# Utilizing earthworm and microbial assays to assess the environmental effects of different mining activities

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***"It may be doubted whether there are many other animals which have played so important a part in the history of the world as these lowly organised creatures"***
***Charles Darwin***
***1881***

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## PREFACE

Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto.

## DECLARATION

The experimental work conducted and discussed in this dissertation was carried out at the School of Environmental Sciences and Development, Zoology and Microbiology, North-West University, Potchefstroom Campus. This study was conducted under the supervision of Prof. Mark S. Maboeta and Prof. Leon van Rensburg.

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Charné van Coller

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**SUMMARY**

Mining has negative impacts on the environment, and is one of the main contributors to environmental pollution. This holds a potential hazard for ecosystems surrounding mining areas and also for public health in the surrounding communities. There is therefore a need for ecotoxicological research in order to assess these possible risks and find ways to minimize the harmful effects. One way in which to assess soil vitality are soil enzymes which are produced by plants and microorganisms and will therefore be more abundant in healthy soils. Earthworms have been proven to be useful bioindicators for metal contamination of soil, as they are able to accumulate metals from their environment into their body tissue. The aim of this study was to use earthworm bioassays, neutral red retention time analysis (NRR-t), enzymatic analysis and RAPD-PCR to determine the effect of mining activity on the environment. This was done by assessing the ecotoxicity of tailings collected from three different mines, *viz.* gold-, opencast chrome- and coal mines. The metals chosen for analyses included four (Cr, Co, Ni and Pb) of the seven (Cd, Cr, Ni, Pb, Zn, Cu, Co) environmentally important trace elements as described for South African soils. Arsenic was also chosen since it is associated with gold mine tailings. Tailings and soil were collected from three sites at each of the mines which included unrehabilitated (TDF-U) and rehabilitated (TDF-R) substrates from tailings disposal facilities (TDFs) and reference soils (RS) in close proximity to TDFs. The physical and chemical properties of these different substrates were determined in terms of pH, particle size as well as metal contents. In addition to this, they were analysed for microbial community function by means of enzymatic activity, which has been proven to be useful in evaluating contamination of soil. The enzymes analysed in this study included dehydrogenase, ß-glucosidase, acid phosphatase, alkaline phosphatase and urease. Earthworms (*Eisenia andrei*) were exposed to different material for 28 days during which their biomass, reproduction, mortality and lysosomal membrane stability were monitored weekly. Hereafter, they were removed from the material while the cocoons were left behind for a further 56 days. The hatched and unhatched cocoons as well as the juvenile worms were then counted to determine reproduction patterns in the materials. Metal concentrations in the substrates and earthworm body tissues were compared to selected benchmarks. Results indicated that when comparing the different materials from each mine, enzymatic activity proved to be a very sensitive parameter. Enzymatic activity showed significant differences ($p < 0.05$) between RS, TDF-R and TDF-U materials. Biomass was not a sensitive parameter ($p > 0.05$) for the worms exposed to the gold and chrome mine tailings, but it was a sensitive parameter ($p < 0.05$) for the coal mine exposed earthworms, showing early differences between the worms from the different sites (RS, TDF-R and TDF-U). The NRR-t assay was very sensitive ($p < 0.05$), indicating clear differences between the worms from each investigated site. In terms of reproduction, the production of cocoons showed clear differences ($p < 0.05$) between the different sites and could therefore be considered a sensitive parameter. Hatching success however, was not a sensitive parameter. The reason being that there were so little cocoons produced that it is not possible to determine the correct percentage of

juvenile worms hatching from, for example, only one or two cocoons. Mortality was also not a sensitive endpoint as it was only observed in the coal mine material. RAPD - PCR results indicated genetic differences between earthworms exposed to the control- and the tailings materials, indicating either DNA alterations due to possible genotoxic effects, or genetic variation between individuals of the same species. Since mine waste materials often contain complex mixtures of metals that might be toxic on their own or in combination with other factors, it is difficult to attribute any observed genotoxic effect to any of the specific metals.

***Keywords:*** *bioassays, biomarkers, earthworms, mining, soil enzymes, tailings disposal facility*

**OPSOMMING**

Mynbouaktiwiteite het negatiewe impakte op die omgewing en is een van die hoof bydraers tot omgewingsbesoedeling. Dit hou 'n potensiële gevaar in vir omliggende ekostelsels asook publieke gesondheid vir gemeenskappe naby myne. Daar is dus 'n behoefte vir ekotoksikologiese navorsing in sulke gebiede om moontlike risiko's te identifiseer en maniere te vind om negatiewe effekte te minimaliseer. Een manier om grondgesondheid te bepaal is deur grondensieme wat hoofsaaklik deur plante en mikroörganismes geproduseer word en gevolglik meer volop is in gesonde grond. Erdwurms is ook bewese bioindikatore van metaalkontaminasie in grond en kan metale in hul liggame akkumuleer. Die doel van hierdie studie was om bioevaluerings toetse, neutraalrooi retensietyd, ensiematiese analise en RAPD-PCR te gebruik om die effekte van mynaktiwiteite op die omgewing te bepaal. Dit is bereik deur die omgewingstoksisiteit te bepaal van uitskot van drie verskillende myne nl. goud-, oopgroef chroom- en steenkoolmyne. Die metale wat gekies is vir analise (Cr, Co, Ni en Pb) is vier van die sewe (Cd, Cr, Ni, Pb, Zn, Cu en Co) omgewingsbelangrike elemente in Suid-Afrikaanse grond. Arseen is ook gekies omdat dit geassosieer word met goudmyn uiskot. Uitskot en grond is versamel vanaf drie terreine by elk van die myne en het ongerehabiliteerde (TDF-U) en gerehabiliteerde (TDF-R) substraat van slikdamme (TDFs) en verwysingsgrond (RS) naby aan slikdamme ingesluit. Die fisiese en chemiese eienskappe van hierdie verskillende substrate is bepaal ten opsigte van pH, deeltjiegrootte en metaalkonsentrasies. Die mikrobiese gemeenskapsfunksie is ook bepaal deur middel van ensiemaktiwiteit wat 'n nuttige manier is om grondbesoedeling te evalueer. Hierdie ensieme het die volgende ingesluit: dehidrogenase, β-glukosidase, suur fosfatase, alkaliese fosfatase en urease. Erdwurms (*Eisenia andrei*) is blootgestel aan die verskillende substrate vir 28 dae en gedurende hierdie tyd is biomassa, voortplanting, mortaliteit en lisosoom membraanstabiliteit (NRR-t) weekliks gemonitor. Hierna is die wurms verwyder vanuit die materiaal en die kokonne agtergelaat vir 'n verdere 56 dae. Die uitgebroeide en onuitgebroeide kokonne, sowel as die jongelinge wat uitgebroei het, is getel. Metaalkonsentrasies in die substrate sowel as in die erdwurms se liggaamsweefsel is vergelyk teen vasgestelde maatstawwe. Resultate het getoon dat wanneer die verskillende materiale van elke myn vergelyk word, ensiemaktiwiteit 'n sensitiewe parameter is. Ensiemaktiwiteit het beduidende verskille ($p < 0.05$) getoon tussen RS, TDF-R en TDF-U substrate. Biomassa was nie 'n sensitiewe parameter ($p > 0.05$) vir die erdwurms wat blootgestel is aan die goud- en chroommyn uitskot nie. Dit was wel 'n sensitiewe parameter ($p < 0.05$) vir die erdwurms wat blootgestel is aan die steenkoolmyn uitskot, aangesien dit vroeë verskille getoon het tussen die wurms van elke substraat (RS, TDF-R en TDF-U). In terme van voortplanting, het die produksie van kokonne duidelike verskille ($p < 0.05$) getoon tussen die verskeie substrate en kon dus oorweeg word as sensitiewe parameter. Uitbroeisukses was egter nie 'n sensitiewe parameter nie. Die rede hiervoor is omdat daar so min kokonne uitgebroei het. Dit is dus nie moontlik om die korrekte persentasie van uitbroeisukses te bepaal van slegs een of twee kokonne nie. Mortaliteit was ook nie 'n sensitiewe eindpunt nie aangesien dit slegs waargeneem is in steenkoolmyn

substraat. Met betrekking tot RAPDs, het resultate variasie getoon tussen kontrole wurms en mynmateriaalwurms, wat demonstreer dat daar óf DNA alterasies plaasgevind het, óf moontlike genetiese variasie tussen individue van dieselfde spesie was. Aangesien myn uitskot dikwels komplekse mengsels van metale bevat wat óf op hul eie óf in kombinasie met ander faktore toksies mag wees, is dit moeilik om enige waargenome genotoksiese effek aan enige spesifieke metaal te koppel

***Sleutelwoorde:*** *bioevaluerings toetse, biomerkers, erdwurms, grondensieme, mynbou, slikdam*

## LIST OF ABBREVIATIONS

| | |
|---|---|
| **BCF** | Bioconcentration Factor |
| **E/W** | Earthworms |
| **ICP/MS** | Inductively Coupled Plasma Mass Spectroscopy |
| **NRR-t** | Neutral Red Retention time |
| **PCR** | Polymerase Chain Reaction |
| **RAPD** | Random Amplified Polymorphic DNA |
| **RS** | Reference site |
| **SMO&MP** | Soil Microorganisms & Microbial Processes |
| **TDF** | Tailings Disposal Facility |
| **TDF-R** | Rehabilitated Tailings Disposal Facility |
| **TDF-U** | Unrehabilitated Tailings Disposal Facility |
| **TIL** | Total Investigation Level |
| **TMT** | Total Maximum Threshold |

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## CHAPTER 1: INTRODUCTION

### *1.1 Mining and the environment*

The mining industry played a key role in the development of civilization and the world's infrastructure (Mbendi Information Services, 2010). Since the late 19th century, the economy of South Africa has been based on production and export of minerals; this contributed largely to the industrial development of the country (Mwape *et al*., 2008). During that period South Africa was the largest producer of chromium and vanadium, as well as a leading producer of gold and other minerals (Rank, 2010), while also being the third largest exporter of coal (Coakley, 1999). The country's global resources include nearly 90% of the platinum group metals, 80% manganese, 73% chrome, 45% vanadium and 41% gold (Department of Minerals and Energy, 2010).

The environmental impact of mining activities include water-, air-, noise-, and soil pollution, land degradation, as well as associated human health problems (Kwolek, 1999). An estimate made by the South African Department of Mineral and Energy Affairs in 1992 showed that more than 25 000 hectares of land are taken up by waste dumps alone (Louw, 1992). In another estimate, taken just eight years later by Fairbanks *et al.* (2000), the amount of land taken up by waste dumps had reached 175 420 hectares. Either the estimation by Louw (1992) was an underestimation, or this number really increased with about 150 420 hectares in such a short time. The total soil lost through erosion in South Africa has also been reported to be more or less 300 to 400 million tons per year (Department of Environmental Affairs, 1992), while 0.14% of the country's land surface is comprised of mines and quarries (Fairbanks *et al.*, 2000). According to Hunt (1983), active mines have an erosion tempo of more than 200 times that of natural grasslands. Therefore, if mining wastes are not managed properly, there is a potential hazard for ecosystems surrounding the area and also for public health in the surrounding communities (Van Tonder, 2008). This is of concern since soil is non-renewable on a human time-scale (Tate, 2000) and as a result, our present actions concerning the environment will have an effect for many years to come.

Soil is of critical importance to life and one of the most valuable natural resources. Without it, no living organism would be able to exist. This is why serious concern exists regarding mining activities that cause environmental degradation (Harris, 2003).

As was elegantly described by Eijsackers *et al.* (2006), soil is a vital system and the awareness of this needs to become the basis of soil policy and -management. There is much more to soil, as all kinds of living and non-living matter, each with its own part and function, can be found therein. This diversity is necessary for the quality and fertility of the soil. Soil quality can be defined as "*the capacity of a soil to function within ecosystem and land-use boundaries, to sustain biological productivity, maintain environmental quality and promote plant, animal, and human health*" (Doran and Safley, 1997). Soil fertility is "*the ability of the soil to supply elements essential for plant growth without a toxic concentration*

*of any element*" (Foth and Ellis, 1996). Both these are extremely important factors. Soil plays a vital role in all the natural ecological cycles, and provides a physical support system as well as nutrients to plants, which in turn provide humans and animals with food, shelter and other necessities. It is therefore important to preserve our soil and to improve its quality and productivity where possible (Izquierdo *et al*., 2005). The unfortunate reality is, however, that people are still ignorant of the fact that this valuable and non-renewable resource (Cornu, 2008) is nearing its limit. Numerous anthropogenic factors are continuing to contribute to these problems with no viable alternative for destroyed soil (Shira, 2010).

The health and stability of soil can be damaged by structural changes as well as by changes in its chemistry (Acton and Coote, 2010), which are both processes that occur during mining activity. Since mining forms an essential cornerstone of South Africa's economy, rehabilitation of mining sites should be the leading method used to decrease the negative impacts of these activities. Soil protection in South Africa was first considered a priority during the 1950's, and only because of new laws compelling all mines to take steps in minimising its amount of pollution (Marsden, 1985). Examples of laws protecting the environment include the Constitutional Law of South Africa (Act 108 of 1996) giving all South Africans the right to a healthy environment and the right to protect the environment, the National Environmental Management Act (Act 10 of 1998) requiring the prevention or minimisation of damage to the environment as well as the rehabilitation of already degraded environments and the Draft Integrated Pollution and Waste Management Policy (White Paper) aiming to minimise the generation of waste and pollution as well as the recycling and re-using of waste (South Africa, 1998).

The formation of soil and the re-establishment of soil biological functions are basic requirements for the recovery of ecologically sensitive ecosystems as active and healthy soil microbiology has a lot of positive effects. It is able to mineralize nitrogen and sulphur and also suppresses nematodes, bacterial- and fungal diseases. These microorganisms actively decompose organic material, while also improving root development. They assist in the recycling of nutrients, making it available for plants, and furthermore they also improve soil physical and chemical conditions (Zhou *et al.*, 2009). In order to improve the quality of the post mining material, it is therefore necessary to have knowledge of the soil ecosystem and its physical and biological composition (Claassens, 2007).

An important factor regarding soils is pH. The grade of acidity or alkalinity of soil is represented by the pH value and it is used as an indicator of soil reactions. Most plants prefer a slight to moderate acidic soil with a pH of between 5.5 and 7 and soil have a tendency to become acidic even under natural conditions, mostly because of rainfall, microbial respiration and nutrient uptake by plants. After prolonged leaching, the pH of soil will therefore reach a pH of 5 - 5.6. Microbial respiration leads to the release of $CO_2$ that produces a weak acid when dissolved in ground water. Soil pH has a very important effect on the availability of certain plant nutrients and the optimal pH for maximum nutrient availability

varies depending on the nutrient. For example, at a pH of less than five, there will be aluminium and manganese toxicity, but a calcium deficiency (Ashman and Puri, 2002).

Sand, silt and clay percentages of materials give an indication of the structure of the soils, but post-mining material do not have the same structure or texture as normal soil. It is usually composed of a uniform material with a specific particle size and structure. Mining activity causes an excess of fine or coarse material, which is unfit for plant growth. It is especially a problem with slopes (Fox and Bryan, 1999). The success rate for rehabilitation is also lower for finer material since it is more susceptible to erosion (Department of Industry Tourism and Resources, 2006).

### *1.2 Risk Assessments*

National programmes and management strategies have been implemented by the South African government. These include Strategic Environmental Assessments, Environmental Impact Assessments, and Environmental Management Plans. The aim is to ensure that the impacts of practices and development are understood, that environmental damage is minimised and that rehabilitation is applied to already damaged environments (DEAT, 2004). Risk assessments are therefore done on mining sites in order to identify possible risks. Hazard identification is the first step in this process. The purpose is to determine whether there should be concern for the environment or human health (USEPA, 1979; Mensink *et al*., 1995; Hart *et al*., 1998), including the potential for neurotoxicity, reproductive toxicity, mutagenicity and carcinogenicity. This process is important for the environment since it is included in the regulation of toxic waste management, environmental emission control and contamination site restoration (Hart *et al*., 1998; Vilanova, 1998). Ecotoxicology, which is a combination of the disciplines toxicology, applied ecology and environmental chemistry, involves the interactions that exist between environmental chemicals and biota. Consequently the main focus is on the effects at different biological organization levels, which is a critical parameter for toxicity. Some soil fauna groups are used as bioindicators, since they are very closely linked to each other and to the soil, plants and microorganisms. If pollutants cause disturbances in the soil, the fauna will experience both quantitative and qualitative changes. As a result soil functioning will also be affected (Cortet *et al*., 1999).

Biomarkers are suborganismal (cellular, tissue, physiological, etc.) changes in living individuals used as early warning signs for imminent organismal effects (Newman, 2010). Contaminated areas have hazardous effects which occur from molecular level to ecosystem level, and since the function and structure of the ecosystem will be affected, all the organisms in that ecosystem will also be affected. *In vitro* cell systems used as biomarkers offer a rapid and sensitive solution to some of the limitations that may exist for chemical analysis (Fent, 2003). Chemical analysis of soils might seem like an obvious method to assess for example metal concentrations to give an idea on possible toxicity. This does, however, not elude for the bioavailability of toxicants toward organisms. For this reason, sentinel

species such as earthworms can be used in toxicity studies, since the effects of contaminants can be monitored on organismal level in these species.

### *1.3 Metal analysis*

A heavy metal is defined as "*any one of a number of elements that exhibit metallic properties, which include transitional metals, lanthanides, actinides as well as metalloids Arsenic and Antimony*" (Hogan, 2011). A list of potentially toxic elements has been compiled by the USA Clean Act Amendment of 1990 (Title III). These include antimony (Sb), arsenic (As), beryllium (Be), cadmium (Cd), chlorine (Cl), chromium (Cr), cobalt (Co), fluorine (F), lead (Pb), manganese (Mn), mercury (Hg) nickel (Ni), selenium (Se), thallium (Th) and uranium (U) (Finkelman, 1994; Huggins, 2002). These elements have also been assessed in some studies, including that of gold mine tailings, chrome mines and coal mines. A study by Da Pelo *et al*. (2009) investigated the capability of toxic or harmful elements associated with a gold mine tailings dam to be released into the environment through acid mine drainage. The elements studied were As, Cd, copper (Cu), Cr, Co and Ni. Another paper by Rashed (2010) also investigated some potentially toxic elements by assessing the impact of tailings of an old gold mine on the heavy metal pollution of soils near and surrounding the mine area. The metals assessed included silver (Ag), gold (Au), As, Cd, Cu, Cr, Co, molybdenum (Mo), Mn, Ni, zinc (Zn), mercury (Hg), and Pb.

The metals associated with seepage water from chromite mine quarries are Cr, Ni, Fe, Co, Mn, Zn and magnesium (Mg) (Dey and Paul, 2010). A study investigating metal contamination of soils around a chromite mine found high levels of Cr, Co, Ni, Cu, Pb and As (Kien *et al.,* 2010). Studies by Watten *et al*. (2005) as well as Wilkin and McNeil (2003) have found that effluents containing significant concentrations of metals such as iron (Fe), aluminium (Al), Mn, Cu, Zn and Pb, are produced by the extraction of mineral coal, and they are hazardous to the environment. In another study, done on an open cast coal mine in India, six metals were reported in high concentrations in effluent from the mine (Mishra *et al*., 2008). These metals were Cu, Cd, Cr, Zn, Ni and Fe. In a study by Herselman *et al*. (2005), baseline concentrations were drawn up for several environmentally important trace elements specifically for South African soils. These environmentally important trace elements include Cd, Co, Cr, Cu, Pb, Ni and Zn. For this study Cr, Co, Ni and Pb were quantified, as they were found to be prominent pollutants in literature studied.

### *1.4 Soil enzymatic activity*

Microorganisms are important in soil fertility and the degradation of organic matter (Renella *et al*., 2006). They form part of all the systems that support life on earth and they conduct all known metabolic reactions in the soil (Ashman and Puri, 2002). Furthermore, some are able to decompose pesticides, preventing them from entering the water and becoming pollutants. The activity of these degrading

microorganisms depend on factors such as contaminant uptake and bioavailability, concentration, toxicity, mobility, access to other nutrients, and activated enzymes. Soil enzymes are mainly produced by plants and microorganisms and they are useful indicators of soil degradation and contamination (Tabatabai, 1994). They have been used in previous studies as indicators on the influence of soil treatments on microbial activity and soil fertility (Aon *et al.,* 2001). It has been proposed that microbial properties of soil are potential indicators of the impact that different management practices have on the soil (Li *et al*., 2004). Enzymatic assays give an indication of the maximum enzymatic activity in the soil (Tate, 2002), e.g. dehydrogenase, ß-glucosidase, acidic- and alkaline phosphatase and urease (Alef and Nannipieri, 1995). These enzymes reflect the microbiological activity and are indicators of soil change (Dick *et al*., 2000). Plants and microorganisms such as bacteria and fungi produce these enzymes (Aon *et al.*, 2001). Enzymatic activity affects soil fertility and is affected by pH which is one of the most important soil properties (Dick *et al*., 2000).

Dehydrogenase activity in soil provides information concerning biological activity as well as microbial populations in the soil. It also measures the microbial oxidative capacity of soil, which in turn acts as an indicator of the viable organisms (Dick, 1994; Taylor *et al.,* 2002). Dehydrogenase activity is mainly localized in the plasma membrane of bacteria or in the mitochondrial membranes of fungi (Aon *et al.,* 2001). ß-Glucosidase is one of the enzymes involved in the saccharification of cellulose (Tabatabai, 1994) and it is commonly found in animals, plants and microorganisms (Dick *et al.*, 1996). Phosphatases and urease are synthesized and secreted extracellularly by bacteria or fungi (Aon *et al.,* 2001). Phosphatases are divided into two groups: alkaline phosphatase which is produced by soil microorganisms, and acidic phosphatase which is mainly attributed to plant roots (Criquet *et al*., 2004). Phosphatases transforms organic and inorganic phosphorus compounds in soil (Amador *et al.*, 1997), and because of its association with organic matter content, acidic phosphatase is a useful indicator for the recovery of degraded soils (Gil-Sotres *et al.*, 2005). Urease is found in all soils (Coyne, 2008) and urease activity varies in different soils depending on the temperature, soil moisture, organic matter content, soil texture and Chemical Electric Conductivity (CEC) of the soil (Coyne, 2008). It plays an important role in the nitrogen cycle and has been found to be correlated with microbial biomass (Klose and Tabatabai, 1999).

### *1.5 Earthworms as bioindicators*

Earthworms play an important role in the enhancement of soil structure, fertility and nutrient availability (Little, 1990). They decompose dead and organic matter while recirculating the nutrients found in the organic matter. Furthermore they also stimulate microbial activity, mix soil, and increase infiltration as well as water holding capacity. Earthworms also provide canals for root growth (Edwards, 1983). Analyses of earthworm casts have shown that they contain several times more nitrogen, potassium, phosphorus, magnesium and calcium than the surrounding soil. These worms also only partially digest the matter, where after soil microorganisms can act on it to release available nutrients (Lapinski and Rosciszewska, 2008). Additionally, earthworms are also good biological indicators since they are involved in the functioning of the soil ecosystem and are widely distributed. They migrate over short distances and are easy to sample. Earthworms also have measurable responses such as pollutant concentration in their body tissue. This has been seen in previous studies where the earthworms accumulated heavy metals from soil in their body tissue, therefore indicating environmental contamination (Lapinski and Rosciszewska, 2008). Furthermore, they are not killed by low levels of pollutants and they have similar responses to the same levels of pollution in different sites (Cortet *et al.*, 1999). Earthworm survival, growth and reproduction rates can also be monitored to give an indication of the effects of the specific type of pollution (Haimi, 2000) as well as the degree of substrate contamination (Lapinski and Rosciszweska, 2008; Christensen and Mather, 1990). Of these three parameters, reproduction is the most sensitive (Maboeta and Van Rensburg, 2002).

Apart from reproduction, biomass, survival and growth, the Neutral Red Retention Time (NRR-t) assay, which is a biomarker, can also be used. The assay is very sensitive and provides a response before biomass changes or mortality can be measured. For this reason it is very useful in toxicity tests (Maboeta *et al*., 2004). The NRR-t assay is used to determine lysosomal membrane stability, which is one of the most integrative parameters for monitoring the adverse impacts of environmental pollutants on cellular functions (Svendsen *et al*., 1996). Previous research has indicated that lysosomes have the earliest detectable responses to both internally and externally imposed stress (Harding *et al*., 2004a; 2004b). There have been successful applications of this assay during field studies in terrestrial systems on earthworms (Svendsen *et al.,* 1996) and in aquatic systems on fish and molluscs (Lowe *et al.,* 1992; Lowe and Pipe, 1994; Svendsen and Weeks, 1995). The NRR-t assay is therefore a useful method to evaluate earthworm responses to environmental, physiological, and mechanical stressors (Lowe and Pipe, 1994; Svendsen and Weeks, 1995).

The capacity of the lysosomes to hold the neutral red dye is dependent on the maintenance of an internal low pH, therefore on the efficiency of membrane-bound proton pumps (Seglen, 1983). The NRR-t assay measures the retention time of the neutral red dye in the lysosomes. According to the cell's membrane integrity, the lysosomes will start leaking after a distinct time period. The dye will leak into the

entire cytosol of the cell and produce a pink colour (Harreus *et al.*, 1997). The unstressed cells' lysosomes will accumulate and hold the dye for a longer time. When the cell is destabilized by a stress response, the dye will leak into the cytosol of the cell through the damaged lysosomal membrane (Moore, 1980; Lowe *et al*., 1995a and 1995b) at a faster rate in stressed coelomocytes than in unstressed ones. Therefore the rate of NRR-t can be correlated to the overall stress of the organism (Harding *et al*., 2004a).

There may also be the need to evaluate the effects of certain contaminants such as heavy metals, on the population level. For this purpose genetic techniques such as DNA fingerprinting methods may also be used together with the previously mentioned assays. These methods offer an approach to assess changes in populations as a result of contaminants (Atienzar and Jha, 2006). Random Amplified Polymorphic DNA Analysis (RAPD) is such a method. This assay could be used in genotoxicity and carcinogenesis studies. Genetic effects caused as a result of chemical exposure include alterations to the structure and function of DNA. This includes DNA adducts, DNA breakage, and also mutations and is described as genotoxic effects (Theodorakis *et al*., 1999). The original use of the RAPD assay included genetic mapping, taxonomy and phylogeny (Williams *et al*., 1990; Welsh and McClelland, 1990; Welsh *et al*., 1991; Bassam *et al.,* 1992). Advantages of the assay are that it is suitable for work on anonymous genomes, while it is also cost efficient (Hadrys *et al*., 1992). The RAPD assay is very useful in experiments where different sampling sites are used, since it can help determine whether sampled populations from the different sites possess a uniform genetic structure. This allows the comparison of residues or other biological properties (Kautenburger, 2006). The RAPD technique employs short (10bp) oligonucleotide primers of arbitrary sequence to produce multiple PCR products with different sizes that are separated on agarose gel (Dupont, 2008). The amplification protocol is different from the standard PCR conditions (Erlich, 1989) in the sense that only a single random oligonucleotide primer is employed (Kautenburger, 2006).

### *1.6 Aim and objectives*

The aim of this study was to determine the ecotoxicity of gold, chrome and coal tailings disposal facilities (TDF). These included comparing reference sites (RS) to rehabilitated (TDF-R) and unrehabilitated (TDF-U) tailings in an attempt to determine the effect of rehabilitation practices on ecotoxicity. Specific objectives to achieve this were:

- Evaluation of physical and chemical properties of the tailings to determine correlations between the different results.
- Metal analyses (Cr, Co, Ni, Pb) on material and earthworm tissue to determine its potential of posing a threat to earthworms and soil microorganisms. Additionally, analysis of As on the gold mine samples.

- Determine the effects of the different tailings on microbial activity i.e. dehydrogenase, β-glucosidase, phosphatases and urease in order to assess its use in risk assessment studies.
- Earthworm bioassays using *Eisenia andrei* to determine sublethal endpoints (growth and reproduction) and biomarkers in the form of the neutral red retention assay.
- Genetic characterization of earthworms exposed to tailings from different mining sites, using RAPD - PCR analysis.

### *1.7 References*

ACTON, D.F. & COOTE, D.R. 2010. Soil Conservation. (*In* The Canadian Encyclopedia. [Web:] http://www.thecanadianencyclopedia.com/index.cfm?PgNm=TCE&Params=a1ARTA0007544 [Date of access: 30 October 2010].)

ALEF, K., NANNIPIERI, P. 1995. Methods in Applied Soil Microbiology and Biochemistry. New York: Academic Press. 576 p.

AMADOR, J.A., GLUCKSMAN, A.M., LYONS, J.B. & GORRES, J.H. 1997. Spatial distribution of soil phosphatase activity within a riparian forest. *Soil Science,* 162: 808-825.

AON, M.A., SARENE, D.E., BURGOS, J.L. & CORTASSA, S. 2001. Microbiological, chemical and physical properties of soils subjected to conventional or no-tillage management: an assessment of their quality status. *Soil and Tillage Research*, 60: 173-186.

ASHMAN, M.R. & PURI, G. 2002. Essential soil science: a clear and concise introduction to soil science. Oxford: Blackwell Science Ltd. 134-135 p.

ATIENZAR, F.A. & JHA, A.N. 2006. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: A critical review. *Mutation Research,* 613: 76-102.

BASSAM, B.J., CAETANO-ANOLLES, G. & GRESSHOFF, P.M. 1992. DNA amplification fingerprinting of bacteria. *Applied Microbial Biotechnology,* 38: 70-76.

CHRISTENSEN, O. & MATHER, J.G. 1990. Dynamics of lumbricid earthworm cocoons in relation to habitat conditions at three different arable sites. *Pedobiologia,* 34: 227-238

CLAASSENS, S. 2007. Measuring rehabilitation success of coal mining disturbed areas: A spatial and temporal investigation into the use of soil microbial properties as assessment criteria. Ph.D. thesis, North-West University, Potchefstroom, South Africa.

COAKLEY, G.J. 1999. The mineral industry of South Africa. [Web:] http://Minerals.usgs.gov/minerals/pubs/country/1999/9235099.pdf [Date of access: 13 November 2011].

CONSTITUTIONAL LAW OF SOUTH AFRICA. 1996. Constitution of the Republic of South Africa as adopted by the Constitutional Assembly on 8 May 1996 and as amended on 11 October 1996. (Act 108 of 1996) (B34B-96) (ISBN: 0-260-20716-7)

CORNU, S. 2008. Modelling pedogenesis: Purpose and overview of the Special Issue. *Geoderma*, 145(3-4): 399-400.

CORTET, J., GOMOT-DE VAUFLERY, A., POINSOT-BALAGUER, N., GOMOT, L., TEXIER, C., & CLUZEAU, D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects. *European Journal of Soil Biology*, 35(3): 115-134.

COYNE, M.S. 2008. Urease: Important facts about Urease. University of Kentucky: Department of Agronomy. [Web:] www.agrotain.com [Date of access: 25 March 2011].

CRIQUET, S., FERRE, E., FARNET, A.M. & LE PETIT, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biology and Biochemistry,* 36: 1111–1118.

DA PELO, S., MUSU, E., CIDU, R., FRAU, F. & LATTANZI, P. 2009. Release of toxic elements from rocks and mine wastes at the Furtei gold mine (Sardinia, Italy). *Journal of Geochemical Exploration,* 100(2-3): 142-152.

DEAT. 2004. Strategic Environmental Assessment, Integrated Environmental Management, Information Series 10, Department of Environmental Affairs and Tourism (DEAT), Pretoria.

DEPARTMENT OF ENVIRONMENTAL AFFAIRS. 1992. Building the foundation for sustainable development in South Africa. National report to the United Nations Conference on Environment and Development. Pretoria: Scientia Printers/Publishers, CSIR. 268 p.

DEPARTMENT OF INDUSTRY TOURISM AND RESOURCES. 2006. Mine Rehabilitation: Leading practice sustainable development program for the mining industry. Australian Government – Department of Industry, Tourism and Resources. Okt. [Web:] http://www.ret.gov.au/resources/Documents/LPSDP/LPSDP-MineRehabilitationHandbook.pdf [Date of access: 11 November 2011]

DEPARTMENT OF MINERALS AND ENERGY. 2010. South Africa's share of the world reserves and production. [Web:] www.SouthAfrica.info. [Date of access: 30 October 2010].

DEY, S. & PAUL, A.K. 2010. Occurrence and Evaluation of Chromium Reducing Bacteria in Seepage Water from Chromite Mine Quarries of Orissa, India. *Journal of Water Resource and Protection,* 2: 380-388.

DICK, R.P. 1994. Soil enzyme activities as indicators of soil quality. (*In*: Doran, J.W., Coleman, D.C., Bezdicek, D.F., Stewart, B.A., editors. Defining soil quality for a sustainable environment, SSSA Special Publication 35, Madison: SSSA. pp 107-124.)

DICK, R.P., BREAKWELL, D.P. & TURCO, R.F. 1996. Soil Enzyme Activities and Biodiversity measurements as Integrative Microbiological Indicators. (*In*: Methods for Assessing Soil Quality, SSSA Spec. Publ. (Doran, J.W., Jones, A.J., Eds.), pp 247-271. SSSA, Madison, WI, USA.)

DICK, W.A., CHENG, L. & WANG, P. 2000. Soil Acid and Alkaline Phosphatase activity as pH adjustment indicators. *Soil Biology and Biochemistry*, 32(13): 1915-1919.

DORAN, J.W. & SAFLEY, M. 1997. Defining and assessing soil health and sustainable productivity. (*In*: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. Biological indicators of soil health. New York: CAB International. p. 1-28.)

DUPONT, L. 2008. Perspectives on the application of molecular genetics to earthworm ecology. *Pedobiologia,* 52: 191-205.

EDWARDS, C.A. 1983. Earthworm ecology in cultivated soils. (*In*: Stachell (Ed), Earthworm Ecology from Darwin to vermiculture. London: Chapman and Hall. p. 123-137.)

EIJSACKERS, H., MABOETA, M & DOELMAN, P. 2006. Vitale bodem als basis voor een duurzaam bodembeheer. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie,* 25(4): 300.

ERLICH, H.A. 1989. PCR technology: principles and applications for DNA amplifications. New York: Stockton Press. 3-35 p.

FAIRBANKS, D.H.K., THOMPSON, M.W., VINK, D.E., NEWBY, T.S., VAN DEN BERG, H.M. & EVERARD, D.H. 2000. The South African Land-cover Characteristics Database: a synopsis of the landscape. *South African Journal of Science,* 96: 69-82.

FENT, K. 2003. Ecotoxicological problems associated with contaminated sites. *Toxicology Letters*, 353-365p.

FINKELMAN, R.B. 1994. Modes of occurrence of potentially hazardous elements in coal: levels of confidence. *Fuel Processing Technology,* 39: 21-34.

FOTH, H.D. & ELLIS, B.G. 1996. Soil Fertility. *Technology and Engineering*, 2nd Ed. Lewis Publ. p.1

FOX, D.M. & BRYAN, R.B. 1999. The relationship of soil loss by interril erosion to slope gradient. *Catena,* 38: 211-222.

GIL-SOTRES, F., TRASAR-CEPEDA, C., LEIRÓS, M.C. & SEOANE, S. 2005. Different approaches to evaluate soil quality using biochemical properties. *Soil Biology and Biochemistry,* 37: 877-887.

HADRYS, H., BALICK, M. & SCHIERWATER, B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology,* 1: 55-63.

HAIMI, J. 2000. Decomposer animals and bioremediation of soils. *Environmental Pollution,* 107: 233-238.

HARDING, J.M., COUTURIER, C., PARSONS, J. & ROSS, N.W. 2004a. Evaluation of the neutral red assay as a stress response indicator in cultivated mussels (*Mytilus* spp.) in relation to post-harvest processing activities and storage conditions. *Aquaculture,* 231: 315-326.

HARDING, J.M., COUTURIER, C., PARSONS, J. & ROSS, N.W. 2004b. Evaluation of the neutral red assay as a stress response indicator in cultivated mussels (*Mytilus* spp.) in relation to seasonal and environmental conditions. *Journal of Shellfish Research*, 23: 745-751.

HARREUS, D., KÖHLER, H.R. & WEEKS, J.M. 1997. Combined Non-Invasive cell Isolation and Neutral-Red Retention assay for measuring the effect of copper on the Lumbricid *Apporrectodea rosea* (Savigny). *Bulletin of Environmental Contamination and Toxicology*, 59: 44-49.

HARRIS, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *European Journal of Soil Science,* 54: 801-808.

HART, J.W., HANSEN, B.G., & KARCHER,W. 1998. Hazard assessment and risk assessment of chemical substances in the EU. (In: Pugh, D.M., Tarazona, J.V., editors. Regulation for chemical safety in Europe: analysis, comment and criticism. Kluwer Academic Publisher, 1998: 113-125.)

HERSELMAN, J.E., STEYN, C.E. & FEY, M.V. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa. *South African Journal of Science,* 101: 509-512.

HOGAN, C.M. 2011. Heavy Metal. The Encyclopedia of Earth. www.eoearth.org/article/heavy_metal?topic=49498 Date of access: 8 November 2011.

HUGGINS, F.E. 2002. Overview of analytical methods for inorganic constituents in coal. *International Journal of Coal Geology,* 50: 169-214.

HUNT, T.C. 1983. Vegetative stabilization of a taconite tailing basin in Wisconson: The effect of mulch, seed mix, seed placement and an amendment. Madison: University of Wisconsin. (Thesis – M.Sc.) 93p.

IZQUIERDO, I., CARAVACA, F., ALGUACIL, M.M., HERNÁNDEZ, G., & ROLDÁN, A. 2005. Use of microbiological indicators for evaluating success in soil restoration after revegetation of a mining area under subtropical conditions. *Applied Soil Ecology,* 30: 3-10.

KAUTENBURGER, R. 2006. Genetic structure among earthworms (*Lumbricus terrestris L.*) from different sampling sites in western Germany based on random amplified polymorphic DNA. *Pedobiologia,* 50: 257-266.

KIEN, C.N., NOI, N.V., SON, L.T., NGOC, H.M., TANAKA, S., NISHINA, T. & IWASAKI, K. 2010. Heavy metal contamination of agricultural soils around a chromite mine in Vietnam. *Soil Science and Plant Nutrition,* 56: 344-356.

KLOSE, S. & TABATABAI, M.A. 1999. Urease activity of microbial biomass in soils. *Soil Biology and Biochemistry,* 31(7): 205-211.

KWOLEK, J.K. 1999. Aspects of geo-legal mitigation of environmental impact from mining and associated waste in the UK. *Journal of Geochemical Exploration,* 66(1-2): 327-332.

LAPINSKI, S. & ROSCISZEWSKA, M. 2008. The impact of cadmium and mercury contamination on reproduction and body mass of earthworms. *Plant, Soil and Environment*, 54(2): 61-65.

LI, Q., ALLEN, H.L., WOLLUM, I. & ARTHUR, G. 2004. Microbial biomass and bacterial functional diversity in forest soils: effects of organic matter removal, compaction and vegetation control. *Soil Biology and Biochemistry*, 36: 571-579.

LITTLE, E.E. 1990. Behavioural toxicology: stimulating challenges for a growing discipline. *Environmental Toxicology and Chemistry*, 9: 1-2.

LOUW, J. 1992. DEPARTMENT OF ENVIRONMENTAL AFFAIRS. Estimate taken on hectares of land taken up by waste dumps. National report to the United Nations Conference on Environment and Development. Pretoria: Scientia Printers/Publishers, CSIR. 268 p.

LOWE, D.M., MOORE, M.N, & EVANS, B.M. 1992. Contaminant impacts on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda. Marine Ecology Progress Series*, 91: 135-140.

LOWE, D.M. & PIPE, R.K. 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in-vitro study. *Aquatic Toxicology,* 30: 357-365.

LOWE, D.M., FOSSATO, V.U. & DEPLEDGE, M.H. 1995a. Contaminant induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincials* from Venice Lagoon: an in vitro study. *Marine Ecology Progress Series*, 129: 189-196.

LOWE, D.M., SOVERCHIA, C. & MOORE, M.N. 1995b. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquatic Toxicology,* 33: 105-112.

MABOETA, M.S. & VAN RENSBURG, L. 2002. Vermicomposting of industrially produced woodchips and sewage sludge utilizing *Eisenia fetida*. *Ecotoxicology and Environmental Safety,* 56(2): 265-270.

MABOETA, M.S., REINECKE, S.A. & REINECKE, J. 2004. The relationship between lysosomal biomarker and organismal responses in an acute toxicity test with *Eisenia Fetida* (Oligochaeta) exposed to the fungicide copper oxychloride. *Environmental Research*, 96(1): 95.

MARSDEN, M.D. 1985. Gold mine reclamation practices as implemented by the Chamber of Mines vegetation unit and developments by the Potchefstroom University – NTC Institute for Ecological Research. Proceedings of the Revegetation Symposium for Mining Groups, Potchefstroom, pp. 53-59.

MBENDI INFORMATION SERVICES. 2010. A Mbendi Profile – World Mining – Overview. [Web:] www.mbendi.co.za [Date of access: 10 November 2010].

MENSINK, B.J.W.G., MONTFORTS, M., EIJKHUIZEN-MASLANKIEWICZ, L., TIBOSCH, H., & LINDERS, J.B.H.J. 1995. Manual for summarizing and evaluating the environmental aspects of pesticides. Report no. 67910022. Bilthoven: National institute of public Health and the Environment, RIVM.

MISHRA, K.V., UPADHYAYA, A.R., PANDEY, S.K. & TRIPATHI, B.D. 2008. Heavy metal pollution induced due to coal mining effluent on surrounding aquatic ecosystem and its management through naturally occurring aquatic macrophytes. *Bioresource Technology,* 99: 930-936.

MOORE, M.N. 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. *Rapports et Proces – Verbaux des Réunions. Conseil International pour l'Exploration de la Mer*, 170: 7-15.

MWAPE, P.; ROBERTS, M.J.; MOKWENA, E.; MUSI, L.; TJATJIE, T.; MNGUNI, M.; MASHABA, P. & KWATA, P.G. 2008. SAMI: South Africa's Mineral Industry. Department: Minerals and Energy, General Review. 25th ed. Republic of South Africa. [Web:] www.dme.gov.za [Date of access: 6 Sept. 2010].

NATIONAL ENVIRONMENTAL MANAGEMENT ACT. 1998. Department of Minerals and Energy, 2nd ed. Environmental Management Plan. (Act 10 of 1998) *Government Gazette*, 16 July 2008. No 31188.

NEWMAN, M.C. 2010. Fundamentals of ecotoxicology. 3rd ed. Virginia: CRC Press. 571p.

RANK, J. 2010 South Africa - Mining. (*In* Encyclopedia of the Nations.) [Web:] http://www.nationsencyclopedia.com/Africa/South-Africa-MINING.html [Date of access: 1 Oct. 2010].

RASHED, M.N. 2010. Monitoring of contaminated toxic and heavy metals, from mine tailings through age accumulation, in soil and some wild plants at Southeast Egypt. *Journal of Hazardous Materials,* 178: 739-746.

RENELLA, G., GELSOMINO, A., LANDI, L. & NANNIPIERI, P. 2006. Soil and Sediment Remediation (SSR), Biochemical parameters and bacterial species richness in soils contaminated by sludge-borne metals and remediated with inorganic soil amendments. *Environmental Pollution,* 144(1): 24-31.

SEGLEN. 1983. Lysosomes. (*In*: Harreus, D., Köhler, H.R. and Weeks, J.M. 1997. Combined Non-Invasive cell Isolation and Neutral-Red Retention assay for measuring the effect of copper on the Lumbricid *Apporrectodea rosea* (Savigny). *Bulletin of Environmental Contamination and Toxicology*, 59:44-49.)

SHIRA, V. 2010. Soil Not Oil: Climate Change, Peak Oil and Food Insecurity. [Web:] www.soilnotoil.com [Date of access: 15 December 2010].

SOUTH AFRICA. 1998. Department of Environmental Affairs and Tourism. The White Paper on Integrated Pollution and Waste Management for South Africa. A Policy on Pollution Prevention, Waste Minimisation, Impact Management and Remediation. *Government Gazette,* 20978

SVENDSEN, C. & WEEKS, J.M. 1995. The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). *Marine Pollution Bulletin,* 31: 139-142.

SVENDSEN, C., MEHARG, A.A., FREESTONE, P. & WEEKS, J.M. 1996. Use of an Earthworm lysosomal biomarker for the ecological assessment of pollution from an industrial plastics fire. *Applied Soil Ecology*, 3: 99-107.

TABATABAI, M.A. 1994. Soil enzymes. (*In:* Weaver, R.W. (Ed), Methods of Soil Analysis. Part 2. SSSA Book, Ser 5. SSSA and ASA, Madison, WI, pp.775-833.)

TATE, R.L. (III). 2000. Soil microbiology. 2nd ed. New York: John Wiley and Sons, Inc. 508 p.

TATE, R.L. (III). 2002. Microbiology and enzymology of carbon and nitrogen cycling. (*In*: R.G. Burns and R.P. Dicks, Editors, *Enzymes in the Environment,* New York: Marcel Dekker, Inc., (2002): 227-248, 508.)

TAYLOR, J.P., WILSON, B., MILLS, M.S., BURNS, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biology and Biochemistry,* 34: 387-401.

THEODORAKIS, C.W., ELBL, T. & SHUGART, L.R. 1999. Genetic ecotoxicology IV: survival and DNA strand breakage is dependent on genotype in radionuclide-exposed mosquitofish. *Aquatic Toxicology,* 45: 279-291.

USEPA. 1979. Toxic Substances Control Act. Discussion of premanufacture testing policies and technical issues: request for comment. Federal Register. 44: 16240-16292.

VAN TONDER, A. 2008. Rio Tinto HSE standard on Mineral waste management. (2), STE08. 3 p.

VILANOVA, E. 1998. Evaluation of neurotoxicity. An example of new approaches needed in risk assessment. (*In*: Pugh, D.M., Tarazona, J.V., editors. Regulation for chemical safety in Europe: analysis, comment and criticism. Kluwer Academic Publisher, 1998: 27-40.)

WATTEN, B.J., SIBRELL, P.L. & SCHWARTZ, M.F. 2005. Acid neutralization within limestone sand reactors receiving coal mine drainage. *Environmental Pollution,* 137: 295-304.

WELSH, J. & McCLELLAND, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research,* 18: 7213-7218.

WELSH, J., PETERSEN, C. & McCLELLAND, M. 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acids Research,* 19: 303-306.

WILKIN, R.T. & McNEIL, M.S. 2003. Laboratory evaluation of zero-valent iron to treat water impacted by acid mine drainage. *Chemosphere,* 53: 715-725.

WILLIAMS, J.G.K., KUBELIK, A.R., LIVAK, K.J., RAFALSKI, J.A. & TINGEY, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* 18: 6531-6535.

ZHOU, Q., LI, K., JUN, X. & BO, L. 2009. Role and functions of beneficial microorganisms in sustainable aquaculture. *Bioresource Technology,* 100: 3780-3786.

## CHAPTER 2: MATERIALS AND METHODS

Methodology given below is applicable to chapters 3, 4 and 5.

### *2.1 Sampling procedure and sites*

Materials were randomly collected in triplicate from three sites respectively, from a gold mine tailings dam, a chromium mine and a coal mine. A minimum of 1 kg from the top cover layer (15 cm) was collected from each site and sealed in a Ziploc® bag. The samples were kept at 4 ˚C to preserve biological properties for the enzymatic assays, which were carried out within 5 days of sampling.

### *2.2 Material properties*

Samples obtained from the different sites were sealed in Ziploc® bags and transported to the Eco-Analytica laboratory in Potchefstroom where chemical analysis was done according to standard procedures. A physical analysis of particle size distribution was done in order to determine gravel-like, sand-like, silt-like and clay-like particles and also to determine pH.

### *2.3 Metal analyses*

Metal analysis was done on the samples collected from each site. The samples from each tailings disposal facility were oven dried at a maximum temperature of 40˚C and then sifted through a 2mm sieve. The samples were prepared at the Eco-Analytica Laboratory in Potchefstroom according to the United States Environmental Protection Agency (USEPA) method 3050 (Edgell, 1988). Two grams of the material/soil samples, prepared as mentioned above, was weighed and 15 ml of nitric acid ($HNO_3$) was added to each sample. The samples were then heated for a minimum of 1 hour. After cooling down, 10 ml of hydrogen peroxide was added to each sample, where after 10 ml of 3N HCl was added. The samples were then heated for at least 1 hour. After this the samples were cooled and filtered and then 50 ml of deionised water was added to each sample. The samples were then analysed by means of Inductively Coupled Plasma Mass Spectrometry (ICP/MS) to determine the total metal concentrations of the four metals analysed. For metal analysis of earthworm body tissue the following procedure was used: Adult earthworms of *Eisenia andrei* were placed in jars containing the sampled material. After 28 days of exposure period, the adult worms were removed from the jars and placed on wet filter paper in petri dishes for 24 hours in order for the earthworms to expel their gut contents. The worms were then placed in small plastic tubes which were marked and sealed, and frozen in a -20˚C freezer. These earthworm samples were then transported to the Eco-Analytica Laboratory in Potchefstroom, where the tissue metal concentrations were determined.

The earthworms were inserted in 2 ml pure nitric acid ($HNO_3$) and kept in it overnight to dissolve. The next day 1 ml of the overnight solution was extracted from each sample and 9 ml deionised water was added to obtain a ten times dilution. This solution was then injected through a 45 µm filter, where after it was analysed by means of the ICP/MS method for heavy metals. The heavy metals analysed were cobalt (Co), chromium (Cr), nickel (Ni) and lead (Pb), on all the sampled material. The reason for these specific metals was that they formed part of the seven environmentally important trace elements in South African soils (Herselman *et al*., 2005). Arsenic (As) concentrations were also analysed on the gold mine tailings material. New total investigation levels (TIL) and total maximum threshold levels (TMT), as described by the above mentioned study, as well as other benchmarks as described by Efroymson *et al.* (1997) were used to measure the samples against.

### *2.4 Soil enzymatic activities*

Before the samples were analysed, they were passed through a 2 mm sieve. Tailings material was kept at field water content (i.e. not air dried) for the dehydrogenase activity analysis while air dried samples were used for ß-glucosidase, urease, acidic phosphatase and alkaline phosphatase analyses (Alef and Nannipieri, 1995).

Since the purpose of the enzymatic assays was to give an indication of enzymatic activity of the materials as they are in their environment, the same control as were used for earthworm assays were not used for enzymatic assays. This assay focused only on the effects of heavy metals on the enzymatic activity in the material. For each sample, material was weighed off in triplicate and placed in bottles marked control, replica one and replica two. Each enzymatic assay had its own protocol on preparing the controls. This will be discussed briefly. To determine the enzymatic activity in the materials, the activity in the control of a specific sample was subtracted from replica one and two of that sample. The average of the two replicas was then used to indicate the enzymatic activity in that sample.

#### 2.4.1 Dehydrogenase activity

The method of Von Mersi and Schinner (1991) as described in Alef and Nannipieri (1995) was followed for the dehydrogenase activity assay. The method is based on the incubation of soil with the substrate iodonitrotetrazolium chloride (INT) at 40˚C for 2 hours followed by colorimetric estimation of the reaction product iodonitrotetrazolium chloride-formazan (INF). Field moist tailings material samples were weighed (1 g), placed in a 100 ml Schott® bottle and mixed with 1.5 ml Tris (hydroxymethyl)-aminomethane (THAM) buffer and 2 ml INT solution.

Controls were prepared with sterilized soil (autoclaved at 121˚C for 20 minutes), thus ensuring that enzymatic activity is kept to an absolute minimum.

The Schott® bottles were sealed and incubated at 40˚C in the dark for 2 h. After incubation 10 ml of N,N-dimethylformamide:ethanol (1:1 v/v) extraction solution was added and the bottles were shaken every 20 minutes for another hour. N,N-dimethylformamide:ethanol was added to terminate the reaction. The tailings suspensions were then filtered through Whatman no. 2v filter paper and the absorbance of the filtrate measured at 464 nm. The calibration curve was prepared by pipetting 0, 1, 2 and 5 ml of INF standard solution into test tubes and adding 13,5 ml extractant solution to each tube. The contents were then mixed thoroughly. The calibration concentrations were 0, 100, 200 and 500 µg INF per test. The dehydrogenase activity is expressed as µg INF $g^{-1}$ dwt 2 $h^{-1}$ and calculated according to the following relationship:

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

Where $S_1$ = INF (in µg) of the test

$S_0$ = INF (in µg) of the control

dwt = dry weight of 1 g moist soil

**2.4.2 ß-glucosidase activity**

ß-glucosidase activity was based on the determination of the released p-nitrophenol after the incubation of tailings samples with p-nitrophenol glucoside (PNG) solution for 1 h at 37˚C (Alef and Nannipieri, 1995).

For the ß-glucosidase assay, 1 g of each tailings sample was weighed and placed in a 100 ml Schott® bottle where after it was mixed with 0.23 ml of toluene, 4 ml of Modified Universal buffers (MUB) (pH 6) and 1 ml PNG solution. The bottles were sealed and incubated at 37˚C in the dark for 1 h. After incubation 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M Tris (hydroxymethyl)-aminomethane (THAM) buffer (pH 12) was added to each sample.

The control was prepared by adding the PNG substrate after incubation and after adding the $CaCl_2$ and THAM buffer, but before filtration of the tailings suspension.

Samples were filtered through Whatman no. 2v filter paper and the absorbance of the filtrate measured at 410 nm. A calibration curve was prepared with 1 ml of standard p-nitrophenol solution diluted to 100 ml with distilled water in a volumetric flask. Aliquots (0, 1, 2, 3, 4 and 5 ml) of the diluted standard solution were pipetted into 50 ml Erlenmeyer flasks and the volume adjusted to 5 ml with distilled water.

The ß-glucosidase activity is expressed as p-Nitrophenol $g^{-1}$ dwt $h^{-1}$ and calculated according to the following relationship:

$$P\text{-}Nitrophenol\ (\mu g\ g^{-1}\ dwt\ h^{-1}) = \frac{C \times v}{dwt \times SW \times t}$$

Where C = measured concentration of p-nitrophenol (µg $ml^{-1}$ filtrate)
dwt = dry weight of 1 g moist sample
v = total volume of the tailings material suspension in ml
SW = weight of samples used (1 g)
t = incubation time in hours

### 2.4.3 Phosphatase (acidic and alkaline) activities

Phosphatase (acidic and alkaline) activities were based on the determination of the released p-nitrophenol after the incubation of tailings samples with p-nitrophenol glucoside (PNG) solution for 1h at 37°C (Alef and Nannipieri, 1995).

The phosphatase assays differed from the ß-glucosidase assays in choice of buffers. For the phosphatase assays, 1 g of each tailings sample was weighed and placed in a 100 ml Schott® bottle where after it was mixed with 0.23 ml of toluene, 4 ml of Modified Universal buffers (MUB) (pH 6.5 for assay of acidic phosphatase or pH 11 for assay of alkaline phosphatase) and 1 ml PNP solution. The bottles were sealed and incubated at 37˚C in the dark for 1 h. After incubation 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH was added to each sample.

The control was prepared by adding the PNP substrate after incubation and after adding the $CaCl_2$ and NaOH, but before filtration of the tailings suspension.

Samples were filtered through Whatman no. 2v filter paper and the absorbance of the filtrate measured at 410 nm. A calibration curve was prepared with 1 ml of standard p-nitrophenol solution diluted to 100 ml with distilled water in a volumetric flask. Aliquots (0, 1, 2, 3, 4 and 5 ml) of the diluted standard solution were pipetted into 50 ml Erlenmeyer flasks and the volume adjusted to 5 ml with distilled water.

Phosphatase activity is expressed as µg of p-nitrophenol $g^{-1}$ dry weight $h^{-1}$ and calculated according to the following relationship:

$$P\text{-}Nitrophenol\ (\mu g\ g^{-1}\ dwt\ h^{-1}) = \frac{C \times v}{dwt \times SW \times t}$$

Where C = measured concentration of p-nitrophenol (µg $ml^{-1}$ filtrate)

dwt = dry weight of 1 g moist sample

v = total volume of the tailings material suspension in ml

SW = weight of samples used (1 g)

t = incubation time in hours

### 2.4.4 Urease activity

Urease activity was assayed according to the procedure of Kandeler and Gerber (1988) as described by Alef and Nannipieri (1995). The method is based on the colorimetric determination of released ammonia after the incubation of tailings material samples with urea solution for 2 h at 37°C. Air-dried samples (5 g) were mixed with 2.5 ml urea solution and 20 ml borate buffer in a 100 ml Erlenmeyer flask where after it was incubated at 37˚C for 2 h. After incubation 30 ml of the KCl solution was added and the flask was shaken for 30 minutes. The suspension was filtered through Whatman no. 2v filter paper and the filtrates were analysed for ammonium content.

Controls were prepared with 2.5 ml distilled water and the urea solution was added at the end of the incubation, before the addition of the potassium chloride solution.

For ammonium determination 1 ml clear filtrate was added to a 50 ml Erlenmeyer flask and mixed with 9 ml distilled water, 5 ml sodium salicylate/sodium hydroxide solution, and 2 ml sodium dichloro-isocyanide solution. The mixture was allowed to stand at room temperature for 30 minutes before measuring the optical density at 690 nm. A calibration curve was prepared by diluting 0, 1.0, 1.5, 2.0 and 2.5 ml ammonium standard solution I with 100 ml potassium chloride solution to prepare ammonium standard solution II. 1 ml Ammonium standard solution II was diluted with 9 ml distilled water and the ammonium determination performed as mentioned above. The ammonium concentrations were 0.1, 1.5, 2 and 2.5 µg $NH_4$ – $Nml^{-1}$.

Urease activity is expressed as µg $NH_4$ – N $g^{-1}$ dwt 2 $h^{-1}$ and calculated according to the following relationship:

$$(\mu g\ NH_4 - N\ g^{-1}\ dwt\ 2h^{-1}) = \frac{\mu g\ NH_4 - N\ ml^{-1}\ x\ V\ x\ 10}{dwt\ x\ 5}$$

Where dwt = dry weight of 1 g moist tailings material

V = total volume of the extract (52.5 ml)

10 = the dilution factor

5 = weight of soil used in the assay

### *2.5 Earthworm assays*

*Eisenia andrei* was used in this study as they can tolerate fluctuations in temperature, acidity and moisture levels (Edwards, 1983), which makes them ideal for ecotoxicological tests. Tailings material and soil were sampled as described in section 2.1. The material was then sifted through a 2 mm sieve to homogenize the samples. Three replicates of each site were prepared. The material was then moistened with distilled water, until it appeared crumbly and moist. Oversaturated medium would have led to problems of aeration for the earthworms and survival would have been low.

Controls were prepared by filling the jars with horse manure (prepared by air drying the manure and moistening it with distilled water). The substrates were then inserted into 500 ml jam jars and filled up to 75% of the volume. The lids were perforated for aeration purposes and plastic circles (cut out from plastic bags) were inserted under the lids to prevent the worms from escaping and also to ensure minimal moisture loss. The jars containing the material were then placed in an environmental chamber set at a temperature of 25˚C for 48 hours during which the material stabilized. For the duration of the experiment, the jars were also kept in the 25˚C chamber to prevent temperature fluctuations from influencing the results. Ten adult earthworms of the species *Eisenia andrei* were individually weighed and placed in each jar, containing the material collected from the mining sites and the control, respectively. This was done in triplicate.

To weigh the worms, they were washed with distilled water, placed on tissue paper and then individually weighed in a petri dish containing distilled water (to prevent the worms from drying out). The earthworms were removed from the jars and weighed every seventh day over a 28 day period to determine their biomass. After weighing the worms, 4 g of dry horse manure were mixed with distilled water and added to each jar.

At the end of the 28 day period, the mature earthworms were removed from the jars to be prepared for metal analysis and RAPD-PCR analysis. The cocoons were left behind and the jars were placed back in the environmental chamber (25˚C) where they remained for another 56 days (during which moisture and manure levels were kept the same as during the experiment with the adult worms). After the 56 days, the hatched and unhatched cocoons as well as the juveniles were counted in order to determine reproductive behaviour and success of worms in the different materials (Hankard *et al*., 2005).

Mortality was also assessed in the replicates every week. Unresponsive earthworms were poked with a syringe needle in the anterior side to determine response. If the earthworm did not respond by pulling back or moving away from the needle, it was considered dead and removed from the substrate. In some cases earthworms might become missing, which may occur as a result of either escaping or dying. Missing earthworms were therefore considered to be dead.

Neutral red retention times were taken every 7 days. The NRR-t assay was used to determine lysosomal membrane stability. The solutions used for this assay were prepared as follows:

Base solution was made up by measuring 0.5 ml of Dimethyl sulfoxide (DMSO) ($C_2H_6OS$) in an Eppendorf container and adding neutral red dye ($C_{15}H_{17}N_4Cl$) to the DMSO. The contents were then shaken to dissolve.

Earthworm ringer solution was made up by weighing 2.72 g $NaCl_2$; 0.1775 g KCl; 0.2088 g $CaCl_2$; 0.1353 g $MgSO_4$; 0.0272 g $KH_2PO_4$; 0.0213 g $Na_2HPO_4$; and 0.1764 g $NaHCO_3$ on a scale. These chemicals were placed in a 500 ml volumetric flask and 500 ml of distilled water was added. The solution was then shaken to dissolve the chemicals. Earthworm ringer solution is used to keep the coelomocyte cells alive while they are viewed under the microscope. It was kept in a refrigerator for the duration of the experiment and allowed to warm up to room temperature before it was used.

Working solution was made up by measuring 2.5 ml ringer solution and adding 10 µl of base solution. This solution was made up every hour (or sooner if crystals started to form before an hour had passed).

Three earthworms of each replicate were cleaned with distilled water and dried on tissue paper. Coelomic fluid was extracted from the region posterior to the clitellum of the mature earthworms by using a 10 µl disposable syringe with a fine needle. Here after the worms were placed back in the material. The coelomic fluid contains lysosome membranes and the NRR-t assay is used to determine the stability of the lysosomal membranes when an organism is subjected to stress (Moore, 1980). 10µl of coelomic fluid was mixed with 10 µl of ringer solution and placed on a microscope slide. The solution was left for 20 seconds to adhere to the glass and then 20 µl working solution was added and the slide covered with a cover slip. The slide was immediately placed under a light microscope where counting proceeded. The numbers of stained and unstained coelomocytes were counted within a 2 minute time period (Figure 1). Two manual counters were used respectively for stained and unstained coelomocytes. After the 2 minute period the slide was put in a Petri dish with wet towel paper for 2 minutes to keep it from drying out. When 50 % of the total amount of cells was stained, observation was stopped and neutral red retention time was noted.

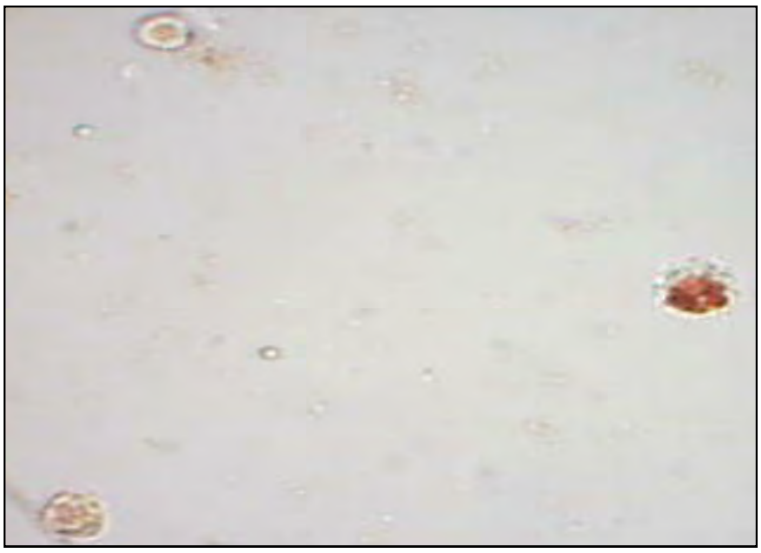

**Figure 2.1.** Coelomic cells, unstained (left) and stained (right) (Charné van Coller)

### *2.6 RAPD PCR analysis*

The extraction of earthworm DNA was done using a peqGOLD Tissue DNA Mini Kit (peqLab, Germany). Frozen earthworms exposed to the different treatments were crushed using a mortar and pestle, as well as liquid nitrogen. Thirty micrograms of the crushed tissue was used, and DNA was extracted as described in the manufacturer's protocol.

Random Amplified Polymorphic DNA (RAPDs) - Polymerase Chain Reaction (PCR) analyses were performed to determine the genetic structure of earthworms. This allowed the comparison of residues and biological properties from the different sampling locations (Kautenburger, 2006).

Isolated DNA was amplified in 25 µL RAPD-PCR mixtures containing the following final concentrations or total amounts: 12.5 µL 2x PCR Master Mix (Fermentas Life Sciences, USA) (resulting in 0.4 mM of each dNTP, 2 mM $MgCl_2$ and 0.625 u *Taq* DNA polymerase in reaction buffer), 8 µL (1-30 ng/µl) of DNA template, 1.5 µL (of a 100 pmol stock solution) primer (Inqaba Biotec, South Africa). An additional 2 µL of a 25 mM $MgCl_2$ was added, as well as 2 units of Kapa Taq (KAPA Biosystems, South Africa). The final volume was adjusted to 25 µL using nuclease free water (Fermentas, USA).

Amplified products were separated via electrophoresis on 2 % (w/v) SeaKem® LE agarose gels (Lonza, USA) in 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA) for 90 minutes at 80 V. 1 µg/mL EtBr was added to the gel prior to casting, enabling visualization under UV light. 20 µL of the samples were loaded using 5 µL 6x Orange loading dye (Fermentas, USA). As a molecular weight marker, 2.5

µL O'GeneRuler™ 1 kb DNA ladder was used (Fermentas, USA). The Gene BioImaging System (Syngene, Synoptics, UK) and GeneSnap software (SynGene) v6.08.04 was used to capture images after electrophoresis for visualisation of the bands that formed.

Images were analyzed using GeneTools v 4.02 (Syngene, Synoptics, UK) to obtain data regarding the band intensities, location and relative quantities. Table 2.1 shows details of the three primers used during this experiment.

**Table 2.1**. Details of the primers that were used in the RAPD PCR analyses on the earthworm DNA samples

| Primer | Sequence 5'-3' | C+G % |
|---|---|---|
| **OPA 4** | AAT CGG GCT G | 60 |
| **OPA 16** | AGC CAG CGA A | 60 |
| **OPA 17** | GAC CGC TTG T | 60 |

The above mentioned primers were initially used only in RAPD analyses on control earthworms numbered 1 to 9 in Figure 2.2. This was done to determine which of the three primers would work best on the earthworms before RAPD analysis was started on the mine exposed earthworms, since samples of exposed worms were limited. Between OPA 4, OPA 16 and OPA 17, the best quality bands were observed (Figure 2.2) with OPA 16, as this primer gave consistency of amplified fragments between 245 and 799 bp. There was also a similar banding pattern observed between the control worms with very little variation except in samples five and nine. The variation in lanes marked "5" and "9" could however also be observed with the other two primers, and therefore it is not regarded as being related to the primer. The primers OPA 4 and OPA 17 also gave variation between the control worms while OPA 16 showed very little variation. Therefore only results from primer OPA 16 were used in further analysis.

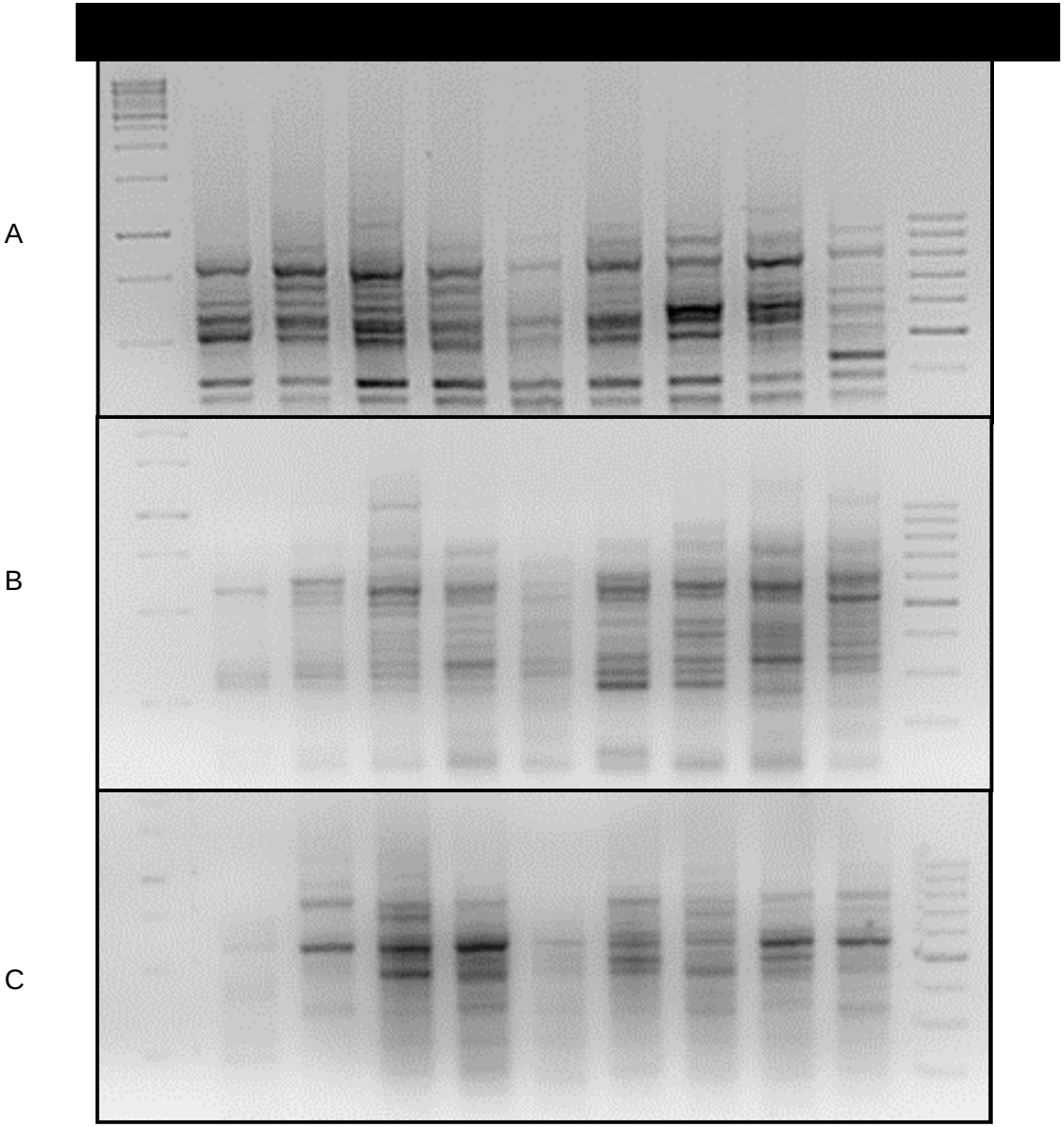


**Figure 2.2.** RAPD PCR optimization on control earthworms (1 to 9), (A) OPA 16, (B) OPA 17 and (C) OPA 4

### *2.7 Statistical analyses*

Statistical analyses for soil properties, enzymatic activity and earthworm assays were done with the SigmaStat® program (SigmaStat for Windows Version 3.11, 2004 Systat Software Inc). Differences between sample groups were determined by using one-way analysis of variance (ANOVA). All the data were presented as the mean ± standard deviation (SD). Mortality was determined by counting the number of survivors and determining the survival rate every week over the 28 day period.

Material and earthworm body tissue were analysed for Co, Cr, Ni and Pb. The metal contents of each sample were measured against different benchmarks. The benchmarks described in the study of Herselman *et al*. (2005) include TIL (the total investigation levels above which further site investigations are recommended) and TMT (the total maximum threshold levels above which soils are considered to be contaminated). Other benchmarks as described by Efroymson *et al*. (1997) were also used. Earthworm benchmarks (E/W)(as derived from a number of acute toxicity tests that are used to benchmark concentrations of chemicals toxic to earthworms), and SMO&MP (Benchmark concentrations for toxicity to soil microorganisms and microbial processes as determined by effects on C-mineralization, N-transformation and enzyme activities).

For RAPD analysis, band positions as well as heights were used as a quantitative measure, within a presence or absence table. Bands were subjected to both computerized and subjective comparison, with a maximum of 5% difference in the estimated molecular weight as calculated by GeneTools Software. This presence\absence table was exported to Statistica v10 (StatSoft, Inc., USA). Absent values were adjusted to 0, after which a multivariate analysis was done using a weighted pair group average approach.

### *2.8 References*


ALEF, K. & NANNIPIERI, P. (eds.). 1995. Methods in Applied Soil Microbiology and Biochemistry. New York: Academic Press. 576 p.

EDGELL, K. 1988. USEPA Method Study 37 – SW-846 Method 3050 Acid Digestion of Sediments, Sludges, and Soils. EPA Contract No. 68-03-3254.

EDWARDS, C.A. 1983. Earthworm ecology in cultivated soils. (*In*: Stachell (Ed), Earthworm Ecology from Darwin to vermiculture, London: Chapman and Hall. p.123-137.)

EFROYMSON, R.A., WILL, M.E. & SUTER II, G.W. 1997. Toxicological benchmarks for contaminants of potential concern for effects on soil and litter invertebrates and heterotrophic process: 1997 revision. U.S. Department of Energy, East Tennessee Technology Park.

HANKARD, P.K., BUNDY, J.G., SPURGEON, D.J., WEEKS, J.M., WRIGHT, J., WEINBERG, C. & SVENDSEN, C. 2005. Establishing principal soil quality parameters influencing earthworms in urban soils using bioassays. *Environmental Pollution*, 133: 199-211.

HERSELMAN, J.E., STEYN, C.E. & FEY, M.V. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa. *South African Journal of Science,* 101: 509-512.

KANDELER, E. & GERBER, H. 1988. Short – term assay of soil Urease activity using colorimetric determination of ammonium. *Biology and Fertility of Soils*, 6: 68-72.

KAUTENBURGER, R. 2006. Genetic structure among earthworms (*Lumbricus terrestris L.*) from different sampling sites in western Germany based on random amplified polymorphic DNA. *Pedobiologia,* 50: 257-266.

MOORE, M.N. 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. *Rapports et Proces – Verbaux des Réunions. Conseil International pour I'Exploration de la Mer*, 170: 7-15.

SIGMASTAT. SigmaStat for Windows Version 3.11. 2004. Systat Software Inc. [Software]

STATISTIKA. Statistika version 10. StatSoft Inc. [Software]

VON MERSI, W. & SCHINNER, F. 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. *Biology and Fertility of Soils,* 11: 216-220.

## CHAPTER 3: UTILIZING EARTHWORM AND MICROBIAL ASSAYS TO ASSESS THE ENVIRONMENTAL EFFECTS OF A GOLD MINE TAILINGS DAM

### *3.1 Introduction*

Nobel Prize winner Dr. Alexis Carell, as quoted by Tompkins (1998), wrote in his book, "Man the unknown", that "*soil is the basis of all life on earth*". Soil disturbances such as the ones caused by pollutants, lead to both qualitative and quantitative changes in the soil fauna, which in turn affect soil functioning (Cortet *et al*., 1999). A major cause of disturbance in soil is mining activities, which have contaminated soil with metals throughout the world (Valery and Eugene, 1998).

Mining is South Africa's largest industry (Geology Department, South Africa, 2000). The country was the world's largest gold producer up until 2007 when China took the lead. However, there are great environmental concerns associated with gold mining (Hilson and Murck, 2001). Over the years, it has had a negative impact through ecological degradation, health costs, loss of agricultural potential, loss of water, contamination of the surface and groundwater, atmospheric pollution and sinkhole formation (Lieferink, 2007). The greatest concern however, is the disposal of the overburden (tailings) on tailings disposal facilities (TDF) (Environmental Literacy Council, 2008), since this waste often contains high metal concentrations which are generally toxic to biota (Ledin and Pedersen, 1996). Furthermore, cyanide solutions are used to dissolve and extract gold (Miltzarek *et al*., 2002), raising environmental concerns, as large quantities are applied to the ore for this purpose (Clayton *et al.,* 1997). Cyanide is a fast acting poison and if tailings dams leak, break or overflow, the toxin is released into the environment where it can have harmful effects on fauna and flora (Donato *et al.,* 2007) and even lead to death (Environmental Literacy Council, 2008). Cyanide spills associated with gold mining have led to fish and wildlife kills as well as water pollution (Anane, 2006), and there have also been reports of the lethal toxicity of a number of cyanide complexes to birds (Davis, 1981; Reece, 1997). Another pollutant associated with gold mining is sulphuric acid, which can also cause pollution in soil and water as well as a low pH (Earthworks, 2007). A consequence of these contaminants is Acid Mine Drainage (AMD) which is considered as the worst environmental problem associated with mining activities (Peppas *et al.,* 2000). It occurs when material that bears sulphide is exposed to oxygen and water, resulting in a product characterized by a low pH as well as high concentrations of heavy metals and other toxic elements (Akcil and Koldas, 2006). Recently AMD has been in the South African media when it reached the Cradle of humankind, a World Heritage Site in Gauteng South Africa. This was as a result of the increased rainfall over the previous months, which raised the level of acidic mine water of the Witwatersrand (Mail and Guardian online, 2011). Ecotoxicological research on gold mine tailings have been done in previous studies and on different types of fauna, e.g. in Canada where meadow voles living in a tailings disposal facility area were captured. Biomarkers of exposure and effect were used to

determine possible sub-cellular effects on them as a consequence of their dietary arsenic exposure (Saunders *et al.,* 2009). Another study (Zorita *et al*., 2006) focused on mussels as bio - indicators after exposure to a copper site gradient, by selecting the digestive gland to serve as biomarker in the study. A wide variety of soil organisms can be used for ecotoxicological research, and include (but are not limited to) microorganisms, earthworms, enchytraeids, springtails, mites, insects and molluscs (Van Straalen and Van Gestel, 1993; Van Gestel and Van Straalen, 1994; Løkke and Van Gestel, 1998; Cortet *et al.,* 1999; Oehlmann and Schutte-Oehlmann, 2003).

Based on this information, the aim of this study was to utilize earthworm assays to assess the environmental effects of gold mining. Because of their close relationship to soil and their ability to accumulate heavy metals from their environment into their body tissue (Lapinski and Rosciszewska, 2008), earthworms are ideal for the assessment of metal rich waste and tailings material. Furthermore, the aim was to utilize microbial assays, since microbial activity is an indicator of the state of the soil or mining material (Tabatabai, 1994). The specific objectives included an assessment done on the tailings dam material which included revegetated tailings (TDF-R), unrehabilitated tailings (TDF-U) and a reference site (RS). This was done to assess the current state of the gold mine area and also to determine possible risks that may be present. A further objective was to assess whether earthworm biomarkers could be utilized to assess metal contamination and its effects on organisms and the ecosystem with regards to gold mining. The reason for this was to verify the bioavailability and the presence of metals in the biota (Bucheli and Fent, 1996).

### *3.2 Site description*

Samples were taken from a gold mine tailings dam in the area of Klerksdorp, South Africa (Figure 3.1). The area normally receives an average of 482mm rain per year, of which most rainfall occurs during summer (saexplorer, 2008). An aerial photo of the TDF is shown in Figure 3.2 and Figure 3.3 shows a part of the dam from the side. Unrehabilitated tailings material (TDF-U) was collected at the top of the dam (Figure 3.4). Samples were taken from a naturally revegetated area (TDF-R) at the foot of the dam (Figure 3.5) and reference site (RS) material was collected 100 meters away from the dam as shown in Figures 3.2 and 3.6.

**Figure 3.1** A map of South Africa showing the Klerksdorp area in the North West province (Nationsonline, 2010).

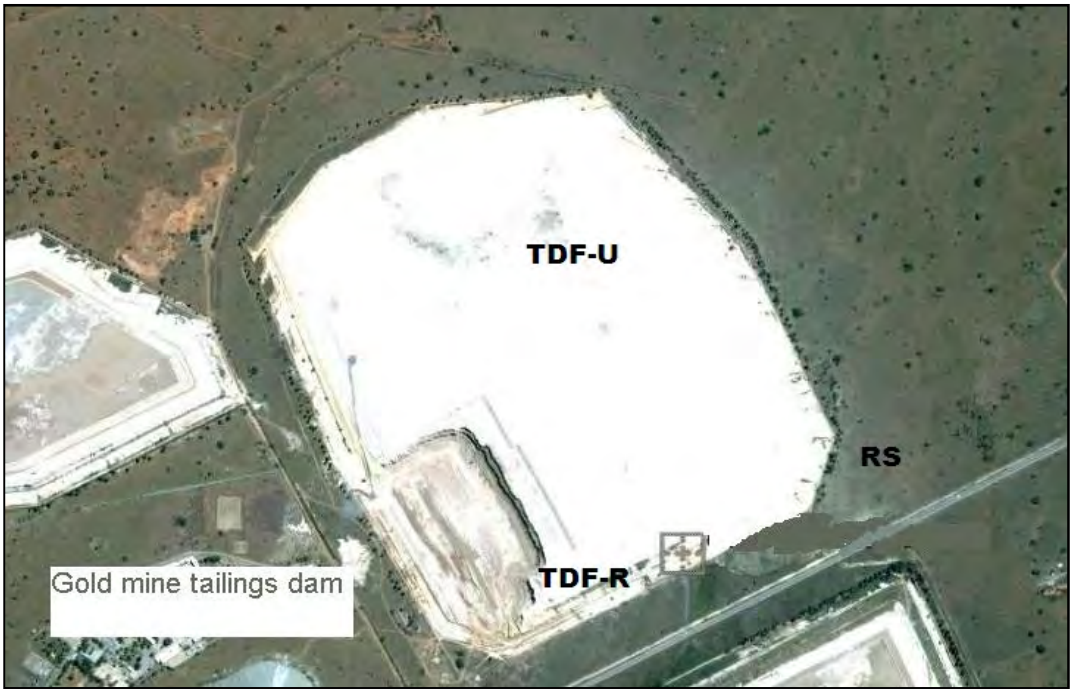


**Figure 3.2.** An aerial photograph taken of the gold mine tailings dam in the Klerksdorp area (Google Earth, 2011).

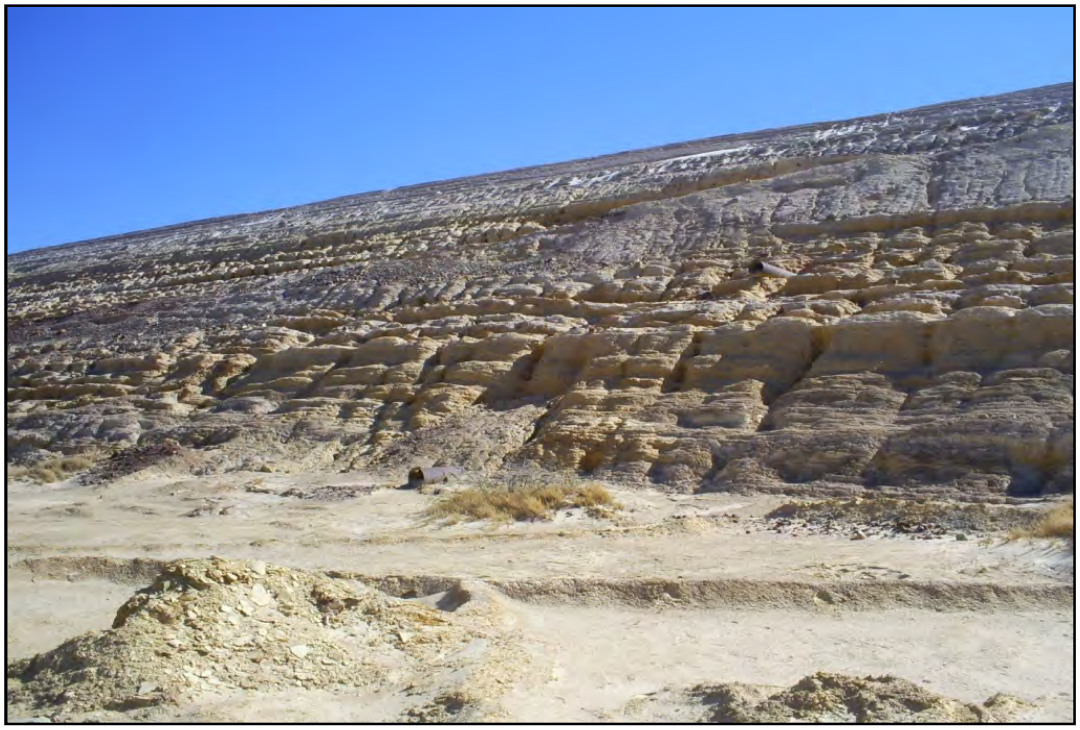

**Figure 3.3.** A photograph taken of the gold mine tailings dam as viewed from the side (Charné van Coller).

**Figure 3.4.** A photograph taken at the top of the gold mine tailings dam where unrehabilitated tailings (TDF-U) were sampled (Charné van Coller).

**Figure 3.5.** The foot of the gold mine tailings dam, where revegetated tailings (TDF-R) was sampled (Charné van Coller).

**Figure 3.6.** The area next to the tailings dam where reference material (RS) was sampled (Charné van Coller).

### *3.3 Materials and methods*

Refer to chapter 2.

### 3.4 Results

### *3.4.1 Material properties*

Gravel-, sand-, silt- and clay-like particles of the material were analysed since TDF's are not composed of real soil. Particle size distribution of the different sites of the TDF are shown in Figure 3.7. Particle sizes were compared between the reference site (RS) material, revegetated tailings (TDF-R) and unrehabilitated tailings (TDF-U) samples. The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

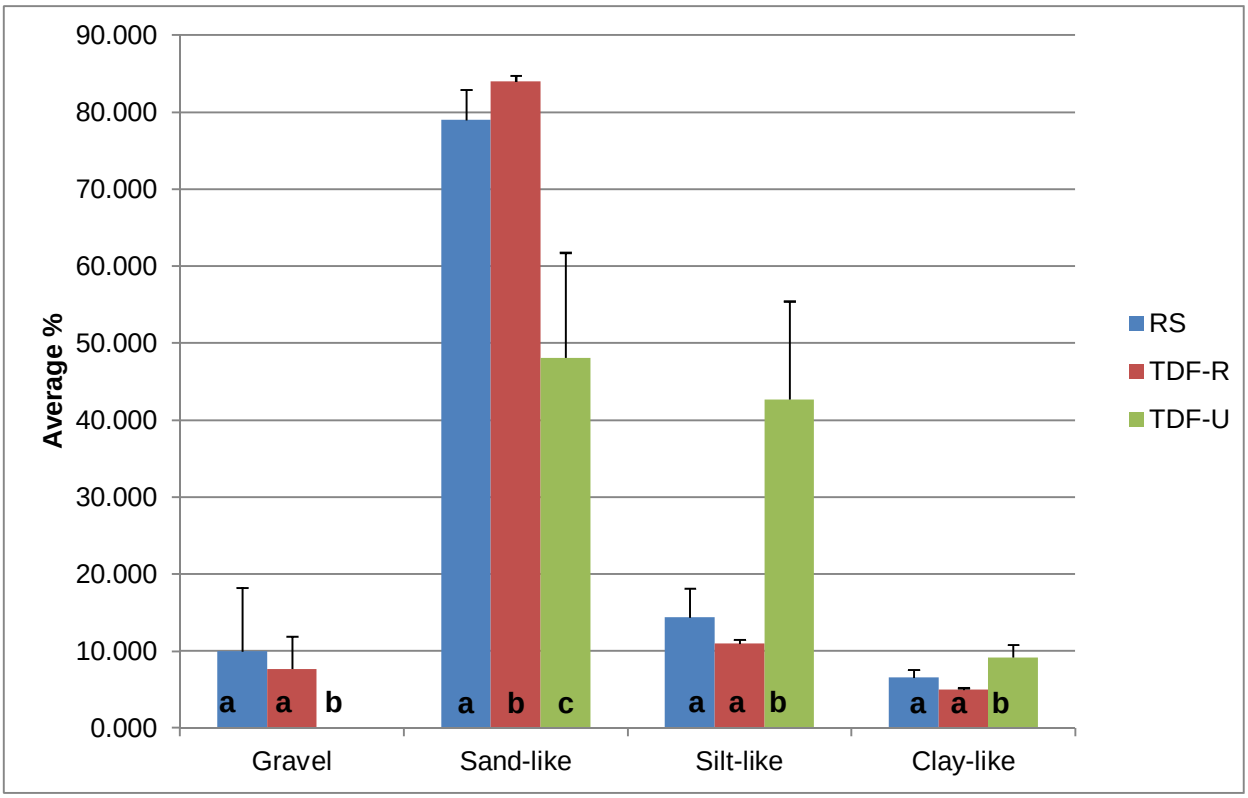


**Figure 3.7.** Particle size distribution (average ± SD) of the materials collected from the different areas [Reference site (RS), revegetated material (TDF-R), and unrehabilitated material (TDF-U)] of a gold mining area.
a-c: Bars with the same letter were not statistically different from each other ($p > 0.05$).

All three sites consisted mostly of sand-like particles. The reference site consisted mostly of sand-like particles with small amounts of clay-like, silt-like and gravel-like material. TDF-R consisted of the highest amount of sand-like particles, with small amounts of silt-like, clay-like and gravel-like material. TDF-U also consisted of a lot of silt-like material, some clay-like particles and very little gravel-like particles.

Figure 3.8 indicates the pH levels measured in the control, RS, TDF-R and TDF-U samples.

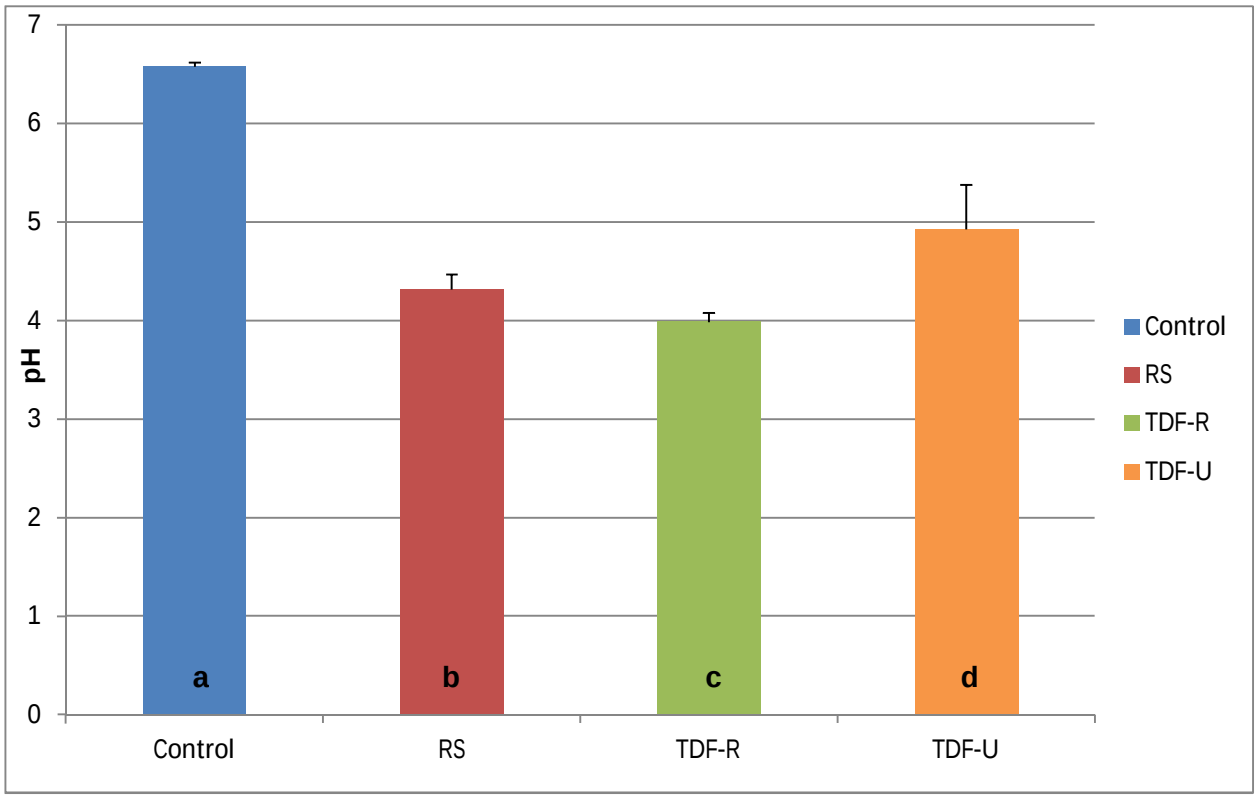


**Figure 3.8.** pH levels of the material [control, reference site material (RS), revegetated material (TDF-R), and unrehabilitated material (TDF-U)] sampled from the gold mine tailings dam.

a-d: Bars with the same letter were not statistically different from each other ($p > 0.05$).

The material collected from all three sites (RS, TDF-R and TDF-U) had an acidic pH. Of the three sites, TDF-U had the highest pH.

### *3.4.2. Metals*

Results from earthworm tissue metal analysis compared to metal analysis on the tailings and soil are shown in Table 3.1. These concentrations were compared to different benchmarks. Total investigation level (TIL), above which further site investigations are recommended, and total maximum threshold (TMT), above which soils are considered to be contaminated, are baseline concentrations that were drawn up for South African soils (Herselman *et al*., 2005). A benchmark indicating the level at which metal concentration in soil is toxic to earthworms, as described by Efroymson *et al.* (1997), is indicated as E/W. Other benchmarks include SMO (benchmark concentrations for toxicity to soil microorganisms) and MP (benchmark concentrations for toxicity to microbial processes) (Efroymson *et al.,* 1997). Metal concentrations were measured in parts per million (ppm). Earthworm body concentration over total soil concentration was calculated to determine the bioconcentration factor (BCF).

**Table 3.1**. Soil metal analysis for control, reference site material (RS), revegetated material (TDF-R) and unrehabilitated material (TDF-U), as measured against the following benchmarks in ppm: Total investigation level (TIL), total maximum threshold levels (TMT) (Herselman *et al.,* 2005), benchmarks for earthworm toxicity (E/W), benchmark concentrations for toxicity to soil microorganisms and microbial processes (SMO and MP), as described by Efroymson *et al.* (1997). Also bioconcentration factor (BCF) (i.e. Earthworm body concentration/total soil concentration).

| | | *Cr* | *Co* | *Ni* | *Pb* | *As* |
|---|---|---|---|---|---|---|
| ***TIL*** | | ***80*** | ***-*** | ***50*** | ***56*** | ***-*** |
| ***TMT*** | | ***350*** | ***-*** | ***150*** | ***100*** | ***-*** |
| ***E/W*** | | ***0.4*** | ***-*** | ***200*** | ***500*** | ***60*** |
| ***SMO and MP*** | | ***10*** | ***1000*** | ***90*** | ***900*** | ***100*** |
| **Control** | **Material** | 0.26±0.03$^{a}$ | 0.04±0.01$^{a}$ | 0.22±0.04$^{a}$ | 0.05±0.01$^{a}$ | <0.01 |
| | **E/w** | 0.06±0.01$^{A}$ | 0.16±0.02$^{A}$ | 0.08±0.02$^{A}$ | 0.03±0.02$^{A}$ | 1.12±1.10$^{A}$ |
| | **BCF** | 0.23 | 4.00 | 0.36 | 0.60 | |
| **RS** | **Material** | 0.59±0.04$^{b}$ | 1.16±0.43$^{b}$ | 8.83±2.88$^{b}$ | 0.10±0.01$^{b}$ | 0.02±0.01$^{a}$ |
| | **E/w** | 0.16±0.05$^{B}$ | 0.63±0.12$^{B}$ | 0.99±0.37$^{B}$ | 0.15±0.09$^{B}$ | 1.26±1.20$^{B}$ |
| | **BCF** | 0.27 | 0.54 | 0.11 | 1.5 | 82.06 |
| **TDF-R** | **Material** | 0.49±0.05$^{c}$ | 6.42±1.51$^{c}$ | 19.08±4.01$^{c}$ | 0.21±0.03$^{c}$ | 0.01±0.00$^{b}$ |
| | **E/w** | 0.13±0.05$^{B}$ | 0.70±0.23$^{B}$ | 0.33±0.09$^{C}$ | 0.07±0.04$^{B}$ | 2.74±1.34$^{C}$ |
| | **BCF** | 0.27 | 0.11 | 0.02 | 0.33 | 336.89 |
| **TDF-U** | **Material** | 0.33±0.02$^{d}$ | 9.33±4.02$^{c}$ | 0.83±0.14$^{d}$ | 0.04±0.01$^{d}$ | 0.025±0.00$^{c}$ |
| | **E/w** | 0.13±0.07$^{B}$ | 1.23±0.39$^{C}$ | 0.60±0.32$^{D}$ | 0.18±0.16$^{B}$ | 4.27±1.43$^{D}$ |
| | **BCF** | 0.39 | 0.13 | 0.72 | 4.5 | 107.67 |

a-d: Statistical comparison of the control material with the material from the sites RS, TDF-R, TDF-U;
A-D: Statistical comparison of the worms from the control site with the worms of RS, TDF-R, TDF-U.
Values with the same letter in superscript were not statistically different from each other ($p > 0.05$).

Concentrations of Cr exceeded the benchmark for toxicity to earthworms in the RS and TDF-R materials. The Co concentrations were higher than 1 ppm in all the mining material but far from the benchmark. Lead bioaccumulated in the RS samples as well as in the TDF-U samples. Arsenic concentrations were extremely high and bioaccumulation occurred in all the mine material.

### *3.4.3 Soil enzymatic activities*

Figure 3.9 shows the enzymatic activities of five different enzymes in the samples collected from and around the gold mine tailings dam. The amount of activity of each enzyme was compared between the

different samples. The grouped data for each of the enzymes was evaluated statistically against one another within the group, and not over the whole graph.

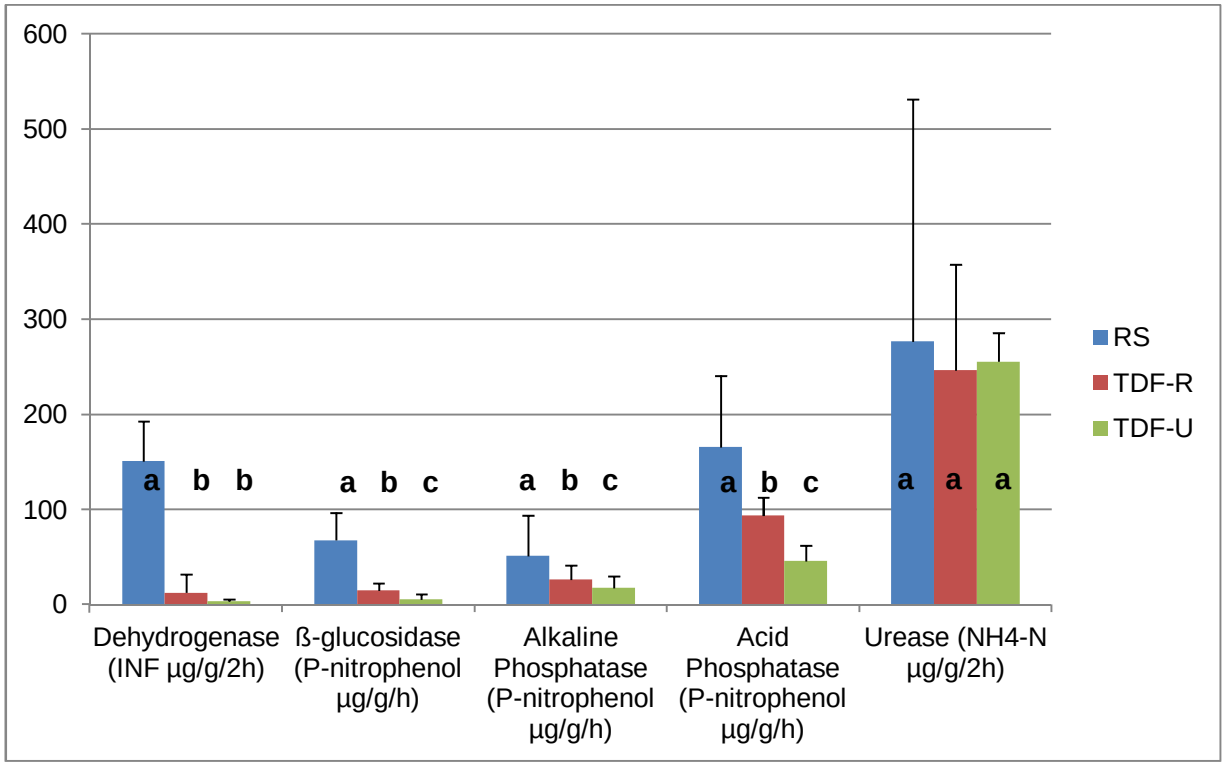


**Figure 3.9.** Enzymatic analyses of the samples collected from and next to the gold mine tailings dam, showing average microbial activity (± SD) in the material collected from the different areas including a reference site (RS), revegetated tailings (TDF-R) and unrehabilitated tailings (TDF-U).

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

Dehydrogenase activity was higher in the RS material than in both the TDF-R and the TDF-U material. B-glucosidase activity differed significantly ($p < 0.05$) between the three sites with the most activity in the RS material. Alkaline phosphatase activity also differed significantly ($p < 0.05$) between the three sites. Urease activity was observed as having the same amount of activity in all three sites with no statistically significant difference ($p > 0.05$) between the sites. It was also observed as the highest of all the enzymes over the three sites (RS, TDF-R and TDF-U). β-glucosidase, acid phosphatase and alkaline phosphatase activity showed statistically significant differences ($p < 0.05$) between the sites, indicating that RS > TDF-R > TDF-U.

### *3.4.4 Earthworm assays*

#### *3.4.4.1 Biomass*

Earthworm biomass over a 28 day period, as monitored weekly, is shown in Figure 3.10. The grouped data (per day) was evaluated against one another within the group, and not over the whole graph.

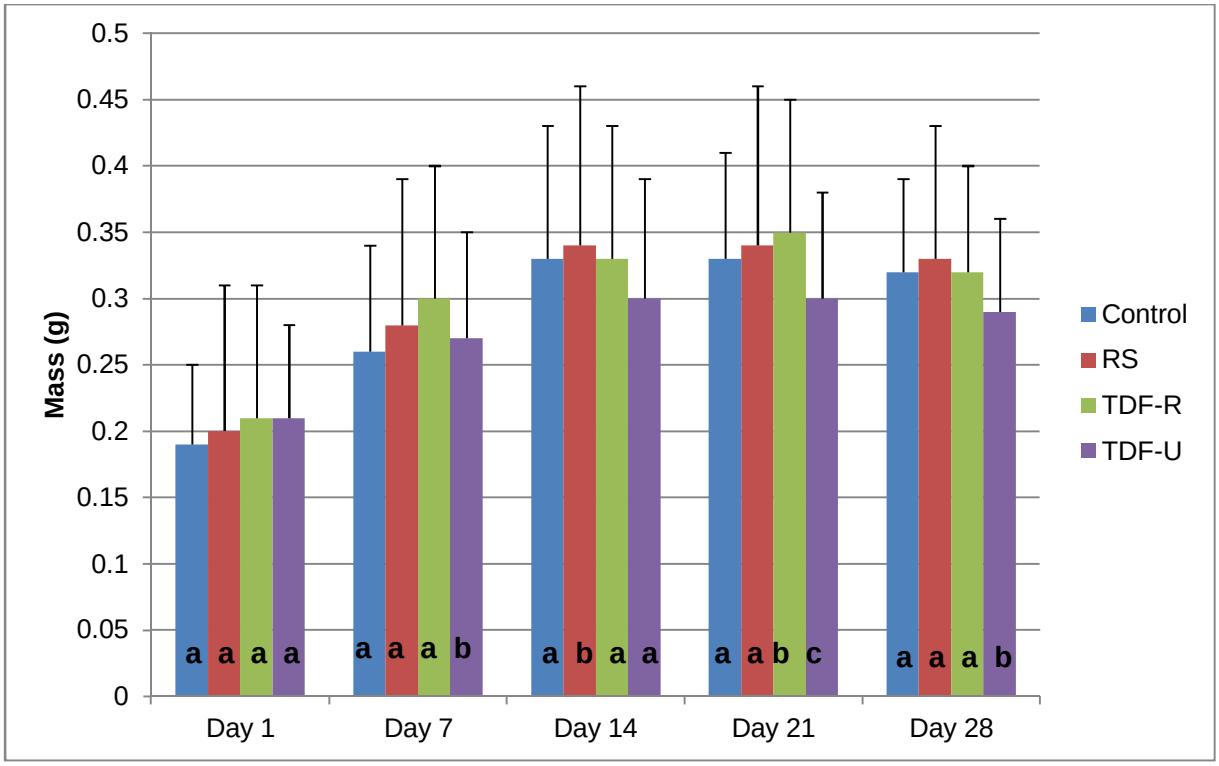


**Figure 3.10.** A histogram comparing earthworm biomass (average ± SD) between worms in the different sites [reference site (RS), revegetated material (TDF-R) and unrehabilitated material (TDF-U)] over the 28 day period.
a-c: Bars with the same letter were not statistically different from each other ($p > 0.05$).

On day 1, before exposure to the material, the biomass of the earthworms placed in the material did not differ significantly ($p > 0.05$). Over the following weeks biomass of earthworms exposed to RS material were notably lower (although not always statistically ($p < 0.05$) lower). On days 21 and 28, the TDF-U exposed earthworms had a significantly lower ($p < 0.05$) average biomass than those exposed to the control, RS and TDF-R material.

### *3.4.4.2 NRR-t*

Figure 3.11 shows the results of NRR-t measured weekly on the earthworms exposed to the control medium as well as the reference site (RS), revegetated tailings material (TDF-R) and unrehabilitated tailings material (TDF-U). The grouped data (NRR-t differences per day) was statistically evaluated against one another within the group, and not over the whole graph.

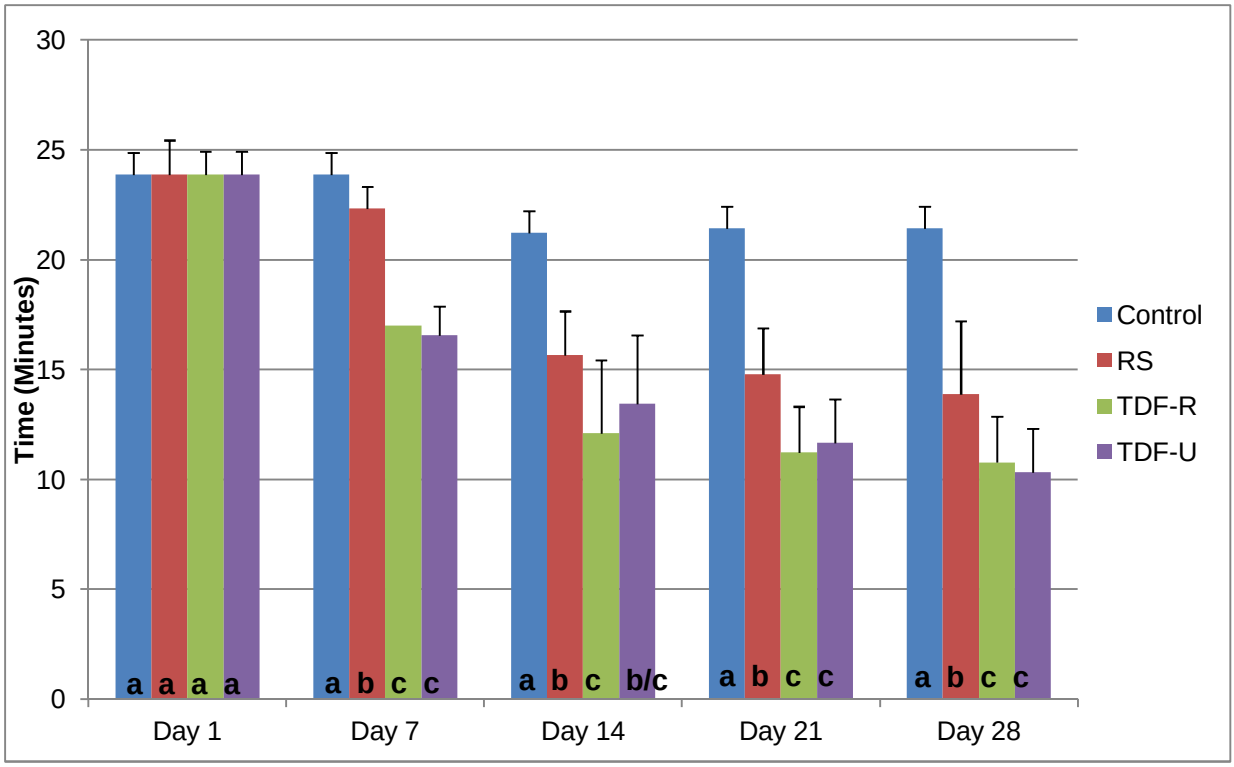


**Figure 3. 11.** Comparison of earthworm lysosomal membrane stability measured in neutral red retention time (±SD) between control worms and worms exposed to the reference site material (RS), revegetated material (TDF-R) and the unrehabilitated material (TDF-U) over the 28 day period.

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

Before exposing the worms to the different materials, NRR-t analysis was done, showing no statistically significant difference ($p > 0.05$) between the worms. They were then placed in the material and monitored weekly. On day seven, there was already a statistically significant difference ($p < 0.05$) between the worms from the control, the RS material and the TDF material. The same pattern could be observed up to day 28 of exposure, indicating that both of the TDF materials caused stress on the earthworms. Statistically significant differences ($p < 0.05$) indicated that C > RS > TDF-R = TDF-U after seven days up until 28 days of exposure.

### *3.4.4.3 Mortality*

No unresponsive or missing earthworms were observed during or after the 28 days of exposure to the different materials.

### *3.4.4.4 Reproduction*

After the 28 days of exposure, all the adult earthworms were removed from the material; the jars were placed back in the environmental chamber for 56 days during which the moisture levels were maintained. After the 56 days, all the juvenile worms and cocoons were counted. These results are shown in Figure 3.12 (average ± SD). The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

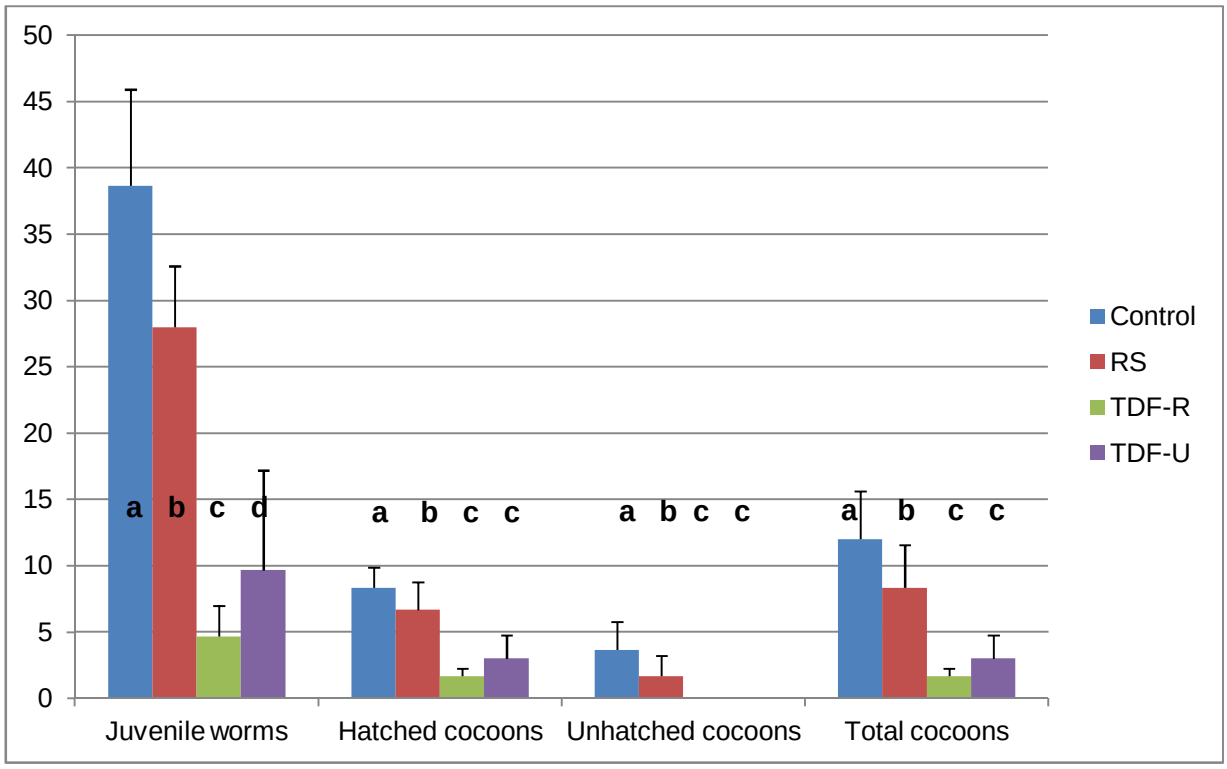


**Figure 3. 12.** Reproduction patterns of the worms in each site [control, reference site (RS), revegetated material (TDF-R) and unrehabilitated material (TDF-U)] by comparing juvenile worms, hatched cocoons, unhatched cocoons and total cocoons that were collected from the jars after the completion of the experiment. a-d: Bars with the same letter were not statistically different from each other ($p > 0.05$).

The number of juvenile worms produced were statistically higher ($p < 0.05$) in the control compared to that in the other material. Statistical data indicated significant differences ($p < 0.05$) as follows:
C > RS > TDF-U > TDF-R. From the results it is evident that the earthworms in the control and RS produced significantly more cocoons ($p < 0.05$) than those in the TDF-R and TDF-U sites.

### *3.4.5. RAPD PCR analysis*

The DNA from the earthworm samples that were exposed to the different tailings types were analysed in order to determine whether there were detectable differences in RAPD profiles between the control samples and those that were exposed to the tailings (containing heavy metals).

RAPD analyses were done on 3 worms from each of the control, RS, TDF-R and TDF-U as shown in Figure 3.13. The details of the bands that formed were summarized in Table 3.2.

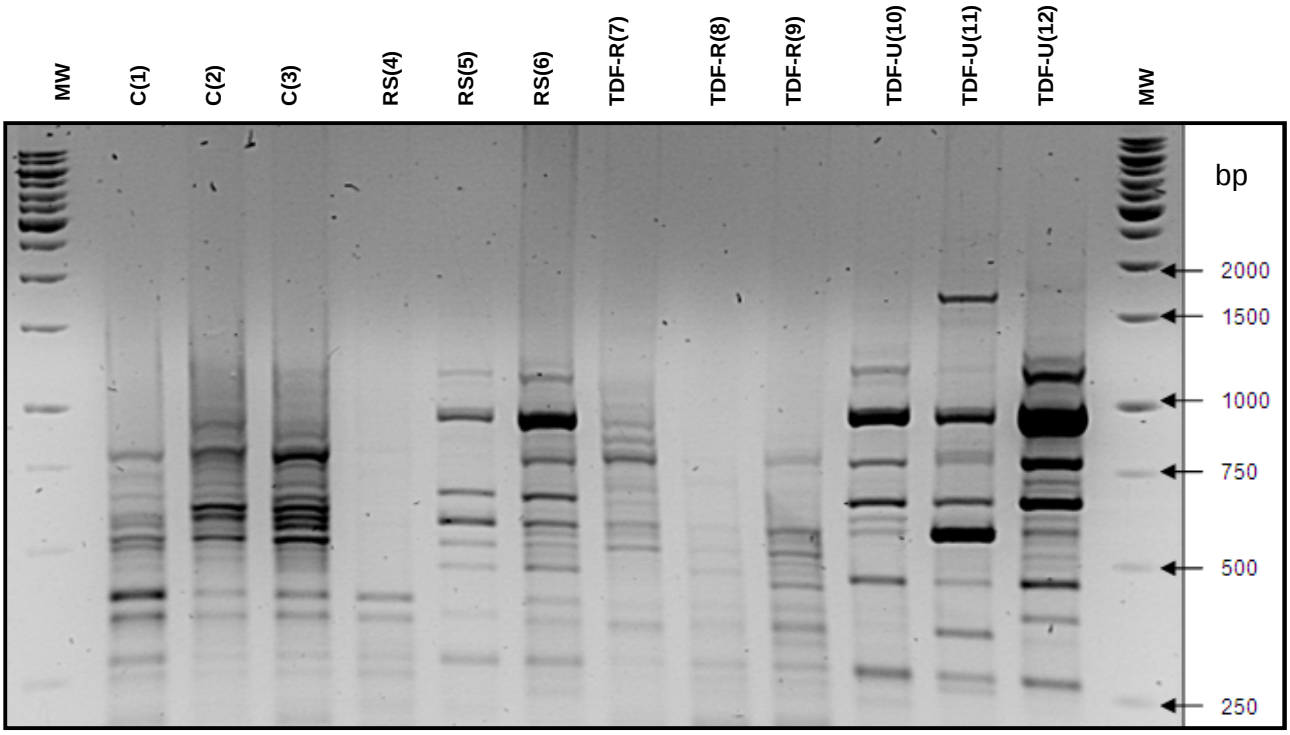


**Figure 3. 13.** RAPD-PCR gel of gold mine samples [worms from the control material (C), the reference site material (RS), revegetated material (TDF-R) and unrehabilitated material (TDF-U).]. MW indicates the use of a 1kb Molecular Weight Marker (Fermentas, USA). Numbers in parenthesis next to sample names indicate the profile number on the gel (excluding the MW).

**Table 3.2.** A summary of RAPD analysis of gold mine related materials on average DNA banding profiles of earthworms exposed to the control medium, reference site material (RS), revegetated material (TDF-R) and unrehabilitated material (TDF-U)

| Primer | Sequence | % G + C | Population | Total Number of Bands | Number of Polymorphic Bands | Bands range |
|---|---|---|---|---|---|---|
| **OPA 16** | AGC CAG CGA A | 60 | **Control** | 32 | 7 | 208-931 |
| | | | **RS** | 24 | 0 | 199-1194 |
| | | | **TDF-R** | 22 | 1 | 199-938 |
| | | | **TDF-U** | 27 | 2 | 241-1765 |

Different numbers of polymorphic bands were detected in all the test sites with a total of 105 bands. In the control samples, a total of seven polymorphic bands were detected in comparison to the zero polymorphic bands from the RS samples, one in the TDF-R samples and two in the TDF-U samples. The bands also differed in size ranging from 199 bp to 1765 bp. The largest band sizes were observed in the RS and TDF-U samples while smaller band sizes were observed in the control and the TDF-R samples.

Figure 3.14 depicts a multivariate analysis, using Statistica (v10 StatSoft, Inc., USA), on the presence or absence table subjectively created with a 5% maximum overlap. Molecular weight, determined from the molecular weight marker, was used as comparison method, whilst the peak heights of all the bands were selected as a quantitative measure in the weighted pair-group average analysis.

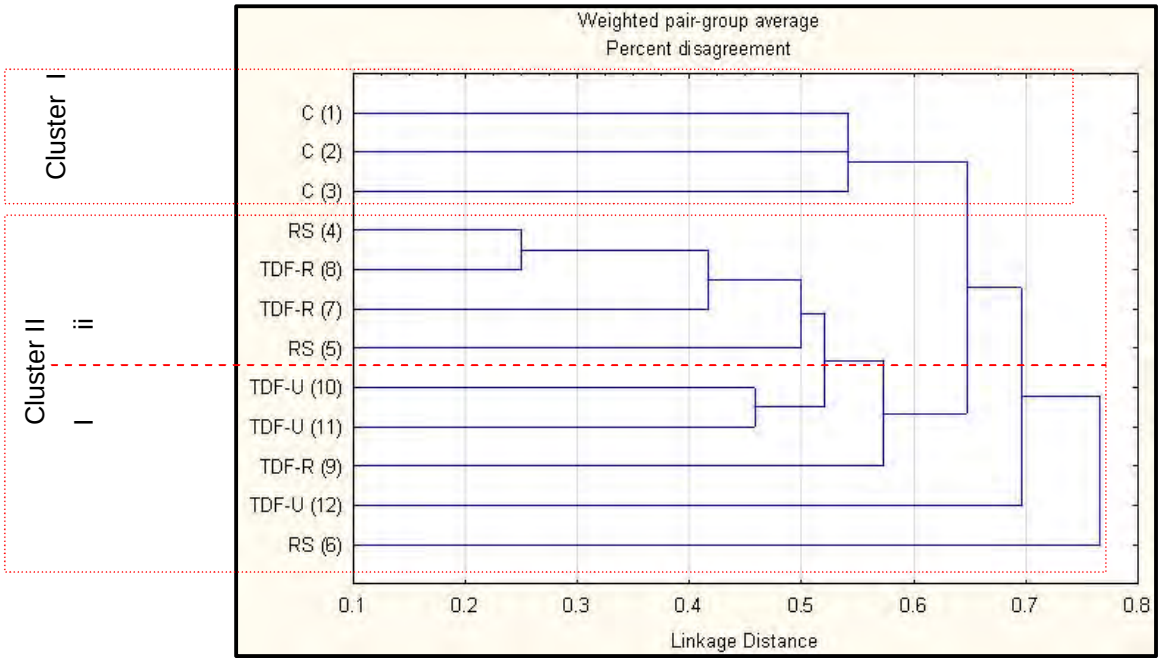


**Figure 3.14.** Dendrogram of gold mine RAPD analysis. The dendrogram can be divided into two clusters, marked I and II. Cluster II can be further divided into two sub clusters, i and ii.

Within cluster I the controls are grouped, while in cluster II most of the samples are clustered together. RS is scattered throughout the dendrogram, however 2 samples of RS and 2 of TDF-U are grouped together (within sub cluster i). Two of the TDF-U samples are also grouped together within sub cluster ii together with the remaining TDF-R sample.

### *3.5 Discussion*

Tailings disposal facilities (TDFs) are environmental control structures with the purpose of providing safe and permanent storage of mining residues (Minerals Council of Australia, 1996). Unfortunately this purpose is not always achieved (Rossouw *et al*., 2010), causing them to have a negative impact on the environment such as wildlife and bird deaths occurring as a result of interaction with the tailings (Donato *et al*., 2007).

Post mining material is usually composed of material uniform in size (unlike soil), and in this case the samples (collected from and next to the tailings dam) consisted mostly of sand-like particles, that has a texture which causes it to have an excessive permeability (Agrawal, 1991) as well as a low water- and nutrient- holding capacity (Ismail and Ozawa, 2007). Finer material is also very susceptible to wind erosion which will furthermore cause dust pollution (Department of Industry Tourism and Resources, 2006), which is especially a problem when there is no vegetation cover.

It is well documented that the pH of soil is a main factor affecting the adsorption – desorption behaviour and therefore the bioavailability of heavy metals in the soil (Wen *et al*., 2004). The pH was acidic in all the material, which has been shown to not be ideal for earthworms as their chance of survival decreases at low pH levels (Spurgeon and Hopkin, 1996; Ohno, 2001). Even though no mortality was observed throughout the experimental period, the pH level of material may have had an effect on earthworm reproduction as observed by Bengtsson *et al.* (1986). This correlates with the high reproduction success in the neutral control medium in comparison to the lower reproduction in the acidic tailings material. A previous study by Maboeta *et al.* (2007) also found an increase in earthworm reproduction with increasing distance from a platinum TDF.

The metals chosen for this study were four of the seven (Cd, Cr, Ni, Pb, Zn, Cu, Co) environmentally important trace elements as described in Herselman *et al*. (2005). Arsenic was also chosen since it is associated with gold mine tailings. The BCF was determined by calculating the ratio of earthworm metal concentration to material metal concentration.

In the control medium and earthworm body tissue, Cr concentration was lower than the benchmarks. In the RS, Cr concentration of the material was 0.59 ppm, which exceeds the benchmark limit of toxicity to earthworms. The TDF-R material was also above the Cr toxicity to earthworm level, with a concentration of 0.49 ppm. However, no bioaccumulation of Cr took place. Cobalt results are hard to explain since no benchmarks are available for Co in South African soils. Concentration of Co in the materials, were higher in the TDF-U than in TDF-R. Although there was some Co bioaccumulation in the control, it was the only medium in which Co bioaccumulation occurred.

Nickel concentrations were low in the control and no bioaccumulation occurred. In the RS samples, Ni was high with a concentration of 8.83 ppm. In the TDF-R material Ni was higher with 19.08 ppm. Bioaccumulation of Pb occurred in the RS and the TDF-U samples. Arsenic bioconcentration factor was high in the RS (82.06) but extremely high in the TDF-R (336.89), and in the TDF-U tailings (107.67). Arsenic has been found to have a negative effect on enzymatic activity and in the studies of Wang *et al*. (2011) and Fernández *et al*. (2005), were found that arsenic had a negative correlation with dehydrogenase, phosphatase and urease activity.

Except for Cr and As, none of the metal concentrations in the sampled material were cause for concern when looked at individually. It is however difficult to make conclusions due to the fact that the metals were present in mixtures. This is said in the light of possible additive, antagonistic or synergistic effects that metals in mixture might have on organisms (Khalil *et al.,* 1996; Chaperon and Sauvé, 2007).

When looking at dehydrogenase, acidic- and alkaline phosphatases as well as β-glucosidase activity, statistical data indicated that there was a significantly higher ($p < 0.05$) amount of microbial activity in the RS than in the TDF-U material for these four enzymes. This might be explained by the vegetation cover in the RS, which creates a niche environment for microorganisms to thrive in. Since acidic phosphatase activity is mainly attributed to plant roots (Criquet *et al*., 2004), the significant differences between the TDF-R and the TDF-U sites can also be explained by the fact that the TDF-R has established some vegetation. Chemical and physical characterization have been combined with enzymatic activity results (Antunes *et al*., 2011) in order to provide complementary information. In general, studies have also shown that with an increase of metal pollution, comes a significant decrease of enzymatic activity (Lee *et al*., 2009; Pereira *et al*., 2006; Papa *et al*., 2010).

From the earthworm biomass results it is evident that the tailings material had a substantial effect on the worms as was also found in Berthelot *et al.* (2008) namely that metals have the potential to impair earthworm growth, reproduction and even NRR-t times. After the 28 days of exposure, biomass of the worms in the TDF-U material was significantly lower than biomass of worms from the other sites. A similar effect was observed in studies of Malecki *et al*. (1982) and Neuhauser *et al*. (1984), indicating that the body mass of earthworms decreased with an increase in metal contamination of the substrate. This might be the reason why the worms in the TDF-U material had a lower body mass than the worms in the RS material or those in the control medium, even though the worms were fed the same amount of horse manure in all the treatments.

NRR-t analysis showed statistically significant ($p < 0.05$) changes from as early as 7 days of exposure. The reason being that coelomocytes of the worms exposed to the tailings material, stained in half the

time it took for coelomocytes of worms in the controls to stain (Harreus *et al.,* 1997). NRR - times were lower for worms exposed to unrehabilitated material than for those in control mediums. This may be observed as a result of metal pollution or another form of pollution as was found in a study by Booth *et al.* (2005) during the evaluation of contaminated sites before and after rehabilitation. In this study, the worms in the TDF material showed the most cellular stress by producing more stained coelomocytes in a shorter period of time during the NRR-t assay. In a study done by Scott-Fordsmand *et al.* (1998) on specifically Ni exposure (which was one of the metals with the highest content in all the material) over a period of 4 weeks, the NRR-times also decreased with increasing Ni concentrations.

Reproduction results indicated a statistically significant ($p < 0.05$) difference between the amount of juvenile worms found in all the material after the 56 days. In the control there was almost 40 worms compared to the almost 30 in the reference material. The amount of juvenile earthworms were however very low in the TDF-R and TDF-U materials. Total cocoon production also showed statistically significant ($p < 0.05$) differences between the different materials, with the most cocoons produced in the control and then in the reference materials. Total juveniles and total cocoon production were sensitive endpoints in this mine as they showed significant differences between the different sites. Due to the low number of cocoons produced in the tailings materials, hatchling success was not a good endpoint to use to make conclusions or recommendations. This was because with so little cocoons, it would be hard to determine the amount of juvenile worms emerging from each cocoon.

RAPD-PCR detected genetic differences between the DNA of the control worms and the worms exposed to the tailings material. This was observed as differences in polymorphic bands. Different numbers of polymorphic bands were detected in all the samples (Table 3.2) with a total of 105 bands. In the control samples, a total of seven polymorphic bands were detected in comparison to the zero polymorphic bands from the RS samples, one in the TDF-R samples and two in the TDF-U samples. The bands also differed in size ranging from 199bp to 1765bp. The RS and TDF-U samples produced the largest band sizes, while smaller band sizes were observed in the control and the TDF-R samples. As demonstrated in the dendrogram (Figure 3.14), the control samples grouped together while the mine samples were scattered. The large polymorphic bands in the RS and TDF-U samples could be explained as genetic variation due to genotoxic effects of the metals, since RS material was sampled in close proximity to a road, which might have played a big role in pollution of the material. Of the metals analysed, Cr concentration of the RS material exceeded the benchmark limit of toxicity to earthworms while bioaccumulation of Ni, Pb and As also took place. For this reason it is possible to assume that the RS and TDF-U samples were most polluted, causing the most genotoxic effects.

### *3.6 Conclusions*

All of the samples had an acidic pH and consisted mostly of sand-like particles.

The metals Co and Ni were present at high concentrations in the material, as well as in the earthworms, while the As concentration was high in the earthworm body tissue. Although Pb had a high bioconcentration factor, the bioaccumulation of As was higher than for all the other metals.
For dehydrogenase, β-glucosidase, alkaline phosphatase and Urease, enzymatic activity was higher in RS material when compared to that in the TDF-R and the TDF-U materials. When comparing the tailings material, enzymatic activity was higher in the TDF-R than in the TDF-U.

After 28 days of exposure, biomass was statistically lower ($p < 0.05$) in the TDF's. NRR-times were very low for the worms exposed to TDF-R and TDF-U materials. No mortality was observed over the test period. Reproduction was higher in the RS material than in the TDF-R and TDF-U material.

RAPD-PCR analysis indicated several genetic differences between the DNA of the control worms and the worms exposed to the tailings material. Differences in polymorphic bands may be as a result of genotoxic effects as metals bioaccumulated in the exposed earthworms.

### *3.7 References*


AGRAWAL, R.P. 1991. Water and nutrient management in sandy soils by compaction. *Soil and Tillage Research,* 19(2-3): 121-130.

AKCIL, A. & KOLDAS, S. 2006. Acid Mine Drainage (AMD): causes, treatment and case studies. (*In Journal of Cleaner Production*. Improving Environmental, Economic and Ethical Performance in the mining industry. Part 2. Life cycle and process analysis and technical issues. 14(12-13): 1139-1145.)

ANANE, M. 2006. Lethal cyanide spill in Ghana outrages gold mining communities. Environmental News Service: 25 July. [Web:] http://www.ens-newswire.com [Date of access: 14 December 2010].

ANTUNES, S.C., PEREIRA, R., MARQUES, S.M., CASTRO, B.B. & GONÇALVES, F. 2011. Impaired microbial activity caused by metal pollution: A field study in a deactivated uranium mining area. *Science of the Total Environment,* 410(411): 87-95.

BENGTSSON, G., GUNNARSSON, T. & RUNDGREN, S. 1986. Effects of metal pollution on the earthworm *Dendrobaena rubida* (Sav) in acidified soils. *Water, Air and Soil Pollution,* 28: 361-383.

BERTHELOT, Y., VALTON, E., AUROY, A., TROTTIER, B. & ROBIDOUX, P.Y. 2008. Integration of toxicological and chemical tools to assess the bioavailability of metals and energetic compounds in contaminated soils. *Chemosphere,* 74: 166-177.

BOOTH, L.H., HEPPETHWAITE & O'HALLORAN, K. 2005. Effects-based Assays in the Earthworm *Aporrectodea caliginosa.* Their Utilisation for evaluation of contaminated sites before and after remediation. *Journal for Soils and Sediments*, 5(2): 87-94.

BUCHELI, T.D. & FENT, K. 1996. Introduction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Review: Environmental Science and Technology*, 25: 201-268.

CHAPERON, S. & SAUVÉ, S. 2007. Toxicity interaction of metals (Ag, Cu, Hg, Zn) to urease and dehydrogenase activities in soils. *Soil Biology and Biochemistry,* 39(9): 2329-2338.

CLAYTON, C., GOLDBERG, M.M. & POTTER, B.B. 1997. Design and analysis of an experiment for assessing cyanide in gold mining wastes. *Chemometrics and intelligent laboratory systems,* 36: 181-193.

CORTET, J., GOMET-DE VAUFLERY, A., POINSOT-BALAGUER, N., GOMOT, L., TEXIER, C. & CLUZEAU, D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects: utilisation de la faune invertebree du sol comme bio-indicateur des effets des pollutants. *European Journal of Soil Biology,* 35: 115-134.

CRIQUET, S., FERRE, E., FARNET, A.M. & LE PETIT, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biology and Biochemistry,* 36: 1111–1118.

DAVIS, R.H. 1981. Cyanide detoxification in the domestic fowl. (*In*: Vennesland, B.E., (Ed). Cyanide in biology. New York: Academic Press, 1981).

DEPARTMENT OF INDUSTRY TOURISM AND RESOURCES. 2006. Mine Rehabilitation: Leading practice sustainable development program for the mining industry. Australian Government – Department of Industry, Tourism and Resources. Okt. [Web:] http://www.ret.gov.au/resources/Documents/LPSDP/LPSDP-MineRehabilitationHandbook.pdf [Date of access: 11 November 2011].

DONATO, D.B., NICHOLS, O., POSSINGHAM, M., MOORE, M., RICCI, P.F.& NOLLER, B.N. 2007. Review article: A critical review of the effects of gold cyanide-bearing tailings solutions on wildlife. *Environment International*, 33: 974-984.

EARTHWORKS. 2007. Costa Rican Mine Suspended Due to Pollution Risks. Groups call on company to disclose information and pay for clean-up; Cyanide, other chemicals may have already leaked into environment. San Jose and Washington, DC, 11 September, Press release, EARTHWORKS.

EFROYMSON, R.A., WILL, M.E. & SUTER II, G.W. 1997. Toxicological benchmarks for contaminants of potential concern for effects on soil and litter invertebrates and heterotrophic process: 1997 revision. U.S. Department of Energy, East Tennessee Technology Park.

ENVIRONMENTAL LITERACY COUNCIL. 2008. Gold Mining. [Web:] www.enviroliteracy.org [Date of access: 12 November 2010].

FERNÁNDEZ, P., SOMMER, I., CRAM, S., ROSAS, I. & GUTIÉRREZ, M. 2005. The influence of water-soluble As (iii) and As (v) on dehydrogenase activity in soils affected by mine tailings. *Science of the Total Environment,* 348(1-3): 231-243.

GEOLOGY DEPARTMENT, SOUTH AFRICA. 2000. Diamonds, Gold, and South Africa. Diamonds and De Beers. [Web:] www.geology.ucdavis.edu [Date of access: 12 November 2010].

GOOGLE EARTH. 2010. GOOGLE INC. Google Earth (Version 4.2.0180.1134) [Software]. Available from http://www.google.com/earth/index.html

HARREUS, D., KÖHLER, H.R. & WEEKS, J.M. 1997. Combined Non-Invasive cell Isolation and Neutral-Red Retention assay for measuring the effect of copper on the Lumbricid *Apporrectodea rosea* (Savigny). *Bulletin of Environmental Contamination and Toxicology*, 59: 44-49.

HERSELMAN, J.E., STEYN, C.E. & FEY, M.V. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa. *South African Journal of Science,* 101: 510.

HILSON, G. & MURCK, B. 2001. Progress toward pollution prevention and waste minimization in the North American gold mining industry. *Journal of Cleaner production*, 9: 405-415.

ISMAIL, S.M. & OZAWA, K. 2007. Improvement of crop yield, soil moisture distribution and water use efficiency in sandy soils by clay application. *Applied Clay Science,* 37(1-2): 81-89.

KHALIL, M.A., ABDEL-LATEIF, H.M., BAYOUMI, B.M. & VAN STRAALEN, N.M. 1996. Analysis of separate and combined effects of heavy metals on the growth of *Aporrectodea caliginosa* (Oligochaeta; Annelida), using the toxic unit approach. *Applied Soil Ecology,* 4(6): 213-219.

LAPINSKI, S. & ROSCISZEWSKA. 2008. The impact of cadmium and mercury contamination on reproduction and body mass of earthworms. *Plant, Soil and Environment*, 54(2): 61-65.

LEDIN, M. & PEDERSEN, K. 1996. The environmental impact of mine wastes – Roles of microorganisms and their significance in treatment of mine wastes. *Earth Science Reviews*, 41(1-2): 67-108.

LEE, S.H., KIM, E.Y., HYUN, S. & KIM, J.G. 2009. Metal availability in heavy metal-contaminated open burning and open detonation soil: assessment using soil enzymes, earthworms, and chemical extractions. *Journal of Hazardous Materials,* 170: 382-388.

LIEFERINK, M. 2007. Poisonous legacy of Gold and Uranium Mining. Wild Nature – poisoned and silent. CANE – Coalition Against Nuclear Energy South Africa. [Web:] www.cane.org.za [Date of access: 4 April 2011].

LØKKE, H. & VAN GESTEL, C.A.M. 1998. Handbook of soil invertebrate toxicity tests. Chichester: John Wiley and Sons.

MABOETA, M.S., VAN RENSBURG, L. & JANSEN VAN RENSBURG, P.J. 2007. Earthworm (*Eisenia fetida*) bioassay to assess the possible effects of platinum tailings disposal facilities on the environment along a gradient. School for Environmental Sciences and Development, North-West University, Potchefstroom Campus, Private bag x6001, Potchefstroom 2520 South Africa.

MAIL & GUARDIAN ONLINE. 2011. Acid mine drainage reaches Cradle of Humankind. [Web:] http://www.mg.co.za/article/2011-01-15-acid-mine-drainage-reaches-cradle-of-humankind [Date of access:27 January 2011].

MALECKI, M.R., NEUHAUSER, E.F. & LOEHR, R.C. 1982. The effect of metals on growth and reproduction of *Eisenia foetida (Oligochaeta Lumbricidae)*. *Pedobiologia*, 24: 129-137.

MILTZAREK, G.L. SAMPAIO, C.H. & CORTINO, J.L. 2002. Cyanide recovery in hydrometallurgical plants: use of synthetic solutions constituted by metallic cyanide complexes. *Minerals engineering*, 15(1-2): 75-82.

MINERALS COUNCIL OF AUSTRALIA. 1996. Tailings storage facilities at Australia gold mines. Submission to the senate environment, recreation, communication and the arts reference committee. Melbourne: Minerals Council of Australia.

NATIONSONLINE. 2010. Map of South Africa's provinces. [Web:] http://www.nationsonline.org/oneworld/map/za_provinces_map2.htm [Date of access: March 2, 2011].

NEUHAUSER, E.F., MALECKI, M.R., & LOEHR, R.C. 1984. Growth and reproduction of earthworm *Eisenia fetida* after exposure to sublethal concentrations of metals. *Pedobiologia*, 27: 89-97.

OEHLMANN, J. & SCHULTE-OEHLMANN, U. 2003. Bioindicators and Biomonitors – Principles, Concepts and Applications. *Trace Metals and other Contaminants in the Environment,* 6: 577-635.

OHNO, M. 2001. Sensitivity of a Japanese earthworm (*Allolobophora japonica*) to soil acidity. *Water, Air and Soil Pollution,* 130: 1019-1024.

PAPA, S., BARTOLI, G., PELLEGRINO, A. & FIORETTO, A. 2010. Microbial activities and trace element contents in an urban soil. *Environmental Monitoring and Assessment,* 165: 193-203.

PEPPAS, A., KOMNITSAS, K. & HALIKIA, I. 2000. Use of organic covers for acid mine drainage control. *Minerals Engineering*, 13(5): 563-574.

PEREIRA, R., SOUSA, J.P., RIBEIRO, R. & GONÇALVES, F. 2006. Microbial indicators in mine soils (S. Domingos mine Portugal). *Soil and Sediment Contamination,* 15: 147-167.

REECE, R. 1997. Cyanide toxicity to birds. ACMRR. Short course notes on management of cyanide mining. Perth: ACMRR, 1997.

ROSSOUW, A.S., ANNEGARN, H.J., WEIERSBYE, I.M. & FURNISS, D.G. 2010. SAAB Annual Meeting Abstracts: Evaluating the functional status of a rehabilitated gold tailings storage facility – A case study in the Witwatersrand. *South African Journal of Botany,* 76(2): 402.

SAEXPLORER. 2008. Klerksdorp rainfall. [Web:] www.saexplorer.co.za/south-africa/climate/klerksdorp_climate.asp [Date of access: 15 December 2010].

SAUNDERS, J.R., KNOPPER, L.D., YAGMINAS, A., KOCH, I. & REIMER, K.J. 2009. Use of biomarkers to show sub-cellular effects in meadow voles (*Microtus pennsylvanicus*) living in an abandoned gold mine site. *Science of the Total Environment,* 407(21): 5548-5554.

SCOTT-FORDSMAND, J.J., WEEKS, J.M. & HOPKIN, S.P. 1998. Toxicity of nickel to earthworm and the applicability of the neutral red retention assay. *Ecotoxicology,* 7: 291-295.

SPURGEON, D.J. & HOPKIN, S.P. 1996. Effects of variations of the organic – matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida. Pedobiologia,* 40: 80-96.

STATISTIKA. Statistika version 10. StatSoft Inc. [Software]

TABATABAI, M.A. 1994. Soil enzymes. (*In*: Weaver, R.W. (Ed), Methods of Soil Analysis. Part 2. SSSA Book, Ser 5. SSSA and ASA, Madison, WI, p.775-833.).

TOMPKINS, P. 1998. Secrets of the Soil. [Web:] www.relfe.com/07/soil_humus_properties.html [Date of access: 12 November 2011].

VALERY, B. & EUGENE, K. 1998. Soil surface geochemical anomaly around the copper-nickel metallurgical smelter. *Water, Air and Soil pollution,* 103: 197-218.

VAN GESTEL, C.A.M. & VAN STRAALEN, N.M. 1994. Ecotoxicological test systems for terrestrial invertebrates. (*In*: Donker, M.H., Eijsackers, H., Heimbach, F., editors. Ecotoxicology of soil organisms. Boca Ranton: CRC Press, Inc.; p. 205-228).

VAN STRAALEN, N.M. & VAN GESTEL, C.A.M. 1993. Soil Invertebrates and micro-organisms. (*In*: Calow, P, editor. Handbook of ecotoxicology I. Blackwell Scientific Publications. p.229-325).

WANG, Q., HE, M. & WANG, Y. 2011. Influence of combined pollution of antimony and arsenic on culturable soil microbial populations and enzyme activities. *Ecotoxicology,* 20:9-19.

WEN, B., HU, X., LIU, Y., WANG, W., FENG, M., & SHAN, X. 2004. The role of earthworms (*Eisenia fetida*) in influencing bioavailability of heavy metals in soils. *Biology and Fertility of Soils,* 40: 181-187.

ZORITA, I., ORTIZ-ZARRAGOITIA, M., SOTO, M. & CAJARAVILLE, M.P. 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. *Aquatic Toxicology,* 78S: S109-S116.

## CHAPTER 4: UTILIZING EARTHWORM AND MICROBIAL ASSAYS TO ASSESS THE ENVIRONMENTAL EFFECTS OF A CHROMIUM MINE

### *4.1 Introduction*

Soil is vital, either directly or indirectly, for plants, animals and humans to survive (Department of Agriculture, 1999). It is however being contaminated by various activities, mining being one of the biggest culprits. Furthermore, South Africa is the largest producer of ferrochrome as it holds about 70 % of the total worldwide chrome reserves and produces 75 % of the ferrochrome needed by the global industry (Mbendi Information Services, 2010). Chromium, which is produced from chromite ores, is a vital and indispensable metal in the modern industry (Vermaak, 1986), some of its uses include the use in ferro alloys, high temperature furnace linings, chemicals products, plating, and as coating on metals or steels to protect against corrosion (Vermaak, 1986).

Mining operations have an undesirable impact on the environment (Chester *et al*., 1989), since the industry is one of the main contributors to the deterioration of the environment in terms of land pollution as well as water and air pollution (Godgul and Sahu, 1995). This can be seen in the increase in mining and industrial activities in the late $19^{th}$ and early $20^{th}$ century, which have led to an increase in metal pollution worldwide (Dey *et al.*, 2007). Chromium specifically, has been found to be hazardous to fauna and flora at elevated levels (Nriagu *et al*., 1988), including having detrimental effects on plant growth and development (Shanker *et al*., 2005).

Ecotoxicological research done on chromium found that hexavalent chromium, Cr(VI), could have genotoxic effects in vertebrates, including the formation of reactive oxygen species (ROS). These ROS in turn have serious effects on the cellular system, damaging the DNA, and could therefore interrupt cellular activity. The *in vitro* and *in vivo* exposure effects of earthworms to Cr(VI) was also examined by Manerikar *et al*. (2008). The *in vitro* exposure was done to determine dose dependent DNA damage, while the *in vivo* exposure was to determine at which concentrations of Cr(VI) DNA damage would occur in the earthworms. Results showed that Cr(VI) affected DNA even at a low (1ppm) concentration. These effects of Cr(VI) as observed using a comet assay in a study by Bigorgne (2010), showed that 491 $mg.kg^{-1}$ was fatally toxic to 100% of individuals of the species, *Eisenia fetida.*

The aim of this study was to utilize earthworm assays to assess the ecotoxicity of Cr mining on the environment. The reasons being that earthworms are closely linked to their soil environment and because they are able to accumulate metals from their environment into their body tissue (Lapinski and Rosciszewska, 2008) making them ideal for the assessment of mining material and soil. Furthermore, to utilize microbial assays, as microbial activity is an indicator of the state of the soil or mining material

(Tabatabai, 1994). Samplings of all three materials were taken from different heaps and not from a rehabilitated or unrehabilitated mine as such. The turf material is the material representative of the soil in the area, and is also used as topsoil in the rehabilitation process. For this reason, it was used as the reference soil. Pyroxenite is found in between the chromium bands and during the mining process those two materials are separated from each other and placed on different heaps: a pyroxenite heap and a silt heap. After mining, the quarries are filled up with the pyroxenite material and covered with topsoil. For this reason and because silt and pyroxenite are both raw, unrehabilitated material, both will be referred to as TDF-U. Silt will be referred to as TDF-U1 while pyroxenite will be referred to as TDF-U2.

The specific objectives included an assessment done on the material collected from the mining area. This was done to get an indication of the state of the area while mining activity is going on, and also to determine the current state of the chromium mining area as well as possible risks that may be present. A further objective was to determine if earthworm biomarkers could be utilized to assess metal contamination as well as its effects on organisms and the ecosystem with regards to chromium mining. This was done by assessing the bioavailability of metals to be taken up by the biota (Bucheli and Fent, 1996). The bioconcentration factor (BCF), which is the ratio between the concentration of a certain pollutant present within the tissue, of either a specific organ or the whole organism, and the environmental concentration, is an import indicator in any ecotoxicological assessment.

### *4.2 Site description*

Samples were collected from different heaps of material at an opencast chrome mine in Rustenburg, South Africa (Figure 4.1). Average rainfall of the area is 513 mm per year, mostly during midsummer (saexplorer, 2010). Silt was collected from a heap as shown in Figure 4.2 and will from here on be referred to as TDF-U1. Chromite occurs in a pyroxenite rock clast, where the chromite rich layers alternate with silicate layers such as pyroxenite (Boyd and Meyer, 1979). Pyroxenite was collected from another heap (Figure 4.3) and will be referred to as TDF-U2. Turf, which is used as topsoil in the area, was collected from a heap next to the pyroxenite heap (Figure 4.3) and will be referred to as the reference site (RS). Figure 4.4 shows the process of opencast mining, while the silt heap is shown in Figure 4.5. The pyroxenite heap is shown in Figure 4.6 and the turf heap in Figure 4.7. In terms of rehabilitation for this mine, the area is naturally rehabilitated i.e. holes are filled up with pyroxenite and covered with turf, and then plants from the natural area are planted.


**Figure 4. 1.** A map of South Africa showing the Rustenburg area in the North West province (Worldmapnow, 2010).


**Figure 4. 2.** The open cast chrome mining area showing the silt heap (TDF-U1) from above (Google Earth, 2010).


**Figure 4. 3.** The open cast chrome mining area showing the turf (RS) and pyroxenite (TDF-U2) heaps viewed from above (Google Earth, 2010).

**Figure 4. 4.** Mining activity taking place at the open cast chrome mine (Charné van Coller).

**Figure 4. 5.** The heap at the chrome mine where silt/unrehabilitated material (TDF-U1) samples were collected (Charné van Coller).

**Figure 4. 6.** The heap at the chrome mine where pyroxenite/unrehabilitated material (TDF-U2) samples were collected (Charné van Coller).

**Figure 4. 7.** The heap at the chrome mine where turf/reference material (RS) samples were collected (Charné van Coller).

### *4.3 Materials and methods*

Refer to chapter 2.

### *4.4 Results*

#### *4.4.1 Physical and Chemical properties*

*The distribution of gravel, sand, silt and clay-like particle sized material*

Gravel, sand, silt and clay-like particle distribution of the different sites' samples is shown in figure 4.8. Particle sizes were compared between RS, TDF-U2 and TDF-U1 samples. The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

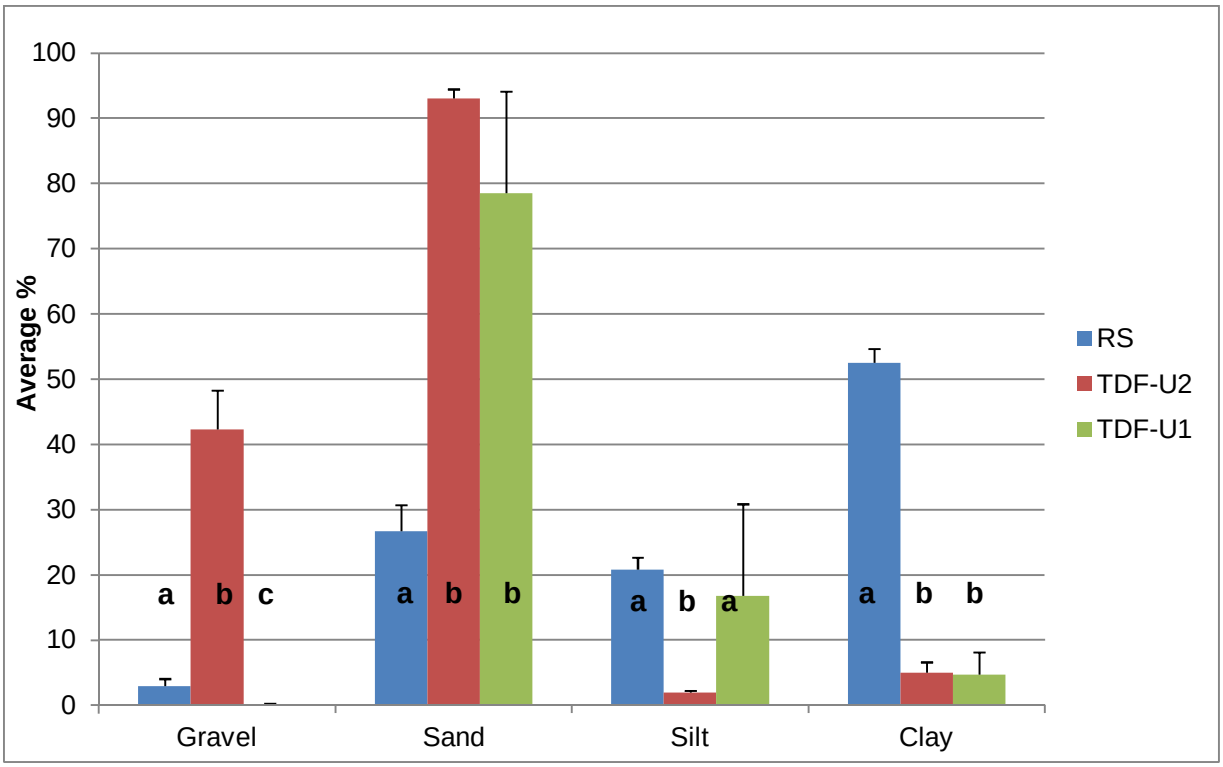


**Figure 4. 8.** Particle size distribution (± SD) of the substrates and soil collected from the different areas [turf/reference material (RS), pyroxenite/unrehabilitated material (TDF-U2) and silt/unrehabilitated material (TDF-U1) of a chromium mining area.

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

RS material consisted mostly of clay-like (<2 µm) particles but also of small amounts of silt-like (20-2 µm) and sand-like (2-0.02 mm) material. TDF-U2 consisted mostly of sand-like particles and gravel-like particles. TDF-U1 consisted mostly of sand-like particles and small amounts of silt-like material. Figure 4.9 indicates the pH of the control, RS, TDF-U2 and TDF-U1 samples.

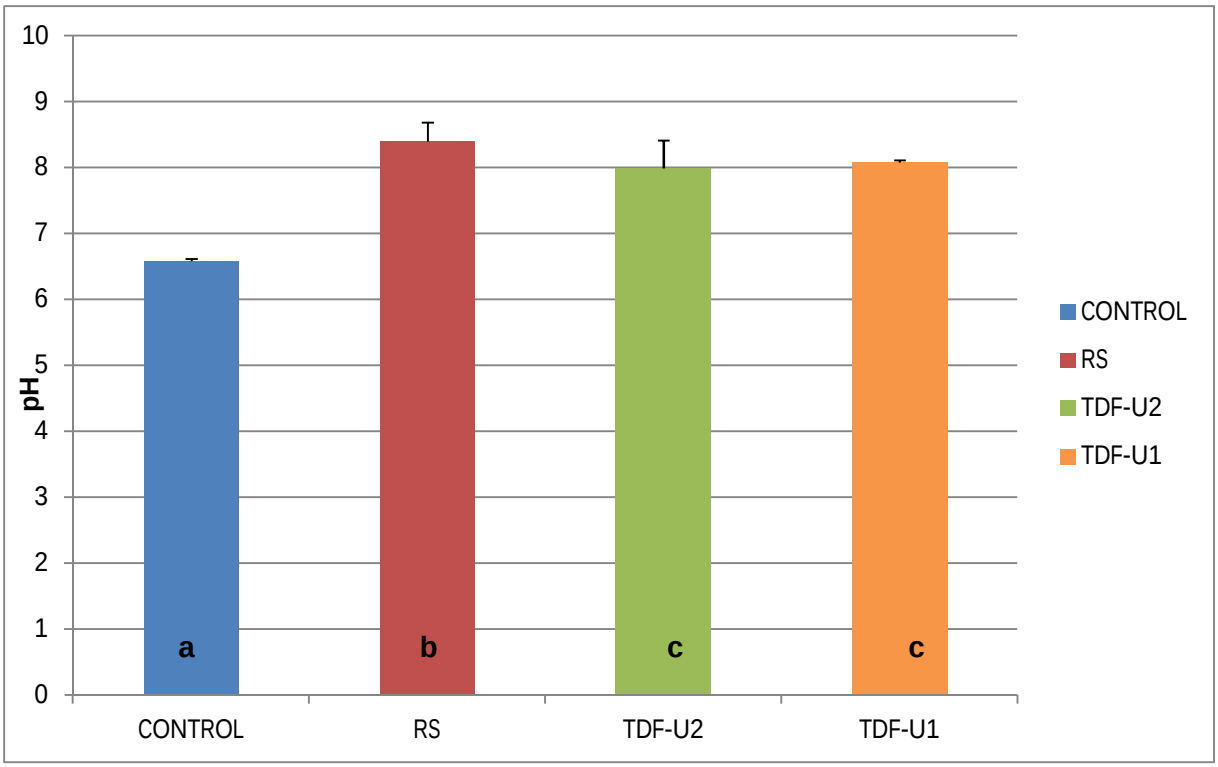


**Figure 4. 9.** pH of the various samples collected from an open cast chromium mine including control, reference site (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U1) material.

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

RS material had the lowest pH and all three the materials had an alkaline pH.

### *4.4.2. Metals*

Results from earthworm tissue metal analysis compared to metal analysis on the mining material are shown in Table 4.1. These concentrations were measured against different benchmarks in ppm.

**Table 4.1**. Soil metal analysis for control, reference site material (RS), unrehabilitated pyroxenite (TDF-U2) and unrehabilitated silt (TDF-U1) measured against the following benchmarks in ppm: Total investigation level (TIL), total maximum threshold levels (TMT) (Herselman *et al.,* 2005), E/W (benchmarks for earthworm toxicity), SMO and MP (benchmark concentrations for toxicity to soil microorganisms and microbial processes, described by Efroymson *et al.* 1997), as well as bioconcentration factor (BCF) (i.e. Earthworm body concentration/total soil concentration).

| | | ***Cr*** | ***Co*** | ***Ni*** | ***Pb*** |
|---|---|---|---|---|---|
| ***TIL*** | | ***80*** | ***-*** | ***50*** | ***56*** |
| ***TMT*** | | ***350*** | ***-*** | ***150*** | ***100*** |
| ***E/W*** | | ***0.4*** | ***-*** | ***200*** | ***500*** |
| ***SMO and MP*** | | ***10*** | ***1000*** | ***90*** | ***900*** |
| **Control** | **Material** | $0.26\pm0.03^{a}$ | $0.04\pm0.01^{a}$ | $0.22\pm0.04^{a}$ | $0.05\pm0.01^{a}$ |
| | **E/w** | $0.06\pm0.01^{A}$ | $0.16\pm0.02^{A}$ | $0.08\pm0.02^{A}$ | $0.03\pm0.02^{A}$ |
| | **BCF** | 0.23 | 4.00 | 0.36 | 0.60 |
| **RS** | **Material** | $3.25\pm0.66^{b}$ | $0.08\pm0.09^{a}$ | $0.50\pm0.09^{b}$ | $0.03\pm0.00^{b}$ |
| | **E/w** | $0.23\pm0.15^{B}$ | $0.47\pm0.39^{B}$ | $0.36\pm0.09^{B}$ | $0.01\pm0.01^{B}$ |
| | **BCF** | 0.07 | 5.88 | 0.72 | 0.33 |
| **TDF-U2** | **Material** | $0.75\pm0.20^{c}$ | $0.02\pm0.01^{b}$ | $0.44\pm0.35^{c}$ | $0.02\pm0.00^{b}$ |
| | **E/w** | $0.17\pm0.12^{B}$ | $0.61\pm0.54^{B}$ | $0.39\pm0.24^{B}$ | $0.01\pm0.00^{B}$ |
| | **BCF** | 0.23 | 30.5 | 0.89 | 0.5 |
| **TDF-U1** | **Material** | $30.56\pm33.42^{d}$ | $0.02\pm0.00^{b}$ | $0.13\pm0.13^{d}$ | $0.03\pm0.01^{b}$ |
| | **E/w** | $0.19\pm0.09^{B}$ | $0.23\pm0.08^{C}$ | $0.21\pm0.06^{C}$ | $0.02\pm0.01^{C}$ |
| | **BCF** | 0.01 | 11.5 | 1.62 | 0.67 |

a-d: Statistical comparison of the control material with the material from the sites RS, TDF-U2 and TDF-U1;
A-D: Statistical comparison of the worms from the control site with the worms of sites RS, TDF-U2 and TDF-U1.
Values with the same letter in superscript were not statistically different from each other ($p > 0.05$).

Chromium concentration was the highest in the TDF-U1 material, but no bioaccumulation took place. Although the Co levels were below the benchmark, bioaccumulation of Co occurred in the worms from the control, the RS, TDF-U2 and TDF-U1. The BCF was however the highest in the TDF-U materials. Bioaccumulation also occurred for Ni in the TDF-U1 material. No bioaccumulation occurred for the other metals.

### *4.4.3 Soil enzymatic activities*

Figure 4.10 shows the enzymatic activities of five different enzymes in the samples collected from the chromium mine. The amount of activity of each enzyme was compared between the different samples. The grouped data for each of the enzymes was evaluated statistically against one another within the group, and not over the whole graph.

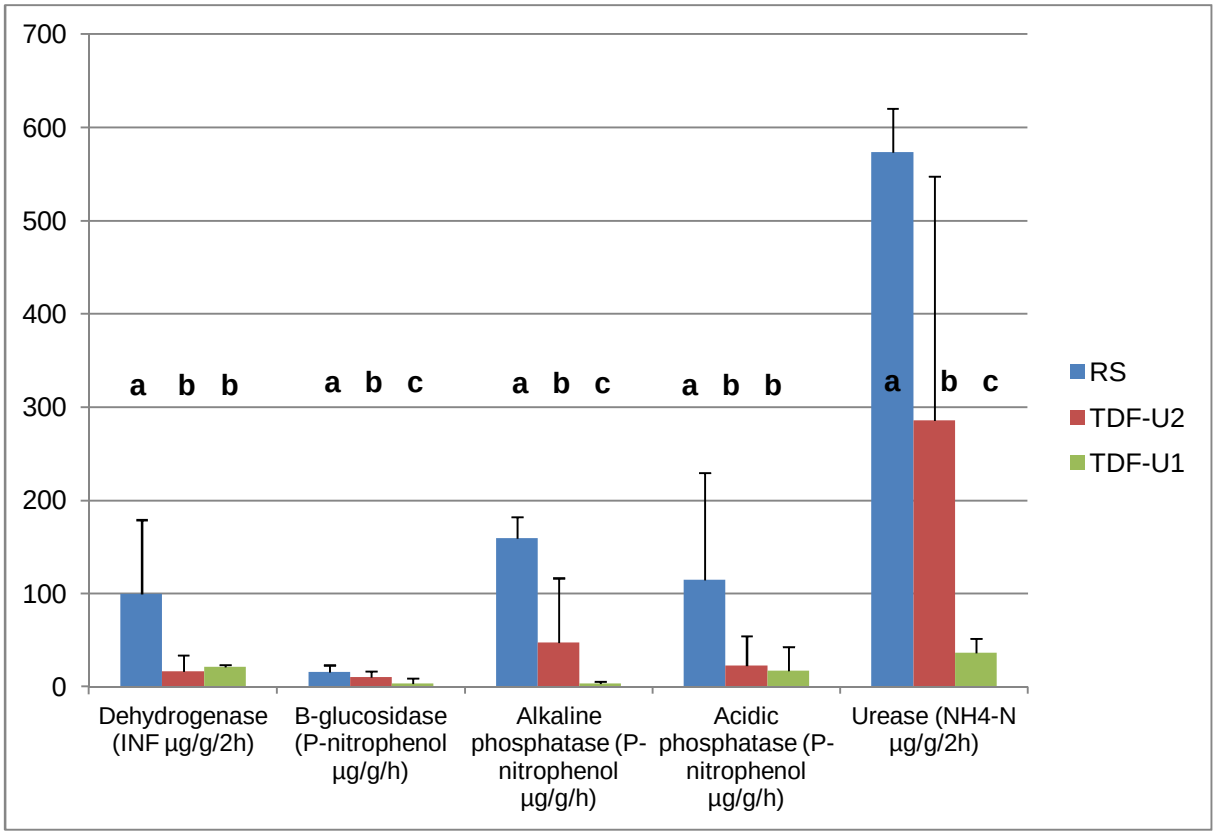


**Figure 4. 10.** Enzymatic analyses of the samples collected from different heaps at a chromium mining area, showing microbial activity (± SD) in the substrates and soil collected from the different areas [turf/reference (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U1) material].

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

Dehydrogenase activity differed significantly ($p < 0.05$) between the RS material and the TDF materials. ß-glucosidase activity was very low in all three materials and differed significantly ($p < 0.05$) between the three heaps. Alkaline phosphatase activity was highest in the RS material and differed significantly ($p < 0.05$) between the three material types. Acidic phosphatase activity was also highest in the RS material and differed significantly between the RS material and the TDF materials. Urease was the enzyme with the highest activity of all the enzymes and differed significantly between the materials from all three heaps.

### *4.4.4 Earthworm assays*

The same control was used for the gold and chromium mining sites as the experiment for both mines were done at the same time, in the same conditions and started at the same time.

#### *4.4.4.1 Biomass*

The mean (± SD) biomass of earthworms that were exposed to the control material and the mining material is shown. The grouped data (per day) was evaluated against one another within the group, and not over the whole graph (Figure 4.11).

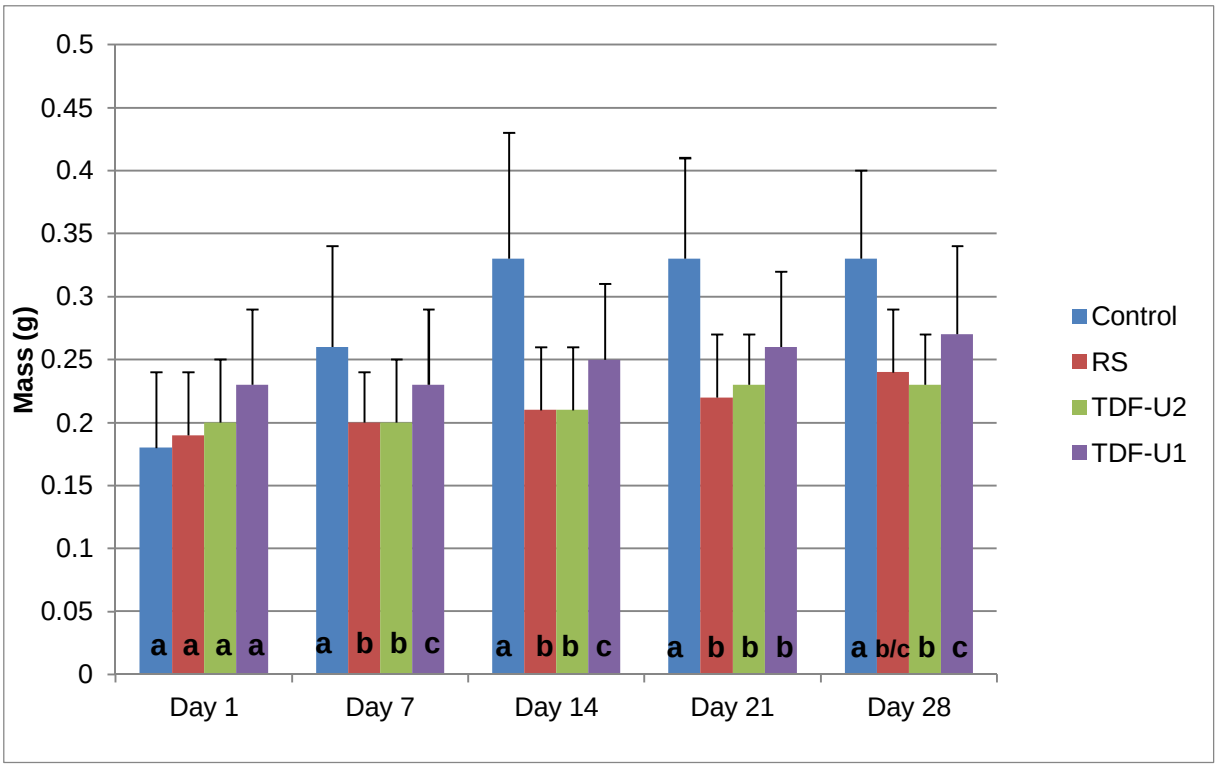


**Figure 4. 11.** A comparison of earthworm biomass (± SD) between worms in the different sites [control, turf/reference (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U1) material] every week over the 28 day period. a–c: Bars with the same letter were not statistically different from each other ($p > 0.05$).

The biomass of the earthworms in all three sites (RS, TDF-U1 and TDF-U2) as well as the control worms, increased over the 28 day period. From day 7, statistically significant differences ($p < 0.05$) could already be observed between the biomass of the control worms and the worms exposed to the other materials.

#### *4.4.4.2 NRR-t*

Figure 4.12 shows the results of NRR-t measured weekly on the earthworms. The grouped data (NRR-t differences per day) was statistically evaluated against one another within the group, and not over the whole graph.

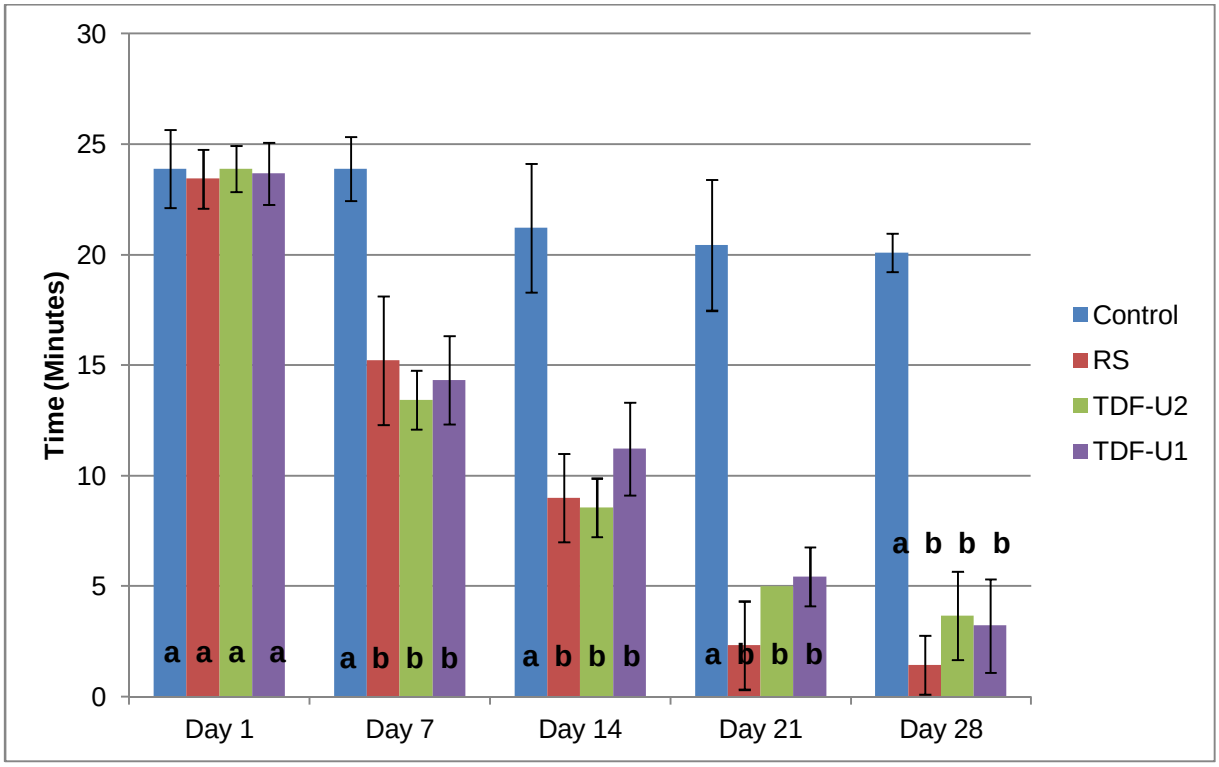


**Figure 4. 12.** A graph comparing earthworm lysosomal membrane stability measured in neutral red retention time (± SD) between worms in the different sites [control, turf/reference (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U2) material] every week over the 28 day period.

a–b: Bars with the same letter in superscript were not statistically different from each other ($p > 0.05$).

The results of the NRR-t analyses are given in time (minutes) it took for the coelomocytes to stain red. Results showed a statistically significant difference ($p < 0.05$) between the cellular stress of the control worms and those of the worms in the different sites. Over the periods of 28 days, the NRR-times became lower with exposure to the contaminated materials. After seven days and also after 28 days, statistical analysis indicated that C > RS = TDF-U1 = TDF-U2.

#### *4.4.4.3 Mortality*

No unresponsive or missing earthworms were observed during or after the 28 days of exposure.

#### *4.4.4.4 Reproduction*

The following figure shows reproductive success by indicating average amounts of juvenile worms produced, hatched cocoons, unhatched cocoons and total cocoons. Results are shown as averages ± SD. The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

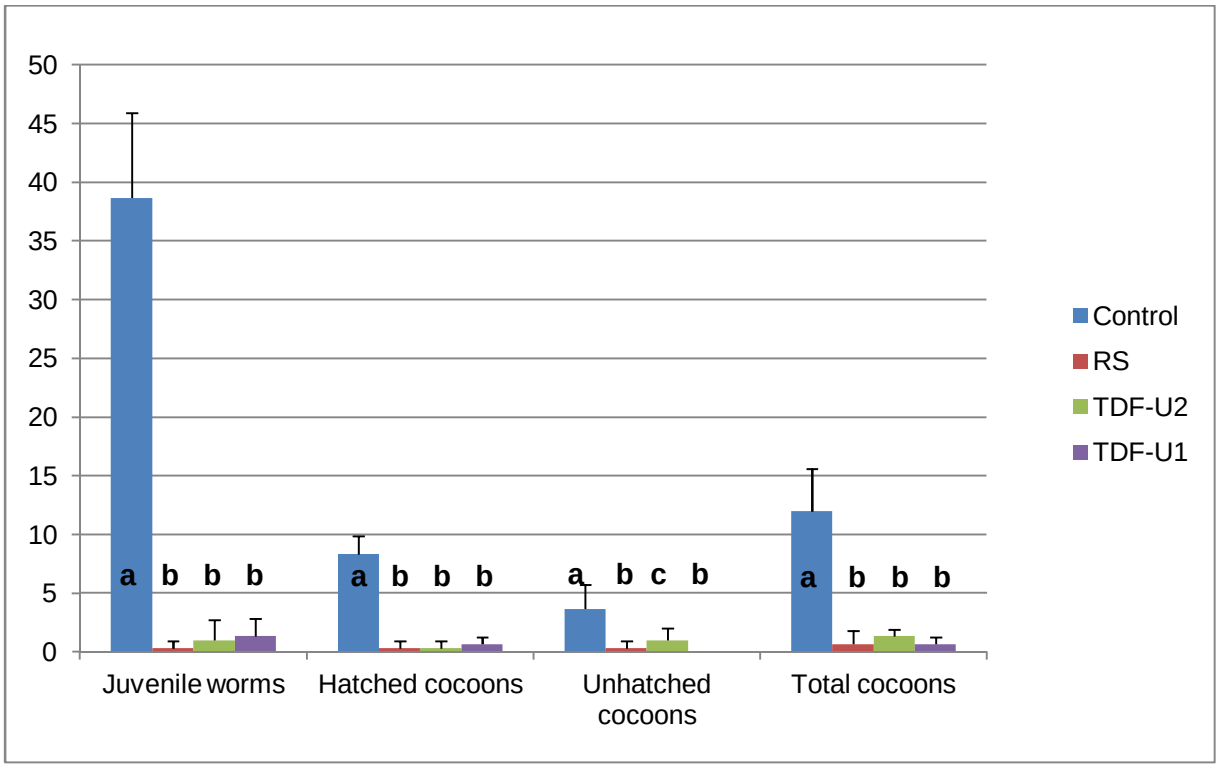


**Figure 4.13.** Reproduction patterns of earthworms placed in mining material from a chromium mine including turf/reference (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U2) material, compared to earthworms in a control medium, by looking at juvenile worms and number of cocoons produced.

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

From the results it is evident that the earthworms in the control samples produced significantly ($p < 0.05$) more cocoons than those in the RS and the TDF materials. The total cocoons produced did not differ significantly ($p > 0.05$) between the RS, the TDF-U1 and the TDF-U2.

### *4.4.5. RAPD PCR analysis*

Genetic variability based on RAPD profiles were observed between the mining area exposed worms and the control sample worms. RAPD analyses were done on 3 worms from each of the TDF-U1, TDF-U2 and RS samples (Figure 4.14). The bands that formed were then compared to determine genetic adaptation of the worms to the material.

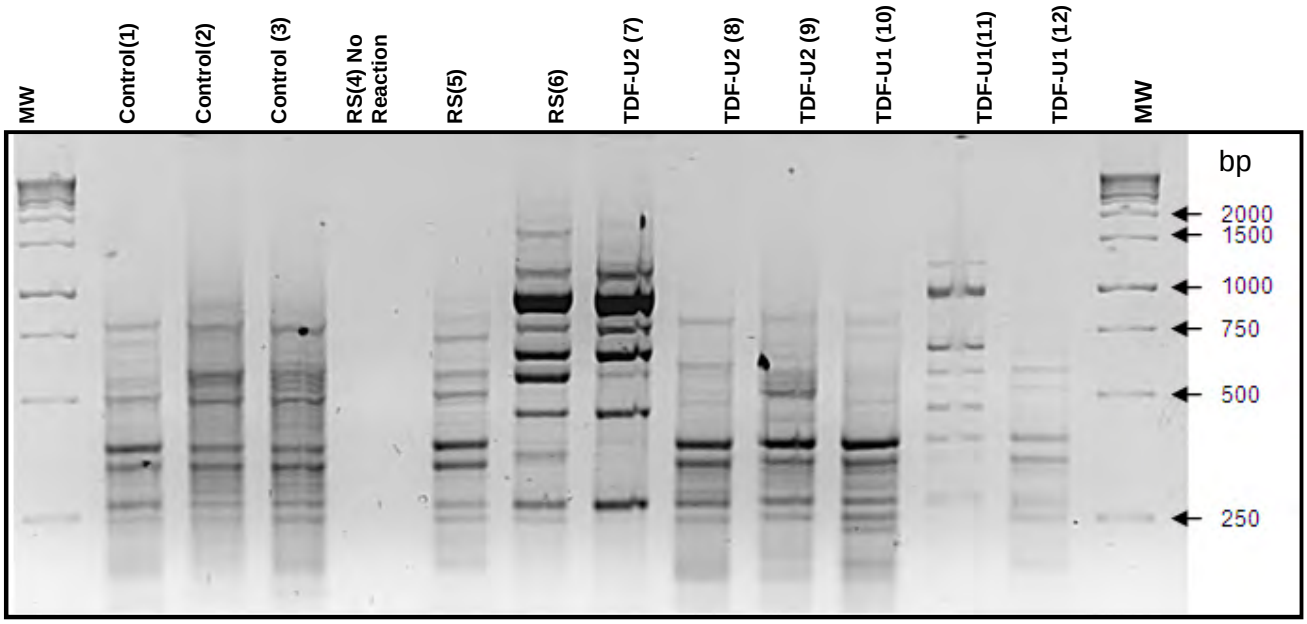


**Figure 4.14.** RAPD-PCR gel of chrome mine samples [including the control, turf/reference site (RS), pyroxenite/unrehabilitated (TDF-U2) and silt/unrehabilitated (TDF-U1) material exposed earthworms]. MW indicates the use of a 1kb Molecular Weight Marker (Fermentas, USA). Numbers in parenthesis next to sample name indicate the lane number on the gel (excluding the MW).

A summary of the banding pattern is given in Table 4.2.

**Table 4.2.** A summary of RAPD analysis of chrome mine related materials on average DNA banding profiles of exposed earthworms

| Primer | Sequence | % G + C | Population | Total Number of Bands | Number of Polymorphic Bands | Bands range |
|---|---|---|---|---|---|---|
| **OPA 16** | AGC CAG CGA A | 60 | **Control** | 19 | 6 | 245-799 |
| | | | **RS** | 18 | 3 | 251-1622 |
| | | | **TDF-U2** | 22 | 2 | 247-1830 |
| | | | **TDF-U1** | 20 | 2 | 250-1219 |

Different numbers of polymorphic bands were detected in all the test sites, totalling 79 bands. In the control samples, a total of six polymorphic bands were detected in comparison to the three bands in the

RS samples and two polymorphic bands at both the TDF-U1 and TDF-U2 samples. Differences in the size of the bands were also apparent, with sizes ranging from 245 bp to 1830 bp. Large band sizes were observed only in the TDF samples while bands from control samples were below 800 bp. Sample RS (4) did not achieve any amplification, as can be seen in Figure 4.14. This might have been due to several factors, including human error (i.e. pipetting error).

These results were used to compile a weighted pair-group average dendrogram (Figure 4.15). Peak heights were compared within the same molecular weight ranges (5%). The controls grouped together, as was expected. All of the other samples were scattered, with some similarity between TDF-U2 and TDF-U1 being observed. RS samples were the furthest removed from the control samples.

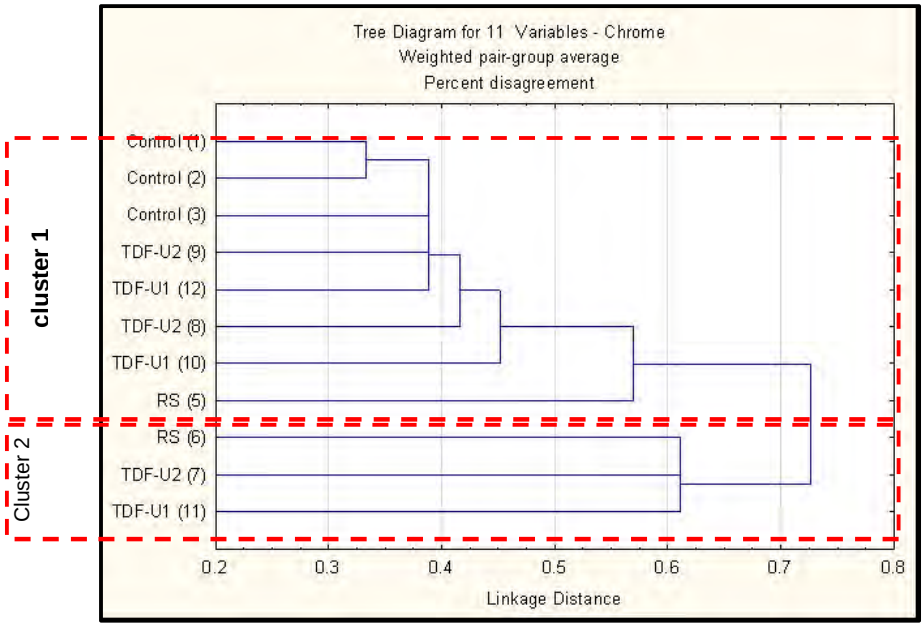


**Figure 4.15.** Dendrogram of chrome mine RAPD analysis. The dendrogram can be divided into two clusters, marked I and II.

Within cluster I the controls are grouped together. Two samples of each TDF-U2 and TDF-U1 are grouped within cluster I. Within cluster II the remaining samples are grouped together.

### *4.5 Discussion*

The opencast mining of minerals has a serious impact on the environment, causing the destruction of natural soils and also by the extraction of volumes of important minerals (Vega *et al.*, 2004). The material that is formed after the extraction of minerals is unstable with an unfavourable texture and structure, exposing it to erosion (Michelutti and Wiseman, 1995).

Physical analysis done on the samples showed that TDF-U1 consisted mostly of sand-like particles and silt-like particles. TDF-U2 consisted mostly of sand-like particles and gravel-like particles. Reference material consisted mostly of clay-like particles, but also small amounts of silt-like and sand-like particles. It is believed that soils with high clay content can have higher concentrations of most trace metals, as opposed to those with low clay content (McBride, 1994). Some studies have found that clay content actually influences heavy metal bioavailability in soil, for example, minimizing the toxic effects that heavy metals could have on soil and on soil fauna (Vega *et al.*, 2004), and affecting bioavailability and toxicity of metals such as Zn and Cu (Owojori *et al.,* 2009; 2010) to earthworms.

The pH of all three materials (RS, TDF-U1 and TDF-U2) was alkaline, while the pH of the horse-manure control was neutral. Since the control medium's pH was neutral, it could not have had any effect on the experiment.

Bioconcentration factor (BCF) was calculated as the ratio of earthworm metal concentration to material metal concentration (Van Gestel *et al.,* 2010). In the control material, Cr concentrations were lower in both the material and the earthworms than the benchmarks. The bioconcentration factor was also below 1, since the concentration of Cr was lower in the earthworms than in the material. In the RS material, Cr concentrations were higher than in the control (3.25 ppm). The Cr concentrations were lower in the worms (0.23 ppm), with a BCF of 0.07. The Cr concentrations in the TDF-U2 material were lower than in the RS material (0.75 ppm in the material and 0.17 ppm in the earthworms). The BCF was 0.23. The TDF-U1 material had a high Cr concentration (30.56 ppm). At this concentration, the material has a negative influence on SMO (soil microbial organisms). Chrome concentrations in earthworms averaged 0.19 ppm and therefore BCF was low at 0.01.

In the control material, the mean Co concentration was 0.04 ppm. In earthworm body tissue, Co concentration was 0.16 ppm. This resulted in a BCF of 4. In the RS material, the BCF was higher than in the control, being 5.88. TDF-U2 and TDF-U1 had a high BCF of 30.5 and 11.5 respectively. Since there are no South African benchmarks available for Co concentrations in soil, it is problematic to interpret this data. According to the benchmark that is available and described by Efroymson *et al.*

(1997) a Co concentration of 1000 ppm can be considered toxic to SMO and MP. Thus, the Co concentrations in RS, TDF-U2 and TDF-U1 samples are considered non-toxic to microorganisms.

In both the control medium and earthworm body tissue, Ni was observed in low concentrations. The BCF was less than 1.00, implying that Ni did not bio-accumulate in the control medium (Falusi and Olanipekun, 2007). In the RS material, the concentration of Ni was higher, but still below benchmarks and a BCF of less than 1.00. In the TDF-U2 material the BCF was 0.89, and in the TDF-U1 samples, Ni bio-accumulated, as the earthworms had a BCF of 1.62

In all the studied materials (RS, TDF-U2, TDF-U1) the Pb concentrations were lower in the material than in the control, as well as lower in the earthworm body tissues than in the control worms' body tissue. Therefore, Pb did not bio-accumulate.

With regards to enzyme activity, dehydrogenase activity differed significantly ($p < 0.05$) between the RS material and the TDF materials. Activity of β-glucosidase was very low in all three materials, and differed significantly ($p < 0.05$) between the three tested sites. Alkaline phosphatase was highest in the RS material, and differed significantly between the three materials, while acidic phosphatase activity was also highest in the RS material and differed significantly between RS and TDF materials. Urease was the enzyme with the highest activity of all the enzymes and differed significantly between materials from all three heaps. The overall enzymatic activity between the different sites as indicated by alkaline phosphatase, urease and β-glucosidase statistically indicated that RS > TDF-U2 > TDF-U1. The RS samples had overall the highest enzymatic activity, and therefore high levels of microbial activity (Alef & Nannipieri, 1995). The RS is a representative material of the natural soil in the area surrounding the mining site and is consequently used as topsoil in the mine's rehabilitation process. The low enzymatic activity and therefore low microbial activity in the TDF-U1 material correlated with previous studies (Rath *et al.,* 2010; Huang *et al.,* 2009) which found a negative correlation between the soil microbial population and metal concentration. The TDF-U1 and TDF-U2 materials had significantly lower enzymatic activity as opposed to RS. This could be due to the possibility that RS had a larger surface, being mainly a clay-like material type. It could also be due to the higher water retention capabilities of the RS material. Both TDF-U1 and TDF-U2 were mostly sand-like particles, which decreases water retention capability, therefore leading to a lower overall humidity and water fraction available to microorganisms.

Earthworm biomass as monitored on a weekly basis was the highest in the control group. This assay was however not a sensitive endpoint for worms exposed the specific mine.
Results showed a statistically significant difference ($p < 0.05$) between the cellular stress of the control worms and those of the worms in the different sites, as was also found in Van Gestel *et al.* (2009) and Hankard *et al.* (2004). From day seven, NRR-t analysis showed no statistically significant difference ($p >$

0.05) between the RS, TDF-U2 and TDF-U1 material. The time noted (in minutes) indicates how long the coelomocytes took to stain red, hence how much the cell walls have been destabilized by a stress response such as heavy metal contamination, therefore explaining why longer exposure time caused more cell damage (Van Gestel *et al*., 2009).

Various factors such as soil pH, organic matter, soil texture and pollution have an effect on the abundance of earthworms in the soil (Tu *et al.,* 2011) and may cause earthworms to either escape out of the material or to die. Among the earthworms placed in all of the site samples of the chrome mining area, however, no mortality was noted throughout the 28 day test period.

Reproduction results also could not explain the different effects of the three different sampled materials as results showed no significant differences ($p > 0.05$) between the RS, TDF-U2 and TDF-U1 test groups. Cocoon production and -hatching were both very high in the horse manure control, but extremely low in the samples collected from the mining area. These results did show a substantial difference between the control and the mine samples, but since there are so little cocoons and juveniles in all the sites, it is hard to interpret the data. Because of the low cocoon production, determining reproductive success would give unreliable results and is therefore not a sensitive or reliable endpoint to be used for discussion or recommendation purposes.

RAPD-PCR detected genetic differences between the DNA of the control worms and the worms exposed to the tailings material since different numbers of polymorphic bands were detected in all the test sites, comprising 79 bands in total. In the control samples, a total of six polymorphic bands were detected in comparison to the three bands in the RS samples and two polymorphic bands at both the TDF-U1 as well as TDF-U2 samples. Differences in the range of the bands were also apparent, with sizes ranging from 245 bp to 1830 bp. The TDF samples exhibited large band sizes while bands from control samples were below 800 bp. It cannot be claimed with certainty whether the bands were due to genetic variation among the earthworm representatives or whether it was due to genotoxic effects of metals on the worms. However, due to several definitive polymorphic regions which could be observed in the OPA primer optimization procedure (Figure 2.2), but were absent from the chrome mine earthworm samples (Figure 4.14), this could be an indication of possible genotoxic effects within the individuals. It is possible that the high Co BCF in RS samples as well as TDF-U2 and TDF-U1 samples had a genotoxic effect on the DNA (De Boeck *et al.,* 2003; Figgitt *et al.,* 2010).

### *4.6 Conclusions*

From the results obtained in the study, the following conclusions could be made:

The pH levels of the RS, TDF-U2 and TDF-U1 materials were alkaline. TDF-U2 and TDF-U1 consisted mostly of sand-like particles while RS consisted mostly of clay-like particles.

Metal bioaccumulation (BCF higher than 1) was observed for the metals Co in all the materials and for Ni in the TDF-U1 material, although metal contents of worms and materials were below the benchmark levels.

RS samples had very high enzymatic activity while lower enzymatic activity was observed in TDF-U2 and TDF-U1 samples. Urease activity was highest of all the enzymes.

Earthworm biomass was highest in the control medium although it was not considered as a sensitive endpoint.

NRR-t showed the same pattern from day seven up to day 28, making it a sensitive parameter for the worms placed in material from the chrome mine.

Reproduction in terms of hatching success was not considered a sensitive endpoint because of the low cocoon production.

RAPD-PCR analysis indicated several genetic differences between the control- and the chrome mine earthworms, indicating either DNA alterations as a result of possible genotoxic effects, or possibly a genetic variation between individuals of the same species.

### *4.6 References*


ALEF, K. & NANNIPIERI, P. (eds.) 1995. Methods in Applied Soil Microbiology and Biochemistry. New York: Academic Press. 576 p.

BIGORGNE, E., COSSU-LEGUILLE, C., BONNARD, M. NAHMANI, J. 2010. Genotoxic effects of nickel, trivalent and hexavalent chromium on the *Eisenia fetida* earthworm. *Chemosphere,* 80: 1109-1112.

BOYD, F.R. & MEYER, H.O.A. 1979. The mantle sample: inclusions in kimberlites and other volcanics. *Volume 2 of Proceedings of the Second International Kimberlite Conference, Henry O.A. Meyer.* Whashington, D.C: American Geophysical Union. 154p.

BUCHELI, T.D. & FENT, K. 1996. Introduction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Review: Environmental Science and Technology*, 25: 201-268.

CHESTER, R., LIN, F.J., & MURPHY, K.J.T. 1989. *Environmental Technology Letters,* 10: 887-901.

DE BOECK, M., KIRSCH-VOLDERS, M. & LISON, D. 2003. Cobalt and antimony: genotoxicity and carcinogenicity. *Mutation Research,* 533: 135-152.

DEY, S.K., DEY, J., PATRA, S. & POTHAL, D. 2007. Changes in the antioxidative enzyme activity and lipid peroxidation in wheat seedlings exposed to cadmium and lead stress. *Brazilian Journal of Plant Physiology,* 19: 53-60.

DEPARTMENT OF AGRICULTURE, RSA. 1999. Soil Erosion. [Web:] www.nda.agric.za [Date of access: 9 May 2011].

EFROYMSON, R.A., WILL, M.E. & SUTER II, G.W. 1997. Toxicological benchmarks for contaminants of potential concern for effects on soil and litter invertebrates and heterotrophic process: 1997 revision. U.S. Department of Energy, East Tennessee Technology Park.

FALUSI, B.A. & OLANIPEKUN, E.O. 2007. Bioconcentration factors of heavy metals in tropical crab (*Carcinus sp*.) from River Aponwe, Ado-Ekiti, Nigeria. *Journal of Applied Sciences and Environmental Management,* 11(4):51-54.

FIGGITT, M., NEWSON, R., LESLIE, I.J., FISHER, J., INGHAM, E. & CASE, C.P. 2010. The genotoxicity of physiological concentrations of chromium (Cr(III) and Cr(VI)) and cobalt (Co(ii)): An *in vitro* study. *Mutation Research,* 688: 53-61.

GODGUL, G. & SAHU, K.C. 1995. Chromium contamination from chromite mine. *Environmental Geology,* 25: 251-257.

GOOGLE EARTH. 2010. GOOGLE INC. Google Earth (Version 4.2.0180.1134) [Software]. Available from http://www.google.com/earth/index.html

HANKARD, P.K., SVENDSEN, C., WRIGHT, J., WIENBERG, C., FISHWICK, S.K., SPURGEON, D.J. & WEEKS, J.M. 2004. Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis. *Science of The Total Environment,* 330(1-3): 9-20.

HERSELMAN, J.E., STEYN, C.E. & FEY, M.V. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa. *South African Journal of Science,* 101: 510.

HUANG, S., PENG, B., YANG, Z., CHAI, L. & ZHOU, L. 2009. Chromium accumulation, microorganism population and enzyme activities in soils around chromium-containing slag heap of steel alloy factory. *Transactions of Nonferrous Metals; Society of China,* 19: 241-248.

LAPINSKI, S. & ROSCISZEWSKA, M. 2008. The impact of cadmium and mercury contamination on reproduction and body mass of earthworms. *Plant, Soil and Environment*, 54(2): 61-65.

MANERIKAR, R.S., APTE, A.A. & GHOLE, V.S. 2008. *In vitro* and *in vivo* genotoxicity assessment of Cr(VI) using comet assay in earthworm coelomocytes. *Environmental Toxicology and Pharmacology,* 25: 63-68.

MBENDI INFORMATION SERVICES. 2010. A Mbendi Profile - Mining in Africa. htm. [Web:] http://www.mbendi.co.za [Date of access: 12 November. 2010].

McBRIDE, M.B. 1994. Environmental Chemistry of Soil. Oxford University Press, Oxford.

MICHELUTTI, B. & WISEMAN, M. 1995. (*In* Lal, R. and Stewart, B.A., *eds*. Environmental Restoration of the Industrial City, Springer-Verlag, Berlin, pp. 135-141).

NRIAGU, J.O., PACYNA, J.M., MILFORD, J.B. & DAVIDSON, C.I. 1988. Distribution and characteristic features of chromium in the atmosphere. (*In*: Nriagu, J.O.; Nieboer, E., editors. Chromium in the natural and human environments. New York: John Wiley: 125-172).

OWOJORI, O.J., REINECKE, A.J. & ROZANOV, A.B. 2009. Role of clay content in partitioning, uptake and toxicity of zinc in the earthworm *Eisenia fetida. Ecotoxicology and Environmental Safety,* 72(1): 99 – 107.

OWOJORI, O.J., REINECKE, A.J. & ROZANOV, A.B. 2010. Influence of clay content on bioavailability of copper in the earthworm *Eisenia fetida. Ecotoxicology and Environmental Safety,* 73(3): 407 - 414.

RATH, M., MISHRA, C.S. & MOHANTY, R.C. 2010. Microbial Population and Some Soil Enzyme Activities in Iron and Chromite Mine Spoil. *International Journal of Ecology and Environmental Sciences,* 36(2-3): 187-193.

SAEXPLORER. 2011. Average rainfall of Rustenburg, South Africa. [Web:] www.saexplorer.co.za/south-africa/climate/rustenburg_climate.asp [Date of access 23 February 2011].

SHANKER, A.K., CERVANTES, C., LOZA-TAVERA, H. & AVUDAINAYAGAM, S. 2005. Review article: Chromium Toxicity in plants. *Environment International,* 31: 739-753.

TABATABAI, M.A. 1994. Soil enzymes. (*In*: Weaver, R.W. (Ed), Methods of Soil Analysis. Part 2. SSSA Book, Ser 5. SSSA and ASA, Madison, Wl, pp.775-833).

TU, C., WANG, Y., DUAN, W., HERTL, P., TRADWAY, L., BR&ENBURG, R., LEE, D., SNELL, M. & HU, S. 2011. Effects of fungicides and insecticides on feeding behavior and community dynamics of earthworms: Implications for casting control in turfgrass systems. *Applied Soil Ecology,* 47(1): 31-36.

VAN GESTEL, C.A.M., KOOLHAAS, J.E., HAMERS, T., VAN HOPPE, M., VAN ROOVERT, M., KORSMAN, C. and REINECKE, S.A. 2009. Effects of metal pollution on earthworm communities in a contaminated floodplain area: Linking biomarker, community and functional responses. *Environmental Pollution,* 157(3): 895-903.

VAN GESTEL, C.A.M., ORTIZ, M.D., BORGMAN, E. & VERWEIJ, R.A. 2010. The bioaccumulation of Molybdenum in the earthworm, *Eisenia andrei:* influence of soil properties and ageing. *Chemosphere,* 82 (11): 1614-1619.

VEGA, F.A., COVELO, E.F. &RADE, M.L. & MARCET, P. 2004. Relationships between heavy metals content and soil properties in mine soils. *Analytica Chimica Acta,* 524: 141-150.

VERMAAK, C.F. 1986. Summary Aspects of the Economics of Chromium with special reference to Southern Africa, 1155-1181. (*In:* Anhaeusser, C.R., and Maske, S., Eds. *Mineral Deposits of Southern Africa,* I. *Geological Society of South Africa*, 1155).

WORLDMAPNOW. 2010. Maps of the world: South Africa Map. [Web:] www.worldmapnow.com/south-africa-map.html [Date of access: 23 February 2010].

# CHAPTER 5: UTILIZING EARTHWORM AND MICROBIAL ASSAYS TO ASSESS THE ENVIRONMENTAL EFFECTS OF A COAL MINE

## *5.1 Introduction*

Mining is South Africa's largest industry and in 2009 the industry employed 556 000 of the economically active population of South Africa. Of these individuals, 12% (66 720) work within the coal industry (MQA, 2009).

On an international scale, coal is the most extensively used primary fuel, and accounts for about 36% of the total fuel consumption of the world's electricity production (Department of Minerals and Energy, South Africa, 2010). South Africa's mineral industry, which is largely supported by gold, diamond, coal and platinum group metals production, has made a significant contribution to the national economy over the past century (De Wit, 2002). In terms of consumption, South Africa is dependent on coal for about 88 % of its total primary energy (Anon, 2010). While coal contributes largely to worldwide energy generation, its environmental impact cannot be ignored.

The impact it has on the environment varies in severity depending on certain factors such as whether the mine is active or not, the mining methods used, and the geological conditions (Bell *et al.*, 2001). In the process of surface coal mining for example, material has to be removed in order to gain access to the coal resource, including topsoil, overburden and waste rock. The estimated land surface area that is disturbed by coal mining is about four hectares for every million tons of coal extracted by opencast mining (Ghosh, 1990). Slope failure, erosion, potential leaching of contaminants into ground water and air pollution are some of the impacts that mining wastes may have on the environment (Bian *et al.*, 2010). Through the process of coal mining, large quantities of metal-rich industrial solid wastes have been excavated from the underground to the surface of the earth. When natural weathering occurs, it may cause the exposed coal mine spoils to break down into small particles that can be released into the environment (Haigh, 1992).

Over the course of the past four decades, an increase in the number of publications on finding a proper approach of assessing both the potential and actual impacts of metals in mining wastes has been observed (Dang *et al.*, 2002). Several metals have been associated with coal mining activities, including Zn, Pb, Fe, Cu (Dang *et al.,* 2002), Ti, Mn, As, Rb, Sr, Nb and Zr (Bhuiyan *et al*., 2010). Most of these elements can be linked to genotoxic effects in various different species.

DNA damage was observed in studies performed on insectivore bats (Zocche *et al.,* 2010), wild rodents (León *et al.*, 2007) and even humans (León-Mejía *et al.*, 2011), all living near open coal mining areas.

Acid mine drainage (AMD) is another problem associated with coal mining (World Coal Association, 2010), and is caused by the oxidation of sulphide minerals, and then the migration of these oxidation products into solution (Pinetown *et al*., 2007). Acid mine drainage is partly the result of a microbial by process, with *Bacillus ferroxidans* being the main culprit. The Loskop Dam and Olifants River Catchment (in Mpumalanga, South Africa) was recently the focus of the national media's attention regarding the severe pollution seen in the area (Manders *et al.,* 2009). This followed after crocodiles started dying off in large numbers in the area, which has been a coal mining area since the 1890's (Cameron, 2009).

Ecotoxicological research of coal include research on an abandoned coal strip-mine where fish biomarkers were used to evaluate acute to semi-chronic heavy metal-induced toxicity in channel catfish (Martin and Black, 1998). Biomarkers are the measurements of organismal or physiological changes in cells or tissue or fluids, which then serve as indicators of environmental contamination (Theodorakis *et al.,* 1992). A wide variety of soil organisms such as earthworms, mollusks and even microorganisms can also be used for ecotoxicological research (Van Straalen and Van Gestel, 1993; Van Gestel and Van Straalen, 1994; Løkke and Van Gestel, 1998; Cortet *et al.,* 1999; Oehlmann and Schulte-Oehlmann, 2003).

Based on the above mentioned information, the aim of this study was to utilize earthworm assays to assess the effects of coal mining activities on the surrounding environment. Earthworms were used because of their close relationship to soil and their ability to accumulate heavy metals from their environment into their body tissue (Lapinski and Rosciszewska, 2008), which makes them ideal for the assessment of mining material and soil. A secondary aim was to utilize microbial assays since microbial activity is an indicator of the state of the soil or mining material (Tabatabai, 1994). The specific objectives included an assessment done on material collected from a rehabilitated area (TDF-R), an unrehabilitated area (TDF-U), and then also from an undisturbed natural area close by (RS), to determine the current state of the coal mine area and also possible risks that may be present. A further objective was to assess if earthworm biomarkers could be utilized to assess metal contamination as well as its effects on organisms and the ecosystem with regards to coal mining. This is to verify the bioavailability and bioaccumulation of metals in the biota (Bucheli and Fent, 1996). The purpose for collecting and analysing material from these three sites were firstly to get an indication of the state of the mining area and secondly to compare both the TDF-U and the TDF-R material to the RS material which is representative of the natural state of the specific environment. These three sites together would then give an indication of the state of the area.

### *5.2 Site description*

Samples were collected from a coal mine in Middelburg (Mpumalanga), South Africa (Figures 5.1 and 5.2) with an average rainfall of 572 mm per year, during summer (saexplorer, 2010). Unrehabilitated (TDF-U) material was collected from an area as shown in Figure 5.3; rehabilitated material (TDF-R) was collected from another area (Figure 5.4), and material from a reference site (RS) was collected from an area (Figure 5.5) in close proximity to the TDF-R area.


**Figure 5.1.** A map of South Africa showing the Middelburg area in the Mpumalanga province (Nationsonline, 2010).


**Figure 5. 2.** The coal mining area in Middelburg from an aerial view (Google Earth, 2010).

**Figure 5. 3.** The area at the coal mine where unrehabilitated material (TDF-U) was sampled (Charné van Coller).

**Figure 5. 4.** The area at the coal mine where rehabilitated material (TDF-R) was sampled (Charné van Coller).

**Figure 5. 5.** The undisturbed part next to the coal mining area used as reference soil (RS) (Charné van Coller).

### *5.3 Materials and methods*

Refer to chapter 2.

### *5.4 Results*

#### *5.4.1 Physical and Chemical properties*

Gravel-, sand-, silt- and clay-like particle distribution of the different sites (RS, TDF-R and TDF-U) of the mining area is shown in Figure 5.6. Averages were used to construct the graph. The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

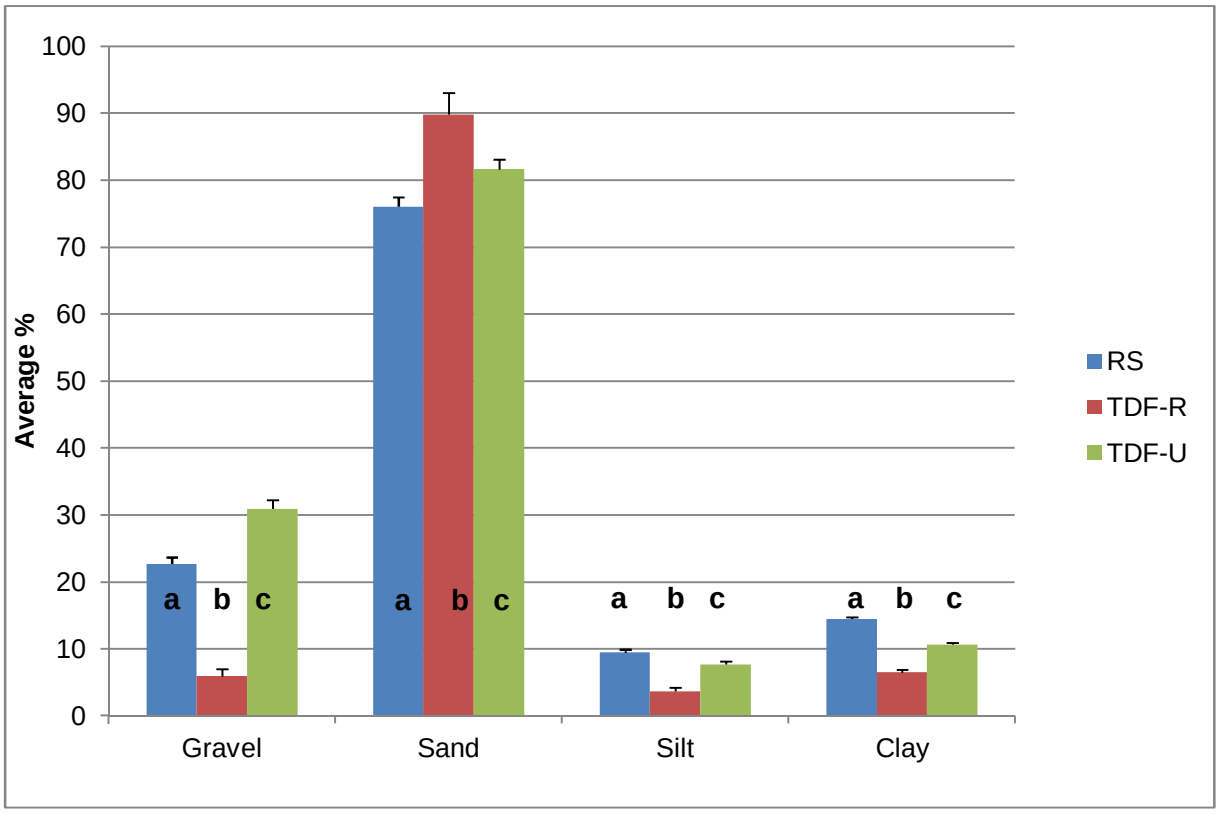


**Figure 5. 6.** Particle size distribution (± SD) of the material collected from the different areas [Reference site material (RS), unrehabilitated material (TDF-U) and rehabilitated material (TDF-R)] of a coal mining area.
a-c: Bars with the same letter were not statistically different from each other ($p > 0.05$).

The materials from all three sites (RS, TDF-R and TDF-U) consisted mostly of sand-like particles (<2mm). Reference site material also consisted of some gravel-like (>2mm) particles and very little silt- and clay-like particles. Material from the TDF-R site consisted 90% out of sand-like particles while the other 10 % was made up of gravel-like, silt-like and clay-like particles. The TDF-U material also consisted mostly of sand-like particles with some gravel-like particles and very small amounts of silt- and clay-like particles. The pH of the materials were also measured and compared in Figure 5.7.

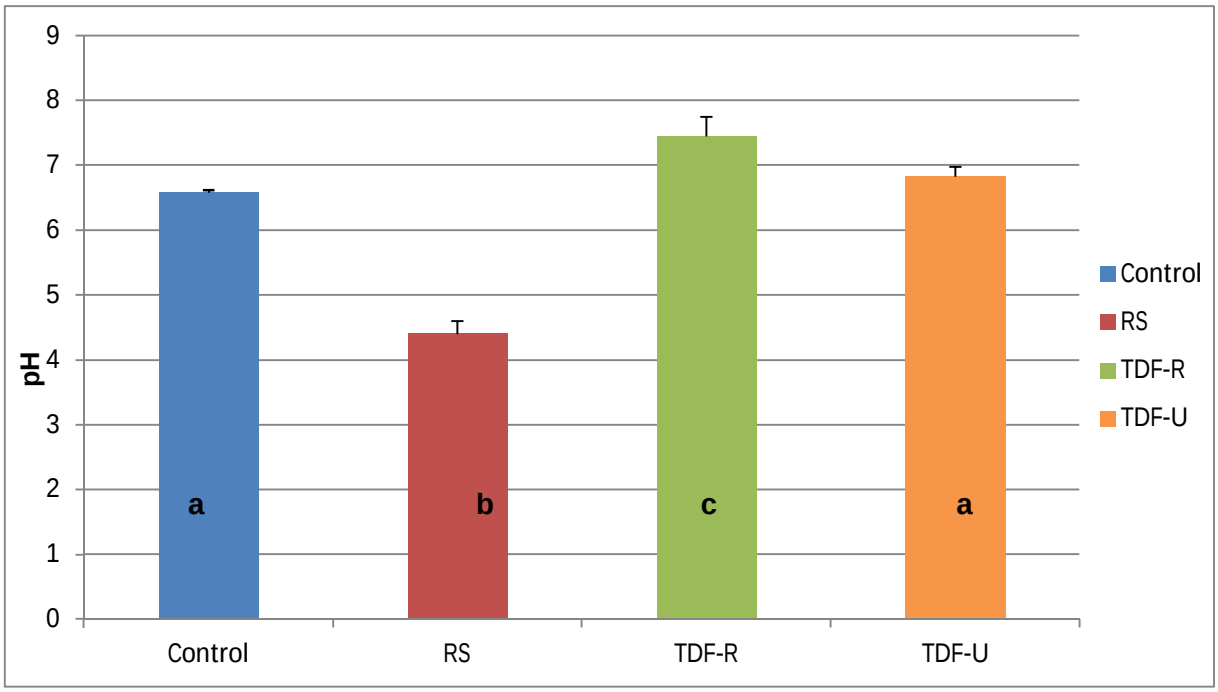


**Figure 5. 7.** pH of the control and the sampled material from the three coal mine sites [reference material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U)].

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

The pH differed significantly ($p < 0.05$) between the materials. It was acidic in the reference site, neutral to alkaline in the TDF-R material and neutral to acidic in the TDF-U material.

### *5.4.2. Metals*

Results from earthworm tissue metal analysis compared to metal analysis on the mining material and soil are shown in Table 5.1. These concentrations were measured against different benchmarks. Earthworm body concentration over total soil concentration was calculated to determine the bioconcentration factor (BCF).

**Table 5.1**. Soil metal analysis for the control, reference site material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U), measured against the following benchmarks in ppm: Total investigation level (TIL) (Herselman *et al*., 2005), total maximum threshold levels (TMT) (Herselman *et al.,* 2005), E/W (benchmarks for earthworm toxicity), SMO and MP (benchmark concentrations for toxicity to soil microorganisms and microbial processes, described by Efroymson *et al.* 1997). The bioconcentration factor (BCF) (i.e. Earthworm body concentration/total soil concentration) was also determined.

| | | ***Cr*** | ***Co*** | ***Ni*** | ***Pb*** |
|---|---|---|---|---|---|
| ***TIL*** | | ***80*** | ***-*** | ***50*** | ***56*** |
| ***TMT*** | | ***350*** | ***-*** | ***150*** | ***100*** |
| ***E/W*** | | ***0.4*** | ***-*** | ***200*** | ***500*** |
| ***SMO and MP*** | | ***10*** | ***1000*** | ***90*** | ***900*** |
| **Control** | **Material** | $0.26\pm0.03^{a}$ | $0.04\pm0.01^{a}$ | $0.22\pm0.04^{a}$ | $0.05\pm0.01^{a}$ |
| | **E/w** | $0.06\pm0.01^{A}$ | $0.16\pm0.02^{A}$ | $0.08\pm0.02^{A}$ | $0.03\pm0.02^{A}$ |
| | **BCF** | 0.23 | 4.00 | 0.36 | 0.60 |
| **RS** | **Material** | $235\pm11.46^{c}$ | $30.83\pm1.44^{c}$ | $89.92\pm0.63^{d}$ | $55.83\pm11.27^{c}$ |
| | **E/w** | $0.19\pm0.08^{B}$ | $1.39\pm0.30^{B}$ | $0.22\pm0.07^{B}$ | $0.12\pm0.04^{B}$ |
| | **BCF** | 0.00 | 0.05 | 0.00 | 0.00 |
| **TDF-R** | **Material** | $303.33\pm11.81^{d}$ | $10.56\pm0.40^{b}$ | $35.33\pm4.02^{b}$ | $21.33\pm0.80^{b}$ |
| | **E/w** | $0.15\pm0.07^{C}$ | $1.60\pm0.84^{B}$ | $0.21\pm0.05^{B}$ | $0.06\pm0.05^{A}$ |
| | **BCF** | 0.00 | 0.15 | 0.01 | 0.00 |
| **TDF-U** | **Material** | $205.83\pm6.29^{b}$ | $47.67\pm1.01^{d}$ | $85.67\pm7.84^{c}$ | $65.17\pm3.30^{d}$ |
| | **E/w** | $0.08\pm0.03^{D}$ | $1.57\pm0.42^{B}$ | $0.36\pm0.25^{B}$ | $0.06\pm0.04^{A}$ |
| | **BCF** | 0.00 | 0.03 | 0.00 | 0.00 |

a-d: Statistical comparison of the control material with the material from the sites N, R and O;

A-D: Statistical comparison of the worms from the control site with the worms of sites N, R and O.

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

All the metals were elevated in concentration. For Cr, the TIL value is 80 ppm. Chrome levels were 235 ppm in the RS material, 303 ppm in the TDF-R material and 205 ppm in the TDF-U material. The Cr concentrations were below the benchmarks for toxicity to earthworms. The lack of benchmarks for Co is problematic for the interpretation of the concentrations. Cobalt did however bioaccumulate in the control worms. For Ni, the TIL value is 50 ppm. The RS material was high above that with 89.92 ppm, and also very high in the TDF-U material with 85.67 ppm. Nickel concentrations were low in the earthworm body tissue and the metal did not bioaccumulate. For Pb, the TIL value is 56 ppm. In the RS material it was 55.83 ppm and in the TDF-U material it was 65.17 ppm. Earthworm body tissue contained very low concentrations of Pb and this metal also did not bioaccumulate.

### *5.4.3 Soil enzymatic activities*

Figure 5.8 shows the enzymatic activities of five different enzymes in the samples collected from a coal mining area. The amount of activity of each enzyme was statistically compared between the different samples.

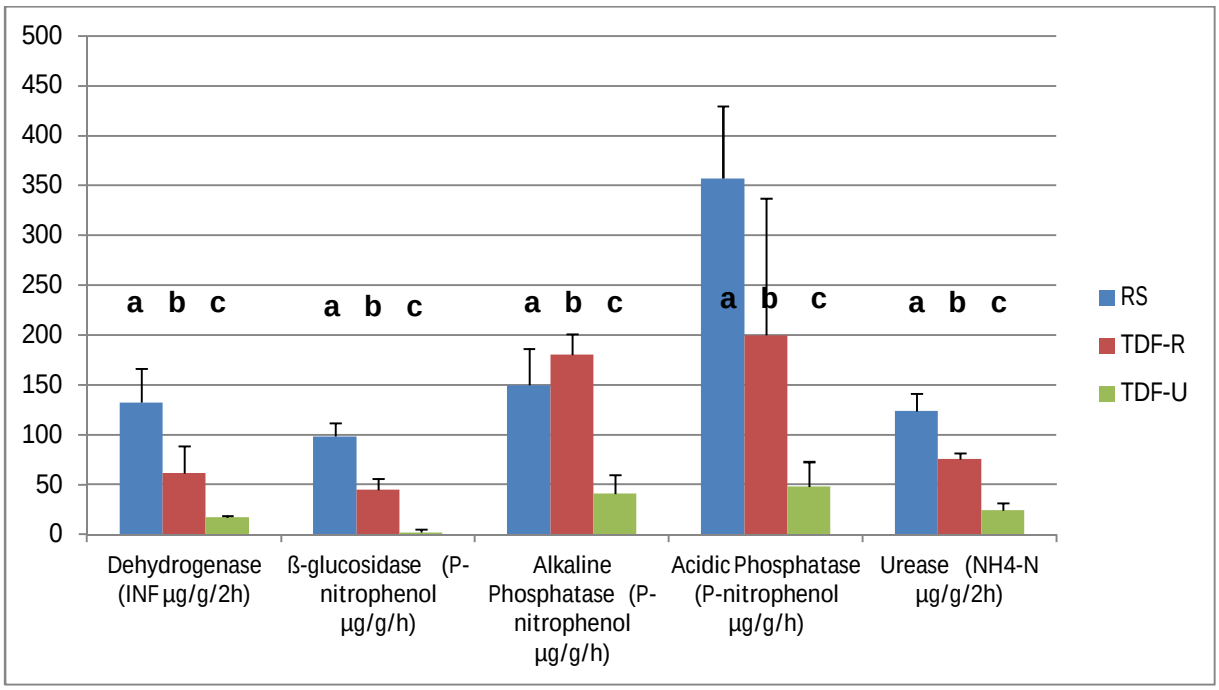


**Figure 5. 8.** Enzymatic analyses of the samples collected from the coal mining area, showing microbial activity (± SD) in the substrates and soil collected from the reference site (RS), rehabilitated site (TDF-R) and the unrehabilitated site (TDF-U).

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

The activity of all five enzymes was very high in the RS material and also high in the TDF-R material. There was a statistically significant ($p < 0.05$) difference between the TDF-R material (which had established soil fauna and flora) and the TDF-U material. Acidic phosphatase activity was the highest of all the enzymes. This enzyme is associated with plant roots (Krämer and Green, 2000), thus explaining why it would have such high activity in the RS material and the TDF-R material against the very low activity in the TDF-U material. Overall enzymatic activity showed statistically significant ($p < 0.05$) differences as RS > TDF-R > TDF-U.

### *5.4.4 Earthworm assays*

#### *5.4.4.1 Biomass*

The mean (± SD) biomass of earthworms that were exposed to the control material and the mining material are shown. The grouped data (per day) was evaluated against one another within the group, and not over the whole graph (Figure 5.9).

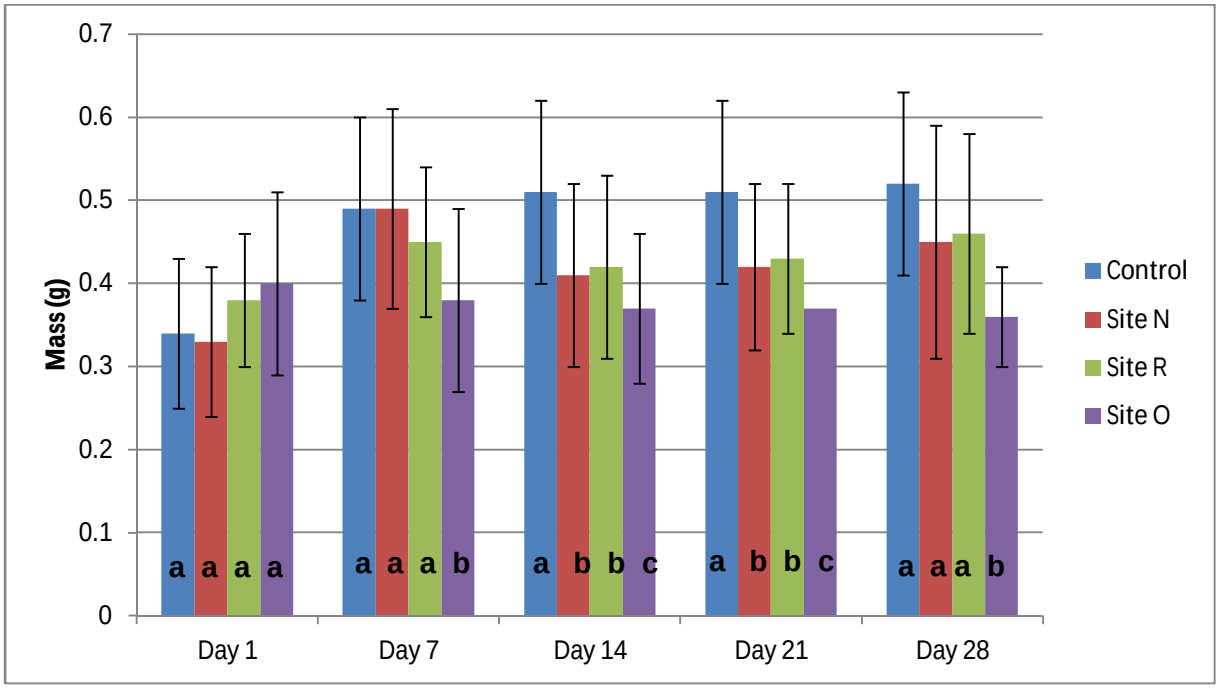


**Figure 5. 9.** Comparison of earthworm biomass (g) averages (± SD) between worms from the control material, the reference site material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U) every week over the 28 day period.

a – c: Bars with the same letter were not statistically different from each other ($p > 0.05$).

Biomass of the earthworms placed in the different samples, started differing from day 7. After 28 days of exposure, the biomass of the earthworms from the TDF-U material were significantly lower ($p < 0.05$) than that of the worms from the other materials. In these materials, biomass was a sensitive parameter of contamination effects.

### *5.4.4.2 NRR-t*

Figure 5.10 shows the results of NRR-t measured weekly on the earthworms: The grouped data (NRR-t differences per day) was statistically evaluated against one another within the group, and not over the whole graph.

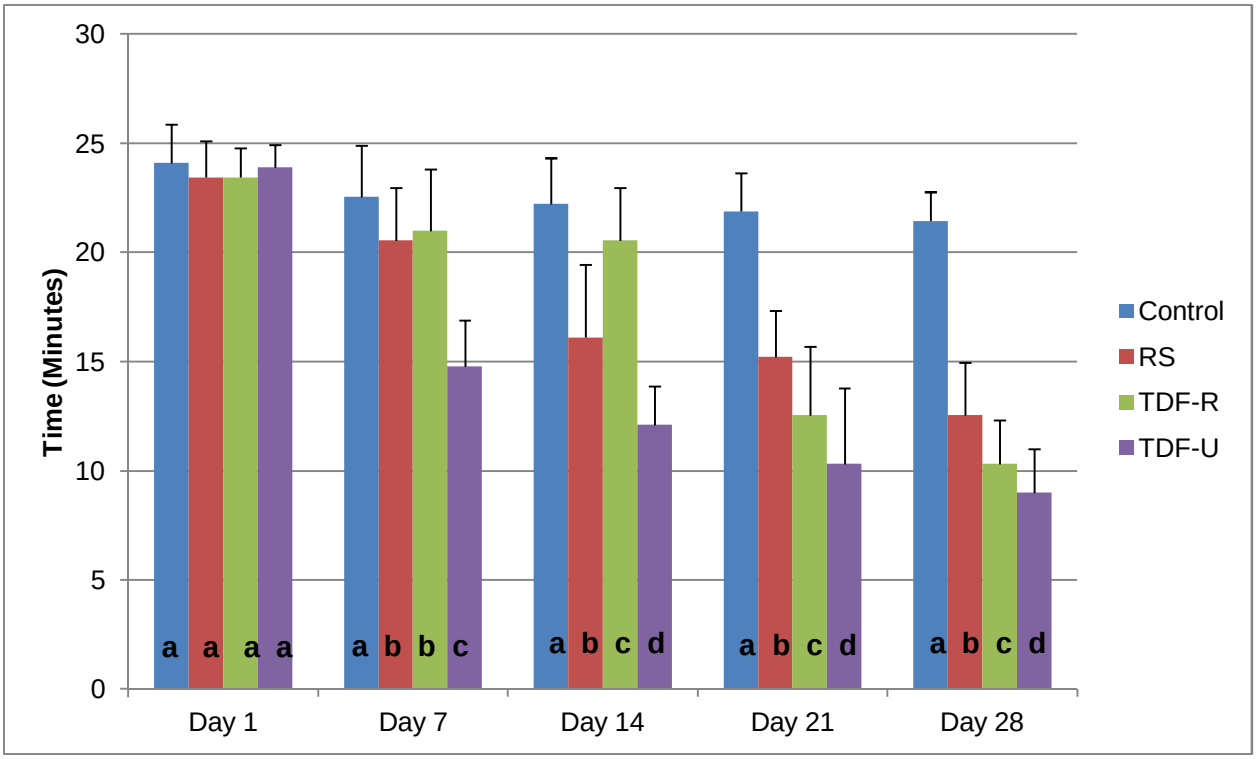


**Figure 5. 10.** A histogram comparing earthworm lysosomal membrane stability measured in neutral red retention time (± SD) between worms in the control, reference material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U) every week over the 28 day period.

a–d: Bars with the same letter were not statistically different from each other ($p > 0.05$).

Results showed a statistically significant ($p < 0.05$) difference between the control worms and those of the worms in the different sites, after seven days of exposure. It furthermore showed that the earthworms placed in the TDF-U samples experienced more cellular stress than the worms placed in the other samples. There was a statistically significant ($p < 0.05$) difference between the NRR-times of the worms in the RS material and those in the TDF-R material, as well as between the worms in the TDF-R material and those in the TDF-U material.

### *5.4.4.3 Mortality*

Mortality was noted after three weeks, with a total mortality of three worms in the rehabilitated samples and one worm in the unrehabilitated samples. This has an effect on interpretation of the earthworm biomass assay, as biomass was measured of all the worms and the average used in the data, therefore giving unreliable results.

### *5.4.4.4 Reproduction*

Figure 5.11 shows reproductive success by indicating the production of juvenile worms, hatched cocoons and unhatched cocoons. Results are shown as averages ± SD. The grouped data was statistically evaluated against one another within the group, and not over the whole graph.

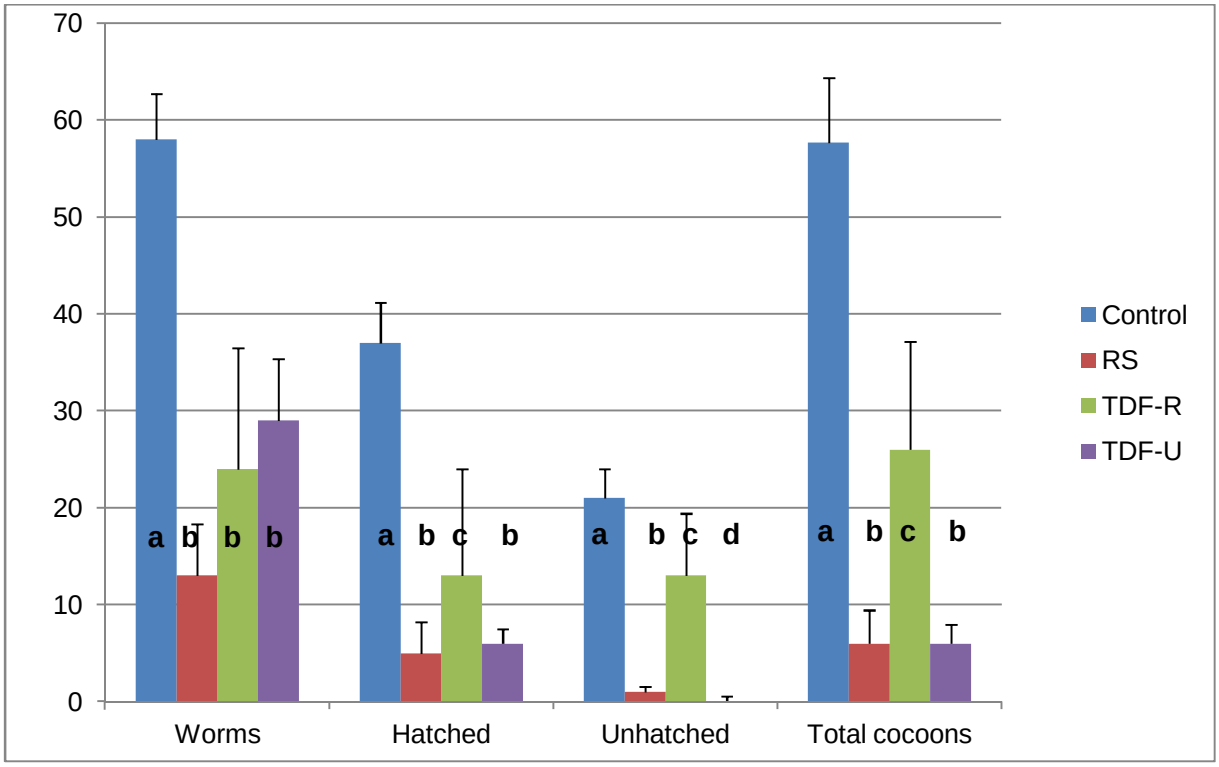


**Figure 5. 11**. Reproduction of the worms observed in the control, reference material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U) samples, by comparing juvenile worms, hatched cocoons, unhatched cocoons and total cocoons after the completion of the experiment.

a-d: Bars with the same letter were not statistically different from each other ($p > 0.05$).

From the results it is evident that the earthworms in the control material produced significantly ($p < 0.05$) more cocoons than those in the RS, TDF-R and TDF-U materials. Worms in the TDF-R produced more cocoons than those in the other materials.

### *5.4.5. RAPD PCR analysis*

Random Amplified Polymorphic DNA Analyses was done on three control worms and on 3 worms from each of the TDF-U, TDF-R and RS samples (Figure 5.12). The bands that formed were then compared between the different samples and summarized in Table 5.2.

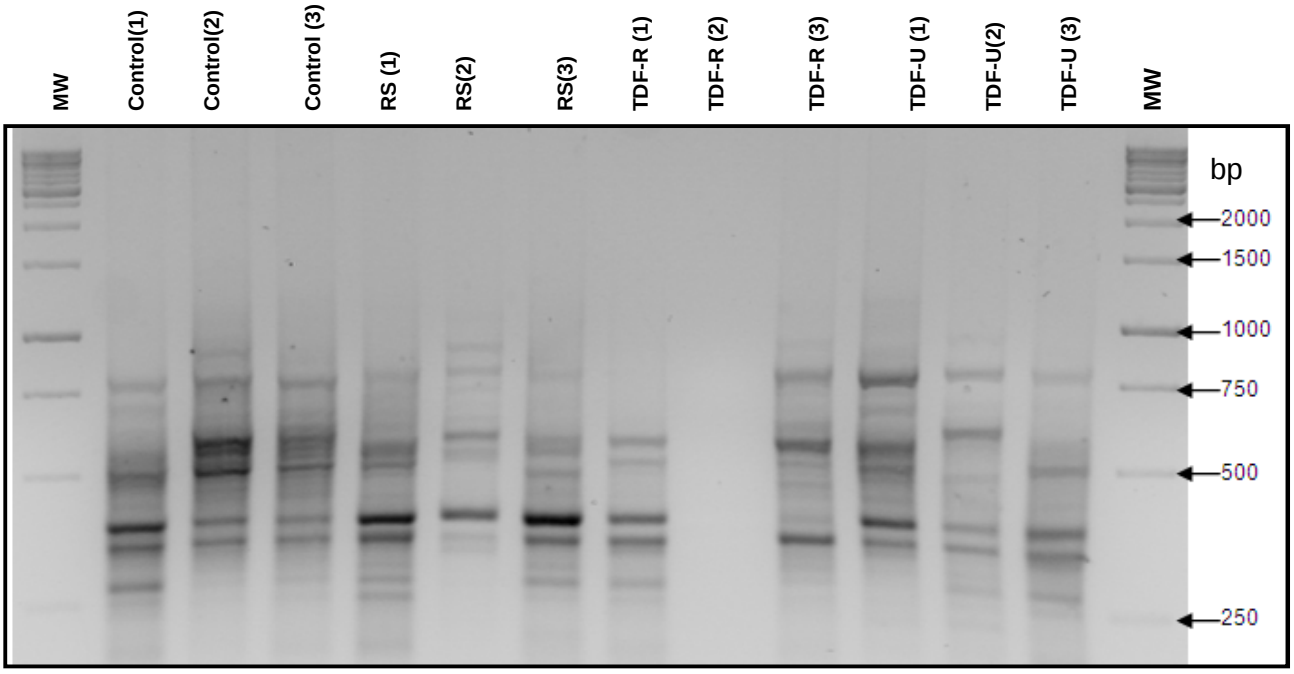


**Figure 5. 12.** RAPD-PCR gel of coal mine samples [worms exposed to the control (C), reference material (RS), rehabilitated material (TDF-R) and unrehabilitated material (TDF-U). MW indicates the use of a 1kb Molecular Weight Marker (Fermentas, USA). Numbers in parenthesis next to sample name indicate the sample number.

**Table 5.2.** A summary of RAPD analysis of coal mine related materials on average DNA banding profiles of exposed earthworms

| Primer | Sequence | % G + C | Population | Total Number of Bands | Number of Polymorphic Bands | Bands range |
|---|---|---|---|---|---|---|
| **OPA 16** | AGC CAG CGA A | 60 | **Control** | 19 | 3 | 99-681 |
| | | | **RS** | 18 | 4 | 92-707 |
| | | | **TDF-U2** | 22 | 3 | 102-570 |
| | | | **TDF-U1** | 20 | 2 | 91-583 |

Polymorphic bands were detected in the samples with a total of 79 bands. In the control and TDF-U2 samples, three polymorphic bands were detected. In the RS four polymorphic bands were detected and in the TDF-U1 samples, two polymorphic bands were detected. The band sizes ranged from 91bp to 707bp. Larger band sizes were observed in the control and RS samples than in the TDF-U samples. Sample TDF-R (2) did not undergo any amplification, as can be seen in Figure 5.12. This might have been due to various factors, including human error (i.e. pipetting error).

These results, together with a presence-absence table with peak heights, were used to compile a weighted pair-group average dendrogram, Figure 5.13. Peak heights were compared within the same molecular weight ranges (5%).

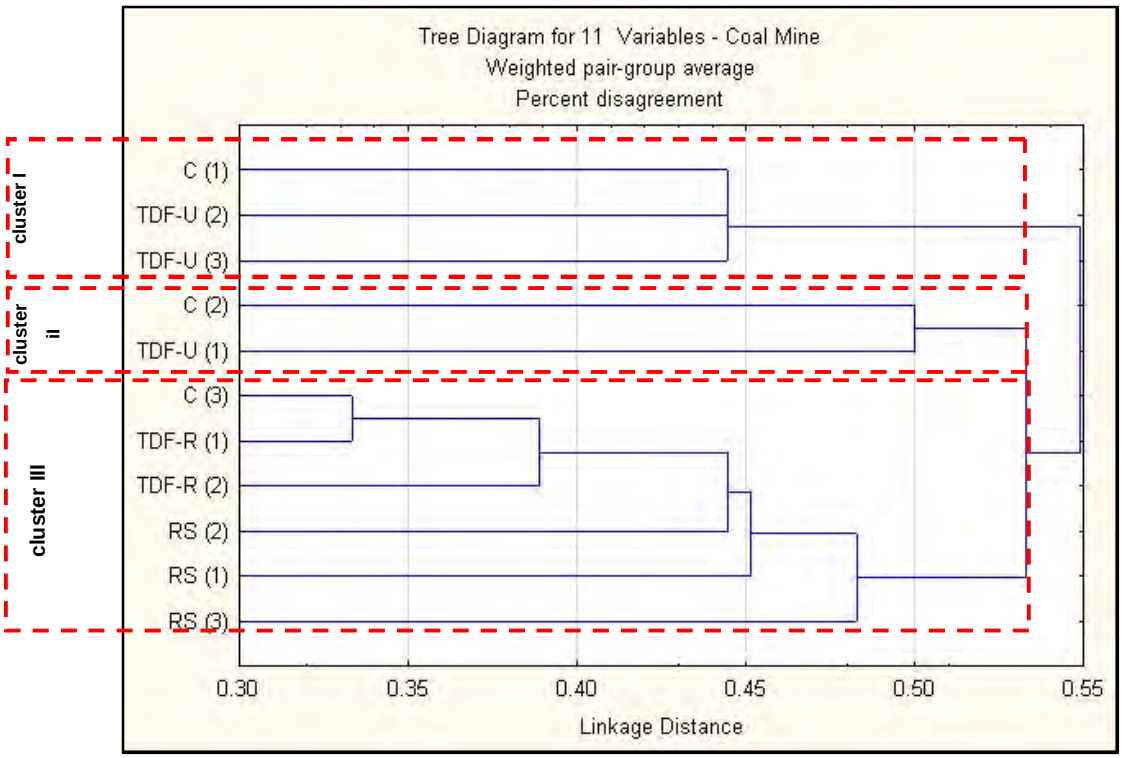


**Figure 5. 13.** Dendrogram of coal mine RAPD analysis. The dendrogram can be divided into three clusters, marked I, II and III.

The results obtained from Figure 5.13 indicated a scattered linkage between the samples, with the exception of RS samples which grouped together. Furthermore, the controls did not group together but were distributed throughout the three clusters.

### *5.5 Discussion*

During the process of open-cast coal mining, massive disturbance is caused on the ecosystems since large amounts of spoil material covering the coal layers are mined and dumped on spoil heaps (Baldrian *et al*., 2008). This affects everything from microorganisms to structure of the material. Physical and chemical analyses showed that all the material collected from the mining site consisted mostly of sand-like (< 2mm) particles. Reference soil had a pH of 4.4, while TDF-R had a pH of 7.45 and TDF-U a pH of 6.83. The pH levels have the potential to influence both metal solubility and speciation. Studies have found that metal accumulation in earthworms were in some cases influenced by pH (Van Nostrand *et al.,* 2005; Spurgeon *et al.,* 2006)

Bioavailability is the proportion of total metals that are available for incorporation into biota. Measuring the total metal concentration does not necessarily correspond with measuring the metal bioavailability within a soil. The control had a low concentration of Cr in both the material and the earthworms. The BCF was also below 1, indicating that there was no bioaccumulation of Cr. In the RS material, Cr was very high and even exceeded the South African TI (total investigation) level. The concentration is also not far under the TMT benchmark, which is the concentration at which the material would be considered to be contaminated. The BCF however, was lower than 1. Cr concentration in the TDF-R material was very high, and also between the TIL and TMT benchmarks for South African soils. No bioaccumulation took place however. In TDF-U samples, Cr concentrations were lower than in the TDF-R and RS material, although it still exceeded the TIL benchmark. Chrome concentrations in the earthworms were low and no bioaccumulation took place. The 3 sites (RS, TDF-R and TDF-U) exceeded the SMO and MP benchmarks, indicating all these materials had a highly negative influence on the microorganisms. This correlates with the enzymatic activity data indicating a statistical difference ($p < 0.05$) between the control and the TDF materials.

Cobalt concentrations in the control medium as well as in the earthworm body tissue were low. Bioaccumulation did however take place, giving a BCF of four in the control. In the RS material, Co concentration was high, with a value of 30.8 ppm being observed. It was however low in the earthworm body tissue, therefore giving a very low BCF of 0.05, indicating that no bioaccumulation took place. The TDF-R material had a Co concentration of 10.56, while earthworm body tissue had 1.6 ppm Co. on average. The BCF was below 1. Cobalt concentrations were high in the TDF-U site with an average 47.67 ppm in the material. No bioaccumulation took place though. Interpretation of Co is however very difficult due to the lack of South African benchmarks.

Nickel concentrations were very low in the control medium and earthworm body tissue, giving a low BCF, and indicating that no bioaccumulation took place. In RS material samples however, very high

concentrations of Ni were observed, both exceeding the TIL benchmark, as well as reaching the SMO and MP benchmarks of toxicity to microorganisms. No bioaccumulation took place though. Although Ni concentration was high in both the TDF-R and TDF-U materials, exceeding the TIL benchmark in TDF-U material, bioaccumulation did not occur within the organisms. The SMO and MP benchmark was almost reached within the TDF-U samples, indicating a negative impact on the microorganisms present in this medium.

Lead concentrations were very low in the control. In RS material, a concentration of 55.83 ppm was observed, almost exceeding the TIL benchmark. No bioaccumulation took place in RS samples however. TDF-U material exceeded the benchmark for TIL, and TDF-R was also on the higher side with 21.33 ppm being observed. No bioaccumulation took place in either the TDF-R or TDF-U material.

As discussed earlier, soil enzymes are mainly produced by plants and soil microorganisms, making them useful indicators of soil degradation and contamination (Tabatabai, 1994). Enzymatic activity of all five enzymes differed significantly between the three materials (RS, TDF-R and TDF-U). Dehydrogenase, β-glucosidase, acidic phosphatase and also urease indicated a statistical difference ($p < 0.05$) of RS > TDF-R > TDF-U among the sites. Activity of the enzymes was the lowest in the TDF-U material, and high in the RS and TDF-R materials. The high phosphatase activity in the RS material and in the TDF-R material can be explained by the lush flora that has been established in the areas, since acidic phosphatase is associated with plant roots (Kramër and Green, 2000).

Earthworm biomass was monitored weekly. After seven days of exposure and again after 28 days the statistical differences ($p < 0.05$) were similar. Biomass of the earthworms exposed to TDF-U material was statistically lower than that of the worms exposed to the control, RS and TDF-R materials. Biomass was a sensitive parameter in the coal mine exposed earthworms, showing early differences between the worms from the different sites (RS, TDF-R and TDF-U).

The biomass results correlated with enzymatic activity results, as TDF-U gave negative results at both endpoints. In terms of differences between the TDF-R and the TDF-U materials, there was a statistically significant difference ($p < 0.05$) between the two materials in both biomass and enzymatic activity.

NRR-t analysis gives an indication of cellular stress and is measured in time it takes for a cell destabilized by a stress response to stain (Moore, 1980). Lower NRR-times indicate that there is higher lysosomal damage (Rocco *et al.,* 2011). The NRR-t data indicated a negative effect of the TDF-U material on the earthworms, as the time it took for coelomocytes to stain red decreased from day 7 right up to day 28. There were statistically significant ($p < 0.05$) differences between NRR-times observed among the worms from the control, RS, TDF-R and TDF-U materials.

Reproduction of the earthworms placed in the coal mine material showed significant ($p < 0.05$) differences between the different materials. The amount of juvenile worms was actually higher in the TDF materials than in the RS material. Total cocoons produced were highest in the control and in the TDF-R material. For this mine, reproduction was a sensitive endpoint as it showed noticeable differences between the materials.

Mortality was observed in the TDF-R (3 individuals of a total of 30) and in the TDF-U material (1 individual out of 30). This gives an indication of the negative effect of the material on soil fauna.

RAPD profiles for coal mine samples were distributed throughout the three clusters (fig 5.13). A possible reason for this might be that little genetic variation existed between the sites. This is supported by a similar number of polymorphic bands that showed little variation between the samples (Fig 5.12, Table 5.2).

### *5.6 Conclusions*

From the results obtained, the following conclusions were made:
RS material had a pH of 4.4, while TDF-R and TDF-U had neutral pH values (7.45 and 6.83 respectively).

All three sites consisted mostly of sand-like particles.

RS, TDF-R and TDF-U exceeded the Cr benchmark for toxicity to microorganisms, while RS and TDF-U exceeded TIL benchmark for Ni. RS also reached the SMO and MP benchmarks for Ni. TDF-U exceeded the TIL benchmark for Pb.

Enzymatic activity was high in both the RS and TDF-R materials, and low in TDF-U material.

Biomass can be correlated with enzymes, which in turn can be correlated with the NRR-t. Thus both biomass and NRR-t were sensitive endpoints. In terms of reproduction, hatching success was however not a reliable endpoint, due to the low cocoon production.

Mortality was observed in the TDF-R (3 individuals of a total of 30) and in the TDF-U material (1 individual out of 30).

In terms of RAPD PCR results, little variation is observed in number of polymorphic bands between the samples, possibly explaining why samples clustered together.

### *5.6 References*


ANON. 2010. Mining Weekly: a brief look at South Africa's coal-mining industry. [Web:] www.miningweekly.com [Date of access: 3 September 2010.]

BALDRIAN, P., TRÖGL, J., FROUZ, J., ŠNAJDR, J., VALÁŠKOVÁ, V., MERHAUTOVÁ, V., CAJTHAML, T. & HERINKOVÁ, J. 2008. *Soil Biology and Biochemistry*, 40(9): 2107-2115. *Special section: Enzymes in the Environment.*

BELL, F.G., BULLOCK, S.E.T., HALBICH, T.F.J. & LINDSAY, P. 2001 Environmental impacts associated with an abandoned mine in the Witbank Coalfield, South Africa. *International Journal of Coal Geology,* 45: 195-216.

BHUIYAN, M.A.H., PARVEZ, L., ISLAM, M.A., DAMPARE, S.B. & SUZUKI, S. 2010. Heavy metal pollution of coal mine-affected agricultural soils in the northern part of Bangladesh. *Journal of Hazardous Materials,* 173: 384-392.

BIAN, Z., INYANG, H., DANIELS, J.L., OTTO, F. & STRUTHERS, S. 2010. Environmental issues from coal mining and their solutions. *Mining Science and Technology,* 20: 215-223.

BUCHELI, T.D. & FENT, K. 1996. Introduction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Review: Environmental Science and Technology*, 25: 201-268.

CAMERON, L. 2009. Olifants river still a hazard to wildlife. *Mining Weekly*: March 20. [Web:] http://www.MiningWeekly.com [Date of access: 3 March 2011].

CORTET, J., GOMET-DE VAUFLERY, A., POINSOT-BALAGUER, N., GOMOT, L., TEXIER, C. & CLUZEAU, D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects: utilisation de la faune invertebree du sol comme bio-indicateur des effets des pollutants. *European Journal of Soil Biology,* 35: 115-134.

DANG, Z., LIU, C., & HAIGH, M.J. 2002. Mobility of heavy metals associated with the natural weathering of coal mine spoils. *Environmental Pollution,* 118: 419-426.

DEPARTMENT OF MINERALS AND ENERGY, RSA. 2010. Coal mining. [Web:] www.dme.gov.za [Date of access: 2 November 2010].

DE WIT, M.P. 2002. The Environmental Impacts of Foreign Direct Investment (FDI) in the mining sector, the case of South Africa. (*In:* CCNM Global Forum on International Investment, Conference on Foreign Direct Investment and the Environment: Draft prepared for OECD conference on FDI and the environment, lessons from the mining sector. Held in Paris on 7 and 8 February 2002, Paris). [Web:] www.oecd.org/dataoecd/37/43/1833897.pdf [Date of access: 16 November 2011].

EFROYMSON, R.A., WILL, M.E. & SUTER II, G.W. 1997. Toxicological benchmarks for contaminants of potential concern for effects on soil and litter invertebrates and heterotrophic process: 1997 revision. U.S. Department of Energy, East Tennessee Technology Park.

GHOSH, A.K. 1990. Mining in 2000 AD – Challenges for India. *Journal of the Institution of Engineers* (India), 39(2): 1-11.

GOOGLE EARTH. 2010. GOOGLE INC. Google Earth (Version 4.2.0180.1134) [Software]. Available from http://www.google.com/earth/index.html

HAIGH, M.J. 1992. Problems in the reclamation of coal – mine disturbed lands in Wales. *International Journal of Surface Mining and Reclamation,* 6: 31-37.

HERSELMAN, J.E., STEYN, C.E. & FEY, M.V. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa. *South African Journal of Science,* 101: 510.

KRÄMER, S. & GREEN, D.M. 2000. Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in semiarid woodland. *Soil Biology and Biochemistry,* 32: 179-188.

LAPINSKI, S. & ROSCISZEWSKA. 2008. The impact of cadmium and mercury contamination on reproduction and body mass of earthworms. *Plant, Soil and Environment*, 54(2): 61-65.

LEÓN, G., ESPITIA- PÉREZ, L., LINARES, J.C., HARTMANN, A. & QUINTANA, M. 2007. Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. *Mutation Research,* 630: 42-49.

LEÓN-MEJÍA, G., ESPITIA- PÉREZ, L., HOYOS-GIRALDO, L.S., DA SILVA, J., HARTMANN, A., HENRIQUES, J.A.P. & QUINTANA, M. 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. *Science of the Total Environment,* 409: 686-691.

LØKKE, H. & VAN GESTEL, C.A.M. 1998. Handbook of soil invertebrate toxicity tests. Chichester: John Wiley and Sons.

MANDERS, P., GODFREY, L., & HOBBS, P. 2009. CSIR: Acid Mine Drainage in South Africa. [Web:] http://www.csir.co.za [Date of access: 3 March 2011].

MARTIN, L.K. Jr., & BLACK, M.C. 1998. Biomarker Assessment of the Effects of Coal Strip-Mine Contamination on Channel Catfish. *Ecotoxicology and Environmental Safety,* 41: 307-320.

MOORE, M.N. 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. *Rapports et Proces – Verbaux des Réunions. Conseil International pour l'Exploration de la Mer*, 170: 7-15.

MQA. MINING QUALIFICATIONS AUTHORITY. 2009. Sector skills plan for the mining and minerals sector: 2005-2010. [Web:] http://www.mqa.org.za/siteimgs/SSP%20Update%202009%20Final%20Signed%20-%20%20Submitted%2031%20Augsut%202009.pdf [Date of access: 15 November 2011].

NATIONSONLINE. 2010. Map of South Africa's provinces. [Web:] http://www.nationsonline.org/oneworld/map/za_provinces_map2.htm [Date of access: March 2, 2011].

OEHLMANN, J. & SCHULTE-OEHLMANN, U. 2003. Bioindicators and Biomonitors – Principles, Concepts and Applications. *Trace Metals and other Contaminants in the Environment,* 6: 577-635.

PINETOWN, K.L., WARD, C.R., & VAN DER WESTHUIZEN, W.A. 2007. Quantitative evaluation of minerals in coal deposits in the Witbank and Highveld coalfields, and the potential impact on acid mine drainage. *International Journal of Coal Geology*, 70: 166-183.

ROCCO, A., SCOTT-FORDSMAND, J.J., MAISTO, G., MANZO, S., SALLUZZO, A. & JENSEN, J. 2011. Suitability of lysosomal membrane stability in *Eisenia fetida* as biomarker of soil copper contamination. *Ecotoxicology and Environmental Safety,* 74: 984-988.

SAEXPLORER. 2008. Average rainfall of Middelburg (ML), South Africa. [Web:] http://www.saexplorer.co.za/south-africa/climate/middelburg_(ml)_climate.asp [Date of access: 15 December 2010].

SPURGEON, D.J., STEPHEN, L., HANKARD, P.K., TOAL, M., MCLELLEN, D., FISHWICK, S. & SVENDSEN, C. 2006. Effect of pH on metal speciation and resulting metal uptake and toxicity for earthworms. *Environmental Toxicology and Chemistry.* 25: 788-796

TABATABAI, M.A. 1994. Soil enzymes. (*In*: Weaver, R.W. (Ed), Methods of Soil Analysis. Part 2. SSSA Book, Ser 5. SSSA and ASA, Madison, WI, pp.775-833).

THEODORAKIS, C.W., D'SURNEY, S.J., BICKHAM, J.W., LYNE, T.B., BRADLEY, B.P., HAWKINS, W.E., FARKAS, W.L., MCCARTHY, J.F., & SHUGART, L.R. 1992. Sequential expression of biomarkers in bluegill sunfish exposed to contaminated sediment. *Ecotoxicology,* 1: 45-73.

VAN GESTEL, C.A.M. & VAN STRAALEN, N.M. Ecotoxicological test systems for terrestrial invertebrates. (*In*: Donker, M.H., Eijsackers, H., Heimbach, F., editors. Ecotoxicology of soil organisms. Boca Ranton: CRC Press, Inc.; 1994. p. 205-28).

VAN NOSTRAND, J.D., SAWDER, A.G., BERTSCH, P.M. & MORRIS, P.J. 2005. Effect of pH on the toxicity of Nickel and other divalent metals to *Burkholderia cepacia*. *Environmental Toxicology and Chemistry,* 24(11): 2742-2750.

VAN STRAALEN, N.M. & VAN GESTEL, C.A.M. 1993. Soil Invertebrates and micro-organisms. (*In*: Calow, P, editor. Handbook of ecotoxicology I. Blackwell Scientific Publications. p.229-325).

WORLD COAL ASSOCIATION. 2010. Coal mining and the environment. [Web:] www.worldcoal.org [Date of access: 2 December 2010].

ZOCCHE, J.J., LEFA, D.D, DAMIANI, A.P., CARVALHO, F., MENDONÇA, R.Á., DOS SANTOS, C.E.I., BOUFLEUR, L.A., DIAS, J.F. & DE ANDRADE, V.M. 2010. Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. *Environmental Research,* 110: 684-691.

## CHAPTER 6: GENERAL DISCUSSION AND CONCLUDING REMARKS

Unlike soil, post mining material is composed of only one sized particles. Our samples, if looked at as a whole, were mostly composed of sand-like particles. This is mainly due to the mining activity in the surrounding areas. The finer texture of the samples makes them excessively permeable and susceptible to wind which may lead to dust pollution. The pH of material has an effect on bioavailability of metals and may even have a negative effect on earthworm reproduction which is why reproduction was higher in e.g. the neutral reference soil than the acidic tailings material.

Four of the seven (Cd, Cr, Ni, Pb, Zn, Cu, Co) environmentally important trace elements were chosen for this study. Arsenic was also added in chapter 3 because it is associated with gold mine tailings. In the gold mine tailings material Co and Ni were present at high concentrations in the material, as well as in the earthworms, while Pb had a high bioconcentration factor. In the chrome mine material, metal bioaccumulation (BCF higher than 1) was observed for Co in all the materials. Nickel also bioaccumulated in the TDF-U1 material. Metal contents of worms and materials were below the benchmark levels. In the coal mine, material from the RS, TDF-R and TDF-U exceeded the Cr benchmark for toxicity to microorganisms, while RS and TDF-U exceeded the TIL benchmark for Ni. RS also reached the SMO and MP benchmarks for Ni while TDF-U exceeded the TIL benchmark for Pb. The fact that soils contaminated with mine waste often contain complex mixtures of metals that might be toxic on their own or in combination with other factors, makes it difficult to attribute any observed genotoxic effect to the specific metal. For future studies I recommend analysing all of the environmentally important metals mentioned above, to draw better conclusions regarding the metals present in the materials. Since the metals were combined in a mixture in the material, a possibility for future studies would also be to measure the metal concentrations in the materials, then take control material and spike it with that concentration of metal and run the same experiment. By doing that, factors such as pH and particle size of the material as well as other metals will not have an influence on the results. In this way, clear results of the effect of each factor can be obtained.

Measuring enzymatic activity, gives an indication of microbial activity. Different enzymes are associated with different factors e.g., dehydrogenase indicating viable organisms, phosphatase and urease being associated with bacteria or fungi, β-glucosidase commonly found in animals, plants and microorganisms. In comparing different materials, enzymatic activity is a very sensitive parameter since it gives an indication of the quality of the material. With the comparison of materials from all three mines with each other, the reference materials had the highest enzymatic activity every time. In the material collected from areas where rehabilitation or revegetation took place, enzymatic activity was also higher. Specifically acidic phosphatase activity, which is associated with plant roots, was high in these flora covered materials. In the unrehabilitated samples from all the mines, enzymatic activity was the lowest

of all the samples. For future studies I would recommend again measuring enzymatic activity after the worms are removed from the material at the end of the experiment, in order to also get an indication of the effect of the worms on the material.

Earthworm biomass was monitored each week and differences were observed between the earthworms from each site. The earthworms from the control medium experienced an increase in biomass over the course of the experiment. The worms placed in the reference materials showed better results in terms of biomass than the worms placed in the TDF-U materials. This occurred even though all the worms were given the same amount of manure and distilled water every week. There may be a few reasons for the low biomass increases (or no increases) in the unrehabilitated material. Metal accumulation may have had an effect. The biomass data correlates with the enzymatic activity data, indicating the difference between reference material and unrehabilitated material. Although biomass was not a sensitive parameter for the worms exposed to the gold mine tailings dam from chapter 3, or the worms exposed to the chrome mine from chapter 4, it was a sensitive parameter in the coal mine exposed earthworms, showing early differences between the worms from the different sites (RS, TDF-R and TDF-U).

Neutral red retention time is a very sensitive endpoint for ecotoxicological studies as was observed in this study. The assay indicates cellular stress and is measured in time it takes for a cell to stain as a result of destabilization by a stress response. These times differed significantly between the different sites and correlated with data from the previous discussed results indicating that TDF-U materials had the most negative effect on the earthworms and therefore on the environment. Different stressors may have had an influence, such as metal pollution or other forms of pollution. The pH may also have been involved in causing a stress response in the acidic materials. In material from all three mines, differences in NRR-times could be observed from as early as seven days of exposure to the material. The control worms stayed at the same time to stain over the 28 days of the experiment and did not show a significant decrease in time for coelomocytes to stain. The RS material worms did however experience a decrease in time of coelomocytes to stain from day seven up to day 28. The worms that were mostly affected were the ones placed in the unrehabilitated materials, showing significant decreases in time for coelomocytes to stain every week.

Reproduction results indicated a statistically significant ($p < 0.05$) difference between the number of juvenile worms found in the control and the sampled material after the 56 days. The total cocoon production also showed statistically significant ($p < 0.05$) differences between the different materials, with the most cocoons produced in the control. For these reasons, total cocoon production and total juveniles were sensitive endpoints in all three mines. Reproductive success however is not as reliable, especially if so little cocoons are hatched. For this reason reproductive success was not a sensitive endpoint for the purpose of making conclusions or recommendations. In future studies I recommend placing each

cocoon in a plate on its own (still containing material and manure) over the 56 days in order to determine the number of juveniles emerging from each cocoon and to avoid missing any cocoons or juveniles in a big jar.

RAPD-PCR results indicated genetic differences between the DNA of the control worms and the worms exposed to the tailings material, observed as differences in polymorphic bands. Differences in the range of the bands were also visible, with band sizes differing between the different samples. This might be explained by either genetic variation among the earthworm representatives, or by genotoxic effects of metals on the worms. For future studies these bands could be cut out and sequenced to obtain more detailed conclusions. RAPD-PCR analysis could also be done again, but with more primers and a larger group of individuals. This will result in a statistically more sound method of applying RAPD technology to the field of ecotoxicology. The problem with individuals of the same species not having exactly the same DNA sequence can be overcome by using a non-lethal method of obtaining earthworm DNA, both before and after treatments.