times ($r^2 = 0.87$; $p=0.07$), Al in soil ($r^2 = 0.88$; $p=0.06$), Zn in solution ($r^2 = 0.87$; $p=0.07$) and dehydrogenase activity after the introduction of earthworms and manure ($r^2 = 0.88$; $p=0.06$). There is a significant relationship between Mn in solution ($r^2 = 0.91$; $p=0.05$) and earthworm masses.

Table 10. Simple linear regression equations $y = b_0 + b_1x$ expressing significant ($p<0.05$) and strong relationships between NRR-T and metals in soil, metals in solution and dehydrogenase on day 28 of the experiment.

	b_0	b_1	r^2	p	Regression
NRR-T	0.35	<0.01	0.87	0.07	Linear
Al in soil	0.50	<-0.01	0.88	0.06	Linear
Mn in solution	0.38	0.23	0.91	0.05	Linear
Zn in solution	0.38	0.38	0.87	0.07	Linear
Dehydrogenase activity (after earthworms and manure)	0.29	<0.01	0.88	0.06	Linear

Linear regressions between metals in soil, metals in solution and dehydrogenase activity are shown in Table 11. The complete table which also includes other bioassay results, soil physical, soil chemical and dehydrogenase can be seen in Table 18 in Appendix I.

There was a significant ($p<0.05$) positive linear relationship between K in soil and NRR-T ($r^2=0.98$; $p=0.01$), between Mn ($r^2=0.99$; $p<0.01$) and Zn ($r^2=0.99$; $p<0.01$) respectively, in solution and NRR-T between dehydrogenase activity and NRR-T ($r^2=0.96$; $p=0.02$).

Table 11. Simple linear regression equations $y = b_0 + b_1x$ expressing significant ($p<0.05$) and strong relationships between metals in soil, metals in solution, and dehydrogenase on day 28 of the experiment.

	b_0	b_1	r^2	p	Regression
K in soil	4.26	0.54	0.98	0.01	Linear
Mn in solution	5.98	56.72	0.99	<0.01	Linear
Zn in solution	5.48	95.97	0.99	<0.01	Linear
Dehydrogenase activity (after earthworms and manure)	-14.89	0.10	0.96	0.02	Linear

Table 12 shows linear regressions between cocoon hatchability and soil physical analysis, metals in soil, metals in solution and metals in *Eisenia fetida*. The complete table which also includes

other bioassay results, soil physical, soil chemical and dehydrogenase can be seen in Table 19 in Appendix I.

Linear relationships of cocoon hatchability indicated a significant negative relationship between clay percentage and cocoon hatchability ($r^2=0.94$; $p=0.03$), a strong negative relationship between Cr in soil and cocoon hatchability ($r^2=0.87$; $p=0.07$), a significant ($p<0.05$) negative relationship between Zn in soil and cocoon hatchability ($r^2=0.99$; $p=0.01$), as well as a significant ($p<0.05$) negative relationship between K in solution and cocoon hatchability ($r^2=0.97$; $p=0.01$). There was also a significant negative linear relationship between cocoon hatchability and metals in *Eisenia fetida* especially Na ($r^2 = 0.98$; $p=0.01$), Al ($r^2 = 0.98$; $p=0.01$), K ($r^2 = 0.98$; $p=0.01$), Cr ($r^2 = 0.94$; $p=0.03$), Mn ($r^2 = 0.95$; $p=0.03$), Ni ($r^2 = 0.92$; $p=0.04$), Cu ($r^2 = 0.95$; $p=0.03$) and Zn ($r^2 = 0.95$; $p=0.02$).

Table 12. Simple linear regression equations $y = b_0 + b_1x$ expressing significant ($p<0.05$) and strong relationships between *Eisenia fetida* cocoon hatchability (no. of cocoons hatched/no. of cocoons produced) and soil physical analysis, metals in soil, metals in solution and metals in *Eisenia fetida*.

	b_0	b_1	r^2	p	Regression
Clay %	97.18	-0.79	0.94	0.03	Linear
Cr in soil	93.90	-0.28	0.87	0.07	Linear
Zn in soil	95.33	-7.57	0.99	0.01	Linear
K in solution	95.89	-0.97	0.97	0.01	Linear
Na in <i>Eisenia fetida</i>	106.72	-205.56	0.98	0.01	Linear
Mg in <i>Eisenia fetida</i>	97.38	-985.95	0.89	0.06	Linear
Al in <i>Eisenia fetida</i>	154.00	-6166.67	0.98	0.01	Linear
K in <i>Eisenia fetida</i>	129.33	-616.67	0.98	0.01	Linear
Cr in <i>Eisenia fetida</i>	97.17	-5460.71	0.94	0.03	Linear
Mn in <i>Eisenia fetida</i>	126.73	-1326.69	0.95	0.03	Linear
Fe in <i>Eisenia fetida</i>	100.51	-277.95	0.87	0.07	Linear
Ni in <i>Eisenia fetida</i>	99.11	-7173.65	0.92	0.04	Linear
Cu in <i>Eisenia fetida</i>	100.45	-10670.1	0.95	0.03	Linear
Zn in <i>Eisenia fetida</i>	105.54	-3257.14	0.95	0.02	Linear

3.3 Microbial analysis

Results from the dehydrogenase activity before additions of earthworms and manure, after additions of manure and after additions of manure and earthworms in the control and sites 1, 2 and 3 are shown in Table 13.

Table 13. Mean dehydrogenase activity (INF $\mu\text{g/g/2h}$) assayed in the control material and tailings material from sites 1, 2 and 3 before the additions of earthworms and manure, after the additions of manure and after the additions of earthworms and manure.

Dehydrogenase activity INF $\mu\text{g/g/2h}$	Before earthworms and manure	After additions of manure	After additions of manure and earthworms
Control	113.57 $\pm$ 49.80 ^a	165.81 $\pm$ 29.38 ^a	527.92 $\pm$ 286.04 ^b
Site 1	21.57 $\pm$ 9.59 ^a	125.76 $\pm$ 36.08 ^a	272.84 $\pm$ 96.44 ^b
Site 2	46.23 $\pm$ 17.49 ^a	64.99 $\pm$ 30.62 ^a	223.72 $\pm$ 89.95 ^b
Site 3	16.36 $\pm$ 6.31 ^a	153.66 $\pm$ 51.06 ^b	226.38 $\pm$ 30.97 ^c

a-c: Values with the same letter in superscript were not statistically different from each other ($p>0.05$).

The addition of manure (organic material) resulted in an increase in microbial activity which was expected since higher organic matter content is correlated to microbial activity (Aira *et al.*, 2002). The additions of manure combined with earthworms had the greatest effect because of their role in conditioning the substrate and altering biological activity (Aira *et al.*, 2002). This is an indication of the sustainable role that earthworms and organic matter play in soils.

There was a statistically significant ($p<0.05$) difference in the dehydrogenase activity in the control material after the additions of manure and earthworms versus before the additions of manure and before the additions of manure and earthworms. The control differed ($p<0.05$) significantly from site 3 after the addition of manure, but there were no significant differences ($p>0.05$) between the control and sites 1 and 2 after the additions of manure and earthworms.

Tailings material from site 1 showed the highest increase in dehydrogenase activity after the addition of earthworms and manure. This material showed statistically significant differences ($p<0.05$) in dehydrogenase activity after additions of manure and earthworms versus before the introduction of earthworms and manure. There was also a significant difference in dehydrogenase activity after the additions of earthworms and manure versus after the addition of manure. There was no statistically significant difference ($p>0.05$) between the dehydrogenase activity after the additions of manure and before the additions of manure and earthworms.

Tailings material from site 2 showed a higher mean initial dehydrogenase activity than those from sites 1 and 3. In tailings material from site 2, a statistically significant difference ($p<0.05$) was found in the dehydrogenase activity after the addition of manure and earthworms versus before the introduction of earthworms and manure. There was also a statistically significant difference

($p < 0.05$) in dehydrogenase activity after the addition of earthworms and manure versus after the addition of manure. There were no statistically significant difference ($p > 0.05$) between the dehydrogenase activity after the addition of manure versus before the addition of manure and earthworms.

Tailings material from site 3 showed the lowest mean dehydrogenase activity prior to any additions of manure or earthworms, compared to the other tailings material samples. Dehydrogenase activity before additions of earthworms and manure, after additions of manure and after introduction of manure and earthworms differed significantly ($p < 0.05$) from one another. Ranking of sites' dehydrogenase activity after the additions of earthworms and manure from highest dehydrogenase activity to lowest dehydrogenase activity: Control > Site 1 > Site 3 > Site 2

3.4 Soil mesofauna

The results of the soil mesofauna studies are shown in Table 14 and Tables 21 & 22 in Appendix III. Species richness for site 1 indicated the presence of 27 species for the summer samples and 16 species for the winter samples. The Shannon-Weaver index calculated for site 1 showed an index of 1.72 for the summer samples and an index of 1.38 for the winter samples. The most abundant species found in the materials from site 1 in summer were *Speleorchestes meyeri* of the family Nanorchestidae (Prostigmata), followed by *Coccotydeus sp.* of the family Tydeidae (Prostigmata) and subclass Acari. In the winter samples, the most abundant species were *Speleorchestes meyeri*, followed by *Coccotydaeolus sp.*

Site 2 showed a species richness of 30 species in the summer and 25 species in the winter. The Shannon-Weaver index showed an index of 2.03 for the summer samples and an index of 2.36 for the winter samples. The most abundant species found in materials from site 2 in summer were *Coccotydeus sp.*, followed by *Tydeus munsteri*, then by *Microtydeus sp.* all belonging to the family Tydeidae. In the winter samples, the most abundant species were the *Coccotydeus sp.* followed by the family Psocidae of the insect order Psocoptera.

Site 3 showed a species richness of four for summer and two for winter. The Shannon-Weaver index for summer was calculated at 1.24 and for winter at 0.50. The most abundant species in the summer was *Coccotydeus sp.* followed by equal abundances of *Bakerdania sp.*, of the family Pygmephoridae, and *Tarsocheylus sp.* (Pseudocheylidae). In the winter the most abundant species was *Coccotydeus sp.* followed by the *Coccotydaeolus sp.*

The soil mesofauna studies indicated the following trend in test sites 1, 2 and 3 from highest species diversity and species richness to lowest species diversity and species richness: Site 2 > Site 1 > Site 3.

Table 14. Numbers of individuals per orders Prostigmata, Mesostigmata, Cryptostigmata, Astigmata and Aranea of the subclass Acari and orders Collembola, Psocoptera, Hemiptera, Isoptera, Coleoptera, Hymenoptera and Diptera of the Class Insecta for summer and winter samples taken at sites 1, 2 and 3. Shannon-Weaver index per site and per season i.e. winter samples and summer samples.

	Acari					Insecta							Shannon-Weaver Index
	Prostigmata	Mesostigmata	Cryptostigmata	Astigmata	Aranea	Collembola	Psocoptera	Hemiptera	Isoptera	Coleoptera	Hymenoptera	Diptera	
Site1													
Winter	471	0	17	0	0	4	2	0	0	0	0	0	1.38
Summer	714	32	52	0	0	9	12	0	1	6	0	0	1.72
Site 2													
Winter	416	11	0	7	3	3	130	18	74	13	1	5	2.36
Summer	2419	22	16	0	4	5	109	127	8	5	1	9	2.03
Site 3													
Winter	5	0	0	0	0	0	0	0	0	0	0	1	0.50
Summer	10	0	2	0	0	0	0	0	0	0	0	0	1.24

CHAPTER 4

4. Discussion

Tailings storage facilities are known to contribute to environmental impacts in the short to medium term, however, the long term environmental effects of these facilities have not been established. A study on the ecotoxicological effects of tailings storage facilities of different ages may provide an indication of the long term effects on the environment and on the sustainability of current rehabilitation techniques.

4.1 Soil physical and chemical analysis

The first level of the ecological risk assessment on platinum tailings material was to screen the soils for contaminants of potential concern (Efroymson *et al.*, 1997), in this case heavy metals. Benchmarks were used in order to determine whether measured heavy metal concentrations were above the toxicological threshold as proposed by the Canadian Council of Ministers of the Environment (CCME) and the Dutch National Institute of Public Health and Environmental Protection (RIVM). Benchmarks for earthworm toxicity and toxicity to soil microorganisms and microbial processes as set by Efroymson *et al.* (1997) were also used. These were used because there are currently no standards proposed in South African legislation regarding polluted soils (Eijsackers *et al.*, 2006). These benchmarks indicate the total metals in soil and were therefore screened against the total metal results obtained from the tailings materials. Contaminants of potential concern are those that exceed both the benchmarks and the background concentration of the soil type (Efroymson *et al.*, 1997). None of the total metal concentrations exceeded the CCME and the RIVM benchmarks, however, earthworm toxicity and toxicity for soil microorganisms and microbial processes were exceeded (Sections 4.2 & 4.3).

Total metal analysis is an indication of the sum of exchangeable and the soluble portion of metals in the soil (Morgan *et al.*, 1992). In general sites 1 and 2 had the highest total metal concentrations. This could be due to a variety of factors like pH, organic matter content and soil texture as will be explained below. Total metal concentration provides an indication of what can be leached out of the soil if conditions deteriorate, e.g. a decrease in pH. If a decrease in pH occurs and the soil structure in terms of colloidal particles is not strong enough there is a possibility that metals will be leached out. Therefore, sites 1 and 2 pose a danger for possible leaching should soil management not take place. Soluble metal analysis is an indication of the metals that are available to plants and organisms for uptake from the soil. In general site 2 indicated higher concentrations of soluble metals than those of the control, sites 1 and 3. This is an indication that site 2 has a higher

bioavailability of heavy metals which can be taken up by plants and animals. However, when these results were compared to that of the BCF, it was not the case.

The pH in the three test sites did not differ significantly from one another. Site 2 had a slightly higher pH than site 1 which could mean that there is a possibility that platinum tailings storage facilities will decrease in pH over time which may lead to leaching of elements. The pH at site 3 (negative control/"raw sludge") was, however, lower than site 1 and site 2. This could be attributed to leaching out of elements due to continuous draining of water. Linear regressions showed that pH had a positive relationship with Mg and Mn in soil, where an increase in pH would result in an increase in Mg and Mn. According to Ashman & Puri (2002), Mg increases with an increase in pH between values of approximately 7.5–9, where after it decreases. The finding in the current study, therefore corresponds to literature as pH ranged from 7.58–7.73. Manganese normally decreases with an increase in pH (Ashman & Puri, 2002). There were no significant ($p > 0.05$) relationships between pH and metals in solution and metals in *Eisenia fetida*.

Physical soil parameters that affect the bioavailability of metals in soils are organic matter content and particle size distribution (Fent, 2004). Linear regressions showed that organic matter content did not play a significant ($p > 0.05$) role in the bioavailability of metals in the tailings materials. Linear relationships were not significant between organic matter content and metals in soil, metals in solution and metals in *Eisenia fetida*. Even though linear regressions did not show significant relationships between organic matter content and bioavailability of metals, a trend was evident between Cu concentrations and organic matter. Soluble metal concentrations showed that Cu concentrations were related to organic matter content, as an increase in Cu concentration was found with an increase in organic matter content. This corresponds to work conducted by Jäggy & Streit (1982), Bengtsson *et al.* (1986), van Gestel (1992) all of whom determined that the bioavailability of Cu is dependent on properties like soil organic matter content.

Soil textures determined with using the USDA textural triangles (Ashman & Puri, 2002) indicated that the control material was loamy sand, due to its extremely high sand content. This is because the material was made up with a high percentage of sand (70%) according to OECD guideline 207 (1984). Although this soil does not have a high percentage of clay particles, the high percentage of organic matter in this material may be sufficient to make up colloidal particles for storage and release of chemicals into the soil (Ashman & Puri, 2002). The USDA textural triangle indicated that site 1 had a loamy sand texture, site 2 had a silt loam texture and site 3 a sandy loam texture. These textures could explain why site 2 had generally higher total and soluble metal concentrations than the control and sites 1 and 3. This is related to the higher clay percentages in this material

resulting in more colloidal particles to which cations can attach themselves, increasing the buffer capacity of soils and thus their ability to store and supply nutrients (Ashman & Puri, 2002). Even though site 2 in general had higher total and soluble metal concentrations, some metals like Al, Fe and Ni concentrations were higher in sites 1 and 3. Aluminium showed higher total metal concentrations in site 1 than in site 2 and higher soluble metal concentrations in the control and site 3. This may be because Al tends to increase with decreasing pH (Ashman & Puri, 2002). Iron concentration tends to decrease with an increase in pH (Ashman & Puri, 2002), which could provide an explanation for the higher concentration of total Fe in site 1 opposed to that found in site 2. However, the pH of the test sites did not differ significantly from one another and therefore may not be the reason for the higher Al and Fe concentrations in materials from test site 1. Linear regressions indicated that clay percentage did not play a significant ($p > 0.05$) role in metal bioavailability in soil, in solution and in *Eisenia fetida*. However, it had a strong negative relationship with cocoon hatchability. This can be explained by the materials with higher clay content (site 2) having higher metals in solution due to their high buffer capacities i.e. their ability to supply the soil with nutrients or in this case heavy metals.

4.2 Earthworm ecotoxicological studies

It has been illustrated that earthworm bioassays can be utilised to determine the extent of soil toxicity (Hankard *et al.*, 2005). Taking the first level of ecological risk assessment into consideration there is a definite potential for platinum tailings material to have an effect on earthworms, especially with regards to Cr concentrations, which exceeded benchmarks for earthworm toxicity in all three tailings storage facilities. Linear regressions indicated that there was a strong relationship between Cr in soil and Cr in solution and a significant ($p < 0.05$) relationship between Cr in soil (total metals) and Cr in *Eisenia fetida*.

Significant differences in earthworm biomasses were measured between days 14 to day 28, where the earthworms exposed to the control material were higher in biomass than those exposed to tailings materials from the test sites. All earthworms exposed to tailings materials experienced an increase in biomass, however not as much as the earthworms exposed to the control materials. Earthworms exposed to tailings material from the oldest test site (site 1) experienced the highest increase in earthworm biomass, followed by those exposed to the tailings material from the negative control/"raw" tailings (site 3) and then those exposed to tailings material from site 2. Earthworms exposed to tailings material from site 3 did not have the greatest effect on biomass due to the initial lack in dehydrogenase activity and organic matter. Therefore, food was not as available to the earthworms exposed to tailings material from site 3 as that experienced in the other test sites. Platinum tailings material thus inhibited the growth of *Eisenia fetida* compared to the

earthworms exposed to the control material. Earthworms did, however, not lose biomass as found in previous bioassay studies conducted on platinum tailings storage facilities using *Eisenia fetida* (Maboeta & van Rensburg, 2003; Maboeta *et al.*, 2007). Linear regressions indicated a strong positive relationship between earthworm biomasses and NRR-T, resulting in a relationship where biomass increased with NRR-T. Earthworms with higher biomass are thus less vulnerable to stress than those with lower biomass. Linear regressions also indicated that earthworm biomass was negatively affected by Al levels in soil and positively affected by Mn and Zn in solution. It has not yet been established what the effects of Al are on earthworms, but similar studies conducted by Maboeta & van Rensburg (2003) indicated that Al at a concentration of 3.35 mg/kg had an effect on growth and reproductive success in *Eisenia fetida*. This concentration is much lower than the concentrations measured in the tailings materials for this study. Therefore, Al could have had a major impact on the growth, reproduction and mortality of *Eisenia fetida*. Manganese and Zn are micro-elements necessary for organism vitality and growth, explaining the positive relationship between biomasses and these two elements.

The hatching success of the cocoons indicated that there was no significant ($p > 0.05$) difference in reproductive success between the earthworms exposed to material in the control and those exposed to tailings material. This parameter normally provides a good representation of reproductive success (Maboeta *et al.*, 2007), however in this case no such conclusions could be drawn. Earthworms exposed to tailings materials from sites 1 and 3, showed the highest reproductive success rate (93%), followed by those exposed to the control material (91%) and then those exposed to tailings material from site 2 (80%). There was no significant difference ($p > 0.05$) between the control and the three test sites in the mean number of cocoons produced and cocoon hatchability and number of cocoons were, therefore not sensitive sub-lethal parameters in this experiment. However, the mean number of juveniles per cocoon and the mean biomass of the juveniles differed significantly ($p < 0.05$) between the control and sites 1, 2 and 3. Juveniles per cocoon indicated that the control had the highest number of juveniles per cocoon, followed by site 2, then site 1 and site 3. Juveniles per cocoon were therefore a sensitive sub-lethal parameter for reproduction. Biomass of juveniles exposed to test sites, differed significantly from the control. Platinum tailings material in this case had an effect on the number of juveniles hatched per cocoon and the masses of the juveniles.

The International Standards Organisation (ISO) guideline for reproduction testing of *Eisenia fetida*, suggests that reproduction is measured solely in terms of cocoon production (Reinecke *et al.*, 2001). According to the latter authors this measurement of reproduction is misleading as contaminants may not specifically affect growth or cocoon production, but may still have an effect

on cocoon viability. Reproduction is vital to maintain and develop populations leading to higher population numbers which are limited by adverse environmental conditions (Begon *et al.*, 1996). There may be a single heavy metal or a combination thereof present in the tailings material that interferes with either the gametes, or fertilization or embryonic development (Reinecke *et al.*, 2001). These effects can be attributed to the strong and significant negative linear relationships between cocoon hatchability and Cr and Zn in soil and K in solution. Previous studies indicated that the 21 day EC₅₀ based on cocoon production for *Eisenia fetida* was at concentrations of 892 mg Cr/kg dry weight (Lock & Janssen, 2002) and 155 mg Cr/kg dry weight (Van Gestel *et al.*, 1992). EC₅₀ is the lethal concentration estimated to kill 50% of the test population (Hoffman *et al.*, 2003). Studies conducted by Molnár *et al.* (1989) indicated that a 50% reduction in reproduction occurred at concentrations of 250 mg Cr/kg wet weight during 56 day reproduction tests. These concentrations are much higher than the concentrations measured in the tailings materials used in this study. Studies conducted by Spurgeon & Hopkin (1996) indicated that Zn affected cocoon production of adults at concentrations of 3600 mg Zn/kg and juveniles' sexual reproduction at concentrations of 1860 mg Zn/kg. Concentrations of Zn found in tailings materials during this study were much lower than these concentrations. Significant negative linear relationships were also determined between Na, Al, K, Cr, Mn, Ni, Cu and Zn concentrations measured in *Eisenia fetida* and cocoon hatchability. This indicates that the higher the amounts of individual metals bioaccumulated in earthworms the greater the effects of these metals on the hatchability of the cocoons. Earthworms exposed to tailings material from site 2 showed significantly ($p < 0.05$) higher concentrations of metals in earthworm tissue compared with the earthworms exposed to tailings materials from sites 1 and 3. This explains the lower reproductive success rate (80%) obtained in earthworms exposed to tailings material from site 2 compared to those exposed to tailings materials from sites 1 and 3 (93%). Accumulation of metals in *Eisenia fetida* thus affected the hatchability of cocoons.

Earthworm mortality was the highest in earthworms exposed to tailings material from site 1 (21%), followed by those exposed to tailings material from site 3 (10%) and then by those exposed to tailings material from site 2 (3%). Bioassay studies conducted by Maboeta *et al.* (2007) on platinum tailings storage facilities using *Eisenia fetida*, showed no mortality. However, these results were obtained from materials taken from the toe (bottom where the foundation of the tailings storage facility meets the ground) of the tailings storage facility and then at 1km intervals from the tailings storage facility and not at the top of the tailings storage facility as in this case. There were no statistically significant ($p > 0.05$) differences between the mortality rate experienced in earthworms exposed to the control material and the mortality rates experienced in earthworms exposed to the tailings materials. Therefore, mortality was not a sensitive sub-lethal indicator for this experiment. There were no significant linear relationships between mortality and earthworm biomasses, neutral

red retention times and cocoon hatchability. There was, however, a trend between increases in mean earthworm biomasses and mortality for earthworms exposed to tailings materials. Earthworms that showed high percentages in mortality also had higher mean biomass increase, e.g. earthworms exposed to tailings material from site 1 had a mortality rate of 21% and a biomass increase of 66%, earthworms exposed to tailings material from site 3 had a mortality rate of 10% and a biomass increase of 63%, followed by those exposed to tailings material from site 2 with a 3% mortality rate and a 58% biomass increase. This could be due to the genetically weaker organisms dying off and allowing the genetically stronger organisms to increase in biomass, i.e. more food resources available for the remaining organisms to survive. Mortality also plays a significant role in earthworm populations and thus has an impact on the continuance of the organism in an ecosystem.

Lysosomal membrane stability measured by means of the NRR-T assay did not show a statistically significant difference ($p>0.05$) from day one to day seven. Statistically significant differences ($p<0.05$) were experienced between the earthworms exposed to the control material and those exposed to the tailings materials from day 14 to day 28. On day 28 the NRR-T of the earthworms exposed to tailings material from site 2 did not differ significantly ($p>0.05$) from the NRR-T of the earthworms exposed to the control, however the NRR-T of the earthworms exposed to tailings materials from sites 1 and 3 differed significantly ($p<0.05$) from the NRR-T of those exposed to the control. This was an indication that earthworms exposed to tailings material from site 1 (oldest tailings storage facility) and 3 (negative control) were under stress. NRR-T is a more sensitive indicator of stress than mortality and biomass change, therefore earlier threshold effects can be obtained by means of the NRR-T assay (Maboeta *et al.*, 2004). The earthworms exposed to tailings material from site 1, showed a vast increase in NRR-T from day 14 (5.00 ± 9.80 minutes) to day 21 (14.33 ± 9.59 minutes). This could be explained by the production of new coelomocytes at all times, allowing for the potential recovery of the NRR-T response as described by Svendsen *et al.* (2004).

Biomarkers are either chemically nonspecific or specific depending on their responses to a range of chemicals. Nonspecific biomarkers respond to a range of pollutants and are useful when the types of toxicants are unknown. Specific biomarkers are highly sensitive to a particular pollutant class or a given pollutant (Maboeta *et al.*, 2004). High sub-lethal toxicity is experienced by exposure to Cd. Moderate sub-lethal toxicity is experienced when exposed to Cu, Zn, and Pb. Low sub-lethal toxicity is experienced when exposed to iprodione. Since Cd was only present in trace amounts in the tailings materials, it is not likely that the earthworms were exposed to a high sub-lethally toxic substance, however levels of Cu, Zn and Pb could have caused moderate sub-lethal toxicity. In this case K has a positive relationship with NRR-T, opposed to a negative relationship experienced for

reproduction. This could indicate that concentrations of K may be sufficient for normal functioning of earthworms, but may be too high for reproduction. This may be an indication that reproduction is more sensitive parameter than NRR-T. Once again pollutants could be synergistic in the effects that they have on organisms, because of the effects of a combination of contaminants. Linear regressions indicate that K in soil has a positive significant relationship with NRR-T. There were significant ($p < 0.05$) linear relationships between NRR-T and Mn and Zn in solution. Manganese and Zn are micro-elements necessary for organism vitality and growth explaining the positive linear relationship between NRR-T and the availability of these metals in solution. Zinc is known to be a stabiliser of membranes and various macro-molecules (Chvapil 1973; Sugarman 1983; Shankar & Prasad 1998), thus the positive relationship between Zn and NRR-T.

In a study by Svendsen *et al.* (2004), the relative sensitivities of endpoints in the order of most sensitive to least were as follows: NRR-T > reproduction > growth > survival. In this current study the measured relative sensitivities indicated NRR-T > reproduction > survival > growth. In order to detect pollution effects before they become irreversible, the biomarker response should be sensitive enough to detect effects before key life cycle parameters such as reproduction and growth are affected (Svendsen *et al.*, 2004). Changes in lysosomal integrity were more sensitive than the ecological life cycle endpoints measured. This is similarly encountered in a number of other studies (e.g. studies conducted by Scott-Fordsmand *et al.*, 1998; Scott-Fordsmand *et al.*, 2000; Svendsen, 2000). According to Kula & Larink (1997), the most sensitive biological response in terms of sub-lethal effects is cocoon production and hatchability. Being the most sensitive endpoint, NRR-T indicated that earthworms exposed to tailings materials from sites 1 and 3 were under stress. However, hatchability and number of cocoons indicated that earthworms exposed to these materials did not significantly differ from those exposed to the control materials. When looking at the results of the juveniles hatched per cocoon, the same pattern arises as for NRR-T. Percentage growth indicated that earthworms exposed to materials from sites 2 and 3 had the lowest biomass increase. The mean biomasses of the earthworms exposed to sites 1, 2 and 3 differed significantly from the control, indicating earthworms exposed to all three sites experienced stress. In studies conducted by Kula & Larink (1997), body weight development was also found to be an insensitive response. After 28 days, the test to determine the survival earthworms indicated that site 1 and 3 experienced the least survival success (i.e. the highest mortality percentage). All endpoints indicated that earthworms from sites 1 (oldest tailings storage facilities) and 3 (raw tailings sludge) experienced the most stress. All endpoints except for growth and reproduction suggested that earthworms exposed to tailings material from site 2 were under the least stress. Cocoon hatchability and number of cocoons did not significantly differ from the control, however when

taking the number of juveniles hatched per cocoon into consideration, site 2 showed the highest number of juveniles hatched per cocoon out of all the test sites.

Metal concentrations measured in earthworms are an indication of what may be bioaccumulated in the food chain. This is important when determining the long term effects of tailings storage facilities on the environment as tailings storage facilities rehabilitated to wilderness areas may pose a risk to the organisms that inhabit these areas. This will also provide an indication of the type of land-use that can be safely recommended for closure. Linear regressions indicated that Cr and Mg in solution have a strong relationship with Cr and Mg respectively in *Eisenia fetida*. This is an indication of bioaccumulation of these metals in this organism. In general, metal concentrations measured in earthworms were the highest for site 2, followed by site 3, then site 1 and finally the control. Even though site 2 showed the highest concentration of metals in the earthworm tissues, site 3 had the highest BCFs, followed by the control, site 1 and then site 2. This indicates that earthworms exposed to materials from site 2 did not bioaccumulate metals through contact with the soil, but could have possibly bioaccumulated these metals through ingestion of microorganisms. Microorganisms would have bioaccumulated heavy metals through ingestion of residual organic matter collected in the sample from the tailings storage facility. Earthworms exposed to tailings material from site 2 also showed the least stress when they were tested by the NRR-T assay. Normally accumulation of metals would increase the stress in earthworms, but this was not the case for site 2 in this study. This can be explained by internal physiological mechanisms adopted by earthworms to deal with toxicants. Studies conducted by Morgan & Morgan (1990) showed that Cd, Pb and Zn tend to accumulate in the posterior alimentary canal of the earthworm, preventing dispersion of these metals to other tissues. As mentioned by the previous authors, this could be a detoxification strategy to immobilise metals by means of accumulation. This also could explain why earthworms exposed to material from site 2 were not under stress despite high tissue metal concentrations.

When comparing the tissue metal concentrations to the soluble metal concentrations, it was evident that higher concentrations of metals in earthworm tissues were related to the soluble metal concentrations. Bioconcentration factors were mostly insignificant because the ratio of metals in earthworm tissue to total metals is proportionately small. This could be an indication that metal toxicity can also be determined by the soluble metal concentration as in the case for toxicity of pesticides (van Gestel & Ma, 1990). From the earthworm tissue metal data, it was clear that K, Ni, Mn and Zn bioaccumulated in earthworm tissue. BCF values were less than one indicating that earthworms were limited in their ability to bioaccumulate metals in their body tissues. This

corresponds with studies conducted by Maboeta *et al.* (2007) on platinum tailings storage facilities using *Eisenia fetida* as a bioindicator. This inability of earthworms to bioaccumulate metals may be attributed to the high pH's of these tailings storage facilities (Maboeta *et al.*, 2007). It is difficult to determine which metals have an effect on earthworms, as metals are assessed in a mixture and toxicity is determined by the bioavailability of toxicants.

4.3 Microbial analysis

Dehydrogenase activity is indicative of the effects of contaminants on microbial activity (Hattori, 1992; Chander & Brookes, 1993; Dick, 1994) and is correlated with microbial biomass (von Mersi & Schinner, 1991). The initial, (before the addition of manure and earthworms) mean dehydrogenase activity in tailings material from site 2 was higher than that of tailings materials from sites 1 and 3. Relating these results to the age of the rehabilitated tailings storage facilities, it is an indication that the older tailings storage facility (site 1) showed more ecological stress than the younger tailings storage facility (site 2). However, when taking site 3 into account this was not the case because the youngest tailings storage facility (6 years old) showed the most ecological stress. The importance of soil management must not be ignored in this case and microbial assays can be related to management practice as was determined in previous studies conducted by Claassens *et al.* (2008), on soil microbial assays in a post-mining chronosequence of rehabilitated coal tailings dumps. Therefore, these results could be due to the high amount of manure and vegetative material administered to site 2. Before additions of earthworms and manure, there was a significant difference ($p < 0.05$) between the dehydrogenase activity in the control and sites 1, 2 and 3. This could provide an indication of the effects of metals on microbial activity on these test sites. The effects of metals are more evident in sites 1 and 3 than in site 2. Metals that could have affected dehydrogenase activity initially were Al, Cr and Fe because these metal concentrations were above the toxicity levels for soil microorganisms and microbial processes as determined by Efroymsen *et al.* (1997).

After manure was added to the samples, an increase in dehydrogenase activity was noticed. This can be explained by the feeding habits of soil microbes and their ability to biochemically degrade organic matter (Aira *et al.*, 2002). There was a statistically significant increase ($p < 0.05$) in dehydrogenase activity after the additions of earthworms and manure opposed to material with only additions of manure. This can be explained by the ability of earthworms to stimulate microbial activity (Binet & Trehen, 1992; Lavelle & Martin, 1992) by increasing the surface area for microbial colonisation of the substrate and enzymatic action through comminuting the organic residues (Edwards & Fletcher, 1988). Earthworm casts deposited on the soil surface during their burrowing activity provide a good environment for soil microorganisms (Coyne, 1999). Microorganisms thrive

from earthworm casts and they also provide a nutrient pool for earthworms (Edwards & Fletcher, 1988). The initial dehydrogenase activity measured in the material from site 3 was the lowest out of all the tailings material samples. This is expected as operational tailings storage facilities are not treated with any manure or ameliorants.

A strong linear relationship exists between dehydrogenase activity and earthworm biomasses and a significant relationship exists between dehydrogenase activity and NRR-T. This is because microorganisms provide a nutrient pool for earthworms (Edwards & Fletcher, 1988). Studies conducted by Maboeta & van Rensburg (2003) indicated that *Eisenia fetida* exposed to platinum tailings materials inoculated with microorganisms had higher biomasses than those that were not inoculated with microorganisms. There was no significant ($p>0.05$) relationship between dehydrogenase activity and cocoon hatchability, therefore microbial activity did not have an effect on earthworm reproduction in this case.

4.4 Soil mesofauna

Soil mesofauna studies are used in ecotoxicology to assess contaminants on the level of community structure and ecosystem functioning (Baird *et al.*, 2001). For this reason species were divided into functional groups according to their feeding habits as described by Wahl (2007). These groups were mycophagous organisms feeding mainly on fungi (MP), predatory organisms (Pred), saprophagous and omnivorous organisms feeding on dead or decaying organic material (SO), micro-algivoracious and bacteriophagous organisms (MB) and plant parasitic and herbivorous organisms (Ppar) feeding off living plant material (Wahl, 2007). It is difficult to place these organisms in clear cut groups as they tend to change feeding habits under certain physical conditions. Placement of these species under certain groups may be contradictory to other publications or views.

The most abundant functional group of individuals found on all the tailings storage facilities were the micro-algivoracious and bacteriophagous (MB) group. The abundance of this functional group is an indication of the level of succession in this community being predominantly pioneer. The most abundant taxon found in the tailings materials was the Prostigmatic mites. This was expected because of the harsh, dry conditions on the tailings storage facilities which significantly stimulates these organisms (Holmstrup *et al.*, 2007). The presence of insects in the tailings materials is most probably a result of an artificial condition as insects do not normally form part of the soil fauna group, except in the form of insect larva. It is suggested that they accidentally fell from the vegetation into the samples during sampling.

The most abundant functional group of individuals in site 1 for summer and winter were MB. This is in contrast to what was found in previous studies conducted by Wahl (2007) on platinum tailings storage facilities. The second most abundant functional group in site 1 was the Pred for both winter and summer samples and the third most abundant functional group was the MP. Site 1 was represented by all the functional groups, however, only two individuals were identified under the Ppar. This indicates that there is a variety of functionalities represented in the most abundant functional groups and that this tailings storage facility will be able to recover after a catastrophic event. *Speleorchestes meyeri* of the family Nanorchestidae, were the most abundant species found on site 1 in the summer, followed by *Coccotydeus sp.*, *Coccotydaeolus sp.* and *Acronothrus sp.* The latter two species are both known to be bacteriophagous (Wahl, 2007). The winter samples indicated that the most abundant species were *Speleorchestes meyeri*, followed by *Coccotydaeolus sp.* These species are fungivorous and phytophagous.

The most abundant functional group in site 2 was MB in the summer and winter samples. In the summer samples, the second most abundant functional group was MP, followed by Ppar. Predators and saprophagous and omnivorous organisms were also well represented in the summer samples. In the winter samples the second most abundant functional group was the SO, followed by the Pred. This trend indicates a drop in numbers of MP in the winter months. This may be due to the drop in moisture in winter months resulting in a decrease in fungi. The most abundant species found in materials from site 2 in summer were *Coccotydeus sp.*, followed by *Tydeus munsteri sp.*, then by *Microtydeus sp.*, all belonging to the family Tydeidae. *Coccotydeus sp.* and *Microtydeus sp.* are bacteriophagous and *Tydeus munsteri sp.* is fungiphagous. In the winter samples, the most abundant species were *Coccotydeus sp.* of the family Tydeidae, followed by the family Psocidae of the order Psocoptera. Psocidae are known to be mycophagous insects, thus feeding mainly on fungi (Walter, 1988).

The most abundant functional group in the summer and the only functional group found in the winter samples for site 3 is also the MB group. This was followed by equal amounts of MP and Pred. No representatives of the Ppar and the SO functional groups were recovered. The most abundant species found in the materials from site 3 in the summer were *Coccotydeus sp.* followed by equal abundances of *Bakerdania sp.* (Pygmephoridae) and *Tarsocheylus sp.* (Pseudocheylidae). *Bakerdania sp.* are known to be mycophagous organisms which feed on fungi and *Tarsocheylus sp.* are known to be predacious. In the winter the most abundant species were *Coccotydeus sp.* followed by *Coccotydaeolus sp.* These are both bacteriophagous species.

Species richness and biodiversity are strong indicators of structural and functional diversity (Eijsackers, 2004). Soil mesofauna studies conducted on sites 1, 2 and 3 indicate that site 2 showed the highest species richness and Shannon-Weaver biodiversity index, followed by sites 1 and 3. This could indicate that site 2 has a more sustainable rehabilitation practice than site 1. This can be corroborated by studies conducted by Andrés & Mateos (2006), which confirm that the richness of taxa is indicative of the maturity of the vegetal community. Site 2 indicated a higher Shannon-Weaver index in winter than in summer. This can be attributed to the high number of species found in winter, although the populations are small.

When comparing the soil mesofauna data to the earthworm bioassay results, it confirms the trend of the NRR-T data (i.e. Site 2 > Site 1 > Site 3) and the reproduction data (juveniles hatched per cocoon). This could be explained by the ability of earthworms to survive and reproduce in habitats with higher species diversity. Dehydrogenase activity before the introduction of manure and earthworms shows the same trend as that of the soil mesofauna. This can be explained by the fact that soil mesofauna feed on soil microorganisms. An increase in the biomass of soil microorganisms, therefore, leads to an increase in diversity of soil mesofauna.

4.5 Evaluation of methodology and results

Table 15 indicates the rankings of the test sites in relationship with the outcome of the experiments. The first stage of the ecological risk assessment indicated that site 1, the oldest site, had the highest concentrations of total metals, followed by site 2, site 3 ("raw sludge") and finally the control. This indicated that total metals increased with the age of the tailings storage facilities. Soluble metal concentrations were the highest in site 2, followed by site 3 ("raw sludge"), the control and finally site 1. Soil physical and chemical analyses were important to assess the potential contaminants as well as their potential bioavailabilities and are important for the first stage of ecological risk assessment. This methodology is however expensive, requires a certain amount of training, skills and technology.

The second phase of the ecological risk assessment indicated that NRR-T, reproduction, dehydrogenase activity and soil mesofauna biodiversity showed the same trend in results. NRR-T was the most sensitive indicator of ecological stress used for the purpose of this study. This methodology is time-consuming, requires a certain amount of training and is fairly expensive. It may be biased due to the use of neutral red dye which may affect the integrity of the lysosome membrane and because some coelomocytes may not be counted due to a lack of adherence to the microscope slide. Earthworm reproduction in the form of number of juveniles hatched per cocoon

was extremely accurate in predicting ecotoxicological effects on community and ecosystem functioning. Growth and mortality did not show the same trend as that of community and ecosystem functioning, but do provide an early warning of possible toxic effects. Earthworm bioassay studies in the form of sub-lethal and lethal endpoints are cost effective, require a minimum amount of skills, are not as time-consuming as NRR-T and soil mesofaunal studies and provide reasonably accurate results in terms of early warning signs of community and ecosystem functioning.

Dehydrogenase assays were extremely accurate in determining the ecotoxicological effects of heavy metals on community structure and ecosystem functioning. They are not as time consuming as the earthworm bioassay studies and soil mesofauna studies, they require a fair amount of training and skills and are also fairly expensive compared to soil mesofaunal studies. They are, however, cheaper than soil chemical and physical analyses and cost more or less the same as earthworm bioassay studies.

Soil mesofauna studies provide an indication of the community structure and ecosystem functioning which provides an indication of the effects of contaminants on this level of organisation. They are extremely time consuming, require a large amount of research to interpret data and require highly skilled personnel to identify organisms.

Table 15. Results of earthworm bioassay studies, dehydrogenase assays and soil mesofauna studies ranked from one to four (best results to poorest results or results indicating the most stressed habitats) and indicating the levels of ecology from cellular to ecosystem functioning.

	Cellular Level	→	Organ	→	Organism	→	Population	→	Community	→	Community structure and ecosystem functioning	
	NRR-T		Reproduction (Juveniles hatched per cocoon)		Growth (Percentage increase in Biomass)		Mortality		Dehydrogenase (Before earthworms and manure)		Soil Mesofauna (Shannon- Weaver index)	Average Score
Control	1		1		1		1		1		1	1
Site 1	3		3		2		4		3		3	3
Site 2	2		2		4		2		2		2	2
Site 3	4		4		3		3		4		4	4

CHAPTER 5

5. Conclusions and Recommendations

5.1 Conclusions

An ecotoxicological study was conducted on tailings storage facilities of different ages (30 years, 20 years and 6 years) in order to determine the effects of heavy metal contamination with age.

First stage ecotoxicity screening indicated that concentrations of certain heavy metals (especially Cr) present in platinum tailings materials could have a potential effect on microorganisms, microbial processes and earthworms. Soil physical analysis was most useful in interpreting data and showed that bioavailability of metals is entirely dependent on these parameters. Organic matter had an effect on the Cu concentrations in solution of tailings materials. Soil texture showed that clay percentage played a major role in total and soluble metal concentrations, whereby materials with higher percentages of clay showed higher concentrations of metals.

There were no trends determined between tailings storage facility ages and results obtained from earthworm ecotoxicological studies. Results from sites 1 (30 years old), 2 (20 years old) and 3 (6 years old) showed that organisms exposed to tailings material from site 3 were under the most stress, followed by those exposed to tailings material from site 1, then those exposed to tailings material from site 2. These results are more likely to be an indication of the rehabilitation and soil management measures administered, because site 3 (6 years old) has not been rehabilitated. Tissue metal analysis of *Eisenia fetida* exposed to tailings material from site 2 indicated that these earthworms had significantly higher concentrations of metals than those exposed to tailings materials from sites 1 and 3. This explains why the hatchability of the cocoons was not as high as the others, because heavy metals are known to affect the viability of cocoons (Reinecke *et al.*, 2001). Bioconcentration factors however showed that earthworms exposed to tailings material from site 2 did not bioaccumulate large concentrations of metals relative to total metal concentrations in the tailings materials. High tissue metal concentrations of earthworms exposed to tailings material from site 2 can be attributed to ingestion of microorganisms contaminated with heavy metals. Earthworms exposed to tailings material from site 2 showed high tissue metal concentrations, but were found to be the least stressed out of all the earthworms exposed to tailings materials. This can be explained by the detoxification strategy adopted by earthworms to immobilise certain metals by means of accumulation. Soluble metal concentrations seemed to correspond more with tissue metal concentrations than total metal concentrations.

Dehydrogenase activity corroborates the earthworm ecotoxicological results and also indicated that environmental impacts of tailings storage facilities might not increase with age, but may provide a stronger indication of the effects of management practices. Earthworms also play a significant role in increasing the number and biomass of soil microbes because significant increases in dehydrogenase activity were noticed after the additions of *Eisenia fetida* to platinum tailings materials. Soil microbes are significant role players in nutrient cycling and thus play a significant role in the sustainability of rehabilitation practices. Assays of microbial activity in combination with earthworm bioassays could provide a more comprehensive view of the status of disturbed ecosystems. Earthworm bioassays can provide an early warning system on the cellular or organism level whereas microbial activity provides a more comprehensive view on community level.

Soil mesofauna studies corroborated the results obtained for the earthworm bioassay studies and the dehydrogenase activity. Species diversity did not show a relationship with age of tailings storage facilities. Once again this trend may be attributed to management practices on the tailings storage facilities.

Ecotoxicological studies conducted on platinum tailings storage facilities of different ages indicated that environmental effects may not be attributed to the age of tailings storage facilities, but are more likely to be affected by management practices.

5.2 Recommendations

The use of chemical and physical soil analyses for ecotoxicological studies is extremely important for screening contaminants of potential concern and interpreting biological test results. Total and soluble metal concentrations provided an explanation of biological results obtained such as reproduction, NRR-T, growth and microbial activity. Soil physical analysis provides a platform for interpretation of bioavailabilities of contaminants, especially in this case pH and clay percentage. Even though soil analysis is expensive, it should remain part and parcel of future ecotoxicological studies.

In South Africa, it is extremely difficult to determine the risks of contaminated soils because of the lack of benchmarks for polluted soil in legislation (Eijsackers *et al.*, 2006). Overseas benchmarks such as Canadian and Dutch benchmarks are often used for screening of contaminants in South African soils. This poses as a potential problem because the background values of South African soils are not taken into consideration and could cause a misinterpretation of the data. There is an urgent need for soil pollution benchmarks in South Africa and it is recommended that government

authorities determine these benchmarks and enact them into South African environmental legislation.

Earthworm bioassays in the form of NRR-T, growth, reproduction and mortality are recommended as early warning indicators of ecological stress and deserve their role in ecotoxicological testing. NRR-T was extremely accurate in predicting the results obtained for community and ecosystem functioning and is recommended for future use in ecotoxicological studies. This corresponds to conclusions drawn by Svendsen & Weeks (1997) about the neutral red assay which was recommended to be used as an early warning to prevent adverse effects on individuals or populations. In terms of reproduction, cocoon viability should take preference over cocoon production in future studies as it is known that contaminants do not only have an effect on cocoon production, but on cocoon viability as well (Reinecke *et al.*, 2001).

Results from this study indicated that tissue metal concentrations correlated stronger with soluble metal concentrations than total metal concentrations. Bioconcentration calculations should thus be calculated by using soluble metal concentrations rather than total metal concentrations. This would provide a more accurate estimation on the tissue metal concentrations.

Dehydrogenase assays are recommended for ecotoxicological studies in the future as they are extremely accurate, are fairly rapidly determined and require only a limited amount of training and skills. Soil mesofauna studies are an excellent indication of the effects of heavy metals on community structure and functioning. There are however drawbacks to using this methodology for ecotoxicological studies. These drawbacks are: extensive time taken for research to interpret data, identification of soil mesofauna requires highly skilled personnel and the shortage of skilled entomologists world wide. However these studies are essential for studies related to total ecosystem functioning.

This study showed that trace amounts of heavy metals from platinum tailings storage facilities are bioaccumulated in earthworms. There is a possibility that other organisms living on rehabilitated tailings storage facilities could also bioaccumulate heavy metals. Further studies are recommended to determine the effects of heavy metals on predators (e.g. predatory birds) currently feeding off prey on platinum tailings storage facilities. Alternative organisms should also be considered to assess bioaccumulation of heavy metals like soil mesofauna studied by Wahl (2007).

Although *Eisenia fetida* increase the microbial activity in platinum tailings materials, they will not survive on tailings storage facilities due to the harsh temperatures and poor moisture content. Earthworm byproducts, such as vermicompost is recommended to be administered to tailings

storage facilities as a growth medium and stimulant for soil microbial activity in an attempt to improve the ability of tailings storage facilities to sustain vegetative growth.

The use of linear regressions to interpret the data obtained from these studies was useful in some instances. However, if not careful can misguide interpretations e.g. in the case of organic matter content. In this instance, linear regressions did not indicate any significant relationships ($p < 0.05$) between organic matter content and metals in soil, metals in solution and metals in *Eisenia fetida*, however trends were evident between Cu and organic matter content. Nahmani *et al.* (2007) also used linear regressions to interpret data of heavy metal contamination on *Eisenia fetida* and results showed some interesting relationships between these parameters. Linear or multiple regressions are useful when determining relationships between criteria, but should however be used with caution.

The ecotoxicological tests indicated that environmental impacts of platinum tailings storage facilities were not related to age, but rather to rehabilitation methodologies used on the tailings storage facilities. This information could be useful when establishing closure costs of tailings storage facilities in the future and could provide governmental departments with an indication of the environmental liabilities of platinum mines in terms of platinum tailings storage facilities.

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Appendix I

**Complete linear regression tables expressing linear relationships
between:**

- 1) organic carbon % and metals;**
- 2) earthworm biomasses and other bioassays, metals and dehydrogenase activity;**
- 3) NRR-T and other bioassays, metals and dehydrogenase activity; and**
- 4) cocoon hatchability and other bioassays, metals and dehydrogenase activity.**

Table 16. Simple linear regression equation ($y = b_0 + b_1x$) expressing relationships between (a) Organic carbon percentage in soil and metals in soil (ppm), (b) Organic carbon percentage in soil and metals in solution (ppm), and (c) Organic carbon percentage in soil and metals (ppm) in *Eisenia fetida* cultured in control material and platinum tailings materials from sites 1, 2 and 3.

	(a) Organic Carbon % and metal in soil					(b) Organic Carbon % and metal in solution					(c) Organic Carbon % and metal in <i>Eisenia fetida</i>			
	b_0	b_1	r^2	p value		b_0	b_1	r^2	p value		b_0	b_1	r^2	p value
Na in soil	42.51	11.96	0.17	0.59	Na in solution	4.31	2.98	0.14	0.63	Na in <i>Eisenia</i>	0.06	0.04	0.36	0.40
Mg in soil	61.83	34.64	0.06	0.76	Mg in solution	3.64	2.51	0.19	0.57	Mg in <i>Eisenia</i>	0.01	0.00	0.16	0.61
Al in soil	1063.31	-315.20	0.08	0.72	Al in solution	3.18	1.01	0.04	0.81	Al in <i>Eisenia</i>	0.01	0.00	0.52	0.28
K in soil	1.15	33.97	0.48	0.31	K in solution	1.57	8.63	0.57	0.25	K in <i>Eisenia</i>	0.06	0.01	0.17	0.59
Cr in soil	8.08	13.72	0.14	0.63	Cr in solution	0.51	0.43	0.06	0.76	Cr in <i>Eisenia</i>	0.00	0.00	0.24	0.51
Mn in soil	5.60	-0.47	0.00	0.94	Mn in solution	0.00	0.28	0.37	0.39	Mn in <i>Eisenia</i>	0.03	0.00	0.28	0.47
Fe in soil	580.41	-151.54	0.04	0.79	Fe in solution	6.69	-1.00	0.02	0.85	Fe in <i>Eisenia</i>	0.03	0.01	0.14	0.62
Ni in soil	8.42	-3.04	0.09	0.70	Ni in solution	0.07	0.03	0.16	0.60	Ni in <i>Eisenia</i>	0.00	0.00	0.24	0.51
Cu in soil	2.70	-0.73	0.04	0.80	Cu in solution	0.03	0.10	0.76	0.13	Cu in <i>Eisenia</i>	0.00	0.00	0.24	0.51
Zn in soil	0.15	1.08	0.53	0.27	Zn in solution	-0.00	0.18	0.43	0.35	Zn in <i>Eisenia</i>	0.00	0.00	0.28	0.48
Pb in soil	0.13	0.05	0.08	0.73	Pb in solution	0.00	0.02	0.61	0.22	Pb in <i>Eisenia</i>	0.00	0.00	0.34	0.42

Table 17. Simple linear regression equations $y = b_0 + b_1x$ expressing the relationships between earthworm masses after the 28 day period and (a) other bioassays, (b) physical soil properties, (c) pH, (d) metal concentrations in soil (ppm), (e) metal concentrations in solution (ppm), (f) metal concentrations in *Eisenia fetida* exposed to tailings material and control material over a period of 28 days (g) dehydrogenase activity (INF µg/g/2h) obtained after the introduction of earthworms and manure. All results compared are the results obtained after the 28 day experimental period except for the reproduction results which were obtained after an additional 56 day period.

	b_0	b_1	r^2	p	Regression
(a)					
NRR-T	0.35	<0.01	0.87	0.07	Linear
Survival	0.07	0.01	0.28	0.47	Linear
Number of cocoons	-0.05	0.02	0.73	0.15	Linear
Hatchability	0.04	<0.01	0.18	0.58	Linear
Number of juveniles hatched per cocoon	0.34	0.05	0.51	0.28	Linear
(b)					
Gravel %	0.37	0.06	0.66	0.19	Linear
Sand %	0.33	<0.01	0.53	0.27	Linear
Silt %	0.48	<-0.01	0.67	0.18	Linear
Clay %	0.44	<-0.01	0.06	0.76	Linear
% C	0.39	0.04	0.13	0.65	Linear
(c)					
pH	6.48	-0.79	0.78	0.12	Linear
(d)					
Na in soil	0.49	<-0.01	0.15	0.62	Linear
Mg in soil	0.47	<-0.01	0.68	0.17	Linear
Al in soil	0.50	<-0.01	0.88	0.06	Linear
K in soil	0.37	<0.01	0.78	0.12	Linear
Cr in soil	0.45	<-0.01	0.49	0.30	Linear
Mn in soil	0.49	<-0.01	0.77	0.12	Linear
Fe in soil	0.48	<-0.01	0.76	0.13	Linear
Ni in soil	0.48	-0.01	0.75	0.13	Linear
Cu in soil	0.48	-0.03	0.67	0.18	Linear
Zn in soil	0.44	-0.03	0.15	0.62	Linear
(e)					
Na in solution	0.45	-0.01	0.13	0.64	Linear
Mg in solution	0.46	-0.01	0.21	0.54	Linear
Al in solution	0.35	0.02	0.70	0.16	Linear
K in solution	0.44	<-0.01	0.12	0.65	Linear
Cr in solution	0.45	-0.04	0.36	0.4	Linear
Mn in solution	0.38	0.23	0.91	0.05	Linear
Fe in solution	0.45	-0.01	0.09	0.70	Linear
Ni in solution	0.43	-0.18	0.01	0.88	Linear
Cu in solution	0.41	0.09	0.01	0.91	Linear
Zn in solution	0.38	0.38	0.87	0.07	Linear

(f)					
Na in <i>Eisenia fetida</i>	0.50	-0.97	0.26	0.49	Linear
Mg in <i>Eisenia fetida</i>	0.47	-6.80	0.42	0.35	Linear
Al in <i>Eisenia fetida</i>	0.63	-20.70	0.11	0.67	Linear
K in <i>Eisenia fetida</i>	0.64	-3.40	0.38	0.39	Linear
Cr in <i>Eisenia fetida</i>	0.46	-32.46	0.33	0.43	Linear
Mn in <i>Eisenia fetida</i>	0.62	-7.17	0.27	0.48	Linear
Fe in <i>Eisenia fetida</i>	0.50	-1.96	0.43	0.34	Linear
Ni in <i>Eisenia fetida</i>	0.47	-40.01	0.30	0.45	Linear
Cu in <i>Eisenia fetida</i>	0.49	-67.07	0.37	0.39	Linear
Zn in <i>Eisenia fetida</i>	0.52	-19.72	0.35	0.41	Linear
(g)					
Dehydrogenase activity (after earthworms and manure)	0.29	<0.01	0.88	0.06	Linear

Table 18. Simple linear regression equations $y = b_0 + b_1x$ expressing the relationships between NRR-T and (a) other bioassays, (b) physical soil properties, (c) pH, (d) metal concentrations in soil (ppm), (e) metal concentrations in solution (ppm), (f) metal concentrations in *Eisenia fetida* exposed to tailings material and control material over a period of 28 days (g) dehydrogenase activity (INF $\mu\text{g/g/2h}$) obtained after the introduction of earthworms and manure. All results compared are the results obtained after the 28 day experimental period except for the reproduction results which were obtained after an additional 56 day period.

	b_0	b_1	r^2	p	Regression
(a)					
Survival	-85.28	3.68	0.44	0.33	Linear
Number of cocoons	-63.90	4.10	0.38	0.38	Linear
Hatchability	-10.16	0.29	0.02	0.88	Linear
Number of juveniles hatched per cocoon	-8.81	15.03	0.83	0.09	Linear
(b)					
Gravel %	0.47	15.18	0.79	0.11	Linear
Sand %	0.26	0.26	0.28	0.47	Linear
Silt %	28.47	-0.41	0.46	0.32	Linear
Clay %	14.19	0.18	0.01	0.91	Linear
% C	5.59	16.86	0.42	0.35	Linear
(c)					
pH	1077.45	-138.71	0.43	0.34	Linear
(d)					
Na in soil	19.76	-0.08	0.01	0.92	Linear
Mg in soil	25.11	-0.11	0.35	0.41	Linear
Al in soil	32.88	-0.02	0.68	0.18	Linear
K in soil	4.26	0.54	0.98	0.01	Linear
Cr in soil	21.48	-0.33	0.22	0.53	Linear
Mn in soil	29.47	-2.53	0.47	0.31	Linear
Fe in soil	29.06	-0.03	0.53	0.27	Linear
Ni in soil	28.99	-1.98	0.57	0.24	Linear
Cu in soil	26.93	-4.84	0.45	0.33	Linear
Zn in soil	16.94	-1.17	<0.01	0.93	Linear
(e)					
Na in solution	20.98	-0.81	0.06	0.75	Linear
Mg in solution	22.83	-1.32	0.09	0.71	Linear
Al in solution	2.68	3.51	0.48	0.31	Linear
K in solution	16.44	-0.06	<0.01	0.97	Linear
Cr in solution	21.52	-7.16	0.22	0.53	Linear
Mn in solution	5.98	56.72	0.99	<0.01	Linear
Fe in solution	26.06	-1.66	0.17	0.59	Linear
Ni in solution	17.61	-19.33	<0.01	0.95	Linear
Cu in solution	9.16	78.32	0.11	0.67	Linear
Zn in solution	5.48	95.97	0.99	<0.01	Linear
(f)					
Na in <i>Eisenia fetida</i>	27.33	-133.33	0.08	0.73	Linear

Mg in <i>Eisenia fetida</i>	24.49	-1029.42	0.18	0.58	Linear
Al in <i>Eisenia fetida</i>	58.00	-4000.00	0.08	0.73	Linear
K in <i>Eisenia fetida</i>	42.00	-400.00	0.08	0.73	Linear
Cr in <i>Eisenia fetida</i>	22.37	-4390.24	0.11	0.67	Linear
Mn in <i>Eisenia fetida</i>	40.36	-862.31	0.07	0.73	Linear
Fe in <i>Eisenia fetida</i>	28.43	-306.89	0.19	0.56	Linear
Ni in <i>Eisenia fetida</i>	23.78	-5657.96	0.10	0.68	Linear
Cu in <i>Eisenia fetida</i>	25.51	-9060.83	0.12	0.65	Linear
Zn in <i>Eisenia fetida</i>	29.03	-2606.38	0.11	0.67	Linear
(g)					
Dehydrogenase activity (after earthworms and manure)	-14.89	0.10	0.96	0.02	Linear

Table 19. Simple linear regression equations $y = b_0 + b_1x$ expressing the relationships between *Eisenia fetida* cocoon hatchability (no. of cocoons hatched/no. of cocoons produced) and (a) other bioassays, (b) physical soil properties, (c) pH, (d) metal concentrations in soil (ppm), (e) metal concentrations in solution (ppm), (f) metal concentrations in *Eisenia fetida* exposed to tailings material and control material over a period of 28 days (g) dehydrogenase activity (INF $\mu\text{g/g/2h}$) obtained after the introduction of earthworms and manure. All results compared are the results obtained after the 28 day experimental period except for the reproduction results which were obtained after an additional 56 day period.

	b_0	b_1	r^2	p	Regression
(a)					
Survival	121.33	1.17	0.25	0.51	Linear
Number of cocoons	51.58	1.93	0.47	0.32	Linear
Number of juveniles hatched per cocoon	92.53	-1.99	0.08	0.72	Linear
(b)					
Gravel %	87.64	2.17	0.09	0.70	Linear
Sand %	77.87	0.19	0.81	0.10	Linear
Silt %	95.47	-0.21	0.63	0.21	Linear
Clay %	97.18	-0.79	0.94	0.03	Linear
% C	93.84	-7.44	0.45	0.33	Linear
(c)					
pH	545.23	-59.59	0.44	0.34	Linear
(d)					
Na in soil	98.61	-0.19	0.24	0.51	Linear
Mg in soil	94.84	-0.07	0.72	0.15	Linear
Al in soil	92.27	<-0.01	0.12	0.65	Linear
K in soil	88.97	0.01	<0.01	0.95	Linear
Cr in soil	93.90	-0.28	0.87	0.07	Linear
Mn in soil	93.29	-0.76	0.23	0.52	Linear
Fe in soil	91.75	-0.01	0.11	0.67	Linear
Ni in soil	90.98	-0.26	0.06	0.76	Linear
Cu in soil	91.19	-0.86	0.08	0.72	Linear
Zn in soil	95.33	-7.57	0.99	0.01	Linear
(e)					
Na in solution	96.02	-1.10	0.63	0.20	Linear
Mg in solution	98.15	-1.72	0.80	0.11	Linear
Al in solution	86.73	0.66	0.09	0.70	Linear
K in solution	95.89	-0.97	0.97	0.01	Linear
Cr in solution	93.37	-5.34	0.68	0.17	Linear
Mn in solution	88.63	3.50	0.02	0.86	Linear
Fe in solution	92.51	-0.54	0.10	0.69	Linear
Ni in solution	97.84	-103.08	0.45	0.33	Linear
Cu in solution	96.70	-85.34	0.73	0.15	Linear
Zn in solution	88.83	3.87	0.01	0.91	Linear
(f)					
Na in <i>Eisenia fetida</i>	106.72	-205.56	0.98	0.01	Linear

Mg in <i>Eisenia fetida</i>	97.38	-985.95	0.89	0.06	Linear
Al in <i>Eisenia fetida</i>	154.00	-6166.67	0.98	0.01	Linear
K in <i>Eisenia fetida</i>	129.33	-616.67	0.98	0.01	Linear
Cr in <i>Eisenia fetida</i>	97.17	-5460.71	0.94	0.03	Linear
Mn in <i>Eisenia fetida</i>	126.73	-1326.69	0.95	0.03	Linear
Fe in <i>Eisenia fetida</i>	100.51	-277.95	0.87	0.07	Linear
Ni in <i>Eisenia fetida</i>	99.11	-7173.65	0.92	0.04	Linear
Cu in <i>Eisenia fetida</i>	100.45	-10670.1	0.95	0.03	Linear
Zn in <i>Eisenia fetida</i>	105.54	-3257.14	0.95	0.02	Linear
(g)					
Dehydrogenase activity (after earthworms and manure)	85.66	0.01	0.07	0.73	Linear

Appendix II

Tables showing average and $\pm$ SD for soluble metal analysis, total metal analysis and earthworm tissue metal analysis for control and sites 1,2 and 3.

Table 20. Mean and $\pm$ SD of total metals, soluble metals and metals accumulated in earthworms for the control and sites 1, 2 and 3

		Li	Be	B	Na	Mg	Al	Si	K	Ca	Sc	Ti
Control	Soil	0.11 $\pm$ 0.01	0.01 $\pm$ <0.01	0.45 $\pm$ 0.04	47.00 $\pm$ 8.66	8.20 $\pm$ 0.90	87.67 $\pm$ 18.82	0.32 $\pm$ 0.01	62.00 $\pm$ 6.24	0.02 $\pm$ <0.01	0.01 $\pm$ <0.01	0.01 $\pm$ <0.01
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.07 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	0.05 $\pm$ <0.01	0.06 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
	Soluble	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	2.37 $\pm$ 0.76	4.13 $\pm$ 0.06	3.50 $\pm$ 0.20	6.83 $\pm$ 0.85	0.38 $\pm$ 0.01	6.00 $\pm$ 0.69	0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
Site 1	Soil	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.27 $\pm$ 0.03	62.67 $\pm$ 13.20	88.00 $\pm$ 19.16	1433.33 $\pm$ 230.94	0.29 $\pm$ 0.01	9.87 $\pm$ 1.86	0.24 $\pm$ 0.05	0.06 $\pm$ 0.03	0.30 $\pm$ 0.05
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.07 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	0.05 $\pm$ <0.01	0.06 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
	Soluble	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.50 $\pm$ 0.04	1.17 $\pm$ 0.40	2.17 $\pm$ 0.64	0.72 $\pm$ 0.42	0.29 $\pm$ 0.01	3.80 $\pm$ 0.96	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
Site 2	Soil	0.02 $\pm$ <0.01	0.01 $\pm$ <0.01	0.46 $\pm$ 0.09	61.67 $\pm$ 8.02	190.00 $\pm$ 34.64	1300.00 $\pm$ 200.00	0.29 $\pm$ 0.01	13.67 $\pm$ 0.58	0.25 $\pm$ 0.01	0.05 $\pm$ 0.02	0.42 $\pm$ 0.14
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.13 $\pm$ 0.05	0.02 $\pm$ 0.01	0.01 $\pm$ <0.01	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
	Soluble	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	1.10 $\pm$ 0.17	11.60 $\pm$ 7.49	9.60 $\pm$ 2.40	2.07 $\pm$ 0.25	0.35 $\pm$ 0.02	16.00 $\pm$ 8.72	0.01 $\pm$ <0.01	<0.00 $\pm$ <0.00	<0.00 $\pm$ <0.00
Site 3	Soil	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.29 $\pm$ 0.02	28.00 $\pm$ <0.01	46.00 $\pm$ 0.01	660.00 $\pm$ 10.00	0.28 $\pm$ 0.01	2.30 $\pm$ <0.01	0.13 $\pm$ <0.01	0.03 $\pm$ <0.01	0.13 $\pm$ 0.01
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.07 $\pm$ <0.01	0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	0.05 $\pm$ <0.01	0.06 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
	Soluble	<0.01 $\pm$ <0.01	<0.00 $\pm$ <0.00	0.63 $\pm$ 0.10	7.63 $\pm$ 0.85	5.43 $\pm$ 0.25	5.57 $\pm$ 2.25	0.38 $\pm$ 0.04	1.63 $\pm$ 0.32	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
		V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As
Control	Soil	0.08 $\pm$ <0.01	0.29 $\pm$ 0.02	1.07 $\pm$ 0.06	43.67 $\pm$ 1.53	0.01 $\pm$ <0.01	0.15 $\pm$ <0.01	0.16 $\pm$ 0.01	0.65 $\pm$ 0.02	0.02 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.08 $\pm$ <0.01
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.03 $\pm$ <0.01	0.02 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01
	Soluble	0.03 $\pm$ 0.01	0.02 $\pm$ <0.01	0.56 $\pm$ 0.08	3.80 $\pm$ 0.26	0.01 $\pm$ <0.01	0.08 $\pm$ 0.02	0.11 $\pm$ 0.01	0.34 $\pm$ 0.13	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.06 $\pm$ 0.02
Site 1	Soil	1.24 $\pm$ 0.67	7.97 $\pm$ 1.55	8.67 $\pm$ 3.21	893.33 $\pm$ 526.24	0.67 $\pm$ 0.21	12.67 $\pm$ 1.53	4.50 $\pm$ 0.56	0.38 $\pm$ 0.14	0.06 $\pm$ <0.01	0.02 $\pm$ 0.01	0.06 $\pm$ 0.01
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.03 $\pm$ <0.01	0.03 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01
	Soluble	0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	2.37 $\pm$ 0.35	<0.01 $\pm$ <0.01	0.03 $\pm$ <0.01	0.02 $\pm$ <0.01	0.02 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01
Site 2	Soil	0.99 $\pm$ 0.19	46.67 $\pm$ 14.57	8.73 $\pm$ 0.51	743.33 $\pm$ 141.54	0.47 $\pm$ 0.07	9.43 $\pm$ 0.98	3.40 $\pm$ 0.46	2.00 $\pm$ 0.98	0.05 $\pm$ 0.01	0.01 $\pm$ <0.01	0.05 $\pm$ <0.01
	E/w	0.01 $\pm$ 0.01	<0.01 $\pm$ <0.01	0.04 $\pm$ <0.01	0.07 $\pm$ 0.02	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.03 $\pm$ 0.02
	Soluble	0.05 $\pm$ <0.01	2.03 $\pm$ 0.32	0.06 $\pm$ <0.01	8.03 $\pm$ 0.85	0.01 $\pm$ <0.01	0.12 $\pm$ 0.01	0.16 $\pm$ 0.02	0.05 $\pm$ 0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01
Site 3	Soil	0.37 $\pm$ 0.02	11.00 $\pm$ 1.00	2.80 $\pm$ 0.10	270.00 $\pm$ 10.00	0.16 $\pm$ 0.01	3.97 $\pm$ 0.06	0.96 $\pm$ 0.02	0.19 $\pm$ 0.01	0.02 $\pm$ <0.01	0.01 $\pm$ <0.01	0.04 $\pm$ <0.01
	E/w	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.03 $\pm$ <0.01	0.04 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	0.01 $\pm$ <0.01
	Soluble	0.02 $\pm$ 0.01	1.02 $\pm$ 0.51	0.07 $\pm$ 0.02	10.10 $\pm$ 3.39	0.01 $\pm$ <0.01	0.10 $\pm$ 0.03	0.06 $\pm$ 0.02	0.03 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01	<0.01 $\pm$ <0.01

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Appendix III

**Complete list of species collected at sites 1, 2 and 3 in winter and
summer months**

Table 21. Species numbers of a summer sample of soil mesofauna extracted from platinum tailings materials from sites 1, 2 and 3. Functional groups are divided into MB (micro-algivorius and bacteriophagous organisms), MP (mycophagous organisms), Pred (predators), Ppar (plant parasitic and herbivorous organisms) and SO (saprophagous and omnivorous organisms).

ACARI		Functional Group	Test 1						Test 2						Test 3	
			1A	1B	1C	1D	1E	1F	1A	1B	1C	1D	1E	1F	1A	1B
PROSTIGMATA																
Nanorchestidae	<i>Speleorchestes meyeri</i>	MB	2	21	37		15	270	48	36	18	37	31			
Alicorhagiidae	<i>Alicorhagia usitata</i>	MP					1									
Tydeidae	<i>Coccotydeus sp.</i>	MB	12	16	66		21	167		62	64	51	177	515		6
	<i>Coccotydaeolus sp.</i>	MB						39		11		29	59	61		
	<i>Microtydeus sp.</i>	MB		6		8				38	82	53	125	143		
	<i>Tydeus munsteri</i>	MP								127	14	14	385	89		
	<i>Pronematus sp.</i>	MB				1				19		14		16		
Eupodidae	<i>Eupodes sp.</i>	MB							2	5						
	<i>Cocceupodes sp.</i>	MB														
Tarsonemidae	<i>Tarsonemus sp.</i>	MP					1			3						
Pygmephoridae	<i>Bakerdania sp.</i>	MP									6					2
Pyemotidae	<i>Pyemotes sp.</i>	Pred														
Cunaxidae	<i>Cyta</i>	Pred						4								
Bdellidae	<i>Spinibdella thori</i>	Pred		6				1				4	4			
Tetranychidae	<i>Eotetranychus sp.</i>	PPar		1						2						
Stigmaeidae	<i>Eustigmaeus sp.</i>	Pred						7			1	3		5		
Cryptognathidae	<i>Cryptognathus sp.</i>	Pred						2								
Rapignathidae	Genus indet.	Pred						1			7			4		
Linotitranidae	Genus indet.	Ppar									28					
Erythraeidae	<i>Abrolophus sp.</i>	Pred														
Anystidae	<i>Anystis baccharum</i>	Pred		3												
Cheyletidae	Genus indet.	Pred					5									
Pseudocheylidae	<i>Tarsocheylus sp.</i>	Pred	1						5		14	8			1	1
MESOSTIGMATA																
Ascidae	<i>Protogamasellus sp.</i>	Pred		17		1				1	7		3			
Rhodacaridae	<i>Rhodacarus sp.</i>	Pred		9	4	1				1				5		
Laelapidae	<i>Cosmolaelaps sp.</i>	Pred										1	1	2		
Phytoseiidae	<i>Lasioseius sp.</i>	Pred										1				

CRYPTOSTIGMATA												
Hypochthoniidae	<i>Brachychthonius sp.</i>	MB	1	5	12		4	9				
	<i>Cosmochthonius sp.</i>	MB	1								2	
Camisiidae	<i>Acronothrus sp.</i>	MP		21					3			
Epilohmanniidae	<i>Epilohmannia sp.</i>	MP										
Scheloribatidae	<i>Scheloribates sp.</i>	MP										
Oribatulidae	<i>Zygoribatula sp.</i>	MP				3	9					
ASTIGMATA												
Acaridae	<i>Rhizoglyphus sp.</i>	SO										
ARANEA												
Lycosidae	<i>Lycosa sp.</i>	Pred							1	2		
INSECTA												
COLLEMBOLA												
Entomobryidae	Genus et sp. indet	MP				9						
Isotomidae	Genus et sp. indet	MP					5					
Poduridae	Genus et sp. indet	MP										
PSOCOPTERA												
Psocidae		MP	1	6	5		15	30	40	11	4	9
HEMIPTERA												
Coccidae		PPar					7	21	68	2	21	8
ISOPTERA												
Hodotermitidae	<i>Hodotermes sp.</i>	PPar		1					8			
COLEOPTERA												
Elateridae (larvae)		Pred							2		1	2
Staphylinidae (larvae)		Pred	1				1	2				
Scydmaenidae (larvae)		Pred		1		1						
HYMENOPTERA												
Formicidae	<i>Pheidole sp.</i>	SO					1					
DIPTERA												
	<i>Brachycera</i>	SO					1					2
	<i>Nematocera</i>	SO					1	2	2			1

Table 22. Species numbers of a winter sample of soil mesofauna extracted from platinum tailings materials from sites 1, 2 and 3. Functional groups are divided into MB (micro-algivororous and bacteriophagous organisms), MP (mycophagous organisms), Pred (predators), Ppar (plant parasitic and herbivorous organisms) and SO (saprophagous and omnivorous organisms).

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