THE ABILITY OF TERRESTRIAL OLIGOCHAETA TO SURVIVE IN ULTRAMAFIC SOILS AND THE ASSESSMENT OF TOXICITY AT DIFFERENT LEVELS OF ORGANISATION

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

Metals are natural elements of the earth crust usually present at low concentrations in all soils. Although many metals such as cobalt, copper, iron and zinc are essential to living organisms, at elevated concentrations most metals are toxic to organisms living in and on soils. Elevated concentrations of metals are caused either by anthropogenic deposition following remobilisation from the earth crust or are of natural origin.

Ultramafic soils do not only pose unfavourable living conditions such as drought and poor organic content, these soils are also characterized by extremely high concentrations of a range of metals known to be toxic under normal circumstances. Ultramafic soils are of high ecological importance as a high proportion of endemic organisms, especially plants, live on these soils.

As it is known that earthworms do occur in ultramafic soils, the aims of the present study were to investigate the abilities of earthworms to survive in these soils and the influences of elevated chromium, cobalt, copper, manganese and nickel levels.

For the evaluation of the metal background conditions, soils originating from ultramafic rocks of the Barberton Greenstone Belt, Mpumalanga, South Africa were collected and different fractions representing different levels of bioavailability were analyzed for arsenic, chromium, cobalt, copper, manganese and nickel. To assess the mobile, readily available metal fraction, i.e. Ca^{2+}-exchangeable metal cations, a 0.01 mol/L $CaCl_2$ extraction was performed. To investigate the mobilisable metal fraction, representing the amount of easily remobilisable complexed and carbonated metal ions, a DTPA (di-ethylene-triamine-pentaacetic acid) extraction was conducted. In relation to non-ultramafic or anthropogenic contaminated soils, a far lower proportion of metals were extractable by the above mentioned extraction methods.

To investigate the availability and effects of these metals on earthworms, two ecophysiologically different species were employed. *Aporrectodea caliginosa* and *Eisenia fetida* were long-term exposed to the ultramafic soils collected at the Barberton region and a control soil from a location at Stellenbosch with a known history of no anthropogenic metal contamination. The responses to the ecological stress originating in the ultramafic soils were measured on different levels of earthworm organisation. As endpoints affecting population development, cocoon production, fecundity and viability were evaluated. On individual level, growth, metal body burden and tissue distribution were investigated. As endpoints on subcellular level, the membrane integrity was assessed by the neutral red retention assay, the mitochondrial activity was measured by the MTT colorimetric assay and as a biomarker for the DNA integrity, the comet assay was performed. Focussing on manganese and nickel, the uptake by *E. fetida* of these metals was investigated with the exclusion of soil related properties using an artificial aqueous medium to draw comparisons to the uptake of these metals in natural soils.

The possible development of resistance towards nickel was tested by exposing pre-exposed (for more than 10 generations) *E. fetida* specimens to ultramafic soils with concentrations of more than 4000 mg/kg nickel.

Abstract

The results showed that, except on the endpoint survival, which was less sensitive than all other bioassays, significant responses to the ultramafic challenge were observed in all earthworm bioassays and on all levels of organisation. The sensitivity of the responses of the earthworms towards the ultramafic conditions was not predictable by the level of organisation.

The two species showed different strategies of metal elimination. In *A. caliginosa*, metals such as nickel, manganese and chromium were transported to the posterior section and the posterior section was subsequently pushed off by autotomization. In *E. fetida*, metals such as chromium and nickel were sequestered in storage compartments in the coelomic cells or fluid. Other metals, such as cobalt, were not taken up at elevated concentrations.

Although an increased accumulation of nickel was observed in *E. fetida* specimens pre-exposed to nickel, development of resistance or cross resistance was not observed in this species. In contrast, pre-exposed specimen exposed to elevated concentrations of nickel showed a higher sensitivity in terms of survival, indicating the absence of acclimatisation or even genetic adaptation.

A comparison of the two species employed indicated that *A. caliginosa* was less suited for the assessment of the ultramafic soils due to the high individual variation in metal body burden, the mass loss observed and the slow reproduction rate even in the control soils. This happened despite the fact that *A. caliginosa* was a soil dwelling species supposed to be better adapted to the soil substrate than the litter dwelling *E. fetida*.

The toxicity of the ultramafic soils was not necessarily related to total or environmentally available amounts of the selected metals. Thus, it can be speculated that either these soils contained unidentified toxicants with resulting interactions between toxicants playing an important role or earthworms were able to remobilize metals occurring in these soils.

As the singular application of an ecotoxicological endpoint did not give reliable results, especially seen over the duration of the exposures, it can be concluded that, when studying soils with such a complex composition, the utilisation of endpoints addressing different levels of organisation is necessary for the assessment of toxic stress emerging from these ultramafic soils.

Opsomming

Swaarmetale is natuurlike elemente wat in die aardkors voorkom in lae konsentrasies in alle gronde. Alhoewel baie metale soos kobalt, koper, yster en sink essensieel is vir lewende organisms, is meeste swaarmetale toksies in hoë konsentrasies vir organismes wat op of in grond leef. Verhoogde konsentrasies word versoorsaak deur antropogeniese deponering wat volg op hermobilisering van die aardkors of kan van natuurlike oorsprong wees.

Ultramafiese gronde skep nie slegs ongunstige leeftoestande soos droogte en arm organiese inhoud nie, hulle word ook gekenmerk deur uiters hoë konsentrasies van ‘n reeks metale wat daarvoor bekend is dat hulle toksies is onder normale toestande. Ultramafiese gronde is van besondere ekologiese belang omdat ‘n groot gedeelte van die endemiese organismes, veral plante, in die gronde leef.

Aangesien dit bekend is dat erdwurms in ultramafiese gronde voorkom, was die doelwit van die huidige studie om die vermoë van erdwurms om in hierdie gronde te oorleef, te ondersoek asook die uitwerkings van verhoogde konsentrasies van chroom, kobalt, koper, mangaan en nickel.

Ten einde die swaarmetaal agtergrondtoestande te evalueer, is grond vanaf die ultramafiese rotse van die Barberton Greenstone Belt, Mpumalanga, Suid-Afrika versamel en verskillende fraksies wat verskillende vlakke van biobeskikbaarheid verteenwoordig is geanaliseer vir arseen, chroom, kobalt, koper, mangaan en nikkel. Om die mobiele, geredelik beskikbare fraksie te bepaal, d.i. Ca^{2+} uitruilbare metaal katione, is ‘n 0.01 mol/L $CaCl_{2}$- ekstraksie uitgevoer. Om die mobiliseerbare grondfraksie wat die hoeveelheid maklik hermobiliseerbare gekomplekseerde en gekarboneerde metaal ione te bepaal, is ‘n DTPA- (di-etileen-triamien-penta-asysnsuur) ekstraksie uitgevoer. Wat nie-ultramafiese of antropogenies gekontamineerde gronde betref, was ‘n baie kleiner gedeelte van die metale ekstraheerbaar met die bogenoemde ekstraksiemetodes.

Om die beskikbaarheid en effekte van hierdie metale op erdwurms te ondersoek, is twee ekofisiologies verskillende spesies gebruik nl *Aporrectodea caliginosa* en *Eisenia fetida.* Hulle is langdurig blootgestel aan ultramafiese grond wat in die Barbertongebied versamel is en ook aan ‘n kontrolegrond vanaf Stellenbosch wat geen geskiedenis gehad het van antropogeniese metaalkontaminasie nie. Die response op die ekologiese stress wat deur die ultramafiese gronde versoorsaak is, is gemeet op verskillende vlakke van organisasie van die erdwurms. As eindpunte wat bevolkingsontwikkeling be-invloed, is kokonproduksie, fekunditeit en uitbroeisukses ge-evalueer. Op die individuele vlak is groei, metaal liggaamslas en weefselverspreiding vam metale ondersoek. As eindpunte op subsellulêre vlak, is membraanintegriteit met die neutraalrooi retensie tegniek ondersoek en mitochondriale aktiwiteit is met die MTT kolorimetriese tegniek ondersoek. As biomerker van DNA integriteit is die komeet-evaluering uitgevoer. Met die fokus op mangaan en nikkel, is die opname van hierdie metale by *E. fetida* ondersoek onder toestande waar grondverwante faktore uitgesluit is deurdat ‘n kunsmatige waterige medium gebruik is om vergelykings te tref met die opname van die metale in natuurlike gronde.

Opsomming

Die moontlike ontwikkeling van bestandheid teen nikkel is getoets deur vooraf blootgestelde (vir meer as 10 generasies) eksemplare van *E. fetida* aan ultramafiese grond met nikkelkonsentrasies van meer as 400 mg/kg bloot te stel.

Die resultate het, behalwe vir die eindpunt oorlewing wat minder sensitief was as alle ander evaluerings, betekenisvolle response op die ultramafiese uitdaging getoon in alle erdwurm bio-evaluerings en op alle vlakke van organisasie. Die sensitiwiteit van die erdwurms se response teenoor ultramafiese gronde was nie voorspelbaar deur die vlak van organisasie nie.

Die twee spesies het verskillende strategieë van metaal eliminering vertoon. By *A. caliginosa* is metale soos nikkel, mangaan en chroom na die stertgedeelte van die liggaam vervoer en daaropvolgend afgesnoer deur middel van outotomering. By *E. fetida* is metale soos chroom en nikkel gesekwestreer in stoorkompartemente in die seloomselle en seloomvloeistof. Ander metale, soos kobalt, is nie opgeneem in verhoogde konsentrasies nie.

Hoewel ‘n verhoogde konsentrasie van nikkel waargeneem is in *E. fetida* eksempalre wat vooraf blootgestel was aan nikkel, is die ontwikkeling van bestandheid of kruisbestandheid nie by die spesie waargeneem nie. In teenstelling, vooraf blootgestelde eksemplare wat aan verhoogde konsentrasise van nikkel blootgestel is het ‘n hoër sensitiwiteit in terme van oorlewing getoon, wat ‘n aanduiding is van die afwesigheid van akklimasie of selfs genetiese aanpassing.

‘n Vergelyking van die twee spesies wat gebruik is, toon dat *A. caliginosa* minder geskik is vir die assessering van die ultamafiese grond as gevolg van die hoë individuele variasies in die metaalladings van die liggaam, die gewigsverlies wat waargeneem is en die stadige voortplantingskoers, selfs in die kontrolegrond. Dit het gebeur ongeag die feit dat *A. caliginosa* veronderstel is om beter aangepas te wees aan grondtoestande as die strooiselbewonende *E. fetida.*

Die toksisiteit van die ultramafiese grond het nie noodwendig verband gehou met totale of omgewingsbeskikbare hoeveelhede van die geselekteerde swaarmetale nie. Daar kan dus gespekuleer word dat hierdie grond of onge-identifiseerde toksikante bevat het en dat wisselwerking tussen toksikante ‘n rol gespeel het of die erdwurms was in staat om die swaarmetale wat in die gronde voorkom te hermobiliseer.

Aangesien die eenduidige aanwending van ‘n enkele ekotoksikologiese eindpunt nie betroubare resultate opgelewer het nie, veral oor die duur van die blootstellings, is die gevolgtrekking dat wanneer gronde met ‘n komplekse samestelling betudeer word, is die gebruik van eindpunte nodig wat verskillende vlakke van organisasie verteenwoordig om die toksiese stress van ultramafiese gronde te assesseer.

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1. General Introduction

1.1 Metals in soils

Soils are extremely complex and dynamic systems containing a mixture of various chemicals interacting with each other, influenced by a broad range of physical and biological effects. Metals are integral parts of soils, involved in many of these chemical, physical and biological processes. Although most metals, apart from those after uranium in the periodic table, are of natural origin, human activities permanently remobilize and redistribute metals and metal compounds in the environment (Baudo et al. 1990). Once remobilised by human activities, metals often undergo fundamental chemical and physical processes transforming them into different chemical forms or speciations which can cause weighty effects. Also, anthropogenic redistribution often induces high levels of metals in soils, sediments and sludge which often have a significant effect on the ecological quality of aquatic and terrestrial environments (Moore et al. 1984, Sastre et al. 2002). Some metals such as copper, cobalt, chromium, iron and zinc are in low concentrations essential for all living organisms, but most of them represent a toxic hazard at elevated concentrations (Rida and Bouché 1997b).

To complicate matters, even in the absence of anthropogenic influences, the natural background concentrations of metals vary tremendously between different sites and areas (Chapman and Wang 2000). With regard to organisms living in soil loaded with elevated metal concentrations it often is difficult to distinguish between the effects of a metal on the organism and other effects occurring in this soil (RIVM 1998).

1.2 Ultramafic soils

Especially in terms of metals, ultramafic soils are very unique in their composition and ecology. They occupy only a small proportion of the earth's land surface, but their biological importance outweighs by far the area in size they encompass, as a high proportion of especially plants living on ultramafic soils are endemic (Proctor and Nagy 1992).

"Ultramafic" is a term used to describe a suite of rock types containing ferromagnesian minerals (Proctor 1999). Due to high contents of ferromagnesian minerals frequently found in ultramafic soils, these soils are also often named "serpentine soils" (Proctor and Woodell 1975). Soils, deriving from ultramafic rocks are strongly influenced by the geochemistry and mineralogy of the parent materials (Lee et al. 2001). A large variety of soils can develop from parent ultramafic material which is due to the manifold processes which themselves depend on climate, time, relief, chemical composition as well as biotic factors (Proctor and Woodell 1975). Common minerals associated to ultramafic soils can be antigorite, quartz, chlorine and olivine (Terlizzi and Karlander 1979), but the chemical composition of ultramafic rocks varies greatly. In general, soils deriving from ultramafic rocks are often rich in chromium, copper, cobalt, iron, nickel and the platinum group elements (PGE) and poor in calcium, phosphorus, potassium and molybdenum (Krause 1958, Naldrett 1989, Harrison et al. 2001).

Frequently, rare and endemic plants adapted to these conditions can be found on ultramafic outcrops (Proctor and Woodell 1975). Caesalpino already in 1538 described a plant growing exclusively on the 'sassi neri' (black stones), the serpentine soils of the upper Tiber valley (Caesalpino 1538). Since then, lots of work has been done on the taxonomy, ecology and physiology of plants growing on ultramafic soils, especially focussed on metal hyperaccumulating plants such as *Berkheya coddi*, *Senecio* ssp. and *Alyssum lesbiacum* (Krämer et al. 1996, Mesjasz-Przybylowicz et al. 1997, Mesjasz-Przybylowicz et al. 2001a, Mesjasz-Przybylowicz et al. 2001b).

In contrast, only little work has yet been done on invertebrates associated with serpentine soils. Apart from the work mentioned by Proctor and Woodell in their review (1975), Schreier and Timmenga (1986) exposed the earthworm *Lumbricus rubellus* to asbestos-rich ultramafic soils, and Marino et al. (1995) measured the concentrations of selected metals in different species of earthworms living in ultramafic soils. Further, Peterson et al. (2003) and Przybylowicz et al. (2002, 2003) studied insects feeding on hyperaccumulating plants endemic to ultramafic soils.

Due to the fact that the disadvantageous living conditions caused by high metal contents, and adverse physical conditions and nutrient shortages require high demands on either plants or animals (Proctor 1999), the study of organisms occurring in and on ultramafic soils offer interesting conclusions for animal adaptation to high metal concentrations in soils.

1.3 Bioavailability of metals in soils

For the assessment of such complex systems as soils, knowledge of the presence of single or few metals by means of chemical analyses only is not sufficient (Weltje 1998). Especially in the case of metals, high proportions are often bound to solid phases in soils and therefore are not bioavailable for organisms living in and on these soils (Peijnenburg et al. 1997), thus, the total amount of a certain metal does not reflect its effects on the soil biota. At this, the bioavailability is defined as the "measure of the potential of an element or substance for entry into biological receptors. It is specific to the receptor, the route of entry, time of exposure and the matrix containing the contaminant" (Anderson et al. 1991, Lanno et al. 2004). Since a pollutant may not be readily bioavailable or assimiliable, the bioavailable concentration is in nearly all cases less than the concentration determined by vigorous extraction (Aubert and Pinta 1971, Tang et al. 2002, Davies et al. 2003b).

1.4 Bioassays for the assessment of metals in soil

Even if the chemical isolation and identification of a metal as (single) source of a noxious effect in soil is often difficult or impossible, the environmental hazard can often be estimated by its effects by means of different bioassays (Claxton et al. 1998). A bioassay can be understood as a test based on the sum of the total effects of compounds in soils on a specific organism. It offers the possibility to show the effects and consequences of all elements, chemical compounds and their

interactions in a defined medium, unlike chemical and/or physical analysis, that is able to cover one or few defined substances only (Tadesse et al. 1994). The advantage of a bioassay is to exclusively indicate the response predetermined by the biological endpoint selected. Consequently, one always has to keep in mind that a positive response does not automatically allow any conclusions towards the contents or the actual hazard potential of a sample.

One way to establish levels and toxicity of pollutants in soils lies in the application of bioassays carried out on soil living key organisms. Regarding this matter, toxicity of metals to soil organisms can be seen as a function of bioavailability, determined by soil chemical and physical properties and the chemical characteristics of a qualified metal (Singh et al. 1996).

1.5 Earthworms as indicator species

For the assessment of the effects of metals to soil, earthworm tests have shown to be a useful tool (Menzie et al. 1992). The investigation of organisms living in and on naturally loaded (contaminated) soils helps to understand the effect of high metal concentrations in general and especially could help in obtaining a better understanding of the effects and risks originating from anthropogenic contaminated soils. Being of high ecological importance, earthworms have for many years been considered as feasible biological indicators of many pollutants in soil. Among soil living organisms, earthworms are not only omnipresent in almost all types of soils and easy to collect and analyse (Rida and Bouché 1997a), but they also constitute the dominant biomass of soil fauna in providing normally 10-200 g of the biomass per square meter of most productive soil types (Laskowski et al. 1988). Due to their way of living, earthworms can be considered as being directly exposed to soil and its bioavailable contents during their complete life cycle. They are directly exposed to the surrounding soil and only separated from it by a thin, moist, permeable body wall, and also take up the assimiliable fraction of soil and excrete it partially. Thus one can conclude that the whole body can potentially respond to the bioavailability of soil contents (Edwards and Bohlen 1996). For that reason, earthworms are regarded as a reference compartment to observe soil contaminant bioavailability (Rida and Bouché 1997b). They are used to evaluate the lethal and sublethal effects of chemical contaminants and pollutants. Therefore they are useful to assess the contaminant fractions which may act on all organisms getting in touch with soil.

Amongst the earthworms, *Eisenia fetida* (Savigny 1826), an European species, has been considered as a suitable test species for tests that is also applied in OECD guidelines (OECD 1984, OECD 2004). *E. fetida* can easily be cultured in the laboratory (Tomlin and Miller 1989), is large in size compared to other soil dwelling organism and shows similar sensitivity to pollutants as many indigenous earthworms in different parts of the world (Laskowski et al. 1988). Besides the OECD guideline 207, guidelines currently existing for *Eisenia fetida* are the ISO 11268-2 (ISO 1998), the EEC 79/831 (EEC 1982) and the OECD Earthworm Reproduction Test - *E. fetida/andrei* (OECD 2004).

E. fetida is an excellent organism to use in tests under laboratory conditions and in soils with a high organic content, but for tests of contaminants with a higher mineral content and less organic material it is often necessary to use other earthworm species (Reinecke and Reinecke 2004a). *E. fetida* is not a soil dwelling species, it prefers compost and dung heaps (Van Gestel and Van Straalen 1994). More suited to soils with a high mineral content than *E. fetida* are endogeic earthworm species like *Aporrectodea caliginosa*, which has been shown to be very susceptible during acute toxicity tests and are relatively sensitive to the effects of metals (Bouché 1992, Spurgeon 1997).

For *E. fetida*, various endpoints in toxicity testing of metals have already been established (Kokta 1992). As an indication of potential sublethal effects, parameters such as mass change and reproduction success are widely used. Although biomass as an endpoint of toxicity often has a limited sensitivity due to high variations (Kula and Larink 1998), many studies have found that metals do affect earthworm growth – and thus the change of biomass – adversely (Rida 1996, Reinecke and Reinecke 1996). Reproductive responses of *E. fetida* are also widely accepted as an indicator of sublethal effects of metal toxicity (Sheppard et al. 1998). In terms of reproduction, it is recommended by Reinecke et al. (2001) to measure cocoon production as well as hatching success, as a specific toxic threat does not necessarily have an effect on cocoon production and growth, but on cocoon viability due to sperm damage, reduced fertilization or problems related to embryonic development.

A challenge in ecotoxicological research is the fact that it is rarely suitable to extrapolate results gained in laboratory studies to field conditions. The few previous studies concerned with the effects of nickel on earthworms were mostly done under laboratory conditions (Saint-Denis et al. 2001, Ribera et al. 2001, Reinecke and Reinecke 2004a, Reinecke and Reinecke 2004b). Further, there are some arguments why field organisms could differ in sensitivity from laboratory organisms (Van Straalen and Denneman 1989, Van Gestel 1997):

- In the laboratory, organisms are tested under optimal conditions
- In the field, bioavailability of chemicals may be lower than in laboratory tests
- In the field, organisms are exposed to mixtures of many chemicals
- In the field, ecological compensation and regulation mechanisms are operating
- In the field, adaptation (genetic manifestation) to chemical stress may occur
- Adaptation often entails costs in ecological performance

From the literature it is known that earthworms can develop different reproduction strategies, for example, parthenogenetic and amphigonic reproduction, which are known to have an influence on adaptation to metal-loaded soils (Cluzeau et al. 1992). Also, ecophysiologically different earthworm species interact differently to different types of pollution (Morgan and Morgan 1992). Three different ecophysiological groups have been described, comprising epigeic (litter dwellers like *E. fetida*), endogeic (horizontal burrowing, like *A. caliginosa*) and anecic (deep burrowing) species (Simms and Gerard 1985). It is known that reactions of endogeic earthworms to certain metals

differ to the reactions of epigeic and anecic species inhabiting the same contaminated soil (Morris and Morgan 1986, Spurgeon and Hopkin 1995), but it is also reported that the general pattern of tissue distribution in the endogeic species *A. caliginosa* is broadly similar to that reported for species from other ecophysiological groups (Morgan and Morgan 1998).

1.6 Aims

1.6.1 Primary aims

Broadly, for the investigation of the "ultramafic challenge" (Proctor 1999) to soil living organisms such as earthworms, three different approaches were applied in this study:

1) For the evaluation of the living conditions of the earthworms in terms of metals, the major metals known to exist in the ultramafic soils of Mpumalanga (Anhaeusser 2001) were quantified. For gaining an insight into the hazardous potential of these metals in the soils of the Barberton Greenstone Belt, a chemical evaluation of the amounts of selected metals in these soils by means of the analysis of different soil fractions, representing different levels of bioavailability for soil living organisms and of earthworms as bioindicators for bioavailability, were conducted.
2) As bioavailability is a dynamic process where one has to distinguish between a physicochemically determined and a physiological determined uptake process into the organism (Peijnenburg et al. 1999), this approach is overlapping to a certain extent with the previous one. The presence of a metal in the body of an earthworm does not necessarily indicate the extent to which this specific metal is bioavailable to the earthworm. Metals can be physiologically compartmentalized inside the earthworm body into inert or biologically unavailable forms (Lanno et al. 2004). Consequently, the presence of metals in an earthworms body does not necessarily has to precipitate in an effect. Thus, to address the effects of the ultramafic conditions to earthworms, a hierarchical approach addressing defined biological endpoints, situated on different levels of organization, was conducted.
3) Third, to study the native Oligochaeta fauna of these soils to investigate what mechanisms the fauna living in these soils has developed to adapt or acclimatize to these extremely disadvantageous living conditions. In general, to evaluate, how and whether ultramafic soils can maintain earthworm populations as it is known that earthworms do occur in ultramafic soils (Marino et al. 1995).

For the first two approaches mentioned above, two ecophysiologically different species of earthworms, *Aporrectodea caliginosa* and *Eisenia fetida* were employed to gather differences in the responses and strategies of these species to cope with the ultramafic challenge.

1.6.1.1 Specific aims

1.6.1.1.1 Background concentrations and bioavailability of metals in ultramafic soils

1) Analysis of the total concentrations of arsenic, chromium, cobalt, copper, iron, manganese and zinc as these metals are known to occur at elevated concentrations in ultramafic soils (Viljoen et al. 1983) and of cadmium and lead for the evaluation of anthropogenic influences in terms of metals into the ultramafic soils.
2) Evaluation of the availability of the above mentioned metals by a sequential extraction procedure using $CaCl_2$ and DTPA to address the mobile and mobilisable amount of these metals (Maiz et al. 1997).
3) For the physicochemically driven uptake process of these metals in relation to the bioavailable fractions in the soils, earthworms were analyzed for these metals.

A substantially lower biological availability in relation to the total concentrations of the metals selected was expected.

1.6.1.1.2 Responses of earthworms to the ultramafic conditions

1) Again, overlapping with the previous aim, metal uptake and distribution in the tissues of earthworms were evaluated by chemical and physical analyses.
2) On a population level, the responses of the earthworms to the ultramafic conditions were recorded by the investigation of mortality and reproductive success.
3) On an individual level, responses to the ultramafic challenge were addressed in mass change and metal body burden.
4) On a cellular/subcellular level, lysosomal membrane integrity (NRR) and cellular proliferation (MTT) were selected as endpoints.
5) On molecular level, the DNA integrity was investigated as an endpoint for the evaluation of ultramafic stress.

An increase in the sensitivity of the responses at decreasing levels of organisation was expected, since the first responses to ecophysiological stress first occur at low levels of organisation (Spurgeon et al. 2005). With regard to the exposure of two ecophysiological different species of earthworms, differences in their responses were expected.

1.6.1.1.3 Evaluation of the native fauna

1) The native Oligochaeta fauna was investigated by the on-site application of standardized field sampling methods and chemical extracting methods.
2) Metal body burden of native earthworms were analyzed to find indications of an acclimative or adaptive advantage of native worms established by differences of metal uptake in comparison to *in situ* exposed earthworms.

1.6.2 Secondary aims

In terms of metals in ultramafic soils, a special focus was placed on manganese and nickel, as both metals do occur at extremely high concentrations in ultramafic soils. At least for nickel, it is known that it is toxic at the total concentrations found in ultramafic soils (Reinecke and Reinecke 2004b). To draw conclusions about uptake and accumulation of metals with regard to manganese and nickel in earthworms, survival and uptake of these metals by the exclusion of soil properties were examined.

The combination of the findings was aimed to reveal if earthworms occurring in ultramafic soils show an increased tolerance or higher resistance to nickel in particular or to ultramafic soils in general or whether earthworms exposed to these soils are able to develop an increased tolerance. Due to the fact that several metals occur in the soil, accruement of a possible cross-resistance, (i.e. that an increased tolerance to one metal renders an increased tolerance to another one) was also examined.

Lastly, the investigation was concerned with which biomarkers may be suitable for the assessment of such complex systems of natural origin such as ultramafic soils.

2. General materials and methods

This chapter provides a general overview of the materials and methods for purposes of orientation. More specific methodologies and procedures that were followed during experiments are presented in the pertaining chapters.

2.1 Study animals

Oligochaetae have a well defined coelomic cavity, a closed vascular system with at least a ventral and a dorsal vessel and a ladder-shaped dorsal nerve cord. They are bilaterally symmetric and externally evenly segmented. The segments have an outer layer of circular muscles and an inner layer of longitudinal muscles and are internally separated by septae. The gonads of the hermaphroditic earthworms are usually located in segments 10 to 13, consisting only of one or two pairs of testis and ovaries. Oligochaeta, as the name literally says - “few setae”, translated from the Greek words ‘oligo’ for few and ‘chaeta’ for seta – usually bears setae in bunches of four on all segments except the first two (Edwards and Bohlen 1996). An exception to that are some species of the genus Megascolecida which have bunches of setae of about 100 (Storch and Welsch 1997). The earthworm species used in this study were *Aporrectodea caliginosa* (Savigny 1826) and *Eisenia fetida* (Savigny 1826). As indicated below, in toxicological studies both species have their advantages and disadvantages.

The specimens of *E. fetida* used in this study were obtained from cultures maintained at the laboratory of the Stress Ecology Group at the Department of Botany and Zoology at the University of Stellenbosch over several years. According to different guidelines such as the OECD-Guideline (OECD 1984, OECD 2004) for the testing of chemicals, EEC (EEC 1982, EEC 1985) and ISO (ISO 1998) *E. fetida* is regarded as a reference species for soil toxicity testing (Edwards and Bater 1992). Under laboratory conditions, it is known as an excellent test organism with a quick reproduction rate. Further, its life cycle is well documented (Figure 1), (Venter and Reinecke 1988).

The specimens of *A. caliginosa* used in this study were collected at the Vergenoegd sports ground, Stellenbosch, Western Cape. As an endogeic earthworm species living in a medium with low organic content, *A. caliginosa* (Figure 4) can be considered as a field relevant species. *A. caliginosa* is also known to be susceptible to toxicity tests, and very sensitive to metal effects (Edwards 1992, Khalil et al. 1996b, Emmerling et al. 1997, Paoletti et al. 1998). Its disadvantages are the slow reproduction rate and *A. caliginosa* is not really suitable for laboratory conditions. Further; there are only little toxicity data available.

2.1.1 *Eisenia fetida*

2.1.1.1 Classification of *Eisenia fetida*

Phylum: Annelida
Class: Clitellata
Subclass: Oligochaeta
Order: Opisthopora
Suborder: Lumbricida
Superfamily: Lumbricoidea
Family:Lumbricidae (Rafinesque-Schmalz 1815)
Subfamily: Lumbricinae (Rafinesque-Schmalz 1815)
Genus: *Eisenia* (Malm 1877)
Species: *Eisenia fetida* (Savigny 1826)
Subspecies: *Eisenia fetida fetida* (Bouché 1972)
after (Storch and Welsch 1997)

2.1.1.2 Morphology of *Eisenia fetida*

The adult *E. fetida* (Figure 2) usually measures between 60 and 120 mm in length, between three and six mm in diameter and has a mean mass of 1.2 g (Haimi 1990). It comprises of about 80 to 120 segments and has a cylindrical body. The colour varies from a light pink to purple red or brown. The ventrum often is entirely unpigmented, the dorsal surface often looks striped (Tiger worm). The four setae per segment are closely paired. The clitellum is saddle-shaped and covers six to eight segments (from 24/25 to 31 to 33). The male pores are on segment 15, four pairs of seminal vesicles are found in segment 9 to 12 and two pairs of spermatecae are in the midline between segments 9 to 10 and 10 to 11 (Simms and Gerard 1985).

2.1.1.3 Lifecycle of *Eisenia fetida*

The life-cycle of *E. fetida* is shown in Figure 1. Usually, the reproduction of *E. fetida* is biparental, but in contrast to *A. caliginosa*, it is also a facultative self-fertilizer (Hartenstein et al. 1980b). *E. fetida* reaches maturity in 40 to 50 days after hatching. On average, four days after mating, cocoon production starts and proceeds continuously at a production rate of between 3.5 cocoons per 10 days (Venter and Reinecke 1988) and 18.5 cocoons per ten days (Edwards 1988). After an incubation period of 14 to 44 days, usually between one and nine hatchlings per cocoon hatch (Venter and Reinecke 1988).

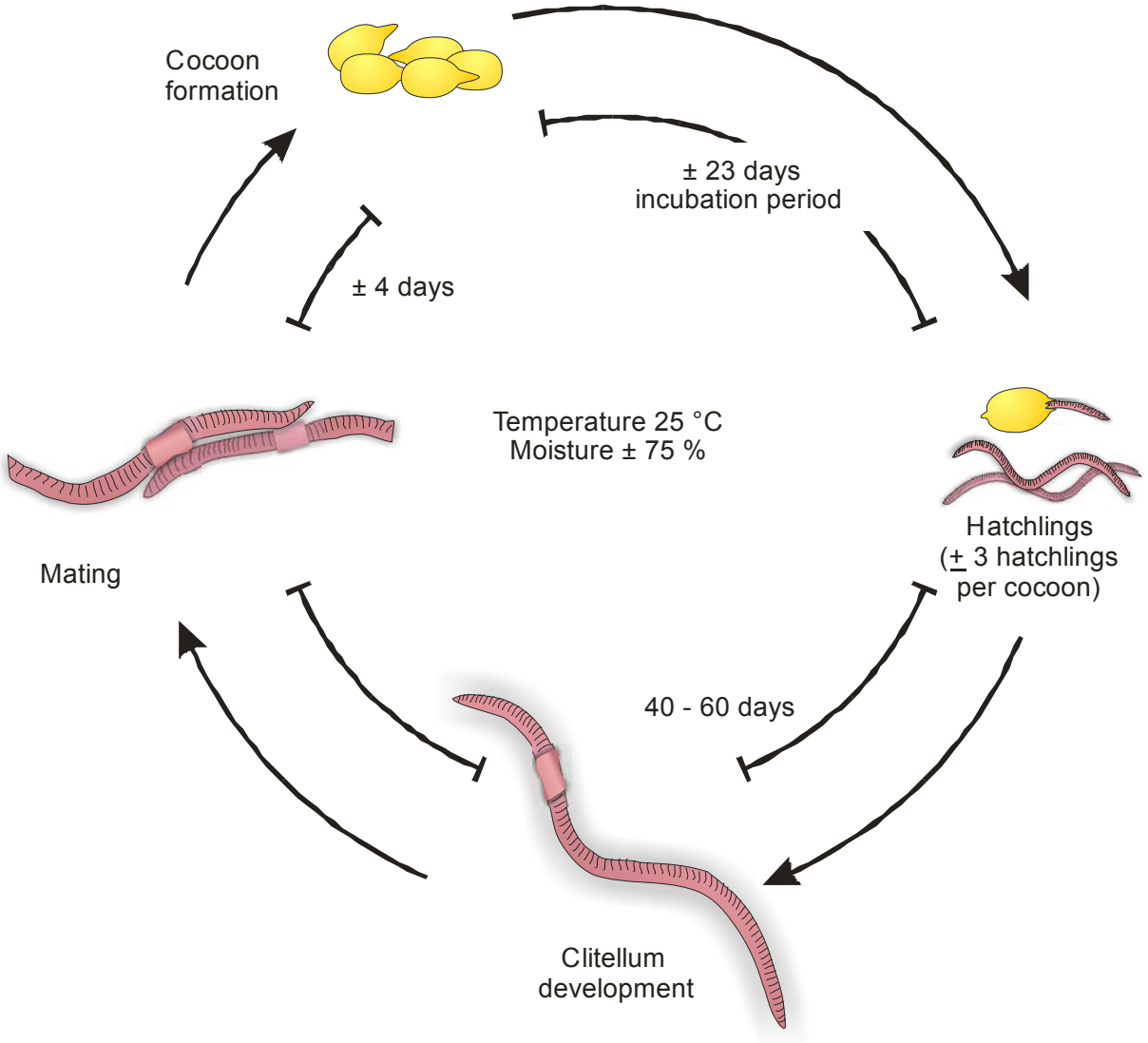

Figure 1: Life cycle of *Eisenia fetida* reared in cattle manure at a temperature of 25°C and a moisture content of 75%; redrawn after Venter & Reinecke (1988)

2.1.1.4 Ecology of *Eisenia fetida*

E. fetida, of palaearctic origin, can now be considered as a cosmopolitan species occurring worldwide except for some sporadic occurrences in the tropics (Simms and Gerard 1985). It is arguably the most domesticated species of Oligochaeta as it is widely bred and sold from earthworm farms as a 'compost worm' or even as a protein supply for pig farms. As an epigeic species (litter dweller), *E. fetida* (Figure 2) prefers a medium with a high organic content (manure, compost) what might cause some limitations in ecotoxicological tests concerning the relevance of field exposures, as *E. fetida* is not really considered to be a field species (Arnaud et al. 2000).

Figure 2: *Eisenia fetida* (Savigny 1826)

2.1.2 *Aporrectodea caliginosa*

2.1.2.1 Classification of *Aporrectodea caliginosa*

Phylum: Annelida
Class: Clitellata
Subclass: Oligochaeta
Order: Opisthopora
Suborder: Lumbricida
Superfamily: Lumbricoidea
Family:Lumbricidae (Rafinesque-Schmalz 1815)
Subfamily: Lumbricinae (Rafinesque-Schmalz 1815)
Genus: *Aporrectodea* (Örley 1885)
Species: *Aporrectodea caliginosa* (Savigny 1826)
after Simms and Gerard (1985)

2.1.2.2 Morphology of *Aporrectodea caliginosa*

The adult *A. caliginosa* ((Savigny 1826), Figure 4) measures between 40 and 180 mm in length, between 3.5 and 7 mm in diameter and has an average mass of 0.8 g (Lofs-Holmin 1983). The body is cylindrically shaped and comprises between 120 and 246 segments. The colour of this polymorphic species is very variable, varying from an unpigmented pale pink in juveniles to a dark brown. The setae are closely paired and the clitellum saddle-shaped, covering at least six segments (27 – 35). Since *A. caliginosa* occurs in many morphological variants, it is still disputed whether the four known possible synonyms (*A. caliginosa*, *A. nocturna*, *A. trapezoides* and *A.*

tuberculata) are separate species or phenotypes of one species (Simms and Gerard 1985, Lowe and Butt 2005).

2.1.2.3 Lifecycle of *Aporrectodea caliginosa*

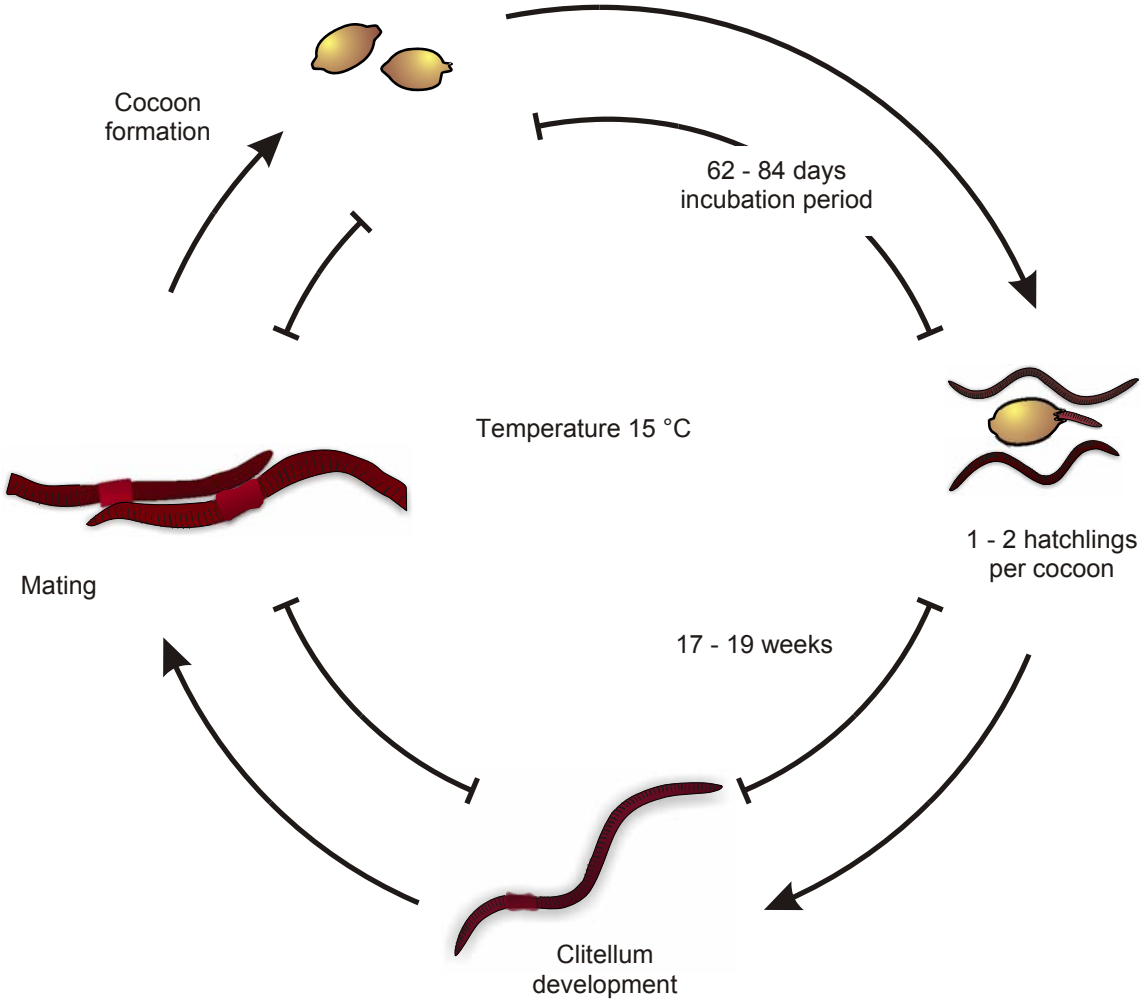

Figure 3: Schematic representation of the lifecycle of *Aporrectodea caliginosa*

A schematic overview over the lifecycle of *A. caliginosa* is shown in Figure 3. The life cycle of this species is not as well documented as the life cycle of *E. fetida*. First, it is strongly dependent on abiotic parameters such as temperature and moisture content (Holmstrup et al. 1991); second, the lifecycle of *A. caliginosa shows* a strong seasonal dynamism (Jensen and Holmstrup 1997); third, a comprehensive study of the species or the species group has not been conducted yet. Characteristic for this species is that the number of hatchlings per cocoon varies between one and two (Holmstrup et al. 1991, Nair and Bennour 1998). With the provision of near optimal conditions *A. caliginosa* grows into a clitellate adult within 17 to 19 weeks (Graff 1953). In contrast to that, according to Nair and Bennour (1998) clitellum development can take more than 35 weeks.

A. caliginosa produces between three and 27 cocoons per year (von Wilcke 1956, Satchell 1967). A strong discrepancy can be found in the literature concerning the incubation time of cocoons. At varying temperatures the incubation times range from 36 to 234 days. Several studies conducted in subtropical regions (el Duweini and Ghabbour 1965, Nair and Bennour 1998) reported an incubation time between 45 to 50 days at 20°C. Holmstrup et al. (1991) reported an incubation time of 62 to 84 days at an optimal culturing temperature of 15°C.

2.1.2.4 Ecology of *Aporrectodea caliginosa*

Figure 4: *Aporrectodea caliginosa* (Savigny 1826)

The endogeic earthworm *A. caliginosa* (Figure 4) prefers neutral to alkaline soils with a pH ranging from 5.9 to 11.1 and usually is the numerically dominant geophageous species in most cultivated areas as well as gardens. Originally from the Western Palaearctic and eastern Nearctic, it was introduced into most temperate regions worldwide (Simms and Gerard 1985). *A. caliginosa* tends to respond to adverse living conditions by becoming quiescent when the substrate is either too dry or to cold or when adequate food is not available (Edwards and Bohlen 1996). The worms empty their guts, construct a cell lined with mucus and roll into a tight ball to reduce water loss (Edwards and Bohlen 1996).

2.2 Field sites

Field sampling was performed on two field localities.

2.2.1 Stellenbosch

As an unpolluted site, a site close to the Vergenoegd sport ground next to the Eersterivier in Stellenbosch (Western Cape, 33°56'28"S, 18°53'22.77"E) was selected. This site has a known history of no application of pesticides and no metal pollution (Maboeta et al. 2003).

2.2.2 Barberton area

The second locality consisted of a group of sites which provided the ultramafic soils used in the experiments.

The ultramafic study area is located in the Barberton area in the Mpumalanga Province (former Transvaal) on volcanic rock outcrops of the Barberton Greenstone Belt dating back 3.5 billion years in the north-eastern part of South Africa.

The Barberton area is characterized by a subtropical climate with a rainy season in summer and a dry season in winter. The temperatures range from 7°C in winter to 30°C in summer (South African Weather Service 2005).

The sampling locations in the Barberton area are shown in Table 1 and in Figure 6. The soil samples from Agnes Mine, Kaapsehoop 3 and Sogimvelo have been collected by J. Mesjasz-Przybylowicz prior to the present study (Mesjasz-Przybylowicz et al. 2001a).

Table 1: Sampling locations in the Barberton area

	m above sea level	Cartesian coordinates	Type
Agnes Mine	1262 m	25°49’S, 30°57’E	Grassland
Barberton Nature Reserve	726 m	25°36’387’’S, 30°58’974’’E	Savannah
Kaapsehoop 1	1327 m	25°33’573’’S, 30°47’399’’E	Grassland
Kaapsehoop 2	1320 m	25°33’573’’S, 30°47’391’’E	Dry river bed
Kaapsehoop 3	1243 m	25°33’S, 30°47’E	Grassland
Songimvelo	1327 m	26°55’033’’S, 31°01’006’’E	Grassland

The locations of examination are on ultramafic soils which are characterised by a very heterogenic soil structure within a small area.

Various soil organisms, amongst them earthworms, are known to occur in these soils. For that reason, one field trip to the Barberton area was undertaken to collect earthworms. The field trip was undertaken in March 2004, after at least three month of intensive rainfall have been reported by the South African Weather Service (Figure 5, (South African Weather Service 2005))

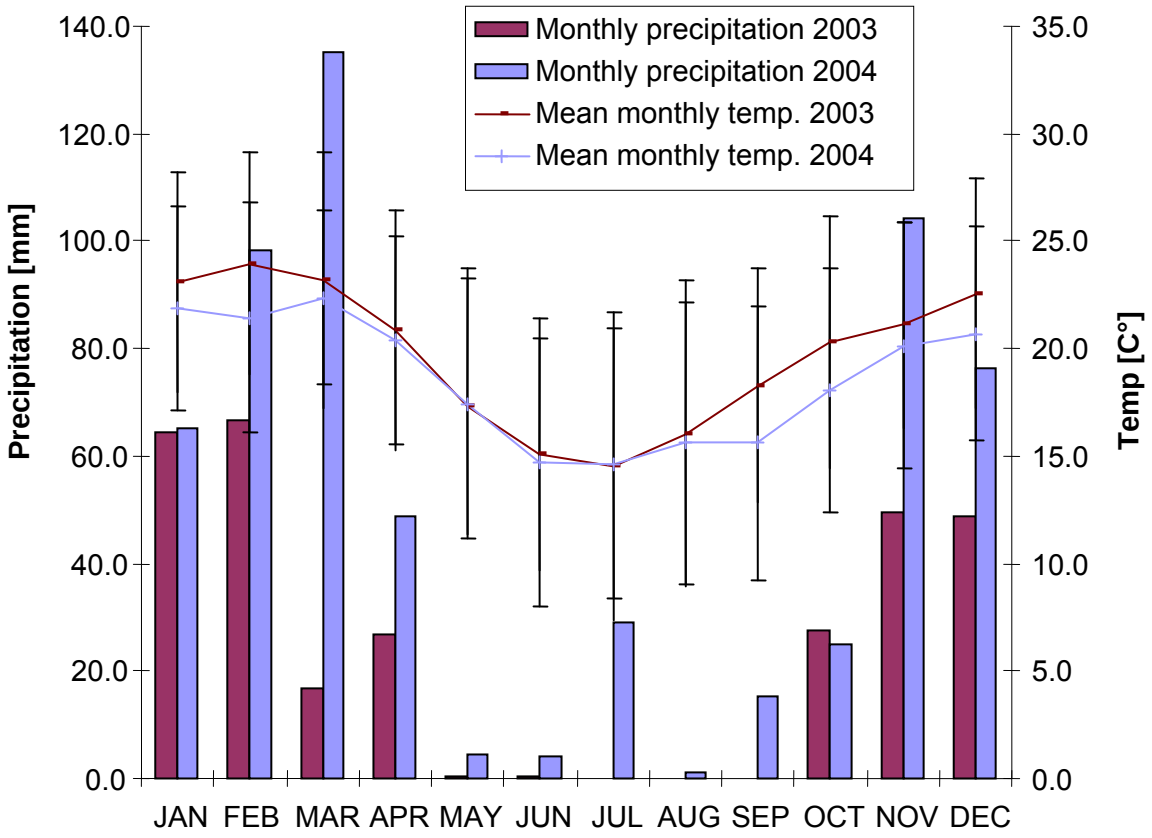

Figure 5: Total monthly precipitation [mm] and mean monthly temperature [°C] for the years 2003 and 2004, Nelspruit, South Africa; data obtained from the South African Weather Service (2005)

Figure 6: Overview map to show the sampling sites in the study area. Songimvelo sampling site lies out of the map area (http://earth.google.com 2005)

2.3 Substrates

2.3.1 Collection of soil samples

Soil samples were collected by randomly extracting five sub samples of 50 cm^3 one meter apart on a plot of 5 m^2. Subsequently, samples were carefully hand sorted to remove the earthworms (Kula 1992).

2.3.2 Preparation of natural soils

Earthworms of the species *Aporrectodea caliginosa* and *Eisenia fetida* were exposed to soil samples collected in the Barberton area (2.2.1). As a reference soil, earthworms were exposed to soil samples collected at the Vergenoegd sports ground, Stellenbosch (2.2.2). Prior to the start of the exposures, soil samples have been prepared by oven drying (48h 70°C; Gallenkamp size one oven BS, model OV-330, Weiss-Gallenkamp Ltd., Loughborough, U.K) and sieving (5- and 2 mm^2 screens; Baird & Tatlock, London, U.K.).

For the exposures of *E. fetida*, to increase organic content 10% (40g to 400 g soil) of dried and ground urine free cattle manure was added to the soil samples. Soil samples were moistened with distilled water to a moisture content of 60-65% of the maximum water holding capacity. Samples were placed into plastic containers (15 x 10 x 5cm) and incubated for 48h to stabilise prior to the introduction of earthworms.

2.3.3 Artificial substrates

As it is known that toxicity of soils to earthworms is also influenced by interactions between soil contents (Schreier and Timmenga 1986), artificial media were used.

2.3.3.1 OECD soil

To standardise the influences of interactions between soil contents, an artificial soil substrate was used (OECD 2004). The artificial soil consisted of a dry weight mixture of 70% silica, 20% Kaolin clay and 10% sphagnum peat moss. The pH was adjusted with calcium carbonate ($CaCO_3$) to pH 7.0±0.5. Soil samples were moistened to a moisture content of 60-65% and incubated for 48 hours at 25°C.

2.3.3.2 Artificial ground water

In order to exclude influences of soil properties like adsorption, artificial ground water was used. Artificial ground water was made up with 100 mg $NaHCO_3$, 20 mg $KHCO_3$, 200 mg $CaCl_2$ * 2H2O and 180 mg $MgSO_4$ per litre distilled water. Also, based on this composition proposed by Kiewiet & Ma (1991), 10 g (1%) normal melting agarose was added. So far, agarose has been used successfully for the cleaning of earthworm guts (Pokarzhevskii et al. 2000)., As an allowance to the artificial soil water, it proposes some advantages like the fact that worms will not be intoxicated so easily by decomposing dead specimen and do not escape so easily as from artificial ground water.

Also preliminary experiments have shown that earthworms can be kept for a longer time in such a mixture.

2.4 Soil analysis

2.4.1 Total metal content

To assess the total metal content of soils, soil samples from all field sampling sites were acid digested (Perkin-Elmer 1979) to determine the total amount of selected metals.

Acid digestion means the transformation of solid samples via acid digestion into solution (De Varies and Tiller 1980). Substrates used for acid digestion were dried for 48 hours at 70°C and weighed. After samples were digested in nitric acid and perchloric acid, the metal dry weight concentration was determined by using flame atomic absorption spectroscopy (Walsh 1955, De Varies and Tiller 1980, Page et al. 1982, Beck and Sneddon 2000).

2.4.2 Mobile metal fraction

Simple and complex metal ions in the soil solution can not be detected only by analysing pore water, the mobile metal fraction of the soils was extracted by a method first described by Lindsay & Norvell (Lindsay and Norvell 1978). Sieved (2 mm^2) and dried soil samples were dissolved 1:10 in a 0.01 mol/L $CaCl_2$ solution and agitated for 2 hours on a shaker plate. After centrifugation and filtration by gravity, the supernatant was analyzed by atomic absorption spectrometry (Lindsay and Norvell 1978, Maiz et al. 1997, Maiz et al. 2000).

2.4.3 Mobilisable metal fraction

To address the mobilisable metal fraction consisting of easily remobilisable, complexed and carbonated metal ions, the residue of the previous fractionation (2.4.2.) was suspended 1:2 with a DTPA extracting solution consisting of 0.1 mol/L TEA ($(HOCH_2CH_2)_3N$), 0.005 mol/L DTPA (di-ethylene-triamine-pentaacetic acid) and 0.01 mol/L $CaCl_2$ at a pH of 7.30 (Lindsay and Norvell 1978, Maiz et al. 1997, Maiz et al. 2000).

2.5 Earthworm sampling

Mature (clitellate) *Aporrectodea caliginosa* individuals were collected at an uncontaminated site near Stellenbosch. To minimize abiotic variability, all animals were collected from a defined circle (Morgan and Morgan 1998).

The presence of earthworms in the ultramafic soils has been noted (Dr. J. Mesjasz-Przybylowicz, personal communication), but no specific collections have been made of these and any other soil-dwelling organisms. It had to be established which species of soil fauna and in what densities - especially Oligochaeta - occur in the area, since literature on the organisms in ultramafic soils of the Mpumalanga area is scarce.

Standardized sampling and preservation methods were used (Bohlen and Edwards 1995, Bohlen et al. 2002) to collect worms from the study sites.

In the field, earthworms were sampled by passive earthworm sampling. Passive earthworm sampling (hand sorting) is known as laborious, but also the most accurate method for the estimation of earthworm populations (Kula 1992, Schmidt 2001). Samples were dug by extracting 50 * 50 * 50 cm wide soil cubes at least one meter apart. As strength and weaknesses of hand sorting methods are well known (Satchell 1971, Axelsson et al. 1971, Springett 1981, Schmidt 2001), also two active (chemical) earthworm extraction methods were applied on sub sample plots of one square meter:

A mustard solution (Gunn 1992, Chan and Munro 2001) consisting of 50 g of mustard powder dissolved in 7 L of water (Colman's traditional hot English mustard powder) was applied on a square of 1 x 1 meter.

As a second method, the formalin method made up by dissolving 0.4% Formalin in 1 L of water was used (Raw 1959, Gunn 1992, Kühle 2003).

2.6 Exposure of earthworms to groundwater and soils

Eisenia fetida specimens were exposed for 48 hours in artificial ground water to different concentrations of $MnSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$. Three replicates per concentration and six worms per replicate were exposed.

Before the start of exposures to the native soil samples, all worms were kept for 48 hours on moist filter paper to clean the guts (Krontowsky and Rumiauzew 1922). 12 worms of the species *E. fetida* and ten worms of the species *A. caliginosa* in three replicates per ultramafic soil sample and four replicates per control soil were exposed. Soil exposures were conducted in accordance with procedures established by Reinecke et al. (1999) and kept under temperature, light and moisture controlled conditions in a climate room.

For the Mpumalanga soil exposures, specimens of *Aporrectodea caliginosa*, collected at the Vergenoegd sports ground at Stellenbosch, and cultured *E. fetida* were used. *E. fetida* were maintained in the laboratory of the Stress Ecology group in the Department of Botany and Zoology of the University of Stellenbosch at a constant temperature of 20-25°C and 70% humidity for several years.

All worms were exposed to the different soil media prepared as described under section 2.3 for the duration of at least 12 weeks. For the exposures to the ultramafic Mpumalanga soil samples, *E. fetida* specimens with and without a previous history of long-term exposure were used. Previously exposed worms were exposed for at least ten generations to cadmium contaminated substrate and fed on a biweekly basis with 0.02% (wet mass) of $CdSO_4 * 8H_2O$ added to their diet consisting of 200 g of urine free cattle manure. A second group of worms was exposed for at least ten generations to nickel, and fed on a biweekly basis with 0.2% (wet mass) of $NiCl_2 * 6H_2O$ added to their diet.

2.7 Earthworm biotests

Earthworm biotests were conducted on different levels of organisation. On the population level, survival respectively mortality, reproduction and fecundity were determined as endpoints. On the individual level, growth (mass change over time) and metal body burden were assessed. On suborganismal level, metal distribution in cells and organs, genotoxicity and cytotoxicity were evaluated.

2.7.1 Life cycle

Mortality was determined by counting the worms in the different containers every two weeks. As worms tend to disintegrate quickly after death, earthworms absent from the containers were considered dead. Further, cocoons were collected and incubated by the water incubation method (Reinecke et al. 2001) in 24 well platters to evaluate hatching success.

2.7.2 Growth

Growth (mass change over time) was measured every two weeks. Worms were removed from the substrate, washed in distilled water and dried on paper towels. Then the worms were weighed singly in water filled weighing boats to avoid desiccation on a Sartorius balance.

2.7.3 Total metal body burden

Three worms were removed every two weeks to determine the metal body burden by acid digestion (Katz and Jenniss 1983). Worms were also cut into three pieces (anterior region, middle part with clitellum, posterior section). The pieces were acid digested and analyzed separately by atomic absorption spectrometry, to evaluate the longitudinal distribution of metal over the body.

2.7.4 Elemental distribution mapping

An elemental imaging analysis of cross sections and coelomic fluid of *Eisenia fetida* exposed to the ultramafic soils from the Barberton Greenstone Belt and the soils collected at the Vergenoegd sports grounds (Stellenbosch) was conducted to determine the elemental distribution of metals. Worms were immobilized by holding them in a plastic tub over liquid nitrogen and dissected under an optical microscope. Cross sections were freeze dried on a piece of formvar foam.

Micro-PIXE (Particle Induced X-ray Emission) measurements were performed using the nuclear microprobe at the Materials Research Group, iThemba Laboratories, Faure, Western Cape, South Africa. A proton beam of 3.0 MeV energy with a current of about 400 pA was focused on a 3μm^2 spot and raster scanned over areas of interest. PIXE and RBS (Rutherford Proton Backscattering) were used simultaneously to create elemental distribution maps (Przybylowicz et al. 2003).

2.7.5 Cellular and subcellular biomarker assays

All cellular and subcellular biomarker assays were conducted with cell suspensions extracted from *Eisenia fetida*.

2.7.5.1 Cell extraction

To find the most suitable method for the cytotoxicological assays and the genotoxicity assays (Chapter 2.7.5.2 and 2.7.5.3), a preliminary test series was conducted with the earthworm *Eisenia fetida*. At this, three different methods of cell extraction for the collection of coelomocytes from individual earthworms were conducted (as described in the Chapters 2.7.5.1.1.-2.7.5.1.3 below).
Viability of cells was tested using the MTT colouring assay as a test for viability (Affar et al. 1998). The quantity of the formazon product accruing after reduction of the MTT salt is directly proportional to the amount of viable cells in the medium (Pan et al. 2003).
Finally, cells were extracted using the ethanol extrusion method as proposed by Eyambe et al. (1991). Using bigger numbers of specimens, it was found that the ethanol extrusion method has in this study proven to be the most feasible method. For all extrusion methods, all specimens exposed in solid medium were kept for 48 hours on filter paper to clean the guts (Krontowsky and Rumiauzew 1922). Then, the hind parts of the worms were carefully massaged to expel remaining gut contents. Afterwards, the worms were washed in distilled water, blotted dry and rinsed in cold PBS (Phosphate Buffered Saline). After massaging again, the worms were blotted dry on tissue paper.

2.7.5.1.1 Alcoholic extrusion

For the ethanol extrusion the procedures proposed by Eyambe et al. (1991) and Diogène et al. (1997) were followed. Worms were incubated for 3 min. at room temperature in a 1.5 mL Eppendorf micro centrifuge tube containing 1 mL of extrusion solution. The extrusion solution was made up by dissolving 50 mg EDTA in 19 mL of PBS (Phosphate Buffered Saline), adding 200 mg of GCE (Guaiacol Gliserol Ether) and 1 mL of EtOH. After 3 min. worms were removed from the Eppendorf vials and returned to the culture. The coelomic fluid was washed three times in cold PBS after extraction at 2000 U/min at °C; first for 5 minutes, then three minutes and finally for one minute. The vials containing the coelomic fluid were then kept on ice until used.

2.7.5.1.2 Cell extraction by punctuation of the coelomic cavity

As described by Reinecke and Reinecke (1999), the coelomic fluid was drawn into a syringe (1 mL) containing temperature adjusted Ringer solution (Lockwood 1963). Then, the body wall was punctured with a disposable syringe with a fine needle, containing 0.2 mL of Ringer solution. Due to the coelomic pressure, a drop of fluid was extruded and 20 µL of this drop drawn into the syringe.

2.7.5.1.3 Ultrasound extraction

In accordance with the method published by Hendawi et al. (2004), each worm was placed in a 1.5 mL polypropylene tube containing 1 mL of cold PBS. The tube was then introduced into an ultrasonic bath for a series of 10 exposures of 2-3 s each, until the worm started to wiggle and the solution became turbid. After removal of the worm, the coelomic fluid was washed three times at 2000 U/min at °C. With the ultrasound method, no cells were found in any of the extractants.

2.7.5.2 Cytotoxicity assays

To survey the toxicity of the Mpumalanga soils on the cellular level, the Tetrazolium salt assay (MTT colouring assay;(3-[4,5-Dimethythiazolium-2-y-2,5-Diphenyl-Tetrazoliumbromide)) and the Neutral Red Retention Assay (NRR) were applied.

2.7.5.2.1 MTT colouring assay

The MTT colouring assay, also called Tetrazolium salt assay, deals with the fact that in metabolically active cells, the MTT dye can be reduced to a blue formazon salt, which leads *in vitro* to a photometric quantifiable change of colour (Mosmann 1983a, Mosmann 1983b, Gerlier and Thomasset 1986, Berridge and Tan 1993). In this assay, the absorption of the blue formazon salt was read photometrically at a wavelength of 570 nm. Due to the fact that the response of the MTT assay is also directly related to the number of viable cells (Pan et al. 2003), the protein content of the coelomic liquid was measured using the Bradford method (Bradford 1976) to exclude influences of high variations of the cell concentration and consequently divided by the returns of the MTT photometric measurement. The results of the photometrical measurement were extrapolated from the absorption values of the controls.

2.7.5.2.2 Neutral Red Retention assay

The NRR assay is a vitality test based on the admission of the water soluble, slightly basic colorant neutral red into the lysosomes of intact cells. Damage of the lysosomal membrane results in diminished retention of the dye in the lysosomes (Barile et al. 1994). As it has been shown that the NRR can potentially be used to link these changes of permeability to ecological relevant live cycle effects, it can be considered as test for the vital function of the cells (Weeks 1995, Svendsen and Weeks 1997a, Spurgeon et al. 2000, Reinecke et al. 2002). The amount of the Neutral Red colorant remaining after a washing step in the cellular lysosomes was read photometrically at a wavelength of 540 nm. The data obtained from the spectrophotometer were processed analogous to these of the MTT assay.

2.7.5.3 Genotoxicity assays

As biomarker of genotoxicity, the Single Cell Gel Electrophoresis (SCGE) and the Micronucleus Assay (MN) were conducted in this study.

2.7.5.3.1 Single Cell Gel Electrophoresis assay

The Single Cell Gel Electrophoresis assay offers the possibility to monitor adverse effects of toxicants on DNA level, i.e. it reports the loss of DNA integrity on cellular level of prokaryotes in detecting strand breakage. It is based on the quantification of the development of a so called comet caused by the migration of DNA fragments out of the cell core during an alkaline electrophoresis step (Singh et al. 1988). In this study, the comet assay was primarily conducted to find out whether the exposure of earthworms to ultramafic soils results in increased DNA strand breaks; further, if it can be indicative whether long-term exposed earthworms are able to develop a genetically based resistance. The procedure of the comet assay followed the description of Singh *et al.* (1988), as slightly modified by Reinecke & Reinecke (2004b).

2.7.5.3.2 Micronucleus Assay

The Micronucleus Assay is based on the identification of micronuclei arising through a splitting off of single chromosomes or parts thereof caused by unrepaired or misrepaired damage during the first cell division (Walker et al. 1996). In contrast to the SCGE assay, assessing damage which subsequently might be repaired by DNA repair mechanisms, the micronucleus formation usually results in permanent genetic damage. The Micronucleus Assay was performed according to a method developed by Evans (1959) and modified by Diekmann et al. (2004).

2.8 Statistical analysis

All statistical tests were performed with the current version of Sigma Stat (7.1; Jandel Scientific) for Windows. As a test for normality, Lilliefors test was used on all numerical data obtained from the different experiments. In case of normal distributed data, a One-Way-ANOVA (analysis of variance) was carried out. If significant differences were found in using ANOVA, a Fisher LSD test was applied as a post-hoc test to compare the mean values of each group to determine which groups differ from one another. In the case of non-normal distributed datasets, a Kruskal-Wallis H-test was used to detect differences among the groups. If significant differences were found, the multiple comparison of z' and p values were conducted (Siegel and Castellan 1988). For the evaluation of dose dependent responses such as mortality related to metal concentrations, a Probit analysis (Finney 1978) was conducted. Further, a generalized linear model was used in the case of comparing different doses or exposures.

3. Metal contents of ultramafic soils

3.1 Introduction

Human activities often mobilise and redistribute harmful substances in the environment which can cause fatal effects (Baudo et al. 1990). Amongst them, metals are considered to be one of the main sources of environmental pollution since they have a significant effect on ecological quality (Sastre et al. 2002). High levels of metals in soils, sediments and sludges often have negative effects on animal and human health (Moore et al. 1984).

There is an increasing economical, political and social interest to make metal contaminated sites available for further human utilization (DETR 1997). Amongst other factors, an essential pre-condition for the re-use of a contaminated site, is a comprehensive assessment of its pollutants to confirm whether their amounts are harmful or not (Griffiths and Board 1992).

Soil is a much more heterogeneous substrate than air or water. Determining the content of a specific substance in soils in general can therefore be much more problematic. The returns of a quantitative measurement by simple analytical measurements is therefore limited and should always be seen in context of the other physical and chemical properties of the specific soil that is to be studied. Apparently, contamination is not considered to be of ecological importance by its sheer presence, but by its effects. A high soil concentration of metals, as determined by conventional soil extraction techniques, may therefore not necessarily pose a toxic threat to the environment because the bioavailability may be low (Tang et al. 2002, Davies et al. 2003b).

Soils deriving from ultramafic rocks are often rich in metals such as chromium, copper, cobalt and nickel, and poor in primary nutrients and calcium (Naldrett 1989). Thus, one can expect that ultramafic soils potentially pose extremely disadvantageous living conditions to soil living organisms (Proctor 1999), depending on the bioavailability of the different toxicants. Ultramafic soil is a tectonically derived rock descended from seafloor alteration found in faulting zones throughout the world (Viljoen and Viljoen 1969). Soil derived from ultramafic rocks contains the highest naturally occurring concentrations of nickel known.

Nickel is a naturally occurring element with an average abundance of 0.018% in the earth's crust (Richardson and Gangolli 1994). In ultramafic soils nickel is usually found at concentrations between 1000mg/kg and 7000mg/kg (Robinson et al. 1999), whereas "normal", non-ultramafic soils have an average background nickel concentration of up to 30mg/kg (Brooks 1983). The rate of the potential availability of nickel in ultramafic soils was determined by various extracts from several examination sites from all over the world to range between 13 and 80% (Robinson et al. 1999).

Nickel, manganese and chromium are metals often coexisting in either ultramafic or metal contaminated soils (Proctor 1971, Reddy and Chinthamreddy 2003). It is also known that metals influence the uptake of other metals, as for example manganese is known to reduce cadmium toxicity (Oste et al. 2001). On the other hand, asbestos increases the uptake of nickel (Schreier and Timmenga 1986). Many organic pollutants as well as metals can form chemical-physical associations with solid phases of different availability (Maiz et al. 1997):

- simple or complex ions in soil solution,
- exchangeable ions,
- linked to organic substances,
- occluded or co-precipitated with oxides, carbonates and phosphates or further secondary minerals, and
- ions in crystalline lattices of primary minerals.

The different chemical-physical associations result in a correlation between soil organic matter content, soil pH and metal bioavailability as shown e.g. by Soon and Bates (1982). As the bioavailability of a certain toxicant can be considered as the key to its (eco-)toxicity, the total metal concentration is not necessarily related to soil organism toxicity (Conder et al. 2001).

3.2 Aims

The aim of this part of the study was the assessment of the potential availability of selected metals by the examination of two different fractions of soils representing different soil compartments in terms of the accessibility of metals to organisms living in and on these soils. It was distinguished between a mobile metal fraction, which represents the “non-specifically adsorbed” or “water-soluble and exchangeable” metal ions in soil and a mobilisable metal fraction, representing the exchangeable and organically bounded trace metals (Beckett 1989).

In this study, the mobile metal fraction was extracted with $CaCl_2$, measuring the cation exchange capacity to assess the readily available exchangeable proportion of metals in soil (Maiz et al. 1997). The ratio behind using 0.01 mol/L $CaCl_2$ was, that the $CaCl_2$-extractable amount of metals includes the metal contents of the pore water and also the easily extractable forms of metals (Novozamsky et al. 1993). The $CaCl_2$-extractable proportion of metals is supposed to be as easily accessible to soil living organisms as pore water, therefore it was refrained from evaluating the metal content of the soil solution (pore water). First developed for the measurement of soil pH, the extraction of metals from soil samples using 0.01 mol/L $CaCl_2$ has been shown to be a useful tool for the assessment of the environmental availability of metals (Houba et al. 1996). The $CaCl_2$-extraction is based on the principle that 0.01 mol/L $CaCl_2$ is an unbuffered neutral electrolyte solution containing calcium as the most important cation on the absorption complex in many soils, cations can be easily exchanged in a comparably diluted Ca-solution (Houba et al. 1990). The 0.01 mol/L $CaCl_2$ extraction was first successfully applied by Hoyt and Nyborg (1971a, 1971b) for the evaluation of phytoavailability of metals.

In the short term, toxicity to soil living organisms can be reduced by adsorption or occlusion to soil surfaces as many organic pollutants as well as metals do have a high affinity to bind on solid phases. Under certain circumstances (such as climatic effects, uptake by plants and soil living organisms and human activities), a contaminant can be remobilized. In this study, the soil phase containing the metals which can be easily remobilized by a number of events or factors was considered as the mobilisable metal fraction. The mobilisable fraction, consisting of complexed,

adsorbed and carbonated metals, was extracted with a DTPA (di-ethylene-triamine-pentaacetic acid) solution first developed by Lindsay and Norvell (1978). Lindsay and Norvell established the DTPA test for the evaluation of the phytoavailability of iron, manganese and zinc in soil. Later, it was also successfully applied for the evaluation of the environmental availability of cadmium, chromium, copper, lead and nickel in anthropogenically contaminated soils (Maiz et al. 1997). Furthermore, a high correlation between the DTPA extractable metals (cadmium, copper, lead and zinc) and the total metal concentrations in earthworms was found (Dai et al. 2004).

3.3 Material and methods

In general, soil samples collected as described in Chapter 2.3.1 of this study at the ultramafic sites at the Barberton region were leached by a sequential extraction procedure. A $CaCl_2$-extraction was followed by a DTPA-extraction; the total amount of metals was evaluated after acid digestion. The fractions gained by the sequential extraction were analyzed spectrophotometrically for aluminium, arsenic, cadmium, chromium, cobalt, iron, manganese, nickel, lead and zinc. Since copper is known to be toxic to earthworm populations (Edwards and Lofty 1972, Streit et al. 1990), and since it is found often coexisting with other metals in ultramafic soils, soil samples from Mpumalanga were also analyzed for copper.

3.3.1 pH

The pH of the soil samples was determined with a Crison Micro-pH 2001 (Crison S.A., Barcelona, Spain) (soil/solution ratio 1g: 30 mL) on a $CaCl_2$ basis.

3.3.2 Mobile metal fraction

The mobile metal fraction was extracted by suspending 3 g (Sartorius handy, Sartorius, Göttingen, Germany) of sieved and oven dried (48 hours at 70°C; Gallenkamp size one oven BS, model OV-330, Weiss-Gallenkamp Ltd., Loughborough, U.K.) soil samples for 2 hours under agitation on a Labcon micro-processor controlled shaker (Orbital Platform Shaker 15 kg, Labmark Ltd. Roodepoort, South Africa) in a 0.01 mol/L $CaCl_2$ solution (1:10; chemically pure, Merck KGaA, Darmstadt, Germany) in a 50 ml centrifuge tube (Sterilin, Barloworld Scientific Ltd., Stone, U.K.). After centrifugation for 15 min. at 4000 rpm (Universal 32R, Andreas Hettich GmbH, Tuttlingen, Germany) and filtration by gravity (Whatman #6, Whatman International Ltd., Maidstone, England) the supernatant was stored in black plastic containers to protect samples from light until atomic absorption spectrometry (Maiz et al. 1997, Maiz et al. 2000).

3.3.3 Mobilisable metal fraction

The mobilisable fraction was extracted by suspending (1:2) the soil residues of the 0.01 mol/L $CaCl_2$ extraction under agitation in a solution consisting of 0.005 mol/L of the chelating agent DTPA ($C_{14}H_{23}N_3O_{10}$; di-ethylene-triamine-pentaacetic acid, Fluka Chemie GmbH, Buchs,

Switzerland), 0.01 mol/L $CaCl_2$ and 0.1 mol/L TEA ($(HOCH_2CH_2)_3N$; triethanolamine, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at pH 7.3 for 4 h at room temperature (Lindsay and Norvell 1978). Also, after centrifugation and filtration by gravity (Whatman #6), the supernatant was stored in black plastic containers until analysis by atomic absorption spectrometry.

3.3.4 Total metal content

Acid digestion means the transformation of solid samples via acid digestion into solution (De Varies and Tiller 1980).

One g of each soil sample was left overnight at room temperature in 10 mL (55%) nitric acid (HNO3, Merck KGaA, Darmstadt, Germany). After heating for two hours at 40-60°C in a Labcon Dual Digestor heating system (Labdesign Engineering Ltd., Roodepoort, South Africa), the samples were heated at 120 °C for one hour before cooling down for another hour at room temperature (Katz and Jenniss 1983). Five mL of perchloric acid (70%) were then added and the samples heated at 120°C again. After cooling down for one hour, five mL of distilled water was added and the samples reheated to 120°C until white fumes developed. Before filtration through Whatman no. 6 filters, the mixtures were left overnight to cool down. Samples were made up to 20 mL with distilled water and filtered through 0.45 µm Sartorius cellulose nitrate microfilters into dark plastic containers. After digestion, the metal dry weight concentration was determined by using a Varian AA-1275 flame atomic absorption spectrophotometer (Varian Inc., Palo Alto, California, USA). The appropriate standards were made up at the Department of Physics at the University of Stellenbosch.

The detection limits for the atomic absorption spectrometry reading were:

0.7 µg/mL for Al, 1 µg/mL for As, 0.01 µg/mL for Cd, 0.06 µg/mL for Cr, 0.07 µg/mL for Co, 0.04 µg/mL for Cu, 0.07 µg/mL for Fe, 0.11 µg/mL for Pb, 0.3 µg/mL for Mn, 0.08 µg/mL for Ni and 0.01 µg/mL for Zn (Varian 1979).

3.3.5 Asbestos

All soil samples were analyzed by scanning electron microscopy (Leo 1430VP Scanning Electron Microscope; Carl Zeiss SMT AG, Oberkochen, Germany) and energy dispersive X-ray spectrometry (Philips 1410-diffractometer, Koninklijke Philips Electronics, Eindhoven, The Netherlands) at the Central Analytical Facility of the University of Stellenbosch for asbestos fibres.

3.4 Results

The pH values of all soil samples varied between 6.2 and 7.0. Further, no asbestos fibres greater than 5µm were found by scanning electron microscopy (SEM) or energy dispersive x-ray spectroscopy.

No arsenic (As) or lead (Pb) was detected in any of the soil samples. Cadmium (Cd) was detected only by acid digestion in the soil samples from Kaapsehoop 3 (67±9.9 mg/kg; n = 4).

3.4.1 Mobile metal fraction

Table 2: Metal contents (mg/kg ± SD, n=4) after $CaCl_2$ extraction of soil samples from different ultramafic sites as measured by atomic absorption spectrometry

	Control	Agnes M.	Barberton	Kaapseh. 1	Kaapseh. 2	Kaapseh. 3	Songimvelo
Al	n.d.	n.d.	0.4±0.44	n.d.	n.d.	n.d.	n.d.
Co	0.31±0.3	0.55±0.11	0.35±0.34	0.31±0.39	0.31±0.36	0.25±0.11	0.4±0.06
Cr	n.d.	0.07±0.13	0.07±0.13	0.07±0.13	0.11±0.19	0.19±0.32	0.15±0.06
Cu	0.15±0.17	n.m.	0.06±0.06	0.06±0.06	0.03±0.06	n.m.	n.m.
Fe	88.55±16.56	28.97±9.61	39.95±1.94	9.57±4.92	2.77±0.92	5.33±3.84	8.8±5.72
Mn	5.57±1.52	16.21±1.96	3.68±0.58	1.93±0.32	0.68±0.54	0.69±0.44	12.54±1.38
Ni	0.95±0.27	3.45±0.23	8.01±0.6	55.5±5.23	14.99±3.52	13.5±0.73	5.76±0.25
Zn	4.40±0	n.m.	3.3±1.56	2.75±0.78	2.75±2.33	n.m.	n.m.

n.d. = not detected

n.m. = not measured

Agnes M. = Agnes Mine

Kaapseh. = Kaapsehoop

The results of the $CaCl_2$ extraction are shown in Table 2 and in Appendix 1-1. The Mann-Whitney-U-test has shown that $CaCl_2$-extractable concentrations of manganese (Mn) and nickel (Ni) were significantly higher in all ultramafic soil samples than in the control soil samples collected at the Stellenbosch site (Control). Additionally, a significantly higher concentration of mobile chromium (Cr) was found at the Songimvelo site. On the other hand, the mobile iron contents (Fe) were significantly higher at the control than at the ultramafic sites. Within the ultramafic samples, no significant difference was found in the mobile soil fraction between the samples collected at Kaapsehoop 2 and Kaapsehoop 3. The contents of mobile nickel and manganese differed significantly between all other ultramafic samples.

3.4.2 Mobilisable metal fraction

Nickel and manganese concentrations of the DTPA extracts were significantly ($p > 0.05$) higher in all of the ultramafic samples than in the control soil collected at Stellenbosch. DTPA extractable aluminium contents were significantly higher in the control than in all ultramafic samples, also, except in the Barberton samples, the mobilisable contents of iron were higher in the control than in all of the ultramafic samples (Table 3). Within the ultramafic samples, significant differences were found in the concentrations of nickel, manganese and chromium (also see Appendix 1-2).

Table 3: Metal contents (mg/kg ± SD, n=4) after DTPA extraction of soil samples from different ultramafic sites as measured by atomic absorption spectrometry

	Control	Agnes M.	Barberton	Kaapseh. 1	Kaapseh. 2	Kaapseh . 3	Songimvelo
Al	20.17±0.64	4.29	0.44±0.40	n.d.	n.d.	0.30±0.24	n.m.
Co	0.22±0.19	1.58±0.06	2.32±3.26	6.86±2.89	3.69±3.16	6.22±0.39	0.40±0.06
Cr	n.d.	0.0 ±0.13	0.18±0.32	0.15±0.13	0.11±0.19	0.36±0.64	0.15±0.06
Cu	5.15±8.82	n.m.	4.56±5.26	4.33±5.01	2.28±3.91	n.m.	n.m.
Fe	190.40±55	57.90±13	261.73±162	94.45±44	101.27±103	53.21±7.09	8.80±5.72
Mn	10.06±3.43	37.69±2.54	73.52±43	82.87±53	35.29±13	74.22±15	12.54±1.38
Ni	2.48±1.45	14.48±1.10	74.68±32	336.16±163	90.68±37	30.72±1.35	5.76±0.25
Zn	28.40±0	n.m.	9.30±1.56	11.75±0.78	16.25±21	n.m.	n.m.

n.d. = not detected

n.m. = not measured

Agnes M. = Agnes Mine

Kaapseh . = Kaapsehoop

3.4.3 Total metal content

Table 4: Metal contents (mg/kg ± SD, n>4) after acid digestion of soil samples from different ultramafic sites as measured by atomic absorption spectrometry

	Control	Agnes M.	Barberton	Kaapseh. 1	Kaapseh. 2	Kaapseh. 3	Songimvelo
Co	0.67±0.15	70.4±22.34	95±4.24	345±4.24	498±40	198.30±51	84.2±8.2
Cr	n.d.	480.7±49	996±28	1635±24	2894±0	1481±55	1251.8±33
Cu	8.15±11	n.m.	16.06±5.9	13.33±7.45	10.18±4.01	n.m.	n.m.
Fe	6672±209	n.m.	40640 ±4221	83720±728	113046 ±12284	n.m.	n.m.
Mn	49.94±7.16	1388±14	458±62	1814±17	1904±136	1993.5±13	1915±120
Ni	8.45±2.33	442.1±85	4408±195	13281±151	13926±34	6898.3±761	1139.2±3.96
Zn	48.4±0	n.m.	36.3±2.69	72.75±2.19	107.25±20	n.m.	n.m.

n.d. = not detected

n.m. = not measured

Agnes M. = Agnes Mine

Kaapseh. = Kaapsehoop

All soil samples collected in the Barberton area had elevated levels of metals (Appendix 1-3). The highest total metal contents were found in the samples collected at the Kaapsehoop 2 sampling site, lowest contents in soils collected at the Barberton area (Figure 7).

Concentrations and availability of metals in ultramafic soils were quite variable, even when comparing soil samples such as the Kaapsehoop 1 and 2 collected at locations only 50 m apart from each other (

Table 4).

Except on chromium, correlations were observed between the total amount (acid digested) and mobilisable amount (DTPA extractable) of all analysed metals, having chi-square values ranging between 4.12 (cobalt) and 59.18 (iron) with corresponding $p < 0.05$. No correlation was observed between the $CaCl_2$-extraction and the DTPA-extraction of aluminium (Chi-square=0.67; p=0.41), cobalt (Chi-square=0.37; p=0.54) and manganese (Chi-square=3.29; p=0.07). The correlations of mobile and mobilisable chromium, copper, iron and nickel had a Chi-square value between 6.65 (Fe) and 59.58 (Ni) with corresponding $p<0.01$.

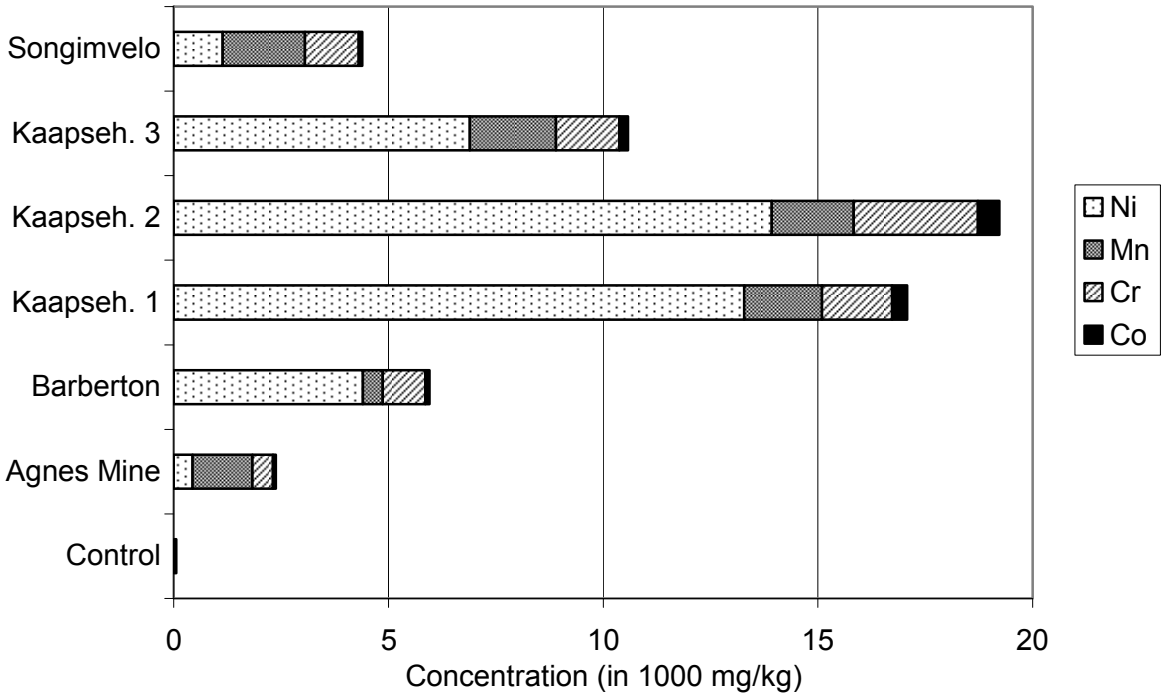

Figure 7: Total concentration of cobalt (Co), chromium (Cr), manganese (Mn) and nickel (Ni) in ultramafic soil samples collected at the Barberton region and a control soil collected at Stellenbosch (Control). Mean values in 1.000 mg/kg.

For chromium, no correlation was found between total metal content and the mobile and mobilisable soil fraction respectively. For nickel, a correlation between all extraction methods was found (Acid – DTPA: Chi-square= 14.02; $p< 0.05$; Acid – $CaCl_2$: Chi-square= 13.50886; $p< 0.05$).

In general, the proportions of metals present in the different soil fractions, in comparison to the total amount of metals measured after acid digestion, were by far higher in the control soils than in all of the ultramafic soils (Figure 8 until Figure 10). The mobile and mobilisable contents of chromium, what was not detected in the control soils, were in all ultramafic soils less than 0.04% of the total amount measured by atomic absorption spectrometry after acid digestions.

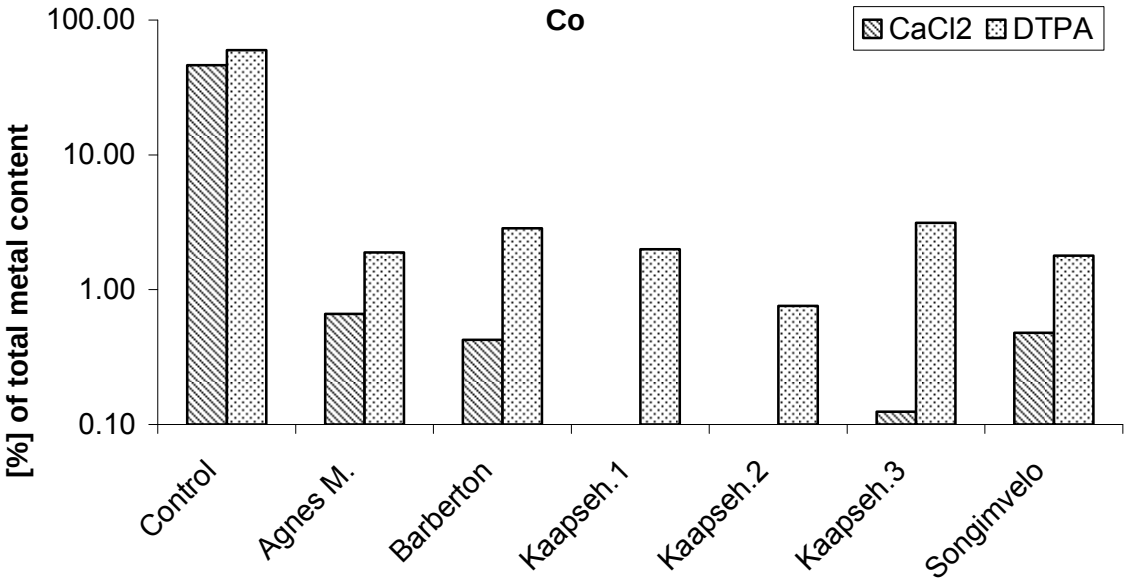

Figure 8: Proportion [%] of metal contained in $CaCl_2$- and DTPA-extract for cobalt (Co). For the purpose of clearness, y-axis is plotted on a logarithmical scale.

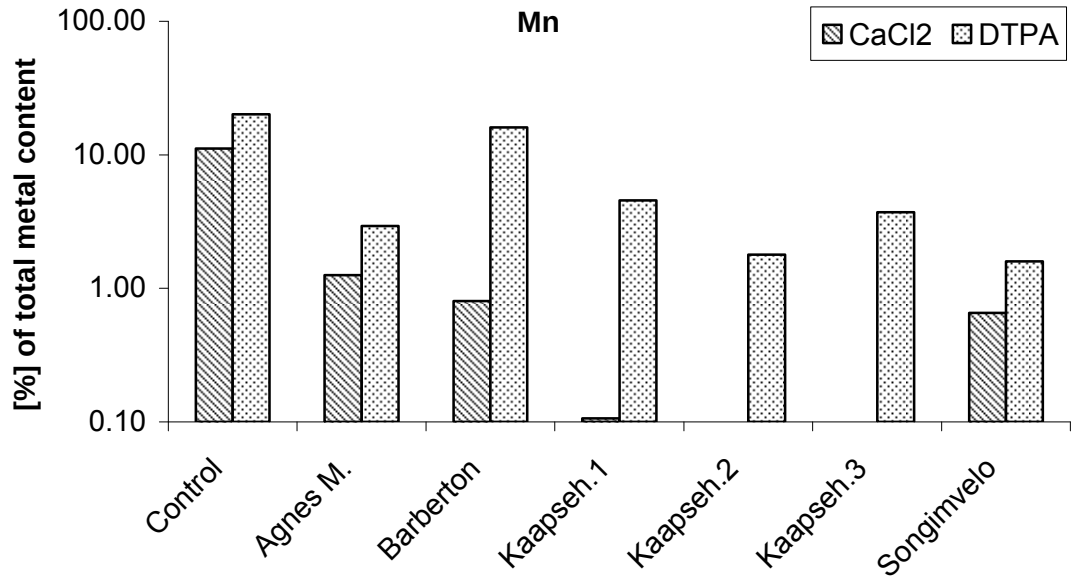

Figure 9: Proportion [%] of metal contained in CaCl2- and DTPA-extract for manganese (Mn). For the purpose of clearness, y-axis is plotted on a logarithmical scale.

Except for cobalt (Figure 8) and manganese (Figure 10), the proportion of metals contained in the presumably bioavailable soil fractions was below 3% of the total amount of metals in the ultramafic soils.

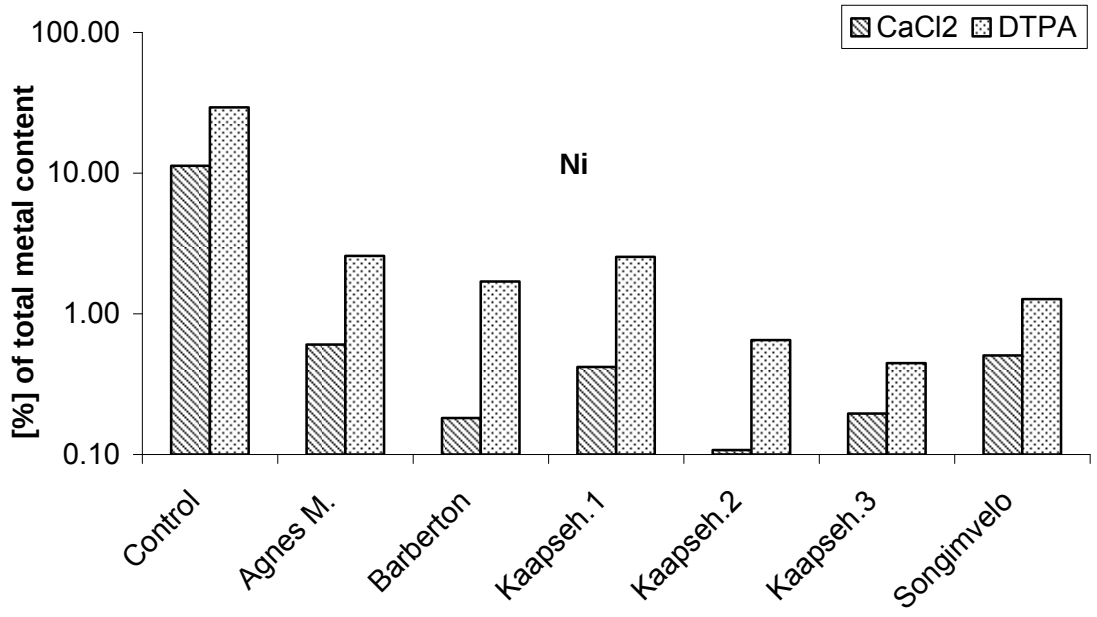

Figure 10: Proportion [%] of metal contained in $CaCl_2$- and DTPA-extract for nickel (Ni). For the purpose of clearness, y-axis is plotted on a logarithmical scale.

3.5 Discussion

From the literature, most of the metals are known to be toxic to soil living organisms at the concentrations measured in the ultramafic soil samples, especially chromium (Lock and Janssen 2002a, Sivakumar and Subbhuraam 2005), cobalt (Fischer and Molnar 1997), manganese (Reinecke and Reinecke 1997b, Gerber et al. 2002, Kuperman et al. 2004) and nickel (Furst et al. 1993, Scott-Fordsmand et al. 1999, Lock and Janssen 2002b, Reinecke and Reinecke 2004b).

The total concentration of the metals analyzed can be considered as exceptionally high (Figure 7), even compared to anthropogenically heavily polluted soils. Notwithstanding, the concentrations measured after acid digestion were within the range reported by numerous publications on ultramafic soils, e.g. by Nemec (1954), Vergnano (1958), Soane and Saunder (1959), Morrey et al. (1994) and Marino et al. (1995). Furthermore, it is widely known that the speciation of most metals is strongly dependent on a wide range of different parameters such as soil pH, mineralogy, humic acid complexing ligands and the presence of silicates and hydrous oxides of other metals (Janssen et al. 2000). For example, the concentration of the divalent nickel ion is strongly influenced not only by the soil pH, but also by the presence of iron and manganese. Especially manganese oxides have a high affinity for metal binding (McKenzie 1977). From pH 5 to 6, the amount of nickel bound to iron oxides strongly increases; whereas from a pH 6 to 7 nickel is increasingly bound to manganese oxides (Theis and Richter 1979). Further, metals known to be often involved in

interactions with other metals are cadmium, chromium, selenium and zinc (Beyer et al. 1982). In ultramafic soils, chromium is assumed to occur mainly as chromite ($FeCr_2O_4$), while small amounts are contained in Fe-Mn concretions (Worst 1960). As chromite is extremely resistant to weathering, only small amounts of the less toxic Cr (III) are hydrolysed to $Cr(OH)_3$ under wet conditions. Cr (III) again is susceptible to oxidation to soluble Cr (VI) only by the reduction of some lower manganese oxides such as hausmannite (Mn_3O_4), braunite (Mn_2O_3) and manganite (MnO_2H), both steps of this two stage-mechanism result in a very slow oxidation of chromium in storage to available toxic Cr (VI) (Cooper 2002), resulting again in a far higher total amount of chromium than the proportion in fact available and – obviously – a strong pH dependency. Due to the unique composition of the ultramafic soils collected at the Barberton area, the proportions of the mobile and the mobilisable concentrations of metals measured in the ultramafic soils are far lower than in the unpolluted, non-mafic soil from Stellenbosch (Figure 8 to Figure 10). This indicates that in spite of the exceptionally high total concentrations of metals, only a comparably small fraction of these metals might be accessible to organisms living in and on the ultramafic soils from the Barberton area. This may explain the presence of earthworms in these soils rather than an increased tolerance or an adapted resistance to the metals found in these soils.

4. Metals in earthworms exposed to ultramafic soils and spiked substrates.

4.1 Introduction

Even though soil can, with respect to the flux rates, not be considered as a transport medium such as water or air, it is often an important source for contamination of groundwater or the atmosphere (Lokke and Van Gestel 1998). Toxicants can be remobilized or immobilized in the process of soil formation, weathering or by anthropogenic action, dissolved in the liquid phase of soil, by atmospheric deposition, or embodied in micro-organisms, plants or animals (Schmitt and Sticher 1991). Often, proportionally high concentrations of metals can be measured by chemical analysis in soil, whereas the total concentrations of toxicants may not necessarily be related to the amount of a toxicant causing an adverse effect on soil organisms (Peijnenburg et al. 1997).

Independent of the concentrations of the metals in soils, the metals need to be taken up into an organism to have a toxic effect (Lanno et al. 1998). This interaction between chemical and target organism is defined as “environmental bioavailability” (Van Straalen and Lokke 1997). The amount of a chemical that is available, or can be made available for uptake, is defined as the “bioavailable fraction” (Peijnenburg and Jager 2003).

4.1.1 Metals in earthworms

Earthworms are not only an integral part of the soil biota, animals from many taxa, including insects, amphibians, reptiles, fishes, birds and mammals, prey on earthworms and thus earthworms are an important pathway for the introduction of soil affiliated toxicants (such as many metals) into higher trophic levels (Gish and Christensen 1973, Eijsackers 2004). It is further known that earthworms do accumulate certain toxicants (Ireland 1979, Ireland 1983, Corp and Morgan 1991, Marinussen et al. 1997, Rida and Bouché 1997b). At this, the toxicant not necessarily has to have an adverse effect on the earthworm itself, but can affect other organisms on higher trophic levels of the food web (Reinecke 1992). Thus, by chemical analysis of earthworms exposed to or collected in soils containing potentially toxic substances such as many metals are, earthworms can serve as an indicator of the bioavailable amount of a selected toxicant (Ma 2005).

4.1.2 Ultramafic soils

Ultramafic soils are shallow, usually low in primary nutrient contents and have a low moisture retaining status (Robinson et al. 1935, Vergnano 1958, Shallari et al. 1998, Parman et al. 2004). These soils deriving from ultramafic rocks are usually loaded with a variety of metals such as cobalt, chromium, manganese and nickel in concentrations toxic to organisms living in and on these soils (Gasser et al. 1994). Earthworms have been found to occur in these soils (Marino et al. 1995, Hubers et al. 2003). The earthworms living in these soils consequently need to be able to overcome the so called “serpentine challenge” (Proctor 1999) - “serpentine” is a term referring to the ferromagnesian minerals characterizing ultramafic rocks (Proctor and Woodell 1975). With regard to the metals in these soils, earthworms either need to be able to tolerate these extremely

high concentrations, or the amount of metals actually bioavailable to the earthworms is substantially smaller than the total amount present in these soils.

4.1.3 Tolerance of earthworms to metal loaded soils

Earthworm populations inhabiting ultramafic soils loaded with high concentrations of various metals over many generations, even centuries or millennia, may be challenged to adapt to these adverse conditions by evolving an increased tolerance towards metals. On individual level, an increased tolerance towards metals can be based on a genetically inherited adaptation or an acclimatory tolerance (Posthuma and Van Straalen 1993). The distinction between an increased tolerance (also sometimes called resistance in the literature) and adaptation on individual level is often difficult (Marino and Morgan 1999). Acclimatory tolerance can also involve behavioural mechanisms, modification of physiological mechanisms and ecophysiological stress may also result in a different gene expression (Stürzenbaum et al. 1998a, Galay-Burgos et al. 2003). Also, although several studies have been conducted on inherited resistance of earthworms towards metals, so far it was impossible to confirm that the tolerance observed was genetically inherited or the result of physiological acclimation (Reinecke et al. 1999, Aziz et al. 1999, Langdon et al. 2003a, Rozen 2006). Independent of the question whether an increased tolerance is the result of a genetic or an ecophysiological resistance, it may influence the uptake and excretion kinetics of metals in earthworms (Posthuma and Van Straalen 1993). A number of studies have demonstrated the presence of cross-resistance, i.e. that a tolerance to one compound renders another, usually toxic, substance harmless or at least influences its toxicity in different invertebrates (Sauphanor and Bouvier 1995, Willuhn et al. 1996, Bisset et al. 1997, Ahmad et al. 2003)., Spurgeon and Hopkin (2000) demonstrated that *E. fetida* pre-exposed to zinc shows a similar increment in tolerance to copper if exposed to elevated concentrations of copper. The perceived “resistance” could actually be the result of changes in the uptake kinetics of metals resulting in changes in body burdens because of interferences or interactions between metals.

4.1.4 Choice of test species

The epigeic (litter dwelling) earthworm *Eisenia fetida* (Savigny 1826) is recommended by different ecological guidelines as a reference test species for soil toxicity testing (EEC 1982, EPA 1982, OECD 1984, EEC 1985, ISO 1998, OECD 2004) and most probably the most commonly used species in soil toxicology. Nevertheless, the selection of *E. fetida* as a test species in toxicity tests is often criticised. *E. fetida* is a compost dweller and therefore as being “not a typical earthworm” (Edwards and Coulson 1992) not seen as representative of many other soil-dwelling species (Weeks et al. 2006), or as too insensitive to many chemicals (Fitzpatrick et al. 1996, Davies et al. 2003a).

Despite a low morphological and taxonomical variability within the suborder Lumbricida (Morgan et al. 1993), several studies show significant differences in sensitivity and metal uptake between

different species of earthworms, sometimes even within the same ecophysiological class (Streit et al. 1990, Stenersen et al. 1992, Marino et al. 1992, Spurgeon and Hopkin 1996b, Morgan and Morgan 1999). For that reason, two ecophysiologically different species of earthworms were used in this part of the present study. As a second species, the endogeic earthworm *Aporrectodea caliginosa* (Savigny 1826), was selected, a variable species living in a broad range of different soils, often found on cultivated land and in gardens (Simms and Gerard 1985). It is considered as a key species in decomposer communities in many soils as it mixes the soil layers by its burrowing activities and by taking its nutrition from the soil organic matter (Edwards and Bohlen 1996). Thus is probably a more relevant species for field related ecotoxicological test setups (Lukkari et al. 2005).

4.1.5 Aims

The primary aim of this part of the study was to determine the extent to which metals in ultramafic soils may be bioavailable for the two ecophysiological different earthworm species *Aporrectodea caliginosa* and *Eisenia fetida*.

In order to do so, the body burdens of metals of the worms were measured after exposure to ultramafic soils. This will provide a more ecologically relevant indication of the direct bioavailability rather than measuring total soil concentration. By determining the actual amount of metals accumulated by the earthworms exposed to ultramafic soils originating from the Barberton Greenstone Belt rock formation a more reliable assessment can be made of the worm's ability to survive in these soils.

To investigate if the two earthworm species differ in the distribution of metals in the body, the anterior region, mid section and posterior sections of specimens of both species were analyzed separately.

To evaluate if a history of previous exposure has an influence on the metal body burden, earthworms of the species *E. fetida* long-term exposed to nickel and cadmium were exposed to the ultramafic soils. Nickel pre-exposed specimens were selected since earthworms in the ultramafic environment may be chronically exposed to high concentrations of nickel. Cadmium long-term exposed worms were selected to investigate if there is an influence specific to a metal occurring in these soils. The aim was also to see whether these influences, if present, could be the result of exposure to other metals as well.

Additionally, to evaluate whether earthworms bioaccumulate nickel, a metal occurring at high concentrations in ultramafic soils. The bioaccumulation rate for *E. fetida* was investigated by exposing *E. fetida* to different concentrations of nickel, and for comparison purposes, to manganese. The comparison of the ratio of a defined metal in soil or substrate to the ratio of the same metal by means of the application of a bioaccumulation factor in soil invertebrates is difficult. A range of edaphic factors strongly influence the biological availability and uptake (Phipps et al. 2002). Hence, most of the influences of soil related edaphic factors were excluded for this test. For

that purpose, this exposure was conducted in reconstituted water resembling ground water (Kiewiet and Ma 1991).

4.2 Material and methods

4.2.1 Animals

The specimens of *E. fetida* used in this study were obtained from a synchronized culture, bred from stock cultures of the Stress Ecology Laboratory of the Department of Botany and Zoology at the University of Stellenbosch.

The specimens with a history of previous exposure were also obtained from cultures of the Stress Ecology Laboratory. One of these cultures was fed for at least three years on a biweekly basis with 0.01% $NiCl_2$ added to their food, the second culture of *E. fetida* biweekly with urine free cattle manure with 0.01 % of $CdSO_4$ added.

Aporrectodea caliginosa specimens were collected at a site close to the Vergenoegd sports ground, Stellenbosch, Western Province, South Africa. This site has a known history of no metal pollution and pesticide application (Maboeta et al. 2003). The specimens of *A. caliginosa* were exposed immediately after gut depuration.

4.2.2 Substrate preparation

4.2.2.1 Soil exposures

The ultramafic soil samples for the soil exposures of the two earthworm species *Aporrectodea caliginosa* and *Eisenia fetida* were collected from different sampling sites in the Barberton area (Mpumalanga, South Africa). Three soil sampling locations were situated close to the Kaapsehoop Chrysotile Mine (Kaapsehoop 1, 2 and 3), one sampling location was in the Barberton Nature Reserve (Barberton). Further, soil samples from two different locations collected by Dr. J. Mesjasz-Przybylowicz, were used in this study. These samples were collected near Agnes Mine (Agnes Mine) (Mesjasz-Przybylowicz et al. 2001a) and close to the Songimvelo Nature Reserve (Songimvelo) (Mesjasz-Przybylowicz et al. 2001b) The ultramafic sampling locations were characterised by a very heterogenic soil structure within a small area.

As an unpolluted control site the site close to the Vergenoegd sport ground next to the Eersterivier in Stellenbosch (Western Cape) was selected (see above).

Soil samples were collected by randomly extracting five sub samples of 50 cm^3 one meter apart on a plot of 5 m^2.

All soil samples were dried (48h, 70°C, Gallenkamp size one oven BS, model OV-330, Weiss-Gallenkamp Ltd., Loughborough, U.K), sieved (5- and 2 mm^2 screens Baird & Tatlock, London, U.K.) and filled into plastic containers (15 x 10 x 5cm). Per replicate, 400g of soil substrate was moistened with distilled water to a moisture content of 60-65% on a dry soil basis and incubated for 48h to stabilise prior to the introduction of earthworms. For aeration, small aeration holes were drilled into the lids of the containers. For the exposure of the earthworm *E. fetida*, 40g (10%) of

grinded and dried urine free cattle manure was mixed as an organic food source into the substrate prior to the start of the exposure. All worms were kept for 48 h on moist filter paper to depurate the gut content before the start of exposure (Krontowsky and Rumiauzew 1922).

4.2.2.2 Artificial ground water

The substrate *for E. fetida* was made up according to the protocol proposed by Kiewiet and Ma (1991). The artificial ground water was made up by dissolving 100 mg $NaHCO_3$, 20 mg $KHCO_3$, 200 mg $CaCl_2$ * $2H_2O$ and 180 mg $MgSO_4$ per litre of distilled water. This substrate was then spiked with 8.5 17.0, 34.1, 68.1 and 136.2 mg/L manganese as manganese sulphide ($MnSO_4$ • $7H_2O$) and nickel as nickel chloride ($NiCl_2$ • $6H_2O$).

4.2.3 Exposure of animals

E. fetida was exposed to the soils from Barberton and Kaapsehoop 1 - 3, whereas the pre-exposed *E. fetida* were exposed only to the Kaapsehoop 3 soils. Twelve individuals per container and four replicates per soil were used for *E. fetida*. Because of the addition of the cattle manure prior to the start of the exposure, worms did not receive additional food for the first three months of exposure.

Of *A. caliginosa*, ten specimens per replicate were exposed to soils from each of Agnes Mine, Songimvelo and Kaapsehoop 3. As a control, specimens of both species were exposed to the unpolluted soils from Stellenbosch.

Six specimens of *E. fetida* were exposed for 48 hours to different concentrations of $NiCl_2$ and $MnSO_4$ in the artificial ground water substrate. All exposures were conducted in a climate and moisture controlled chamber at 20°C without light.

4.2.4 Metal analysis

Prior to the start of the exposure and every second week of exposure thereafter, at least three worms were removed from each soil exposure for analysis of body metal concentrations. Of the *Eisenia fetida* specimens exposed to the artificial ground water, four specimens exposed to every concentration of nickel and manganese and the control were used for analysis.

Additionally, after four weeks of soil exposure, specimens of both species, eight per replicate of *A. caliginosa* and four per replicate of *E. fetida*, were cut into three pieces. The “anterior section” comprised the anterior region excluding the clitellum (the first 25-30 segments of *A. caliginosa* and the first 23 segments of *E. fetida*), the “middle section” including the clitellum (up to segment 80 – 120 depending on the number of segments of *A. caliginosa,* and up to segment 50-80 of *E. fetida*) and the “posterior section” comprising the remaining posterior region. Every section was analyzed separately for cadmium, cobalt, chromium, manganese and nickel to determine the longitudinal distribution of metals in the earthworm body.

The metal body burden was determined by atomic absorption spectrometry after acid digestion (Katz and Jenniss 1983). The acid digestion followed the same principle as described in Chapter

3.2.4. First, the samples were left overnight in 10 mL of 10% nitric acid (HNO_3). The following day, samples were heated twice, first for 2 hours at 40-60°C and thereafter for 120-130°C for an hour. Then 1 mL of 70% perchloric acid ($HClO_4$) was added and the vials were reheated to 120-130°C for about an hour. After cooling down, 5 mL of distilled water was added to the samples. After reheating to 120-130°C, samples were left overnight to cool down and finalize digestion. The solutions were then filtered through Whatman #6 filter paper into 20 mL flasks, and the flasks were filled to the 20 mL mark with distilled water. The 20 mL solutions were then microfiltered through 0.45 µm Sartorius cellulose nitrate filter paper into polyvinyl containers. Samples were stored in light protected, dark containers until spectrophotometrical analysis.

The worms were analyzed spectrophotometrically for aluminium, arsenic, cadmium, chromium, cobalt, copper, iron, manganese, nickel, lead and zinc (Varian AA-1275 flame atomic absorption spectrophotometer; Varian Inc., Palo Alto, California, USA). One blank, consisting of 10 mL nitric acid, 1 mL perchloric acid and 5 mL of distilled water was measured per ten samples. The appropriate standards made up at the Department of Physics at the University of Stellenbosch. The detection limits for the atomic absorption spectrometry reading were: 0.7 µg/mL for aluminium, 1 µg/mL for arsenic, 0.01 µg/mL for cadmium, 0.06 µg/mL for chromium, 0.07 µg/mL for cobalt, 0.04 µg/mL for copper, 0.07 µg/mL for iron, 0.11 µg/mL for lead, 0.3 µg/mL for manganese, 0.08 µg/mL for nickel and 0.01 µg/mL for zinc (Varian 1979).

4.2.5 Mathematical and statistical analysis

The metal content of earthworms was calculated by:

$$Amount \quad in \quad worm \quad [mg/kg] \quad = \frac{AA-reading \quad * \quad volume \quad of \quad sample \quad [mL]}{mass \quad of \quad sample \quad [g]}$$

According to Van Hook (1974), metal biomagnification occurs if the ratio between the metal concentration in the tissue and in the substrate exceeds 1.0. This definition has some weaknesses as it does not take cognisance of the fact that a toxicant can be present at far higher concentrations in a substrate than the amount actually biologically available, nor of the interactions between different soil components or the normal physiological level of a metal already in the body (Hartenstein et al. 1980a). Nevertheless, this definition was used with regard to the exposures to the artificial ground water as most of the edaphic factors are assumed to be excluded. Thus, the biomagnification factor was calculated by dividing the amount of metal in the earthworm by the amount of metal in the aqueous substrate (Ireland 1975, Cortet et al. 1999).

The Lillifors test for normality was conducted to test for parametric/non-parametric distribution. For datasets normally distributed, a one-way-ANOVA was applied and as a post-hoc test, Fishers LSD test was conducted. For datasets not normally distributed, a Kruskal-Wallis ANOVA was applied to determine significant differences in the body load between the exposures. As a post-hoc test for the Kruskal-Wallis-ANOVA, a multiple comparison of z' and p-values was conducted (Siegel and

Castellan 1988). For the evaluation of significant differences between the content of the different metals within the worms, a generalized linear model was applied. As a test for significance, the Wald statistic was used. The Wald statistic, as a test of significance of the regression coefficient, is based on the asymptotic normality property of maximum likelihood estimates and tested against the Chi-square distribution (Dobson 1990). Differences were regarded significant if $p > 0.05$ for all statistical tests.

4.3 Results

4.3.1 Metal content of earthworms collected at the Barberton area

Two earthworms, collected on a previous field trip by Dr. J. Mesjasz-Przybylowicz in the Barberton area, were, as a preliminary test, analyzed for cadmium, cobalt, chromium, manganese and nickel (Table 5). Unfortunately, both worm samples were frozen for an undefined period of time and the specimens were too disintegrated to be identified or to determine the genus. Further, the location of the sampling site is not known exactly. No direct comparisons to the earthworms exposed in the laboratory to the ultramafic soils, collected at the Barberton area, can be drawn. Consequently, no information about the background metal concentrations at the specific collecting sites were available, as the ultramafic soils are extremely heterogeneous in their content.

Table 5: Metal concentrations [mg/kg] in earthworms of two unidentifiable species collected in ultramafic soils in the Barberton area. Collection site refers to the names of sampling locations as given by Mesjasz-Przybylowicz (personal communication).

Collection site	Cadmium	Chromium	Cobalt	Manganese	Nickel
Barberton	n.d.	4.71	9.65	57.65	28.24
Kaapsehoop	n.d.	33.85	10.77	283.08	126.15

n.d.: not detected

4.3.2 Metal concentrations in earthworms exposed to ultramafic soils

In the six specimens of *A. caliginosa* and *E. fetida* analyzed for chromium, manganese and nickel prior to the exposure, no metals were detected.

4.3.2.1 Cadmium in the earthworms exposed to ultramafic soils

With the analytical measurements conducted with *A. caliginosa* exposed for 24 weeks, no cadmium was detected in any of the samples. In the soils the earthworms were exposed to, cadmium was detected only in the Kaapsehoop 3 sample (67±9.9 mg/kg Cd; Chapter 3.3). Due to the fact that no worms survived the whole exposure period of 24 weeks in the Songimvelo soils, no measurements on worms exposed to these soils were conducted at week 24. The concentrations of cadmium are shown in Appendix 2-1 and in Figure 11.

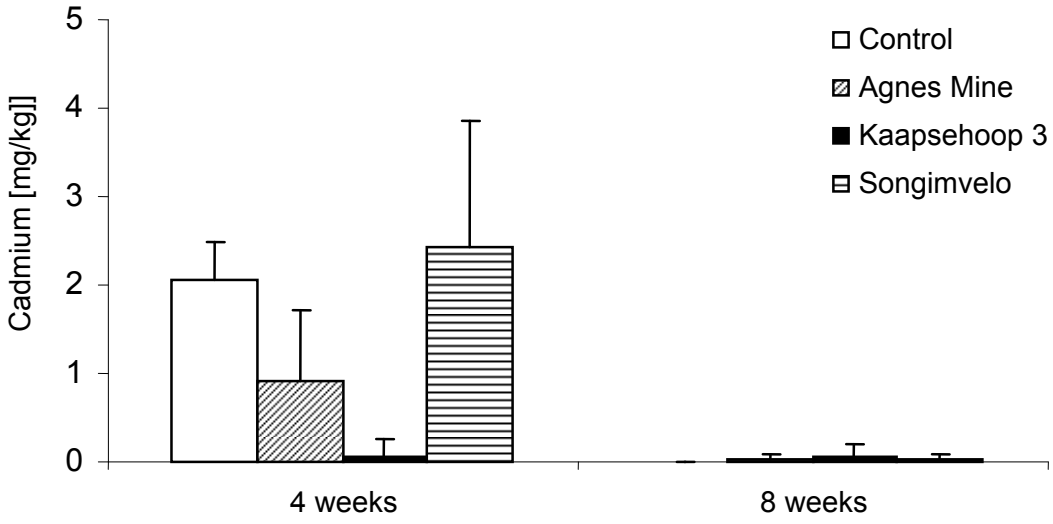

Figure 11: Mean cadmium concentrations (mg/kg) measured in *Aporrectodea caliginosa* specimens after being long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control) over a period of 24 weeks; no cadmium was detected after eight weeks. Whiskers indicate standard deviation.

In general, the concentrations of cadmium were low; the highest concentration, 4.77 mg/kg Cd, was measured after an exposure duration of four weeks in the *A. caliginosa* specimens exposed to soil collected at Songimvelo. After four weeks, only the cadmium concentrations in the earthworms exposed to the Kaapsehoop 3 soils were found to be significantly different from all the other exposures ($H=20.71$; $p<0.05$). After eight weeks, the Kruskal-Wallis-ANOVA on ranks did not reveal any difference between the exposures. Further, except at the exposures to Kaapsehoop 3 soils ($Z<0.1$; $p>0.05$), there were significant decreases of cadmium in all exposure groups from week four to week eight ($H=28.65$; $p<0.05$).
No cadmium was detected in any of the *E. fetida* neither in the ultramafic soils nor in the control soil from Stellenbosch.

4.3.2.2 Chromium in earthworms exposed to ultramafic soils

The Chromium concentrations measured in the bodies of *A. caliginosa* are shown in Appendix 2-2 and in Figure 12.
Between the exposure groups and the control group of *A. caliginosa*, pooled over the exposure period, the Multiple Comparisons of mean ranks for all groups, conducted as a post-hoc test for the Kruskal-Wallis ANOVA revealed a significant difference between the tissue concentrations in animals exposed to the control soils and the tissue concentrations in the animals exposed to the ultramafic soils ($p<0.05$). The control group had the lowest chromium concentrations after four weeks (4.25±0.93 mg/kg) and eight weeks (1.41±1.53), whereas after 24 weeks, no chromium was detected in any of the worms exposed to the Kaapsehoop 3 soils. After four and after eight weeks,

the concentrations of chromium in the *A. caliginosa* specimens exposed to the soils collected at Agnes Mine and at Kaapsehoop 3 were significantly higher than in the controls (H=12.75 for four weeks, H=12.18 for eight weeks; $p<0.05$), and after 24 weeks, only the chromium concentrations in the earthworms exposed to Agnes Mine were significantly different to those in the other groups (H=14.09; $p<0.05$).

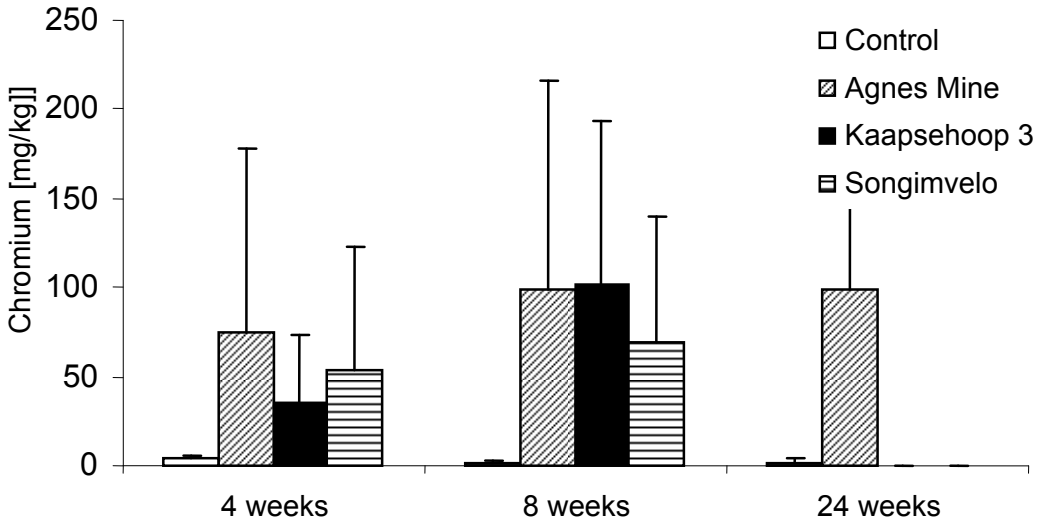

Figure 12: Mean chromium concentrations (mg/kg) measured in *Aporrectodea caliginosa* specimens after being long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control) over a period of 24 weeks. Whiskers indicate standard deviation.

During the exposure period, the multiple comparisons of mean ranks revealed a significant decrease in chromium tissue concentration only in the Kaapsehoop 3 sample from week four to week eight and to week 24 (H=14.43; $p<0.05$). Comparing the concentration of chromium in the tissue of *A. caliginosa* with the $CaCl_2$, DTPA and acid extractable amount of chromium in the soils (Chapter 3.3), the concentrations in the worms exposed to the ultramafic soils were significantly higher than in the $CaCl_2$ and DTPA extractable soil fractions, but also significantly lower than in the total, acid extractable soil fraction except in the Kaapsehoop 3 exposure after 24 weeks, where no chromium was detected in these worms. The Spearman Rank Order Correlation did not reveal any correlations between extraction methods and body burden of chromium in *A. caliginosa*.

The chromium concentrations measured in *A. caliginosa* exposed to the ultramafic soils were continuously characterized during the whole exposure period by extremely high individual variations. In most cases the standard deviation was observed to be higher than the mean concentrations and large differences between median and means were recorded.

The concentrations of chromium in the *E. fetida* specimens exposed to the ultramafic soils collected at the Barberton area and to the unpolluted Stellenbosch soils are shown in Appendix 2-3 and Figure 13.

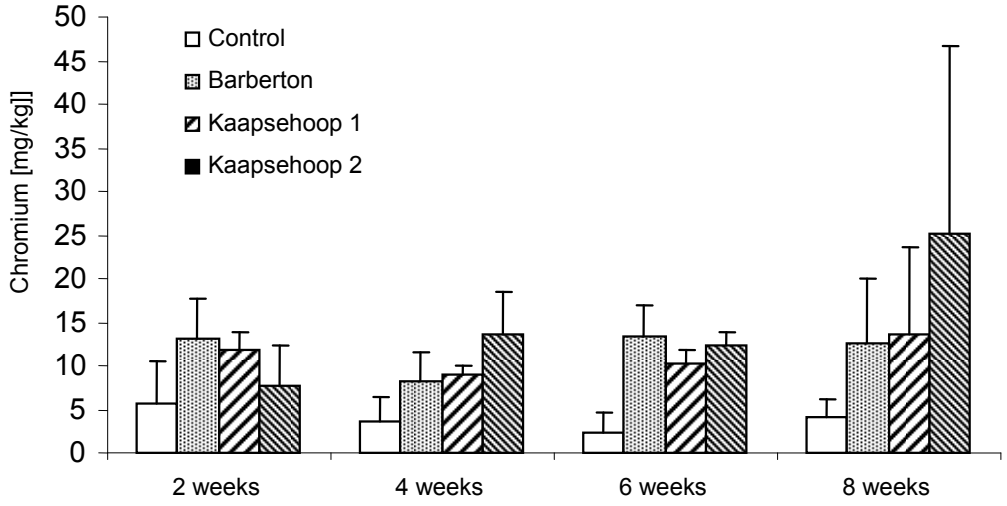

Figure 13: Mean chromium concentrations (mg/kg) measured in *Eisenia fetida* specimens which were long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control). Whiskers indicate standard deviation.

For chromium, the mean concentrations in *E. fetida* ranged between 2.25±2.27 and 25.10±21.45 mg/kg, the mean concentration of all groups was 10.74±8.93 mg/kg Cr (Appendix 2-3). The Kruskal-Wallis ANOVA showed that the chromium concentrations in the worms exposed to the control was significantly different to those of the worms exposed to the ultramafic soils ($H=27.74$; $p<0.05$). After eight weeks of exposure, the mean chromium concentration in the worms exposed to the Kaapsehoop 2 soils increased from 12.26±1.51 to 25.10±21.45 mg/kg chromium (Appendix 2-3). No significant differences ($p>0.05$) were recorded within the different groups of worms exposed to any of the soils during the course of the exposures. No correlation between the chromium concentration in the earthworms and in the different soil fractions was found. Except in the control soil, the concentrations of chromium measured in the earthworms were in all cases significantly higher than concentrations of mobilisable (DTPA-extractable) chromium and significantly ($p<0.05$) lower than in the acid digested soil samples.

4.3.2.3 Cobalt in earthworms exposed to ultramafic soils

After 24 weeks of exposure, no cobalt was detected in any of the *A. caliginosa* specimens.

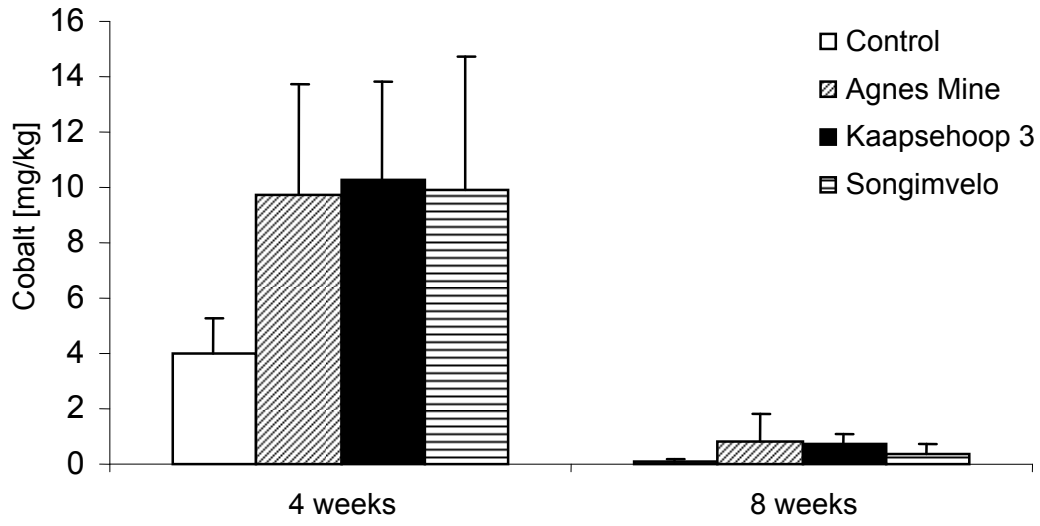

Figure 14: Mean cobalt concentrations (mg/kg) measured in *Aporrectodea caliginosa* specimens after being long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control) over a period of eight weeks. Whiskers indicate standard deviation.

Appendix 2-4 and Figure 14 show the cobalt concentrations in the specimens of *A. caliginosa* over the observation period of eight weeks. After four weeks of exposure, the mean concentration of cobalt in the earthworms exposed to the control soils was with 3.98±1.33 mg/kg, significantly lower than in the *A. caliginosa* exposed to ultramafic soils ($H=15.15$; $p<0.05$). After eight weeks, the Kruskal-Wallis Analysis of Variance on Ranks did not reveal any significant differences between the different ultramafic exposures and the control. From week four to week eight, in all exposures a significant decrease ($p<0.05$) was observed using the Mann-Whitney-U-test. No correlation between cobalt concentrations in worms and in the different soil fractions were found, neither after four weeks of exposure nor after eight weeks of exposure. Also, no relation between cobalt concentrations in *A. caliginosa* and the different soil fractions was found.

The concentrations of cobalt in *E. fetida* after an exposure duration of 14 weeks varied between 1.64 mg/kg in the control and 7.12 mg/kg in the worms exposed to the Kaapsehoop 2 soils and the Spearman Rank Order Correlations showed a correlation to the DTPA extractable and the acid extractable amount of cobalt in the soils ($R=0.52$ for the DTPA extraction, and $R=0.97$ for the acid extraction). The cobalt concentrations in the worms exposed to the ultramafic soils at the end of the exposure were found to be in about the same range as the amounts measured after DTPA extraction (Appendix 1-2).

In the control soil cobalt was detected at a concentration of 1.63 mg/kg only after three to four worms were pooled together to a total weight of approximately 1 g for acid digestion at the end of the exposure (after 14 weeks). Due to the fact that not enough animals were left to analyse more pooled groups, only a single analysis of pooled worms was conducted and thus no statistical analysis was possible.

4.3.2.4 Copper in earthworms exposed to ultramafic soils

No copper was detected in *A. caliginosa*.

In *E. fetida,* copper was only detected after three to four worms were pooled together for acid digestion at the end of the exposure (after 14 weeks). Alas, not enough animals were left to conduct further analysis of more pooled groups. Thus, only a single analysis of pooled worms was conducted and thus no statistical analysis was possible (Table 6).

The copper concentrations of *E. fetida* after 14 weeks varied between 1.9 mg/kg in the worms exposed to the Kaapsehoop 2 soils and 2.94 mg/kg in the worms exposed to the Stellenbosch control soils. In general, the copper concentrations were found to be in the same range as the DTPA extractable amount from the soil samples (Appendix 1-2), but using the Spearman Rank Order Correlation, no correlation between the copper concentrations in the earthworms and in the soil was found.

Table 6: Copper concentrations in *Eisenia fetida* after 14 weeks of exposure and total, DTPA and $CaCl_2$ extractable metal contents (adapted from Chapter 3) of the soil samples (mg/kg)

Exposure	Worm [mg/kg]	$CaCl_2$ [mg/kg]	DTPA [mg/kg]	Acid [mg/kg]
Control	2.94	0.15±0.17	5.15±8.82	8.15±10.55
Barberton	2.13	0.06±0.06	4.56±5.26	16.06±5.89
Kaapsehoop 1	2.67	0.06±0.06	4.33±5.01	13.33±7.45
Kaapsehoop 2	1.90	0.03±0.06	2.28±3.91	10.18±4.01

4.3.2.5 Manganese in earthworms exposed to ultramafic soils

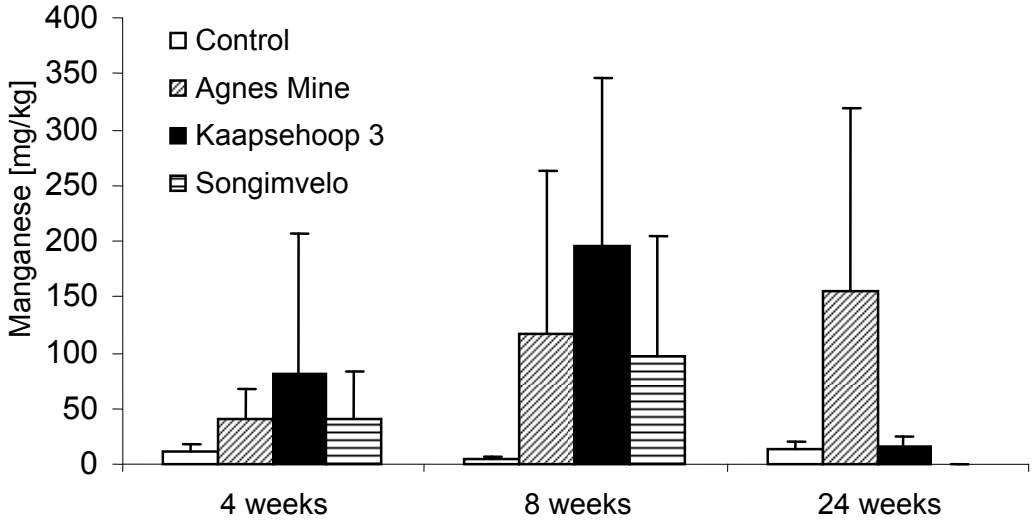

Figure 15: Mean manganese concentrations (mg/kg) measured in *Aporrectodea caliginosa* specimens after being long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control) over a period of 24 weeks. Whiskers indicate standard deviation.

The manganese concentrations (mg/kg) in *A. caliginosa* are shown in Appendix 2-5 and in Figure 15. After log-transformation, the concentrations of manganese in *A. caliginosa* were normally distributed, thus a one-way- ANOVA was conducted ($F=4.52$; $p<0.05$) and as a post-hoc test, Fishers LSD test was applied (Appendix 2-6). After four weeks of exposure, no statistically significant differences between the control group and the ultramafic exposures were recorded, but after eight weeks of exposure, the tissue concentrations of manganese in all ultramafic exposures were significantly ($p<0.05$) higher than in the control. After 24 weeks of exposure, only the body load of the worms exposed to the soils collected at Agnes Mine were still significantly higher than in the control.

A significant increase of the manganese concentrations in the earthworm tissues within the different exposure groups was reported over the period of the exposure only from week four to week eight ranging from 81.11±126.59 mg/kg manganese to 195.42±151.01 mg/kg in the *A. caliginosa* specimens exposed to the Kaapsehoop 3 soils (Appendix 2-5). A significant decrease was observed in the worms exposed to the same soils from week eight to week 24 (15.99±7.91 mg/kg). Due to the extremely high variation, it was not possible to evaluate any correlation between manganese concentration in the earthworms and in the different soil fractions. With regard to Chapter 3.3, one can see that the manganese concentrations in *A. caliginosa* were substantially lower than the total amounts of manganese in the corresponding soils.

The concentrations of manganese in the *E. fetida* specimens exposed to the ultramafic soils collected at the Barberton area and to the unpolluted Stellenbosch soils are shown in Appendix 2-7 and in Figure 16.

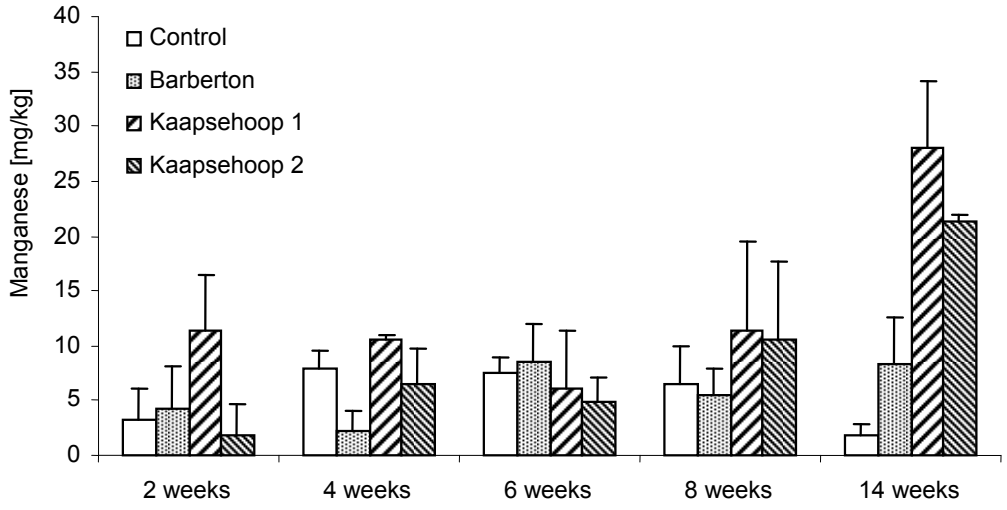

Figure 16: Manganese concentrations (mg/kg) measured in *Eisenia fetida* specimens which were long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control). Whiskers indicate standard deviation.

The lowest mean concentrations of manganese were measured at the end of the exposures in the worms exposed to the control soils (1.73±1.20 mg/kg Mn); the highest concentrations, 27.98±6.09 mg/kg Mn were found at the end of the exposure in the *E. fetida* exposed to the soil sample collected at Kaapsehoop 2 (Appendix 2-7). The Kruskal-Wallis ANOVA revealed that the manganese concentrations on week two were only significantly different between worms being exposed to the Kaapsehoop 1 and 2 soils ($H=10.59$; $p<0.05$). The Median Test, performed simultaneously by the statistical program package, rejected a significant difference within the exposures at week two. At week four, a significant difference was computed by the post-hoc test following Kruskal-Wallis One Way ANOVA between Kaapsehoop 1 and Barberton ($H=8.23$; $p<0.05$). Also in this case, the Median Test did not find significant differences ($p<0.05$). On week six and eight, no significant differences in concentration between exposed worms and/or control worms were observed. Only after 14 weeks of exposure the manganese concentrations in the earthworms exposed to the Kaapsehoop 1 sample were significantly different ($H=14.12$; $z'=3.56$; $p<0.05$) to those of the control. Further, no correlation between the manganese concentrations in the *E. fetida* and the different soil extraction methods was found.

4.3.2.6 Nickel in earthworms exposed to ultramafic soils

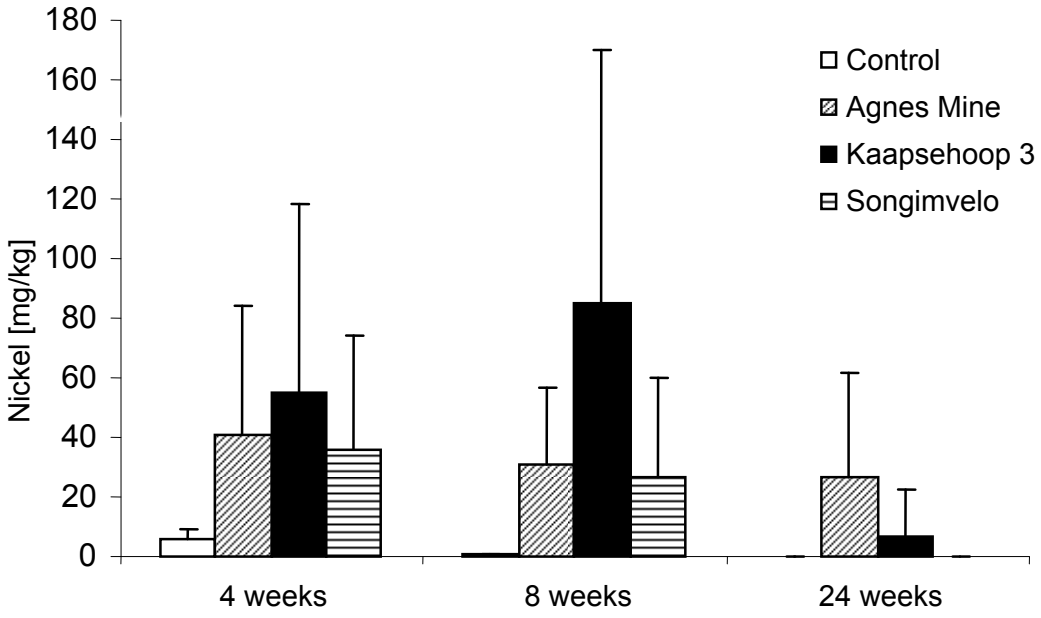

Figure 17: Mean nickel concentrations (mg/kg) measured in *Aporrectodea caliginosa* specimens after being exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control) over a period of 24 weeks. Whiskers indicate standard deviation.

The mean nickel concentrations measured in the tissues of *A. caliginosa* ranged between 85.02±84.63 mg/kg Ni measured in specimens exposed for eight weeks to Kaapsehoop 3 soils and concentrations below detection limit as measured after 24 weeks of exposure in worms from the control soils (Figure 17 and Appendix 2-8). Like the body concentrations of manganese in the *A. caliginosa* specimens exposed to the control soil from Stellenbosch and the ultramafic soils from the Barberton region, the concentration values for nickel were log-transformable to a normal distribution. Thus, also in this case, a one-way ANOVA and a Fisher's LSD test, as a post-hoc test, was applied (Appendix 2-9). For data not log-transformable, a Mann-Whitney-U-test was applied. The Fishers LSD test showed that after exposure periods of four and of eight weeks, the concentrations of nickel were significantly higher in the earthworms exposed to the ultramafic soils than in the worms exposed to the control soils ($p<0.05$). After 24 weeks, no difference between control and ultramafic exposures was revealed. During the whole exposure period, no significant difference between the ultramafic exposures was determined. Also, in relation to the exposure time, no significant change over time was detected in the ultramafic exposures. An extremely high standard deviation was observed in the concentration of nickel in the body of the different specimens of *A. caliginosa* exposed to the same ultramafic soils. In most cases, the standard deviation was higher than the median of the concentration indicating a high individual variability.

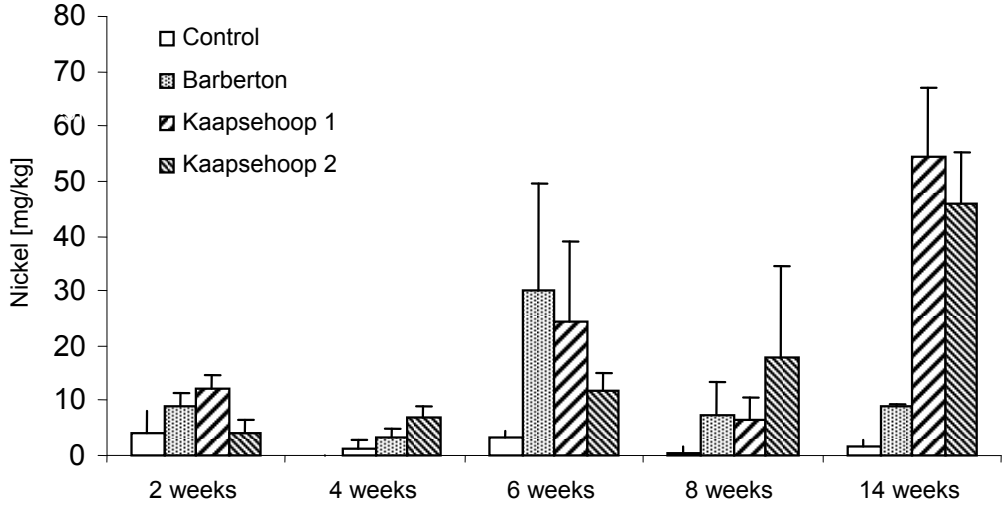

Figure 18: Mean nickel concentrations (mg/kg) measured in *Eisenia fetida* specimens which were long-term exposed to different ultramafic soils collected at the Barberton area and an unpolluted soil from Stellenbosch (Control). Whiskers indicate standard deviation.

The nickel concentrations of *E. fetida* specimens are shown in Figure 18 and Appendix 2-10. The lowest values, below detection limit were recorded after four weeks of exposures in the control, the highest nickel concentrations, 54.22±12.98 mg/kg were recorded at the end of the exposure in the Kaapsehoop 1 sample. After two weeks of exposure, the nickel concentrations in the bodies of *E. fetida* specimens exposed to Kaapsehoop 1 were significantly higher than in the control ($z'=3.27$) and in the specimens exposed to the Kaapsehoop 2 ($z'=3.18$) sample ($H=15.17$; $p<0.05$). After four weeks, the Kruskal-Wallis ANOVA showed a significant difference between the Kaapsehoop 2 exposures (6.91±2.11 mg/kg Ni) and the control (no nickel detected), but the Median Test did not support a significant difference, similar to the nickel body burden analysis after six weeks, where no difference was observed by means of the Median test, but the Kruskal-Wallis ANOVA found a significantly higher nickel concentration in the worms exposed to the Barberton soils compared to the control ($H=9.95$; $z'=2.90$; $p<0.05$). After eight weeks, the body load of the earthworms exposed to the Kaapsehoop 2 soils was found to be significantly higher than in the control ($H=10.85$; $z'=3.02$; $p<0.05$). At the end of the exposure, after 14 weeks, the concentrations of nickel in the bodies of *E. fetida* exposed to the two soil samples containing the highest total amount of nickel, Kaapsehoop 1 and 2, were measured significantly different ($H=13.06$; $p<0.05$) than in the specimens exposed to the control soils and to the soils collected at the Barberton Nature Reserve. For the nickel concentrations in the earthworms after 14 weeks of exposure, a significant correlation between the body burden and the concentration measured after all three different extraction methods in the soils (Chapter 3 and Table 7) was obtained. The concentration of nickel in the earthworms was found to be comparable to the $CaCl_2$ extracted concentrations in the

Barberton and the Kaapsehoop 1 exposure. The concentration in the control worms was found to be comparable to the DTPA extract. No similarities between the body burden of the worms exposed to the Kaapsehoop 2 soil and any of the extraction methods were revealed.

Table 7: Nickel concentrations in *Eisenia fetida* after 14 weeks of exposure to ultramafic soils and a control soil from an unpolluted site and total, DTPA and $CaCl_2$ extractable metal contents (values adapted from Chapter 3) of the soil samples (mg/kg)

Exposure	Worm [mg/kg]	$CaCl_2$ [mg/kg]	DTPA [mg/kg]	Acid [mg/kg]
Control	1.69±1.17	0.95±0.27	2.48±1.45	8.45±2.33
Barberton	8.87±0.64	8.01±0.6	74.68±32.68	4408±195.16
Kaapsehoop 1	54.22±12.98	55.5±5.23	336.16±163.57	13281±151.32
Kaapsehoop 2	45.95±9.22	14.99±3.52	90.68±37.82	13926±33.94

It also should be mentioned that some individuals of *A. caliginosa* were observed to become quiescent during the exposure period, showing the same symptoms as described by Edwards and Bohlen (1996). The worms formed a mucus-lined ball with the two ends of the worm protected in the centre.

4.3.2.7 Comparison of metal contents between *Aporrectodea caliginosa* and *Eisenia fetida*

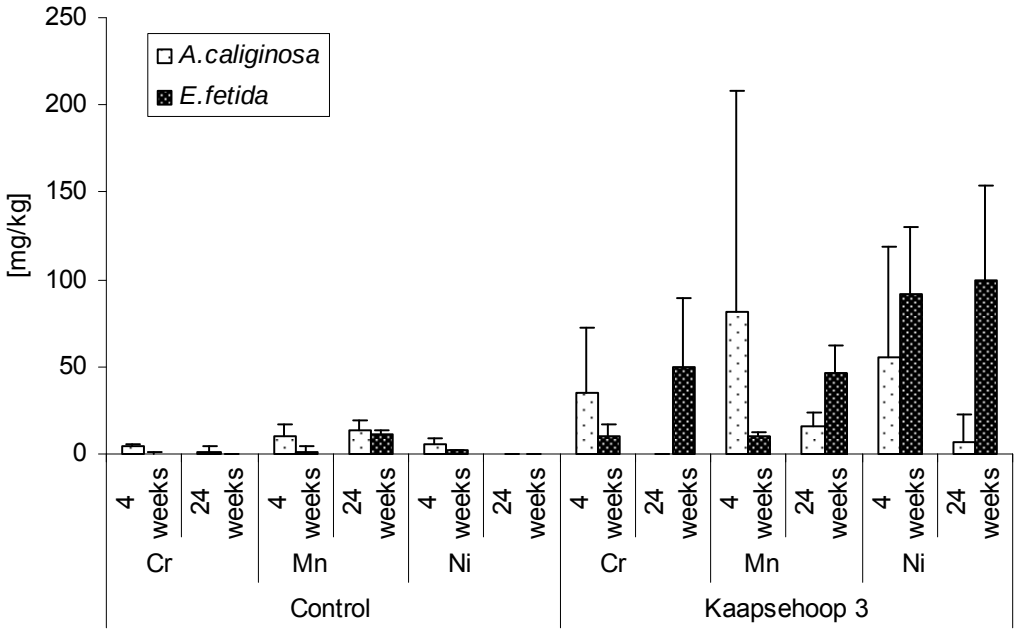

Figure 19: Mean concentrations in mg/kg of chromium (Cr), manganese (Mn) and nickel (Ni) in *Aporrectodea caliginosa* and *Eisenia fetida* after four and 24 weeks of exposure to soil from Stellenbosch and Kaapsehoop 3. Whiskers indicate standard deviation.

A direct comparison of the metal content of both species was made in the case of the specimens of both species exposed to the Kaapsehoop 3 soil and to the control soils from Stellenbosch after four weeks and after 24 weeks.

The concentrations of chromium, manganese and nickel in *A. caliginosa* and *E. fetida* exposed to Kaapsehoop 3 ultramafic soil and to control soil from Stellenbosch are shown in Figure 19 and in Appendix 2-11. The LSD test did not reveal any significant differences in the metal concentrations of both species exposed to the control soil. After four weeks of exposure to the Kaapsehoop 3 soils, the chromium, manganese and nickel concentrations were significant different between *A. caliginosa* and *E. fetida* ($F=11.00$; $p<0.05$). The chromium and manganese concentrations were higher in *A. caliginosa,* and the nickel concentrations were higher in *E. fetida* (Figure 19). After 24 weeks of exposure, the manganese and nickel concentrations in the two species were significantly different ($p<0.05$). Due to the fact that the data were log-transformed to obtain a parametric dataset for the one way ANOVA, and, with no chromium detected after 24 weeks of exposure in *A. caliginosa*, the LSD test did not return any valid p-values. For that reason the Mann-Whitney-U test was applied, which returned a significant difference between the chromium concentrations in *A. caliginosa* and *E. fetida* after 24 weeks ($z=-2.99$; $p<0.05$).

4.3.3 Longitudinal distribution of metals in the earthworm body

No cadmium or cobalt was detected in any of the analyzed worm sections, thus both of these metals will not be mentioned further.

4.3.3.1 Longitudinal distribution of metals in *Aporrectodea caliginosa* exposed to ultramafic soils

The mean values, standard deviation, maxima, minima and the median of the distribution of chromium, manganese and nickel are shown in Appendix 2-12.

In the specimens of *A. caliginosa* exposed to the ultramafic soils from Agnes Mine and Kaapsehoop 3, a significantly higher amount of chromium ($p<0.05$; $H=34.84$) and manganese ($p<0.05$; $H=31.77$) was measured compared to the control, but no significant difference was found between the nickel concentrations in the bodies of the earthworms in the different exposures. Especially in the case of the nickel concentrations in the body of the *A. caliginosa* specimens exposed to the ultramafic soils, the standard deviation was observed in all body sections to be higher than the mean values.

In the *A. caliginosa* exposed to the soils from Agnes Mine, nickel was detected in the posterior sections of four out of eight worms (89.09±8.48 mg/kg Ni). In three out of these four worms, nickel was also proven in the anterior section (29.09±5.66 mg/kg Ni) and in the middle section (62.78±5.87 mg/kg Ni).

Also in the Kaapsehoop 3 exposures, nickel was proved in 50% of the *A. caliginosa* only. Nickel at a mean concentration of 51.72±9.52 mg/kg was detected in the posterior sections of four out of

eight of the specimens. In two out of the four worms, nickel was also found in the anterior (34.61±3.08 mg/kg Ni) and the middle section (60.00±1.13 mg/kg Ni).

The log-linked generalized linear model revealed a negative slope (-0.40; $p<0.05$) between posterior metal content and anterior metal content, indicating an increase of all metals from anterior to posterior and a positive slope (0.38; $p<0.05$) between manganese content and nickel content in the different body sections of the *A. caliginosa*, indicating that with increasing nickel body concentrations the manganese concentrations in the earthworm bodies increased.

4.3.3.2 Longitudinal distribution of metals in *Eisenia fetida* exposed to different ultramafic soils

The metal content of the different body sections in *E. fetida* specimens exposed to the ultramafic soils collected at the Barberton Nature Reserve and the two ultramafic soils collected at Kaapsehoop, Kaapsehoop 1 and 2 and the unpolluted control soil from Stellenbosch are shown in Appendix 2-13. Although significant differences were observed between the control and the exposures for all metals analyzed ($p<0.05$, $F=9.53$), and also in the case of nickel between the worms exposed to the soils from the Barberton Nature Reserve and to those exposed to soil collected at Kaapsehoop 3 ($p>0.05$; $F=10.71$), no significant differences were found between the different body sections in any of the earthworm exposed to the ultramafic soils or to the control soil.

4.3.4 Concentration of metals in *Eisenia fetida* specimens with and without a history of exposure after exposure to ultramafic soils.

The mean, median, standard deviation and the minima and maxima of the metal concentrations in the *E. fetida* specimens with a history of previous exposure are shown in Appendix 2-14 and Figure 20. Comparing the specimens of *E. fetida* exposed to the ultramafic soils to those not exposed (Controls), the Fisher LSD post-hoc test (ANOVA $F=7.60$; $p<0.05$) recorded significantly different concentrations of nickel after four and after eight weeks in the groups of *E. fetida* exposed to the ultramafic soils compared to the control.

The highest concentration of nickel was 199.25 mg/kg observed in the nickel pre-exposed worms exposed to the Kaapsehoop 3 soil, the lowest concentration with no detection of nickel was recorded in the control group (Appendix 2-14 Figure 20).

Further, after 24 weeks the nickel concentration in the specimens pre-exposed to nickel was significantly ($p<0.05$) different from the concentrations in the specimens in all other exposures. Cadmium was detected only in the worms previously exposed to cadmium. The cadmium concentration varied between 18.11 mg/kg measured after four weeks and 125.75 mg/kg cadmium measured in one earthworm after 24 weeks. No significant reduction in the cadmium body burden of the pre-exposed *E. fetida* was found between the measurements after four and 24 weeks. The cadmium concentration was significantly different in the cadmium pre-exposed earthworms ($H=48.02$; $p<0.05$) compared to all other exposures.

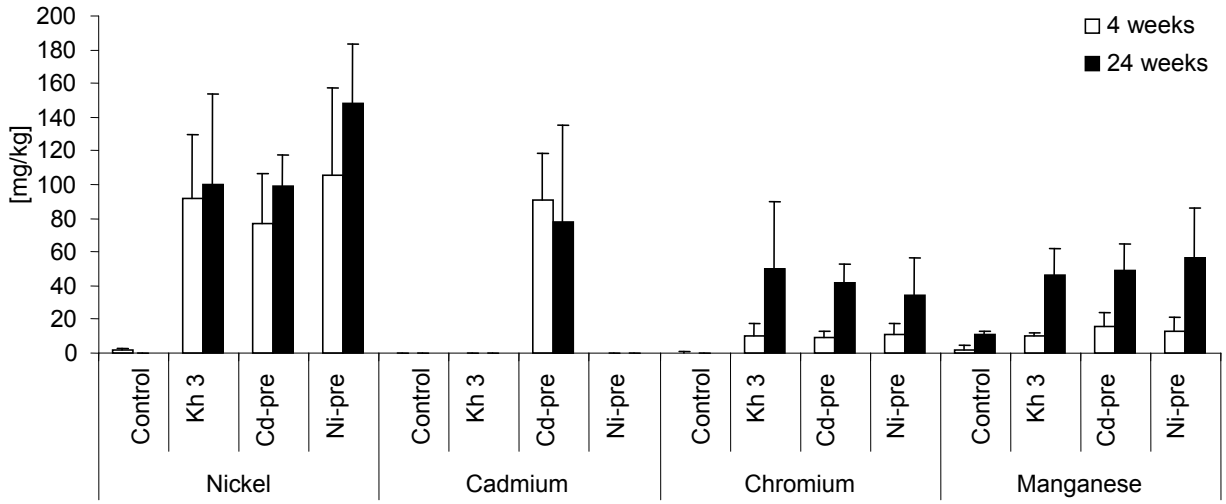

Figure 20 : Concentrations of nickel, cadmium, chromium and manganese [mg/kg] in *Eisenia fetida* without and with a history of exposure (Kh 3=no history of pre-exposure; Cd-pre= previously exposed for >10 generations to $CdSO_4$ * $7H_2O$; Ni-pre= previously exposed for >10 generations to $NiCl_2$ * 6H20). Whiskers indicate standard deviation.

For the chromium concentrations in the earthworm tissue, significantly different concentrations after an exposure duration of four weeks were observed in the tissue of the worms exposed to the Kaapsehoop 3 soils ($H=10.90$; $p<0.05$), when compared to the *E. fetida* specimens in the unloaded control soil. No difference was observed between the different pre-exposures. After 24 weeks of exposure, no significant difference between control and the ultramafic exposed specimens was recorded. Although not significant different from the other exposure groups, the highest concentrations of chromium were observed in the worms not previously exposed after 24 weeks of exposure (Appendix 2-14) with a maximum amount of 119.82 mg/kg Cr compared to no detected chromium in the controls. Remarkable here was the high deviation within the *E. fetida* group (Figure 20). Also, during the course of the exposure experiment, chromium concentrations increased significantly in the worms from the ultramafic exposures from week four to week 24 ($H=12.89$; $p<0.05$).

The concentrations of manganese, after four weeks of exposure, in the earthworms with a history of exposure was significantly higher than in the worms exposed to the Stellenbosch control soils ($H=11.40$; $p<0.05$). Also, a significant increase was reported during the course of the exposure between week four and week 24 ($H=25.60$; $z'=5.06$; $p<0.05$); and between the controls and the cadmium pre-exposed worms ($H=9.02$; $p<0.05$). Further, a high standard deviation was observed in most samples (Appendix 2-14).

4.3.4.1 Longitudinal distribution of metals in *Eisenia fetida* with and without a history of previous exposure

The mean concentrations of cadmium, chromium, manganese and nickel in the different body sections of *E. fetida* exposed for four weeks to a control soil and to ultramafic soil samples collected at the Kaapsehoop 3 site are shown in Appendix 2-15. The Kruskal-Wallis-ANOVA on Ranks did not find any significant ($p<0.05$) differences between the concentrations of any metal in the different body sections.

4.3.4.2 Manganese and nickel in *Eisenia. fetida* after being exposed for 48 h to artificial ground water

The wet mass of the specimens used for the exposure to artificial ground water ranged between 184 mg and 762 mg. No significant change of body mass was observed. Body concentrations and accumulation factors are given in Appendix 2-16 and Appendix 2-17.

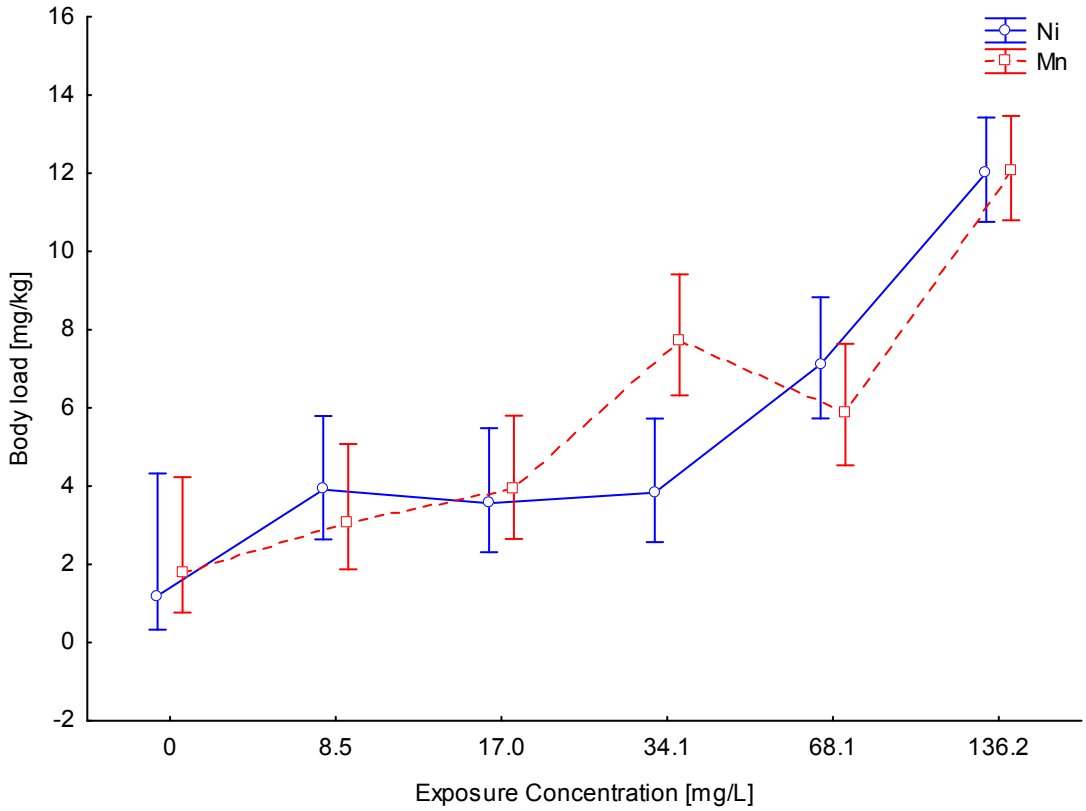

Figure 21: Mean manganese and nickel concentrations in different groups of *Eisenia fetida* specimens after exposure for 48 hours to linearly increasing concentrations of manganese and nickel. Body load [mg/kg] is plotted against exposure concentration [mg/kg]; $p<0.05$; n=6. Whiskers indicate standard deviation.

The highest amount of nickel, 12.02±1.03 mg/kg, was measured in the worms exposed to the highest exposure concentration, 136.29 mg/L Ni as $NiCl_2 \cdot 6H_2O$. The lowest concentration, 3.91±0.42 mg/kg nickel was observed in the exposure containing the lowest concentration, 8.5

mg/L Ni. For manganese, also the highest concentration, 12.06±5.2 mg/kg Mn, was measured at the highest exposure concentration of 136.22 mg/L Mn as $MnSO_4 \cdot 7H_2O$, the lowest concentration with 3.08±0.43 mg/kg manganese was found at the lowest concentration of 8.51 mg/L Mn. After 48 hours of exposure to nickel and manganese, the content of both metals in the animals increased significantly (Chi-Square = 12.75; $p<0.05$) with increasing metal concentrations in the substrate (Figure 21). Also, the generalized linear model showed that the accumulation pattern of both metals correlated with each other.

The bioaccumulation factor for nickel administered as nickel chloride varied between 0.09±0.01 at a concentration of 136.29 mg/L nickel, and 0.46±0.05 at a concentration of 8.5 mg/L nickel in the artificial ground water substrate (Figure 22). The Spearman Rank Order correlation showed a negative correlation with the concentration of nickel in the substrate ($R=-0.85$; $p<0.05$).

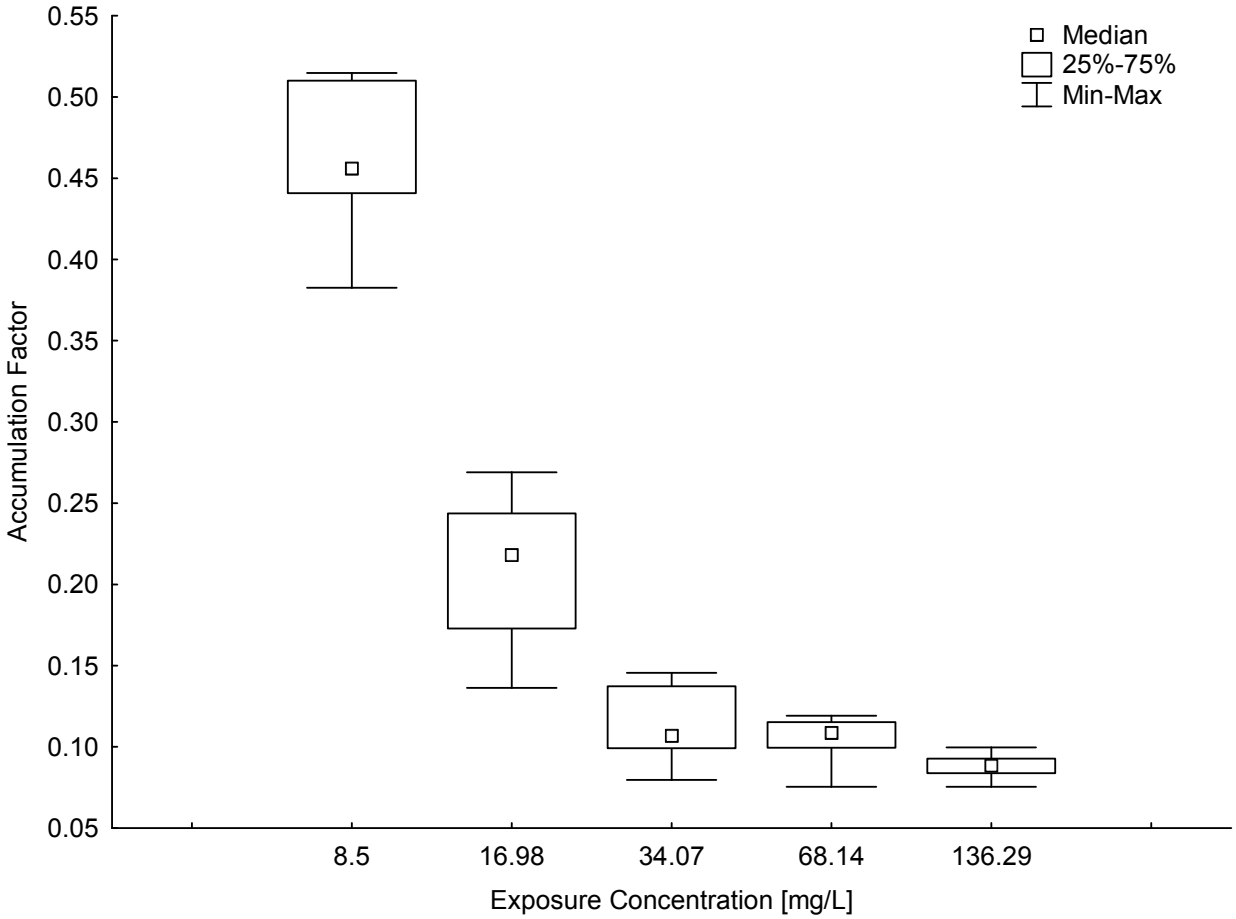

Figure 22: Accumulation factor of nickel in *Eisenia fetida* exposed in artificial ground water to $NiCl_2$ for 48 h ($n=6$; $H=24.88$; $p<0.05$).

Also, for manganese sulphide, a negative correlation between metal concentrations in the substrate and the bioaccumulation factor was observed ($R=-0.87$; $p<0.05$; Appendix 2-17 and Figure 23). The bioaccumulation factor for Manganese as $MnSO_4$ varied between 0.1±0.05 for the

highest concentrations of 68.11 mg/L Manganese and 136.22 mg/L Manganese and 0.36±0.05 at a concentration of 8.51 mg/L.

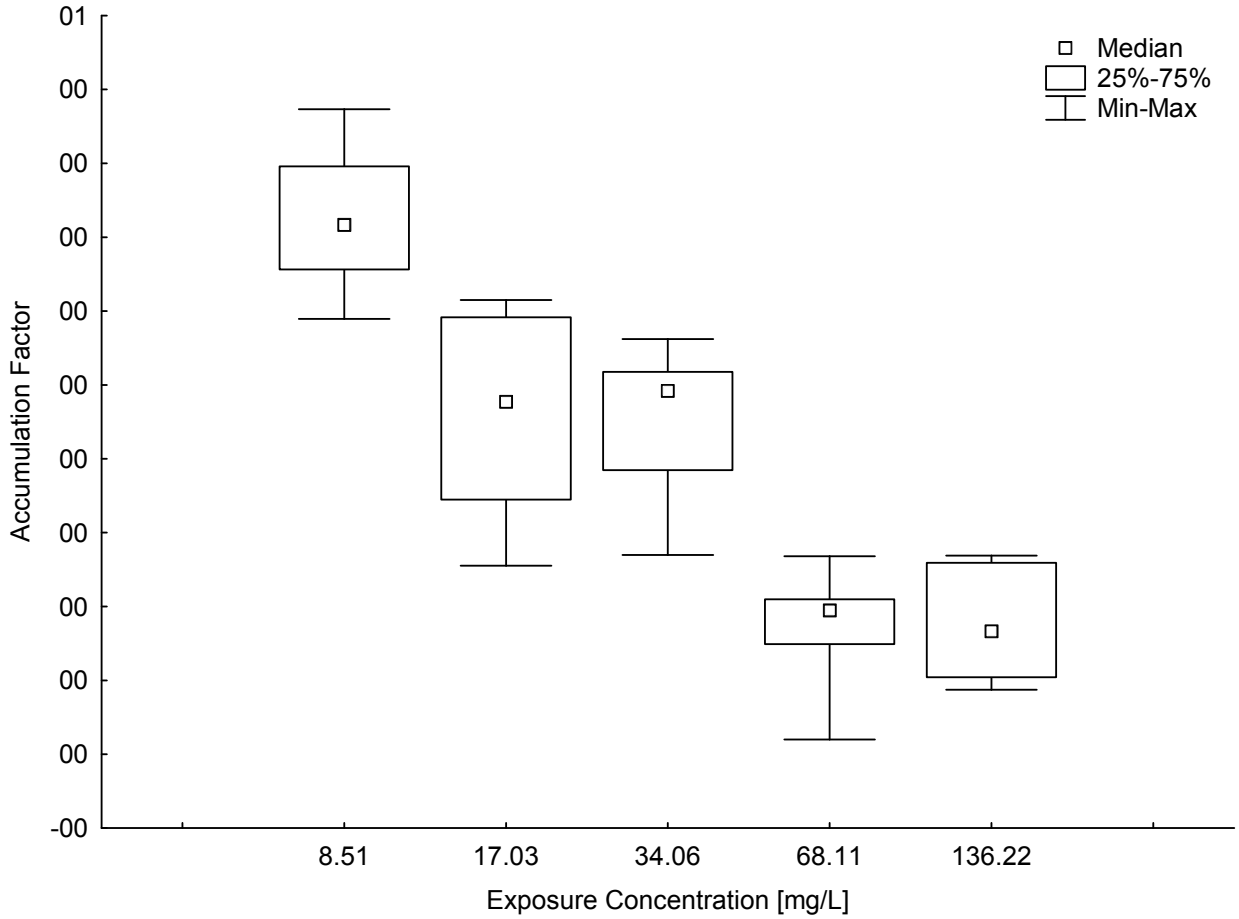

Figure 23: Accumulation factor of manganese in *Eisenia fetida* exposed in artificial ground water to $MnSO_4$ for 48 h (n=6; H=25.04; p<0.05).

4.3.4.3 Longitudinal distribution of manganese and nickel in *Eisenia fetida* exposed to artificial ground water

The distribution of manganese and nickel in the three different body sections, anterior (‘anterior’), middle section and posterior section (tail), is shown in Appendix 2-18. No detectable levels of metals were found in the body sections of the worms exposed to clean medium. The ANOVA revealed no significant differences between the different sections neither for manganese nor for nickel (F=0.42; p>0.05). For nickel, the mean concentration in the body of *E. fetida* ranged from 4.61±0.56 mg/kg Ni in the anterior section (without clitellum) to 5.51±0.90 mg/kg Ni in the middle section. The mean concentration of manganese ranged between 5.77±2.20 mg/kg Mn in the middle section to 6.39±1.38 mg/kg in the posterior section.

Also, no statistical differences were found in the bioaccumulation factor between the different body sections (Appendix 2-19). The mean accumulation factor for nickel was between 0.14±0.02 mg/kg Ni in the anterior sections (without clitellum) and 0.16±0.03 mg/kg Ni in the middle section. For

manganese, the accumulation factor ranged between 0.17±0.06 mg/kg Mn in the middle section and 0.19±0.04 mg/kg Mn in the posterior section.

4.4 Discussion

4.4.1 Metal content of earthworms collected at the Barberton area

Regarding the fact that the concentrations of chromium, cobalt, manganese and nickel in the earthworms collected from the unpolluted site at Stellenbosch, were below the detection limits, one can conclude that both of the earthworm fragments collected at the Barberton area had elevated concentrations of these metals.

Although the fragments of both unidentifiable species were from two different locations in the area, it is not known, whether the differences measured in the amounts of chromium, manganese and nickel in the bodies of these two earthworms (Table 5) can be related to species specific differences in uptake, background concentrations, abiotic conditions, differences in the bioavailability of these metals or intra species-specific differences. All these effects are well documented for earthworms (Terhivuo et al. 1994, Peijnenburg et al. 1999, Van Straalen et al. 2001). As no detailed information about the sampling area and thus the metal background was available, it remains unclear to what extent the metal body burden reflects the bioavailable amount of these metals in these ultramafic soils. These records do, however, demonstrate that earthworms may be able to survive in these soils in spite of the generally high concentrations of these metals.

Reports of metal concentrations in earthworms inhabiting ultramafic soils are scarce. In fact, the only available study was conducted by Marino et al. (1995) who analyzed earthworms collected in ultramafic soils in Galicia, Spain for cadmium, copper, lead, nickel and zinc and found high concentrations of nickel in the earthworms. Marino et al. (1995) collected different earthworm species such as *Allolobophora georgii*, *Aporrectodea caliginosa*, *Dendrobaena madeirensis*, *D. octaedra*, *Eiseniella tetraedra*, *Lumbricus eiseni* and *Lumbricus friendi* and found higher absolute concentrations of nickel in these worms than in earthworms living in non-ultramafic soils. They also observed, that the amount of nickel in worms in proportion to the nickel in the soils was very low (Marino et al. 1995). Other authors also sampled for earthworms in several ultramafic soils, but either did not find any earthworms (Beyer and Cromartie 1987), or focussed their studies on other parameters such as species diversity and population abundance (Hubers et al. 2003).

4.4.2 Earthworms exposed to ultramafic soils

The reasons for the quiescence observed in *Aporrectodea caliginosa* can be considered as a direct response towards adverse conditions such as drought or unfavourable temperatures (Edwards and Bohlen 1996). Both factors can be excluded in this study as both were controlled and close to the conditions known as optimal for that species (Lowe and Butt 2005). Another possibility for the quiescence observed might be the lack of adequate food or adverse conditions originating from the ultramafic soils. Although no individuals currently in quiescence were analyzed, it can not be

excluded that some of the individuals were just preparing to enter quiescence or had just left quiescence. No studies about the metal uptake during quiescence are known, but it is known that the ecophysiological status of the animal changes considerably (Morgan and Winters 1991) which could probably affect the uptake as well. Also, it is known that tissue concentrations of some metals such as cadmium and zinc are in quiescent *A. caliginosa* significantly lower than in active worms (Morgan et al. 1993). Notwithstanding the physiological changes, as the worms do not feed during quiescence, no oral uptake may take place. As far as dermal uptake is concerned, which is suspected as the more important pathway for the metal uptake of earthworms (Vijver et al. 2003), it is not known, to what degree metals are able to penetrate the mucus layer protecting the earthworm skin during quiescence.

4.4.2.1 Cadmium

Cadmium was only detected in one soil sample, the Kaapsehoop 3 sample, (67±9.9 mg/kg; Chapter 3.3). In contrast to that, cadmium was detected in some of the earthworms of the species *Aporrectodea caliginosa* exposed to the ultramafic soil samples and the putative unpolluted soils from Stellenbosch (Appendix 2-1).

From the presence of cadmium in the worms but not in the soils, one can conclude that due to the fact that *A. caliginosa* biomagnifies cadmium (Morgan and Morgan 1999, Dai et al. 2004), cadmium, although below the detection limit of the atomic absorption spectrometer in the soil samples, was present in low concentrations either in the soil samples or prior to the exposure already present in the bodies of the worms (detection limits are given in Chapter 4.2.4). Unfortunately, the worms were analysed for a range of metals prior to the exposure, but not for cadmium as, according to Maboeta et al. (2002) it was assumed that these soils did not contain cadmium. One can only speculate about the significant decrease of cadmium the bodies of *A. caliginosa* in all soil exposures, except the Kaapsehoop 3 soils, during the course of the exposure. During the course of the exposure, the amounts of cadmium “imported” by the earthworms into the exposures could have been slowly eliminated from the bodies of the worms. In this case, the decrease in cadmium in the worms exposed to the control soils can be explained, very speculatively, by the break-off of a small, but still constant immissions of cadmium into the control soils in the cadmium-free conditions in the laboratory. For example, waste incineration and fuel combustion are important sources for airborne cadmium (Wren et al. 1995), both might be sources for a constant entry of low amounts of cadmium into the control soils on-site. In contrast to that, the increase in the cadmium body burdens of the worms exposed to the Kaapsehoop 3 soils can be explained more reliably by the cadmium present in these soils and the constant accumulation of cadmium by the worms exposed to these soils. Another explanation might be that a decrease of cadmium uptake is influenced by the presence of metal binding soil contents, e.g. Oste et al. (2001) showed that the cadmium uptake of *Lumbricus rubellus* decreased with the addition of manganese oxide to the substrate. No cadmium was found in the *Eisenia fetida* specimens

exposed to the ultramafic soils and the control soil from Stellenbosch. This also might be an indication that the cadmium found in the *A. caliginosa* may have been introduced from the field where these individuals were collected.

4.4.2.2 Chromium

Both species, *Aporrectodea caliginosa* and *Eisenia fetida*, accumulated significantly more chromium in the ultramafic exposures than in the control soils. Especially in *A. caliginosa*, the results showed high individual variations in the body burden of chromium (Appendix 2-2, Figure 12). No correlation between the chromium concentrations in both of the earthworm species and the concentrations in the soils or the soil extracts were found. This finding is in agreement with previous studies (Beyer and Cromartie 1987, Sample et al. 1999). However, the maximum chromium concentrations in both species were substantially higher than the amounts of chromium extracted from the corresponding soils by DTPA extraction (Appendix 1-2), i.e. the complexed or adsorbed chromium. This might be caused either by bioaccumulation of chromium by the earthworms or the DTPA extraction underestimates the amounts of chromium that are actually bioavailable. As the DTPA extraction is well established also for chromium (Maiz et al. 1997), one can only speculate that chromium is remobilized by either the earthworms, microbial activity in the soils or the earthworms themselves, or by physicochemical factors (Edwards and Bohlen 1996, Kersanté 2004).

With regard to the biomagnification, Van Gestel et al. (1993) reported a significant accumulation of chromium in *Eisenia andrei*, but in other studies, no biomagnification was found (Ma 1982, Beyer et al. 1990, Sample et al. 1999). This may indicate that not only metal speciation, but several other environmental factors can influence the bioavailability of this metal.

Without knowing the speciation of chromium in these ultramafic soils, it is difficult to assess to what extent the elevated concentrations found in both of the earthworm species might be toxic to the animals. Trivalent chromium (Cr III) is considered as essential to all living organisms and toxic only at comparably high concentrations (Gauglhofer and Bianchi 1991). Hexavalent chromium is non-essential and toxic (Sivakumar and Subbhuraam 2005). The concentrations measured are known to be not toxic to *E. fetida* (Appendix 2-3) (Molnar et al. 1989, Sivakumar and Subbhuraam 2005) even under the worst case assumption that the chromium present in the soil was exclusively the more toxic Cr (VI). Furthermore, *E. fetida* is able to reduce the more toxic Cr (VI) to Cr (III) (Arillo and Melodia 1991). It is not known, if this ability is restricted exclusively to designated species or if all or most earthworm species are able to reduce hexavalent chromium. For *A. caliginosa*, no data about the toxicity of chromium were available. Besides the differences in toxicity of the different chemical speciation, chromium toxicity also is dependent on abiotic factors such as substrate pH (Sivakumar and Subbhuraam 2005). The interaction of chromium with nickel might be important. Hunter and Vergnano (1953) found, that chromium enhances the nickel uptake in plants.

4.4.2.3 Cobalt

In *Aporrectodea caliginosa*, the highest concentration of cobalt measured was 17.78 mg/kg in one specimen exposed to soil from Songimvelo after four weeks of exposure. Afterwards, the amount of cobalt in *A. caliginosa* decreased significantly (Figure 14). In *Eisenia fetida* exposed to the ultramafic soils, no cobalt was detected. The results obtained in this study suggest a loss of cobalt in *A. caliginosa* during the course of the exposure. As an integral part of the structure of vitamin B_{12}, cobalt is an essential micronutrient for earthworms and added in small amounts to the diet, it has positive effects on growth of *E. fetida* (Neuhauser et al. 1984b).

Thus one might suspect that cobalt was present in the earthworms, but below the detection limits of the atomic absorption spectrometer.

A sound explanation for the loss of cobalt in *A. caliginosa* (referring also to the amount below the detection limit of cobalt in *E. fetida)* is delivered by Crossley et al. (1995), who suggest that *E. fetida* has a low uptake rate for cobalt and is not able to assimilate cobalt from mineral soil. Furthermore, they suggest that the equilibration rate of cobalt for *E. fetida* is about 500 hours or 21 days. This implies that *A. caliginosa* has a slower cobalt metabolism than *E. fetida*. It could explain the decrease of cobalt in *A. caliginosa* and the fact, that after 24 weeks of exposure, no cobalt was detected any longer in *A. caliginosa*. Another explanation might be the involvement of cobalt in a sequestration process. One mechanism to eliminate harmful concentrations of metals is by subcellular sequestration (Posthuma and Van Straalen 1993). The incidental loss of essential metals by sequestration was suspected by Marino and Morgan (1999) in the case of zinc in cadmium adapted groups of earthworms.

4.4.2.4 Copper

Copper was detected only in *E. fetida* after three to four exposed worms were pooled for the metal analysis (Table 6). As copper is an essential metal (Morgan and Morgan 1990, Spurgeon et al. 2004), it can be concluded that the copper concentrations in *A. caliginosa* were below the detection limit. The copper concentrations in the substrate were in a similar range to those given by several previous studies for unpolluted or uncontaminated soils respectively, with regard to *E. fetida* for earthworms exposed in these studies to these soils (Morgan and Morgan 1999, Ma 2005, Lukkari and Haimi 2005). Thus it can be concluded that atomic absorption spectrometry was not sensitive enough to detect the copper in individual specimens of *A. caliginosa* and *E. fetida* before pooling the worms together. The detection limits stated in Chapter 4.2.4 obviously have to be multiplied by a factor of 20, the amount of liquid added during the process of acid digestion to the earthworm samples.

4.4.2.5 Manganese

Compared to the control, the manganese concentrations in the bodies of *A. caliginosa* exposed to the ultramafic soils increased significantly from the beginning of the exposure until week eight

(Appendix 2-6), but instead of reaching an equilibrium, the concentrations of manganese decreased afterwards. Similar to the concentrations of chromium in the body, the concentrations of manganese in the *A. caliginosa* specimens also showed a high individual variation (Figure 15). As in the case of chromium, these variations might be coherent with the different physiological status of the different individuals of *A. caliginosa*. It can only be speculated that worms, which do not feed and only scarcely move because they are preparing for quiescence, do take up less metals than actively feeding and burrowing specimens. Another possibility might be that the individual worms take up a certain amount until a distinct threshold is reached and then actively start eliminating the metal from their body. That would also serve as an explanation for the decrease in manganese after eight weeks of exposure of *A. caliginosa* to the ultramafic soils. This might occur by the transport into internal storage compartments such as granules which might be excreted subsequently (Morgan and Morgan 1998, Stürzenbaum et al. 2001) or via excretory glands. For manganese, it has been observed that this metal is stored in excretory glands (Andersen and Laursen 1982), which supports that speculation.

Except in the case of the *E. fetida* analyzed after 14 weeks of exposure to the Kaapsehoop 1 soils, no significant differences in the manganese concentrations between the ultramafic soils and the control soils in the bodies of *E. fetida* were found (Figure 16). The concentrations of manganese in both species were found to be independent from the manganese concentrations extracted with the different extraction methods in the soil. Manganese is an essential trace metal (WHO 2004). It is known that essential trace metals are physiologically regulated (Peijnenburg et al. 1999, Lukkari et al. 2004), which might serve as an explanation for the variation of the concentrations in the earthworms and in the different soil fractions.

4.4.2.6 Nickel

Similar to manganese, the concentrations of nickel in *Aporrectodea caliginosa* showed a high individual variation and an increase of body load followed by a decrease (Figure 17). Similar to manganese, the decrease of the nickel body burden might be caused by the institution of an elimination process after a certain period of exposure in the earthworms or be related to the phenomenon of quiescence.

In *Eisenia fetida*, until after six weeks of exposure, the nickel concentrations in the bodies of the specimens exposed to the ultramafic soils increased. Afterwards, from week six to week eight, a decrease was observed in the concentrations of nickel in the bodies of the earthworms exposed to the soils collected in the Barberton Nature Reserve and Kaapsehoop 1 (Figure 18). This indicates the presence of a regulatory mechanism, regulating the nickel body burden in *E. fetida* exposed to these soils. After 14 weeks of exposure, in both of the Kaapsehoop exposures, the highest amounts of nickel were measured. One reason for this might be that the internal regulation mechanism showed signs of overburdening. Another reason for the fluctuating nickel concentration might be traced back to soil physiochemical effects. After a certain amount of time, the nickel

bounds to the biologically unavailable soil fraction and releases only slowly from this solid fraction, resulting in a diminished availability and a slower uptake rate.

For nickel, two concentrations in *E. fetida* were positively related to the DTPA extracts of the same ultramafic soil sample, but in one ultramafic soil and in the control soil the concentration of nickel in the earthworms were in no relation to either the total amount of nickel or the two extracts representing fractions of different availabilities. An explanation might be the fact, that the DTPA measurements only access a static phase, but the uptake mechanisms of the earthworms are dynamic. For predicting a dynamic mechanism, an equilibrium as previously described (Sheppard et al. 1997, Spurgeon and Hopkin 1999) between internal concentration and external concentration does not necessarily have to be reached. Concerning the establishment of an equilibrium, there often has to be distinguished between essential and non-essential metals (Spurgeon and Hopkin 1999). In the case of nickel, this might be difficult as the essentiality to earthworms is difficult to assess. A natural nickel deficit is rare and the physiological requirements for nickel are considerably low (Cartana et al. 1991). So far no studies about the essentiality of nickel to earthworms are available (Phipps et al. 2002). Transferred to the findings in this study, the nickel uptake rather gives an indication that nickel is non-essential as it takes at least more than 14 weeks for the establishment of an equilibrium, if ever (Figure 18).

4.4.3 Comparison of Cr, Mn and Ni uptake of *Aporrectodea caliginosa* and *Eisenia fetida*

After four weeks of exposure of *A. caliginosa* and *E. fetida,* a higher uptake of chromium, manganese and nickel was observed in the ultramafic exposures compared to the control soil. The concentrations of all three metals differed significantly between both the earthworm species during the whole observation period (Figure 19). This is an indication that these ecophysiologically different species differ in the uptake of these three metals. This finding was expected, as even closely related earthworm species differ in their uptake and excretion abilities (Morgan et al. 1993). Furthermore, the *A. caliginosa* specimens were collected directly from the field and therefore most probably differed in age and physiological status, which might also serve as an explanation for the fact that within that species, high individual differences in body metal loads were observed in the different exposures (Phipps et al. 2002).

4.4.4 Longitudinal distribution of metals in *Aporrectodea caliginosa* and *Eisenia fetida*

A probably stronger explanation of the high variation of the metal concentrations in *A. caliginosa* compared to *E. fetida* is provided by the analysis of the different body sections (Appendix 2-12 and 2-13). In *E. fetida*, all metals were shown to be more or less equally distributed over the whole body. In spite of the small number of individuals used, the results in this study indicate that *A. caliginosa* may be using another mechanism of metal elimination which might also be responsible for the high variation measured in this species. *A. caliginosa* apparently tends to transport metals to the posterior section and consequently pushes-off the posterior section by autotomization. It was

observed in this study that *A. caliginosa* is able to autotomize the posterior section or parts thereof (Figure 26 and Chapter 5.3.4). In support of this finding, autotomization in context with toxic stress was observed e.g. by Reinecke et al. (1999) for *E. fetida*, but autotomization in general also has been observed for other species such as *Bimastos zeteki* (Edwards and Bohlen 1996) and *Diplocardia mississippiensis* (Vail 1972). In general, the findings in this study, that these species differ strongly in the pattern of metal accumulation is confirmed by numerous previous studies comparing the uptake pattern of different earthworm species, e.g. (Morgan and Morgan 1992, Marino et al. 1992, Pizl and Josens 1995, Edwards and Bohlen 1996, Ma 2004).

4.4.5 Concentrations of metals in *Eisenia fetida* with a different history of previous exposure

In the bodies of *E. fetida* specimens previously exposed to cadmium, even after 24 weeks high concentrations of cadmium (77.79±57.50 mg/kg Cd; Figure 20 and Appendix 2-14) were still measured. This finding is an indication of a slow elimination process of cadmium from the body of *E. fetida*. It is supported by the findings of Reinecke et al. (1999), who exposed worms from a similar cadmium pre-exposed group of *E. fetida* for 35 days to clean, non contaminated soils and still measured concentrations of 1598±610 mg/kg cadmium in the worms. It is generally accepted that the elimination of non-essential metals takes substantially longer than the elimination of essential metals (Spurgeon and Hopkin 1999, Vijver et al. 2005). Furthermore, one can speculate that due to the ecophysiological stress originating from the presence of other harmful metals such as nickel or chromium and additional disadvantageous edaphic conditions originating from the ultramafic substrate, the elimination time of cadmium may be increased. But to confirm that speculation, also specimens with a history of cadmium exposure should have been exposed to the soils collected at Stellenbosch. As this study did not focus on the elimination of cadmium, this was not performed. Nevertheless, this might be one reason for the fact that the elimination times, specifically biological half-life times of cadmium in the literature, are lower than those observed in this study (Sheppard et al. 1997, Spurgeon and Hopkin 1999, Vijver et al. 2005). Another reason for the slower elimination time might be related to differences in the species used and in the elimination between cadmium pre-exposed *E. fetida* in the sense of an exposure over several generations and specimens only exposed for a comparatively short time span.

In terms of nickel, the results of this study show that the uptake of nickel in *E. fetida* with a history of previous exposure to nickel is significantly higher than the uptake of nickel by ‘clean’, non pre-exposed worms in relation to time (Figure 20 and Appendix 2-14). In previous studies conducted on other metals, it was also observed that earthworm populations that are acclimatized to a metal do accumulate higher amounts of this metal than populations that may not be resistant. A similar phenomenon as in this study was observed where earthworms previously exposed to cadmium accumulated higher amounts of cadmium than worms without a previous exposure history

(Reinecke et al. 1999). Similar observations were also made in animals of other taxonomic groups (Bryan and Hummerstone 1971, Nagel and Voigt 1989).

In contrast to the findings on cadmium in worms, it has been observed that isopods from a nickel contaminated site accumulated significantly lower concentrations of nickel than specimens from a clean reference site (Alikhan 1995). In this study, it is uncertain whether a comparatively higher accumulation of nickel can be equated with an increased tolerance or even the development of a genetically based resistance. An adaptation towards a certain toxicant is often linked with "costs", e.g. a reduction in cocoon production or a shorter life cycle (Reinecke et al. 1999, Langdon et al. 2003a). In preview of the results presented in chapter 6, showing that in terms of mortality, nickel pre-exposed *E. fetida* were actually more sensitive to nickel than 'clean' worms, one can suspect that an increased mortality might not really be the price to pay for an adaptation. Thus the higher accumulation of nickel in the nickel pre-exposed *E. fetida* exposed to the ultramafic soils is more likely caused by a less well buffered threshold level, or the nickel in the ultramafic soils can be considered as an "add up" to the nickel already present in the worms from the previous long-term exposure.

A pre-adaptation to nickel in zinc tolerant plants was recorded (Cox and Hutchinson 1983). Soil inhabiting bacteria can develop a resistance against nickel (Schmidt and Schlegel 1989), and also a range of different plant species can develop a resistance or tolerance against nickel (Mesjasz-Przybylowicz et al. 1997), but that has not been reported for earthworms yet.

According to the results obtained in this study by exposing cadmium acclimated worms in these ultramafic soils (Figure 20), no connection was found between an acclimatization to cadmium and the uptake of nickel. Also, no possible cross-acclimatisation between nickel or cadmium and chromium or manganese was detected. Previously, a cross-resistance or cross-adaptation between different toxicants was observed on different taxonomic levels (Sauphanor and Bouvier 1995, Willuhn et al. 1996, Bisset et al. 1997, Spurgeon and Hopkin 2000), but with regard to nickel in this study, it seems not to be the case.

The investigation of the longitudinal distribution of chromium, manganese and nickel in the pre-exposed worms (Appendix 2-15) did not reveal any differences in the uptake behaviour (see Chapter 4.4.4).

4.4.6 Manganese and nickel in *Eisenia fetida* after exposure for 48h in artificial ground water

The concentrations of manganese and nickel measured in the bodies of earthworms of the *Eisenia fetida* specimens exposed to artificial ground water were clearly dependent on the concentration in the medium (Figure 21). Both metals showed a similar accumulation pattern. The longitudinal distribution pattern (Appendix 2-17 and 2-18) showed no differences in the uptake of both metals into the different body sections, which, for *E. fetida*, is in agreement with the results obtained from

the analysis of the different body sections of *E. fetida* exposed to the soils collected in the Barberton area and the control soil from Stellenbosch.

Also, in this study *E. fetida* did not biomagnify either manganese (Figure 22) or nickel (Figure 23), as the accumulation factor for both metals smaller was than 1, but a negative correlation between the accumulation factor and exposure concentration was observed for both metals.

In particular for the direct comparison of the accumulation of manganese at the ranges of concentrations used in the substrate, no previous data were available. Notwithstanding this, previous studies, conducted on ecophysiologically different species of earthworms exposed to manganese, including *E. fetida*, have shown that earthworms do not biomagnify manganese (Ireland 1979, Reinecke and Reinecke 1997b, Dai et al. 2004, Maddocks et al. 2005). This is in agreement with the findings of the present study.

The finding of this study that nickel is not biomagnified in *E. fetida* is supported for earthworms in general by the studies of Neuhauser et al. (*A. caliginosa* (1995)), Gish and Christensen (undisclosed species (1973)), Sample et al. (undisclosed species (1999)). For *E. fetida* in particular it is supported by Maboeta and Van Rensburg (2003) and the statement of Phipps et al. (2002) that the bioaccumulation factor for nickel in the earthworm *E. fetida* is generally less than 1 (0.07-1.2).

There seems to be no general agreement in the literature on the bioaccumulation of nickel. Chmielewska et al. (1998) found an average bioaccumulation factor of 8 in undisclosed earthworm species of the family Lumbricidae exposed to emissions of an oil refinery. Barrera et al. (*Allolobophora chlorotica* and *A. caliginosa* (2001)) calculated the bioaccumulation factor in relation to the total nickel concentration in soil and also to the DTPA extractable concentration and found a bioaccumulation factor lower than 1 for the total metal content, but higher than 1 for the DTPA extractable concentration. An explanation for these contradictions might again be the fact that even closely related earthworm species differ in their uptake and excretion abilities (Morgan et al. 1993). Also, most of the available data were obtained in field experiments where many varying factors may influence uptake. It is known that especially the bioavailability of nickel is strongly influenced by abiotic factors such as pH, redox potential and moisture content, and also by the concentrations of other metals present such as e.g. manganese (Theis and Richter 1979).

4.5 Conclusion

Especially with regard to *Aporrectodea caliginosa*, but also, seen over the course of the exposure of *Eisenia fetida*, the concentrations of the metals in the earthworms showed a high variability which makes it difficult to conclude that these values reflect soil concentrations of the metals. Nevertheless, elevated concentrations of chromium, manganese and nickel were detected in *E. fetida* at all stages of the exposure, which shows that these metals could have been bioavailable for *E. fetida* during the whole exposure period. Furthermore, this finding leads to the conclusion that this species might serve as a monitoring species to indicate the presence of bioavailable amounts of these metals, in spite of the fact that is primarily known as a compost dwelling species.

A. caliginosa seemed to be less suited for the biomonitoring of these metals, as in some of these specimens none of the above mentioned metals were detected after 24 weeks of exposure. This might be due to a different elimination/ uptake mechanism when compared to *E. fetida,* or due to the ability to avoid elevated concentrations of metals by entering quiescence.

From the analysis of the different body sections it can be concluded that both ecophysiological different species differed strongly in the distribution of chromium, manganese and nickel in their bodies. *A. caliginosa* seems to concentrate these metals preferentially in the posterior section, which is probably linked to the phenomenon of autotomization as means of getting rid of the metals. In contrast to that, no differences in the longitudinal distribution of the metals in the body of the *E. fetida* were observed.

In *E. fetida*, a history of exposure to nickel caused an increase in the uptake of nickel, which might be caused by an add-up to the nickel already present in the worms. No indication of acclimation or adaptation of historically pre-exposed worms to nickel was found.

It is clearly shown in this study that *E. fetida* does not biomagnify manganese and nickel, two metals found at high concentrations in ultramafic soils. The accumulation factor was dependent on the concentration of manganese and nickel in the aqueous substrate. That finding, in turn, makes it difficult to assess the amount of bioavailable manganese or nickel solely by analysing the earthworms.

5. Growth and reproduction of earthworms in ultramafic soils

5.1 Introduction

For the assessment of the effects of metals in soil, earthworms have shown to be a useful tool (Menzie et al. 1992). It is also known that they respond negatively to asbestos content in soil (Schreier and Timmenga 1986). Among the earthworms, *Eisenia fetida* (Savigny 1826) can be considered as a model species for the evaluation of the effects of metals in soils (Spurgeon and Hopkin 1996a). For this species, various endpoints in toxicity testing of metals have been established (Kokta 1992).

As an indication of potential sublethal effects, parameters such as mass change and reproduction success are widely used as endpoints. Although biomass as an endpoint of toxicity often has a limited sensitivity due to high variations (Kula and Larink 1997), many studies have found that metals do affect earthworm growth – and thus the change of biomass – adversely (Rida 1996, Reinecke and Reinecke 1996). Reproductive responses of *E. fetida* are also widely accepted as an indicator of sublethal effects of metal toxicity (Sheppard et al. 1998). In terms of reproduction, it is recommended by Reinecke et al. (2001) to measure cocoon production as well as hatching success, as a specific toxic threat does not necessarily have an effect on cocoon production and growth, but on cocoon viability due to sperm damage, reduced fertilization or problems related to embryonic development. According to different guidelines, for example the OECD-Guideline (OECD 2004) for the testing of chemicals and ISO guidelines (ISO 1998), *E. fetida* is regarded as a reference species for soil toxicity testing. It is known as an excellent test organism under laboratory conditions and has a fast reproduction rate. Further, its life cycle is well documented (Venter and Reinecke 1988). As an epigeic species (litter dweller), *E. fetida* prefers a medium with a high organic content (manure, compost) which might cause some limitations concerning the relevance of field exposures (Arnaud et al. 2000).

Hence, for tests of contaminants in substrates with a higher mineral content and less organic material, it is often necessary to use other earthworm species (Reinecke and Reinecke 2004a) more suited to such soils than *E fetida*. An endogeic earthworm species, which has been proven to be very susceptible in acute toxicity tests and relatively sensitive to the effects of metals, is *Aporrectodea caliginosa* (Savigny 1826) (Spurgeon 1997). Thus, *A. caliginosa* can be considered as a field relevant species. The disadvantages are its slow reproduction rate and the fact that *A. caliginosa* is not really suitable for laboratory conditions. Only limited toxicity data are available for this species.

The aim of this part of the study was to evaluate the sublethal effects of the so called “serpentine challenge” (Proctor 1999), consisting of a sum of potential threats, such as a mixture of metals, on the reproduction and viability of cocoons of two ecophysiologically different species of earthworms. Also, to evaluate, how and whether ultramafic soils can maintain earthworm populations as it is known that earthworms do occur in ultramafic soils (Marino et al. 1995).

5.2 Materials and methods

5.2.1 Field work

Field work was performed on two field localities. As an uncontaminated site, a site at Stellenbosch (Western Cape, South Africa) was selected (See Chapter 2.2).

The second locality was the ultramafic study site in the Barberton area in the Mpumalanga Province on Ni-rich outcrops in the north eastern part of South Africa. Soil samples were collected at three locations at Kaapsehoop: Kaapsehoop 1 (25°33.573' S, 30°47.399' E); 1327 m above sea level, on grassland and in a dry river bed; Kaapsehoop 2 (25°33.573' S 30°47.391' E); 1320 m above sea level on grassland and at Kaapsehoop 3 near the Kaapsehoop Chrysotile Mine (25°33´S 30°47´E), 1243 m above sea level. Soil was also collected in the Barberton Nature Reserve (25°36.387' S 30°58.974' E) 726 m above sea level, in savannah land. Various soils were collected near Agnes Mine (25°49´S, 30°57´E), 1262 m and Songimvelo (26°55.033 S, 31.016' E) 1327 m above sea level.

The sampling locations (Chapter 2 and Figure 6) are situated on characteristic ultramafic soils of the Barberton Greenstone Belt which are characterized by a very heterogenic soil structure within a small area.

5.2.2 Preparation of substrates and food

As feeding material and as an organic additive for the *Eisenia fetida* exposures, urine-free cattle manure was sun dried for approximately one week, ground with a laboratory mill (Christy & Norris Ltd., Chelmsford, England) and sieved through a 0.5 mm^2 sieve. Soil samples were sieved through 0.5- and 0.2 mm^2 screens and oven dried for 48 hours at 110°C before the start of exposures. 400 g of soil and 40 g of ground urine free cattle manure for *E. fetida*, and chicken feed for *A. caliginosa*, were respectively placed into plastic containers (15 x 10 x 5cm), moistened with distilled water to a moisture content of 60-65% on a dry soil basis, and incubated for 48 hours to stabilise prior to the introduction of earthworms.

5.2.3 Culture of earthworms

The specimens of *E. fetida* used in this study were obtained from a synchronized culture bred from stock cultures maintained for several years at the ecotoxicology laboratory of the Department of Botany and Zoology at the University of Stellenbosch. Cocoons were harvested from the stock cultures and placed singly in 24-well-plates filled with distilled water and incubated in an environmental chamber at 25°C to obtain worms of a same age and maturity. On a daily basis, hatchlings were collected and placed in containers filled with 400g finely sieved OECD soil (OECD 2004) with 40g of urine-free cattle manure, wetted with distilled water to a moisture content of 60-65%. After reaching an age of 60 days, mature (clitellate) worms were removed for exposures. For a preliminary experiment, two cultures of mature *E. fetida* with a history of previous exposure were used and earthworms from the above mentioned stock cultures. The worms in the nickel pre-

exposed culture were fed for at least three years on a biweekly basis with 0.2% $NiCl_2$ added to their food, the *E. fetida* pre-exposed to cadmium were fed biweekly with urine free cattle manure with 0.01 % of $CdSO_4$ added.

Mature (clitellate) specimens of *A. caliginosa* used in this study were collected from the above mentioned uncontaminated field site at Stellenbosch. The worms were used for exposures immediately after collection.

5.2.4 Exposures of earthworms

Exposures were conducted in accordance with procedures established by Reinecke et al. (1999). Before the start of exposure, worms were kept for 48 h on moist filter paper to depurate the gut content (Edwards and Bater 1992). Both species were exposed separately. For the exposures of *E. fetida*, twelve individuals per exposure were placed in each container and for *A. caliginosa*, ten specimens were used. As controls, specimens of both species were exposed to soils from Stellenbosch with a known history of no metal pollution (Maboeta et al. 2002). *E. fetida* was exposed to ultramafic soil samples from Barberton, Kaapsehoop 1 and 2. Ten of the pre-exposed *E. fetida* and the *E. fetida* from the stock cultures were exposed to unpolluted soils from Stellenbosch and to soil from Kaapsehoop 3. Three replicates per exposure were conducted. *A. caliginosa* was exposed to soils from Agnes Mine, Songimvelo and Kaapsehoop 3. For aeration, small aeration holes were drilled into the lids of the containers. Due to the addition of organic matter to the substrate (cattle manure or chicken feed), worms were not fed otherwise during the duration of the exposure of eight weeks. In contrast to that, to the *E. fetida* with a history of exposure used in the preliminary experiments no organic matter was added to the substrate, but the worms were fed from week 6 onwards biweekly with fresh, urine free cattle manure. At the beginning of the exposure, and thereafter every second week, three worms were removed from each container for analysis of body metal concentrations. For the three different groups of *E. fetida* exposed to the ultramafic soils from Kaapsehoop 3, mass and mortality was recorded after 28, 42, 56 and 168 days. Cocoon production and hatching success was recorded only after four and six weeks.

5.2.5 Mortality, growth and reproduction of earthworms

Mortality was determined by counting the worms in all containers every two weeks. As worms disintegrate quickly after dying, earthworms absent from the containers examined were considered dead. Growth was measured by weighing the earthworms on a biweekly basis. Cocoons were collected on a biweekly basis by hand picking of the soil samples and incubated according to the water incubation method (Reinecke et al. 2001) in 24-well-plates filled with 3 mL of distilled water in an environmental chamber at 25°C to evaluate hatching success; hatchlings were counted and removed every two days.

5.2.6 Soil analyses

The pH of the soil samples was determined with a Crison MicropH 2001 (soil/solution ratio 1g : 30 mL) on a $CaCl_2$ basis. The background levels of selected metals in the soil samples used were determined by a Varian AA-1275 atomic absorption spectrophotometer. Substrates used for soil analyses were sieved and dried for 48 hours at 70°C.

For the determination of total metal content, soil samples from all field sampling sites were acid digested (Perkin-Elmer 1979). 1 g of each sample was left overnight at room temperature in 10 mL (55%) nitric acid. After heating for two hours at 40-60°C, the samples were heated to 120 °C for one hour before cooling down for another hour at room temperature. Five ml of perchloric acid (70%) were then added and the samples heated at 120°C again. After cooling down for one hour, five ml of distilled water was added and the samples reheated to 120°C until white fumes developed. Before filtration through Whatman no. 6 filters, the mixtures were left overnight to cool down. Samples were made up to 20 mL with distilled water and filtered through 0.45 µm Sartorius cellulose nitrate microfilters into dark plastic containers until atomic absorption spectrometry analysis.

The mobile soil fraction was extracted by suspending the soil samples for 2 hours under agitation in a 0.01 mol/L $CaCl_2$ solution (1:10). The mobilisable fraction was extracted by suspending the ground and dried soil samples under agitation in 0.005 mol/L of the chelating agent DTPA, 0.01 mol/L $CaCl_2$ and 0.1 mol/L TEA at pH 7.3 (1:2) for 4 hours at room temperature (Lindsay and Norvell 1978). Soils were analyzed for cadmium, chromium, cobalt, copper, manganese and nickel. Further, all soil samples were analyzed by scanning electron microscopy (SEM, Leo 1430VP Scanning Electron Microscope) and energy dispersive X-ray spectrometry (EDS, Philips 1410-diffractometer) for asbestos fibres.

5.2.7 Statistical analyses

All statistical tests were performed with the current version (7.0) of Sigma Stat™ (Jandel Scientific) for Windows. As a probability level for statistical significance $p<0.05$ was chosen. As a test for normality, Lillifors test was used. For parametric data, for the comparison of two samples, Students t-test was applied, for the comparison of more than two samples a one-way-ANOVA was conducted. For non-parametric data, to compare a set of two samples, the Mann-Whitney-U-test was performed; in case of more than two sample sets, a Kruskal-Wallis ANOVA was conducted and as a post-hoc test, Fisher's LSD-test was selected.

5.3 Results

5.3.1 pH and background concentrations of metals

All soil samples collected in the Barberton area had elevated levels of metals. The highest total metal contents were found in the samples collected at the Kaapsehoop 2 sampling site, lowest contents in soils collected at the Barberton area (Chapter 3.4.3). Concentrations and availability of

metals in ultramafic soils were quite variable, even when comparing soil samples such as the Kaapsehoop 1 and 2 collected at locations only 50 m apart from each other (Chapter 2.2). Nevertheless, total metal concentrations measured in the ultramafic soil samples were within the range reported by previous publications on ultramafic soils (Nemec 1954, Vergnano 1958, Soane and Saunder 1959, Morrey et al. 1994, Marino et al. 1995). No cadmium and copper were detected in any of the samples. The pH values of all samples varied between 6.2 and 7.0. Further, no asbestos fibres greater than 5µm were found by scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy.

5.3.2 Mortality and growth of *Eisenia fetida*

In the earthworms exposed to the Kaapsehoop 1 soil, a significantly increased mortality ($p < 0.01$) compared to the control group and the group exposed to the soil collected at the Barberton Nature Reserve was observed after an observation period of eight weeks (Table 8; $F=6$; $p < 0.01$).

Table 8: Individual growth and mortality of *Eisenia fetida* and *Aporrectodea caliginosa* exposed for 8 weeks to soils from different sites (Mean ± SD; n=48 for *E. fetida*; n=30 for *A. caliginosa*;'*' indicates $p < 0.05$)

Sampling site/ treatment (soil)	Mean individual biomass [mg]		Mass change after 8 weeks [%]	Mortality after 8 weeks [%]
	At start	At end		
E. fetida Control	133.8 ± 22	426.7 ± 76	226.1 ± 77.5	0
E. fetida Barberton	155.7 ± 35.7	430.3 ± 65	190.9 ± 92	0
E. fetida Kaapsehoop 1	133.6 ± 26.6	263.7 ± 100.1*	98.3 ± 40*	18.75 ± 7.98*
E. fetida Kaapsehoop 2	126.7 ± 7	276.7 ± 78.3*	122.5 ± 64.3*	10.42 ± 12.5
A. caliginosa Stellenbosch	660 ± 110	570 ± 81.8*	- 13.4 ± 3.9	0*
A. caliginosa Agnes Mine	660 ± 87.2	470.4 ± 50.1*	-28.4 ± 5.9*	10*
A. caliginosa Songimvelo	857 ± 167.7	516.2 ± 112*	- 39.9 ± 2.9*	13.3 ± 5.8*
A. caliginosa Kaapsehoop 3	573 ± 55.1	370 ± 17.3*	-35.3 ± 3.4*	46.7 ± 5.8*

More complete data on growth of *E. fetida* exposed to the ultramafic soils is also shown in Appendix 3-1. A significant smaller growth was observed after 8 weeks in both of the groups of *E. fetida* exposed to the 2 Kaapsehoop-samples; Kaapsehoop 1 and 2 (Table 8, $F=4.61$; $p < 0.05$). No significant difference was observed between the two Kaapsehoop groups.

After an exposure period of four weeks, the mass gain was significantly ($p<0.05$; $F=131$) higher in the worms exposed to the control soils, compared to the worms exposed to the ultramafic soils. No significant difference was recorded between the different ultramafic samples after four weeks.

Further, after six weeks of exposure, a decrease in total biomass was observed in the specimens exposed to the Kaapsehoop 1 and 2 ultramafic soil samples (Figure 24). A significant difference in growth was observed only between the specimens exposed to the soils from the Barberton Nature Reserve and the specimen exposed to the soils collected at Kaapsehoop 1.

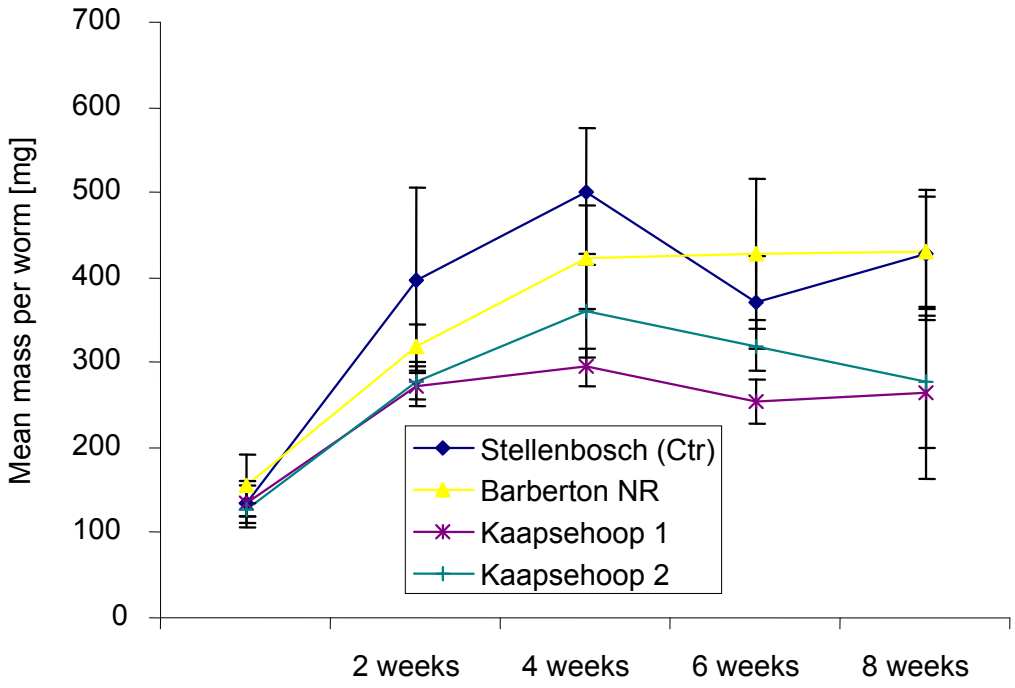

Figure 24: Mean individual biomass (wet mass) of *Eisenia fetida* exposed to control soils (Stellenbosch) and ultramafic soil samples collected at the Barberton Nature Reserve (Barberton NR) and Kaapsehoop (Kaapsehoop 1 and Kaapsehoop 2) over an exposure period of 8 weeks (n=48).

After the whole observation period, the individuals of *E. fetida* in the control group and those in the soils from the Barberton Nature Reserve showed a similar final mass (Figure 24) and significantly higher growth than specimens exposed to the Kaapsehoop 1 and 2 samples (Table 8). No proper explanation was found for the mass loss of *E. fetida* after exposure duration of six weeks in the control groups. Although statistically not significant, it was observed in all control groups in all test intervals of about six months. For the mobile soil fraction ($CaCl_2$), no correlation in terms of mortality and growth was recorded. The highest amounts of mobilisable (DTPA) nickel and cobalt correlated with the highest mortality in *E. fetida*. A correlation was also found between the total amounts of chromium, cobalt, nickel and manganese, in Kaapsehoop 1 and 2 soils. Concerning the mortality of the mature *E. fetida* with a history of exposure, after six weeks of exposure, one single worm of the group with no history of exposure died. That resulted in a mortality of 3.33% for this group. As this part of the study was conducted with mature worms of an unknown age, it is

also possible that this specimen died of age. At the end of the exposure after eight weeks, no worms were dead or absent in the control, one of 48 worms was dead in the exposure of *E. fetida* with no history of exposure to the Kaapsehoop 3 soils and two of 48 specimen in each of the groups previously exposed to nickel and to cadmium were dead or absent. The mass of *E. fetida* with a history of exposure are shown in Appendix 3-2 and Figure 25

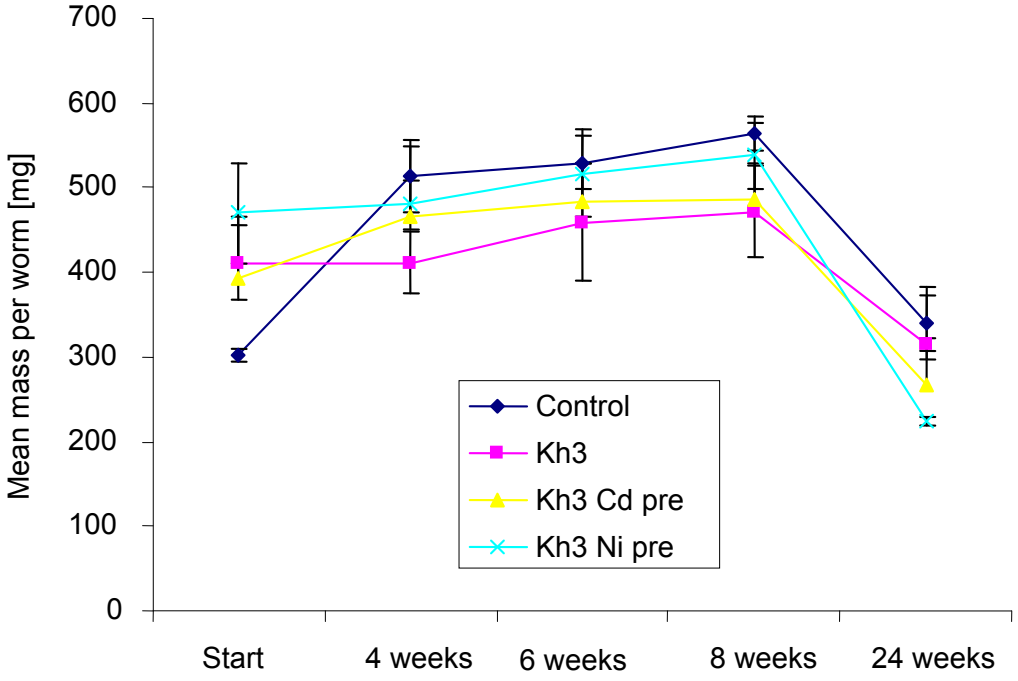

Figure 25: Mean individual mass (wet mass) of *Eisenia fetida* exposed to control soils (Stellenbosch) and ultramafic soil samples collected at Kaapsehoop 3 over an exposure time of 18 weeks (n=30); Kh3: *E. fetida* without a history of pre-exposure exposed to Kaapsehoop 3 soils; Kh3 Cd pre: *E. fetida* long-term (more than 10 generations) exposed to cadmium; Kh3 Ni pre: *E. fetida* long-term (more than 10 generations) exposed to nickel.

In general, no significant difference ($p>0.05$) was observed within the different exposures. Comparing the control to the exposures, except at the start mass ($F=5.52$, $p<0.05$), a significant difference in mass to the ultramafic exposures was observed only at the end of the exposure ($F=9.92$, $p<0.05$).

5.3.3 Cocoon production and hatching success of *Eisenia fetida*

Over the whole observation period of eight weeks, the number of cocoons collected from the Kaapsehoop 2 soil samples was significantly ($z=2.02$; $p < 0.05$) smaller than the number of cocoons collected from the other exposures (Table 9 and Appendix 3-3). A significant increase in

cocoon production in the course of the exposure was observed in the Barberton Nature Reserve ultramafic soil sample, from one cocoon produced by 48 worms after two weeks to 12 cocoons from 28 worms collected at week six up to 119 cocoons per 24 worms after eight weeks.

No significant difference in the mean number of cocoons produced per worm over the whole observation period was observed (Table 9). However, after two weeks of exposure a significant difference was reported between the cocoon production of the worms in the control group (0.35 ± 0.18 cocoons/individual) and of those exposed to the Barberton Nature Reserve samples (0.02 ± 0.04) as well as to the worms exposed to the soil samples of Kaapsehoop 2 (0.04 ± 0.08 cocoons per worm), indicating a lag in cocoon production in the two ultramafic samples. Also, within the exposures, the number of cocoons per worm increased significantly during the exposure time in all substrates.

Concerning the number of cocoons of the specimens of *E. fetida* with a history of exposure (Appendix 3-4), no significant differences ($p<0.05$) between the exposures was observed, but the number of cocoons was at both counting dates significantly higher in the control than in all of the exposures.

The hatching success of *E. fetida* cocoons (mean percentage of cocoons hatched, Appendix 3-3) was found to be significantly higher ($p<0.05$, $f=7.4$) in the Stellenbosch control soils than in the Barberton ultramafic soils and the Kaapsehoop 1 ultramafic soils (Table 9).

The total number of hatchlings was far lower in the exposures than in the control (Table 9, $f=61.8$, $p < 0.05$). The mean number of hatchlings per cocoon of the worms exposed to the Barberton Nature Reserve soil samples and to the Kaapsehoop 1 samples was significantly lower ($p<0.05$, $f=234.6$) than the number of hatchlings per cocoon produced by the worms exposed to the Kaapsehoop 2 soil samples and to the control samples from the Stellenbosch site. Further, a time related decrease of fertility was observed. At the Kaapsehoop samples, the hatching success of cocoons remained constant with 2.5 ± 0.45 hatchlings per cocoon after an exposure duration of eight weeks, whereas in all other samples, including the control a significant ($p<0.05$) decrease was found.

The highest decrease was reported in the Barberton sample, from 2.58 ± 2.5 at the first collection of cocoons to 1.24 ± 0.18 hatchlings per cocoon from cocoons collected after eight weeks, in the control sample a decrease from 3.27 ± 0.52 to 1.77 ± 0.21 hatchlings per cocoon was observed. For the exposures of the *E. fetida* with a history of exposure, no differences in terms of hatching success or number of hatchlings per cocoon were recorded (Appendix 3-4).

Table 9: Mean cocoon production and reproduction success of *Eisenia fetida* and *Aporrectodea caliginosa* in soils from the different sites over an observation period of eight weeks (Mean ± SD; n=48 for *E. fetida*; n=30 for *A. caliginosa* at start of the exposure; '*' indicates p> 0.05).

Sampling site/ treatment	Total # of cocoons	Total # of hatchlings	Mean cocoons per worm	Hatchlings per cocoon	Mean % Hatch
E. fetida Control	226	519*	8.1±2.5	2.2±0.4	91.6±6
E. fetida Barberton	132	180	5.3±1.6	1.3±0.1*	68.3±7.7*
E. fetida Kaapsehoop 1	128	218	5.4±3.5	1.7±0.5*	64.3±13.3*
E. fetida Kaapsehoop 2	94*	190	4.4±1	2.5±0.5	81.7±8.3
A. caliginosa Control	11	6	0.4± 02	0.5±0.1	46.7±5.8
A. caliginosa Agnes Mine	1	0	0.1±0.2	0*	0
A. caliginosa Songimvelo	27	0	0.8±0.3	0*	0
A. caliginosa Kaapsehoop 3	4	5	0.1±0.1	1.2±0.3	100

5.3.4 Mortality and growth of *Aporrectodea caliginosa*

In the control group, no mortality was recorded after an exposure time of eight weeks (Table 8). Compared to the groups of *A. caliginosa* exposed to the ultramafic soils, mortality and mass loss were significantly ($p<0.05$) lower in the control.

Also, the specimens exposed to Agnes Mine and to Songimvelo showed a significantly lower mortality than those in the Kaapsehoop 3 samples (Table 8). Furthermore, after 98 days only 8% of worms survived. The lowest mass losses within the exposed groups were reported in the Agnes Mine soil, lower than the mass loss at Kaapsehoop 3 and significantly lower than the loss of biomass in Songimvelo soil (Table 8).

Further, in the worms exposed to substrates from Kaapsehoop and Songimvelo it was observed that *A. caliginosa* autotomizes the posterior section and consequently loses body length and mass (Figure 26), but was subsequently able to regenerate segments of the hind body.

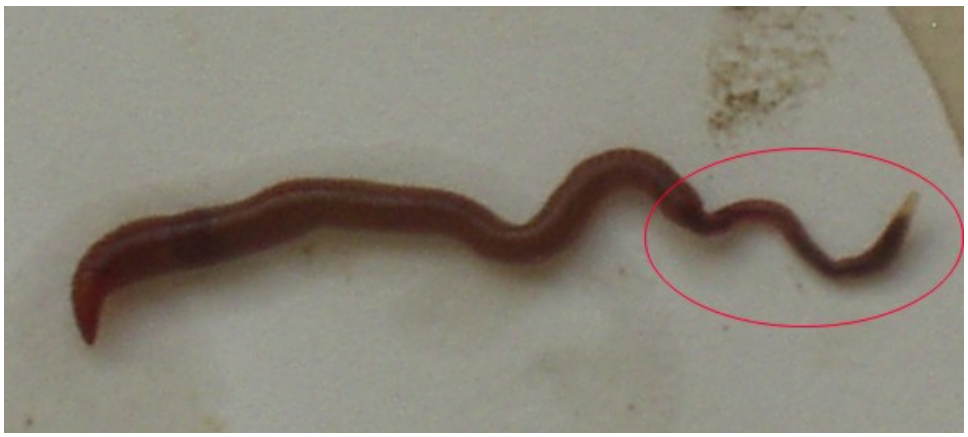

Figure 26: Possible regeneration taking place after autotomization of *Aporrectodea caliginosa* exposed to ultramafic Kaapsehoop 1 soil samples.

5.3.5 Cocoon production, hatching success and hatchling numbers of *Aporrectodea caliginosa*

Due to the small number of cocoons of *Aporrectodea caliginosa* found in the ultramafic soils, no time related change in cocoon production or hatching success was recorded. Over all, only 11 cocoons were produced by the worms exposed to the control soils, resulting in a mean cocoon production over a period of eight weeks of 0.36 ± 0.15 cocoons per worm. Nevertheless, the Songimvelo exposures and the control group showed a significantly higher production ($p<0.05$) of cocoons than the *A. caliginosa* exposed to soil samples from Agnes Mine and Kaapsehoop (Table 9).

In total, only one cocoon was found over an observation period of eight weeks in the samples from Agnes Mine and four cocoons were found in the sample of Kaapsehoop 3 soil (Table 9).

The fertility of cocoons, expressed in the number of cocoons hatched compared to the total number of cocoons, differed significantly ($p<0.05$) in all ultramafic soil samples from the control group. In the control group, 46.7 ± 5.8 % of the cocoons hatched, whereas in the exposures to the Agnes Mine soils and the Songimvelo soils no cocoons hatched, but in the Kaapsehoop samples, all cocoons hatched. The number of hatchlings per cocoon also differed significantly between the *A. caliginosa* exposed to the Kaapsehoop soil samples and the specimens exposed to the control soils (Table 9).

5.4 Discussion

Due to the fact that *A. caliginosa* was considered to be a more field relevant species, preferring soils with a low organic content, it was initially planned in this study to focus on *A. caliginosa*. For that reason, preliminary testings were conducted only with A. caliginosa on the soils from Agnes Mine, Songimvelo and Kaapsehoop 3. A second test, using *A. caliginosa* in the same substrate as *E. fetida*, was not undertaken due to the poor fecundity and growth of this species, even in the

control. Hence, due to the difference in experimental setup, a direct comparison between the two species was not possible.

5.4.1 Mortality and growth

In terms of the total amount of metals, *E. fetida* tolerated far higher concentrations of cobalt, manganese and nickel than are known from laboratory studies such as those conducted by Neuhauser et al. (1985) on various metals, and by Kuperman et al. (2004) on manganese. The toxicity of chromium strongly depends on the chemical speciation of chromium; Cr(VI) is considered far more toxic to *E. fetida* than Cr(III); no conclusions can be drawn about the effects of the total amount of chromium, as it is not known, what speciation predominantly occurs in these ultramafic soils. In general, the bioavailable amount of chromium gathered via the analysis of the mobile ($CaCl_2$) and the mobilisable soil fraction (DTPA) was far lower than the concentrations of Cr(IV) known to cause toxic effects in earthworms (Sivakumar and Subbhuraam 2005). Also, the amounts of $CaCl_2$ and DTPA extracted Co, Mn and Ni were lower than the amounts known to have an effect on survival and growth (Neuhauser et al. 1985, Lock and Janssen 2002b, Kuperman et al. 2004), indicating that *E. fetida* is able to remobilise biologically non-available metals.

Besides the known toxic effects of nickel to *E. fetida*, it also cannot be excluded that the survival of earthworms in this study could have been influenced by the presence of cobalt. Although some essential trace elements do stimulate growth and reproduction at low concentrations, it is known that especially cobalt is extremely hazardous at higher concentrations. Neuhauser et al. (1984b) also stated that cobalt is at comparable concentrations far more toxic to earthworms than nickel.

During the course of the exposures, a slower growth rate was observed for worms in all ultramafic soils. Only for *E. fetida* exposed to the Barberton Nature Reserve soils containing the lowest total, as well as estimated environmentally available concentrations of metals, no significant reduction in final mass was seen (Figure 24). This finding indicates retarded growth of *E. fetida*, probably caused by stress originating from the ultramafic soils. Although *E. fetida* is able to respond at lower concentrations of metals to these ultramafic conditions, it would possibly need a certain time span to accommodate. The decrease in biomass that took place in worms in the Kaapsehoop samples after the exposure period of four weeks can be related to a slowly affecting toxicity. It is known that the uptake kinetics of metals are rather slow (Spurgeon 1999), but this finding might also be an indication of the role of non-metal toxicants such as e.g. asbestos. Although no asbestos fibres larger than 5 µm were identified, it is known that asbestos occurs in the soils of the Barberton Greenstone Belt (Ward 1995). Furthermore, Schreier and Timmenga (1986) stated that asbestos only affects earthworms after an exposure of several weeks.

For *Aporrectodea caliginosa*, the highest mortality was found in the soils with the highest contents of metals measured in all soil fractions. Thus there were no indications of which specific metal can be considered as the main cause of toxic effects. Due to the mass loss observed in the control soils, the mass loss of *A. caliginosa*, observed in all soil substrates might have been caused by

insufficient and inadequate feeding. In addition to this, one can conclude that the significant additional loss of mass was caused by the ultramafic conditions in the soils. The lowest additional mass loss of *A. caliginosa* can be correlated with the lowest metal concentrations in the ultramafic soils, whereas the difference between the soils containing the higher concentrations of metals was not significant to identify a certain metal as main cause of toxicity. Also, in the Kaapsehoop samples, the loss of body mass might be correlated with the observed autotomization of the posterior section. *A. caliginosa* seems to be able to biomagnify metals in the posterior section and discards this body part after reaching a certain concentration of toxic materials.

5.4.2 Cocoon production and fertility

A significant difference in the total cocoon production was observed between the control groups and the groups of *E. fetida* exposed to both of the Kaapsehoop soil substrates containing high levels of metals. Apparently sublethal concentrations or sublethal conditions retarded the production and the viability of cocoons, as seen in the Barberton Nature Reserve sample. Although no significant difference to the other exposures was observed in the mean number of cocoons produced in the Barberton Nature Reserve soils in total, a significant increase in production over the time of exposure was observed. On the other hand, a significant decrease of hatching success during the time of exposure was observed in this sample as well as in the Kaapsehoop 1 soil. A significant difference in the mean cocoon production over the whole time was found only between the Kaapsehoop 1 soils and the control group. Also, the highest amount of DTPA-extractable nickel and manganese was found in this soil, which may indicate that these metals are more available for uptake by soil living organisms. Furthermore, results lead to the conclusion that the assessment of the number of cocoons alone is an insufficient parameter for the evaluation of toxic threats emerging from ultramafic and also most probably anthropogenically contaminated soils. This finding is also supported by previous findings by Reinecke et al. (2001), indicating that toxic metals may affect cocoon viability, but that cocoon production itself is not always a good indicator for the determination of sublethal effects. The mean number of hatchlings per cocoon after eight weeks of exposure was significantly higher in the group of *E. fetida* exposed to the highest concentrations of metals than in all other groups, including the control group (Table 9). One possible explanation might be that under certain conditions of toxic stress, *E. fetida* may be able to respond to this condition by increasing the production of offspring to offset mortality. A stimulation of cocoon production by metals was reported before by Reinecke & Reinecke (1997a) for *E. fetida* exposed to low concentrations of lead. The results also indicate that the reproductive ability is not affected severely under these conditions, in contrast to growth.

A similar increase in cocoon production compared to the control was found for *Aporrectodea caliginosa* in the soil sample from Songimvelo containing lower concentrations of metals than the Kaapsehoop 3 sample, whereas at higher levels of metal load, a decrease of cocoon production was found. The fact that cocoon production not necessarily correlates with fecundity, was shown in

this group of worms exposed to ultramafic soils from Songimvelo. Although they produced the highest number of cocoons, all cocoons were not viable, which means that two levels of reproduction were affected differently, cocoon production positively by stimulation and hatching success negatively. The comparatively low number of cocoons overall can be explained by the fact that the availability of food plays an important role in cocoon production (Reinecke et al. 1990). Also a contributing factor is the slow reproduction of *A. caliginosa* in general (Holmstrup et al. 1991, Nair and Bennour 1998, Lowe and Butt 2002). Due to the relatively low organic matter content of the soil samples, worms were fed additionally with chicken feed, which, referring to the observed mass loss, seems to be less suitable for this purpose.

5.4.3 Conclusions

In contrast to these findings, Spurgeon et al. (1994) did not find significant effects of metals on the viability of cocoons of *E. fetida*. Besides differences in the experimental design, Spurgeon et al. exposed their specimens to different metals than those found in the ultramafic soils. On the other hand, it is in agreement with the findings of Reinecke et al. (2001) who observed a lowered hatching success for cocoons produced by earthworms exposed in OECD soil to different metals. Concerning the production of cocoons, Spurgeon et al. (1994) found it more sensitive than mortality which is in agreement with our findings with the exposures of *E. fetida*. Extrapolated to population levels, it is obvious that the hatching success is more important for the maintenance and increase of earthworm populations than simply the production of cocoons, as seen for *A. caliginosa* in the Songimvelo soil samples and in the Kaapsehoop 2 samples for *E. fetida*.

Ultramafic soils, just as anthropogenically contaminated soils, do pose a manifold threat to soil living organisms. Ecotoxicological endpoints can be considered as tools to assess the threats emerging from these conditions as a sum of different stresses.

As it was shown in this study, endpoints such as growth, cocoon production and viability do respond to the sum of toxic factors. Due to its limited effects on population dynamics, cocoon production alone was not suited in this study as an endpoint for the determination of effects at the population level; in terms of population development the evaluation of the hatching success is a better indicator. No correlation between growth and cocoon viability was found, indicating that on the population level and consequently for the evaluation of a soil *in toto* as a biotope, the validity of individual growth as an endpoint is limited. Also survival as an endpoint might only be useful for substrates with high concentrations of bioavailable toxicants.

By comparing the two ecophysiological different species of earthworms, we can confirm the finding of Spurgeon and Hopkin (1995), that responses of endogeic earthworms to certain metals differ from the reactions of epigeic species inhabiting the same contaminated soil. Furthermore, *E. fetida* seems to be more suited for the assessment of these extremely toxic conditions, due to the lesser but still sensitive response of life cycle parameters. It is in agreement with previous findings

(Holmstrup et al. 1998, Spurgeon et al. 2000) that *E. fetida* is more robust than *A. caliginosa*. The extent and the basic mechanisms underlying this difference need further investigation.

6. Acute toxicity of nickel to *Eisenia fetida*

6.1 Introduction

Even though the behaviour of one toxicant can, especially in soil, not be understood separately due to the fact that the physical and biochemical properties of substances change due to interactions with other substances (Pokarzhevskii and Van Straalen 1996), laboratory exposures focussed on a single toxicant are still important for the investigation of mechanisms and specific effects of toxicants on soil inhabiting organisms such as earthworms. Thus, for the evaluation of a defined toxicant in soil, a detailed knowledge about effects and the uptake kinetics on a stand alone basis is also required. To exclude the interactions between soil contents to focus only on the effects of a selected toxicant, the use of artificial, defined substrates has been established (EEC 1982, OECD 2004). As there is evidence that the bioavailability of chemicals to earthworms is largely determined by the soil solution concentration, Kiewiet and Ma (1991) developed a simplified model based on the assumption that uptake experiments may be done in aqueous media to exclude the influences of soil properties such as, for example, absorption processes.

The gut content in earthworms, consisting mostly of soil and organic materials, may vary between individuals (Martin 1986) and since bioavailability of potentially toxic substances is often determined by the amount of pollutant in the body of an earthworm (Lanno et al. 2004, Ma 2005), different methods have been developed to void the guts of earthworms before experiments or analyses (Jöst 1897, Krontowsky and Rumiauzew 1922, Bouché 1966, Martin 1986, Springett and Gray 1992, Knight et al. 1992, Dalby et al. 1996). Most of these methods do have certain disadvantages; either having the risk of physically damaging the earthworms or, e.g. when using the filter paper method, a small amount of soil remains in the gut. The use of agar was proposed by Pokarzhevskii et al. (2000) to replace the soil. Agar is established as a culture medium for many organisms, such as Protozoa and also for cells, but has also been used as a medium for experiments with nematodes (Williams and Dusenbery 1988) and enchytraeids (Westheide et al. 1991, Arrate et al. 2002). It has been mentioned by Pokarzhevskii et al. (2000) that the earthworm *Perionyx excavatus* (Perrier 1872) was able to live in an agar environment for six months. In light of the above mentioned facts, an exposure of *Eisenia fetida* (Savigny 1826), a species widely used in laboratory experiments, to a medium consisting of a combination of the methods proposed by Pokarzhevskii et al. (2000) and the reconstituted ground water developed by Kiewiet and Ma (1991) to selected metals, was conducted to merge the advantages of both methods and to exclude some of the disadvantages both methods provide. Especially at higher concentrations of toxicants, it has been observed that worms often try to escape from the reconstituted ground water exposures. In reconstituted water resembling natural ground water, it is recommended that earthworms should be exposed singly in glass containers with aerated reconstituted water (Kiewiet and Ma 1991). Depending on the number of exposure concentrations and the number of replicates needed for consecutive tests, space and laboratory equipment also can become a limiting factor. Further, if not exposed singly, it has been observed that worms often get affected by dead

specimens not removed fast enough from the artificial ground water exposures. This may lead to false results in the evaluation of lethal concentrations of a specific toxicant. On the other hand, it is proposed that by the addition of micronutrients and essential trace elements to agar, a reconstituted solid resembling natural ground water can be simulated.

As this study deals in its broader frame with the ability of earthworms to survive in ultramafic soils, a special focus was set on the effects of nickel on earthworms. Nickel occurs in abundance in these soils and is toxic at elevated concentrations.

For nickel, the essentiality is not revealed in detail yet (Phipps et al. 2002), but its toxicity to soil organisms is well documented by numerous studies using different endpoints such as mortality (Furst et al. 1993), growth (Malecki et al. 1982), reproduction (Lock and Janssen 2002b), cytotoxicity (Scott-Fordsmand et al. 1998) and genotoxicity (Reinecke and Reinecke 2004b). In terms of mortality, Neuhauser et al. (1985, 1986) recorded a LC_{50} of 757 mg/kg Ni for *E. fetida* exposed in OECD soil and LC_{50} values ranging between 17 mg/kg for nickel acetate and 25 mg/kg for nickel sulphate in *E. fetida* using the contact paper test (EEC 1982).

Furst et al. (1993) injected saline solutions consisting of different nickel salts subcutaneous into *E. fetida* and graphically determined a LC_{50} for different nickel salts between 52 mg/kg ($NiCl_2$) and 69 mg/kg (nickel acetate) after 48 hours of exposure.

Scott-Fordsmand et al. (1998) obtained a LC_{50} value of 684 mg/kg Ni and recorded no survival at 1000 mg/kg Ni in adult *E. veneta* exposed to nickel as $NiCl_2 \cdot 6\ H_2O$ in a loamy sandy soil. Lock and Janssen (2002b) exposed *E. fetida* to different concentrations of $NiCl_2$ in OECD soil (OECD 1984) and at the highest concentration they used, 1000 mg/kg Ni dry wt., they did not find any mortality.

Important in the frame of the present study was to find out if *E. fetida* is able to develop an increased acclimatisation (physiologically based) or even an adaptation (genetically based) to nickel as may be expected to happen in ultramafic soils. An increased tolerance to different metals has previously been described (Reinecke et al. 1999). Also, a cross-resistance, meaning that earthworms which developed an increased tolerance to one metal can also become tolerant to another one, was found (Spurgeon and Hopkin 2000).

No evidence of such an acquired resistance to nickel is known so far. As manganese is also found at high concentrations in ultramafic soils, another aim of this part of the study was to evaluate if earthworms exposed over several generations to this metal show an increased tolerance to nickel. Naturally, manganese does not occur as a base metal but it is a component of more than 100 different minerals. Anthropogenic sources for manganese pollution are mining and mineral processing, particularly nickel mining, waste water discharging, sewage sludge and emissions of industrial production (WHO 2004). Although manganese is an essential nutrient for micro-organisms, plants and animals (Hurley and Keen 1987), it is at elevated concentrations toxic to soil living organisms (Diaz-Lopez and Mancha 1994).

6.2 Materials and methods

The worms used in this study were pre-clitellate specimens of the species *Eisenia fetida.* They had a history of long-term pre-exposure in stock cultures maintained in the ecotoxicology laboratory of the Department of Botany and Zoology at the University of Stellenbosch, Western Cape. Three different cultures were used, all kept for several years in darkness at a constant temperature of 25°C, a moisture content between 73% and 78% and a pH between 6 and 7 in a climate controlled room. The substrate consisted of ground and sieved cattle manure. All cultures were fed on a weekly basis with fresh urine-free cattle manure. Worms from the first culture have not had exposure to manganese or nickel for at least eight years. Due to the fact that the average life cycle of *E. fetida* is about three to four months (Venter and Reinecke 1988), they were kept for at least 20 generations in these cultures. The other two groups were long-term exposed to manganese or nickel. The cultures exposed to $MnSO_4$ were exposed for at least ten generations (more than four years). In the case of the cultures exposed to nickel, the exposure period was substantially longer. On a biweekly basis, these pre-exposed cultures have been fed with urine free cattle manure containing 0.02% nickel as $NiCl_2 \cdot 6\ H_2O$ and respectively 0.8% of manganese as $MnSO_4 \cdot 4\ H_2O$.

For the assessment of the acute effects of nickel by excluding soil properties, an LC_{50} evaluation was conducted on a medium designed as proposed above. For purposes of comparison, reconstituted water resembling natural ground water was used. Reconstituted water resembling natural ground water consists of 100 mg $NaHCO_3$, 20 mg $KHCO_3$, 200 mg $CaCl_2 * 2H_2O$ and 180 mg $MgSO_4$ per litre distilled water. Additional to this composition proposed by Kiewiet & Ma (1991), 15 g per litre (1.5%) normal melting agarose were added to combine the media used successfully for the cleaning of earthworm guts by Pokarzhevskii et al. (2000), as a new medium of exposure. For the experimental setup, reconstituted water was carefully heated, 1.5% of normal melting agarose (bacteriological grade, Merck KGaA, Darmstadt, Germany) was dissolved and the different concentrations of nickel were added. The agarose medium was then filled into 500 ml plastic containers. After cooling down and solidifying, the agar gel was cut in small cubes.

At least three replicates per metal concentration and six worms of the species *E. fetida* per replicate were exposed for 96 hours in the artificial ground water gel and for 48 hours in 500 mL of artificial ground water to different concentrations of nickel by addition of the specific concentrations of $NiCl_2 \cdot 6\ H_2O$. In detail, a range of 11 concentrations between 13.5 mg/L as lowest concentration and 2180 mg/L as the highest concentration of nickel (as nickel chloride) was used. As a control, four replicates consisting of six worms were exposed for the same time span to clean substrate.

During the period of exposure, both media of exposure were kept in a dark, climate-controlled (20°C) room and covered with black plastic foil. The plastic containers containing agar were closed with a perforated lid for aeration and the artificial soil water was continuously aerated with an aquarium pump.

All worms (pre-clitellate) ranged in live (wet) mass between 297 to 378 mg. After 96 hours in the agarose containing medium and after 48 hours in reconstituted water resembling artificial ground water, the surviving worms were collected, counted and weighed.

6.2.1 Statistics

Mortality was determined and the LC_{50}-values were calculated with the Probit Program Version 1.5 published by the Environmental Protection Agency (Cincinnati, Ohio, USA). Tests were considered valid if the test for heterogeneity was not significant (the calculated chi-square smaller than the tabular value) at 0.05 level (EPA 1993) and if survival in the control passed or exceeded 90%. Exposures were considered significantly different if no overlaps in the 95% confidence limits of the corresponding LC values were observed. If one of these above mentioned criteria was not fulfilled, a trimmed Spearman Karber method was applied (Hamilton et al. 1977). Further, although not recommended in case of partial mortalities (EPA 1993), the LC_{50} concentration was also determined graphically as a backup respectively for purposes of comparison by simply graphically plotting the percentage of mortality against the concentration.

6.3 Results

In general, mortality of *E. fetida* increased with increasing nickel concentrations; also, all worms exposed to concentrations higher than 1000 mg/L died. No mortality was observed in any of the control groups.

6.3.1 *Eisenia fetida* exposed to nickel

Table 10: Estimated LC_{50} values and confidence limits after Probit analysis of *Eisenia fetida* (n>18) with different histories of exposure and then exposed to nickel as $NiCl_2 \cdot 6H_2O$ (mg/L) in agar medium and in artificial ground water.

	Pre-exposure	LC_{50}	95% confidence limits	
			lower	upper
E. fetida in artificial ground water	none	164.24	131.39	210.58
	$NiCl_2$	44.28	38.63	51.23
	$MnSO_4$	161.20	127.86	208.84
E. fetida in agar-medium	none	151.49	122.95	189.72
	$NiCl_2$	50.73	42.96	61.24
	$MnSO_4$	138.19	111.12	178.12

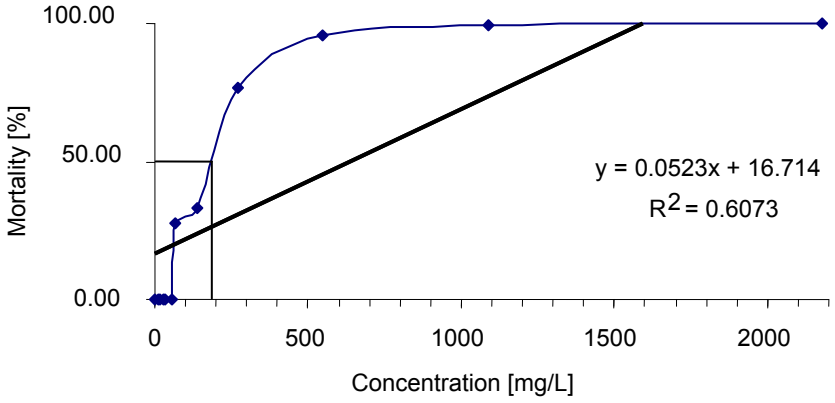

Figure 27: Mortality [%] of *Eisenia fetida* exposed for 48 hours in artificial ground water plotted against exposure concentration of nickel [mg/L]; n>18

The raw data (without control) of *E. fetida* exposed for 48 hours to artificial ground water are shown in Appendix 4-1, the LC-values are shown in detail in Appendix 4-2 and the Probit and regression line is shown in Appendix 4-3. For the *E. fetida* without a history of exposure and then exposed in artificial ground water (Figure 27), a LC_{50} of 164.24 mg/L was calculated by Probit analysis (Table 10). Graphically, a LC_{50} of 185 mg/L nickel as $NiCl_2$ • 6 H_2O was determined. No mortality was observed in the control groups.

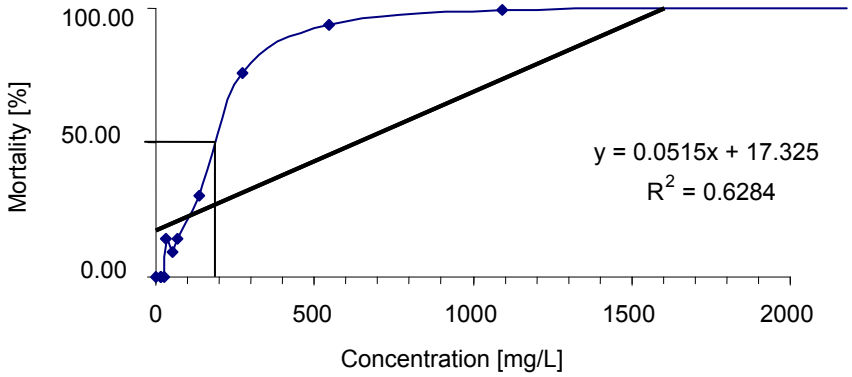

Figure 28: Mortality [%] of *Eisenia fetida* exposed for 96 hours in artificial ground water gel plotted against exposure concentration of nickel [mg/L]; n>18

Mortality and exposure concentrations (without control) of *E. fetida* specimens exposed for 96 hours to artificial ground water gel are shown in Appendix 4-4; detailed results of the Probit analysis are shown in Appendix 4-5 and the plot of adjusted Probit versus predicted regression line is shown in Appendix 4-6. For *E. fetida* exposed in the gel, an LC_{50} of 151.49 mg/L was calculated by Probit analysis (Table 10). Graphically, a mortality of 50% was determined at a concentration of 185 mg/L (Figure 28). No mortality was observed in the control. Even after 9 month of exposure,

90% of the earthworms in the control groups were still alive in the gel substrate although no reproduction was observed.

6.3.2 Manganese pre-exposed *Eisenia fetida* exposed to nickel

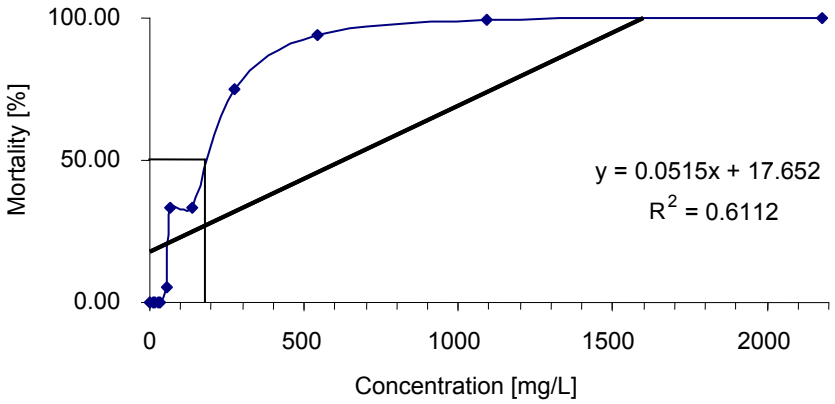

Figure 29: Mortality [%] of manganese pre-exposed *Eisenia fetida* exposed for 48 hours in artificial ground water plotted against exposure concentration of nickel [mg/L]; n>18

Exposure concentrations and mortality values for *E. fetida* specimens previously exposed to manganese and then exposed in artificial ground water to nickel, are shown in detail in Appendix 4-7. Detailed LC values and statistics are presented in Appendix 4-8 and a plot of the predicted regression line and adjusted Probits are shown in Appendix 4-9. The LC_{50} of nickel for *E. fetida* pre-exposed to manganese in artificial ground water was calculated by the Probit analysis at a concentration of 161.20 mg/L Ni as $NiCl_2$ • 6 H_2O (Table 10), the graphical determination revealed an LC_{50} of 182 mg/L (Figure 29).

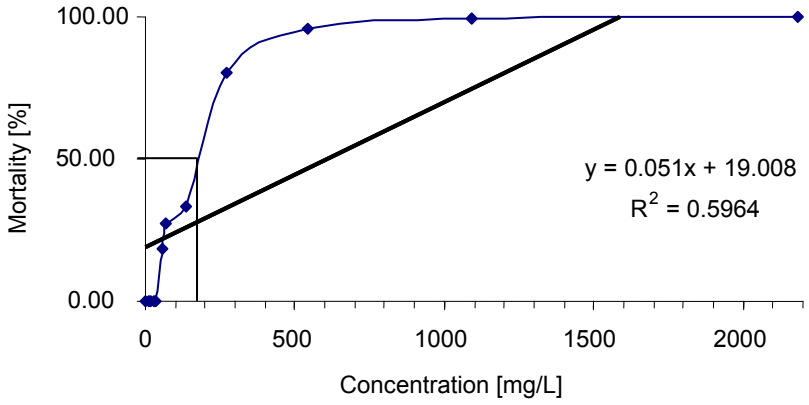

Figure 30: Mortality [%] of manganese pre-exposed *Eisenia fetida* exposed for 96 hours in artificial ground water gel plotted against exposure concentration of nickel [mg/L]; n>18

Mortality (without control) of manganese pre-exposed *E. fetida* specimens exposed for 96 hours in artificial ground water gel is shown in detail in Appendix 4-10. Detailed LC-values are shown in Appendix 4-11 and a plot of adjusted Probit versus predicted regression line is shown in Appendix 4-12. The LC_{50} calculated by Probit analysis was 138.19 mg/L Ni as $NiCl_2$ • 6 H_2O, graphically, a LC_{50} of 178 mg/L Ni was detected.

6.3.3 Nickel pre-exposed *Eisenia fetida* exposed to nickel

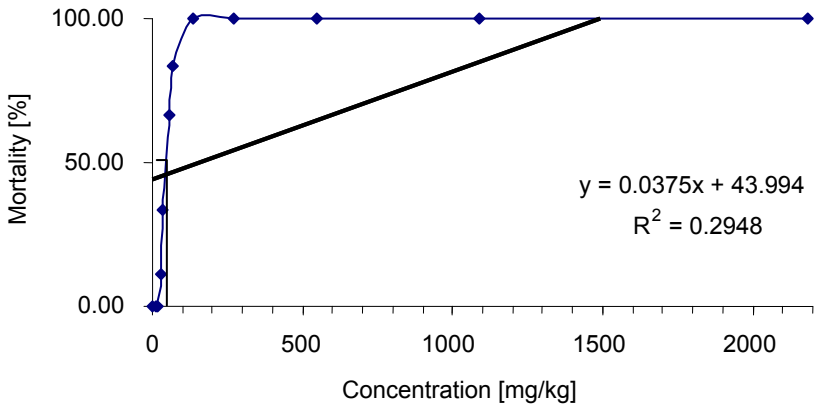

Figure 31: Mortality [%] of nickel pre-exposed *Eisenia fetida* specimens exposed for 48 hours in artificial ground water plotted against exposure concentration of nickel [mg/L]; n>18

In Appendix 4-13, the mortality of the nickel pre-exposed *E. fetida* specimens exposed to nickel in artificial ground water is shown. In Appendix 4-14 the detailed LC_{50} values are given and in Appendix 4-15 a plot of the adjusted Probit is shown. The Probit analysis calculated a LC_{50} of 44.28 mg/L Ni as $NiCl_2$ • 6 H_2O Table 10 and an LC_{99} of 115.85 (Appendix 4-14). The graphical determination revealed a LC_{50} of 45 mg/L Ni (Figure 31).

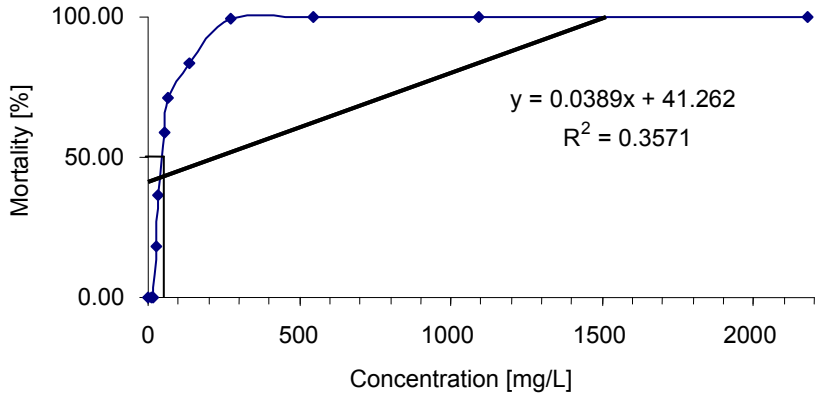

Figure 32: Mortality [%] of nickel pre-exposed *Eisenia fetida* specimens exposed for 96 hours in artificial ground water gel plotted against exposure concentration of nickel [mg/L]; n>18

For the nickel pre-exposed *E. fetida* exposed in agarose gel medium to nickel (Figure 32), the mortalities in detail are shown in Appendix 4-16, the LC values and statistics in detail are shown in Appendix 4-17 and predicted regression line and adjusted Probits are shown in Appendix 4-18. The Probit analysis revealed a LC_{50} value of 50.73 mg/L Ni as $NiCl_2$ • 6 H_2O and graphically, a LC_{50} of 45 mg/L was determined.

All Probit analyses were considered valid as none of the mortalities in the control groups passed or exceeded 10%. No mortality was recorded in the control groups and at the 0.05 level, no significant heterogeneity was observed.

No overlap between the confidence limits of the LC_{50} values of nickel pre-exposed worms and worms not pre-exposed, as well as the *E. fetida* specimens pre-exposed to manganese, was observed. Strong overlaps between the artificial ground water exposures and the agarose gel exposures were revealed by the Probit analysis within the different groups with a different history of exposure. Also, an overlap was recorded between the manganese pre-exposed specimens and the specimens without a history of pre-exposure.

6.4 Discussion

As shown in Figure 27 to Figure 32, a clear dose relationship was observed in both media. At increasing concentrations of nickel, the mortality of *Eisenia fetida* increased linearly. No LC_{50} values for the exposure of *E. fetida* in the same or comparable substrate were found in previous studies; previous studies have used either different substrates or different species or different nickel salts. In relation to the findings obtained in this part of the study, Reinecke and Reinecke (2004b) exposed specimens of a similar group of the *E. fetida* as used in this study for the same exposure time in reconstituted ground water to nickel chloride and found, that non pre-exposed *E. fetida* did not survive concentrations higher than 250 mg/L. In their study, no LC_{50} was determined. It was aimed to assess sublethal effects where mortality was undesired and thus it remains unclear if all of their specimens died at 250 mg/L or if they only observed a partial mortality. Other studies do not only cover a broad range of different LC_{50} values for nickel on earthworms, all presently available studies were conducted under conditions even less comparable to the present study. It is generally accepted that soil and substrate characteristics do have a great influence on the availability and toxicity of metals (Lock et al. 2000, Van Gestel and Weeks 2004), comparisons with the values obtained in the above mentioned previous studies are therefore difficult.

Although in direction of the reconstituted ground water a slightly higher mortality at the corresponding LC values was observed, the confidence intervals of all corresponding mortalities overlapped. A direct comparison of the exposure of *E. fetida* to the two different exposure media, reconstituted ground water (Kiewiet and Ma 1991) and reconstituted water with gel thus revealed a comparable mortality. Both media seemed therefore equally suited for the evaluation of intrinsic effects of nickel on earthworms under the exclusion of soil adsorption and other soil-related effects.

With regard to the possible development of a resistance or an adaptation by *E. fetida* long-term exposed to nickel, i.e. after exposure for more than ten generations, the results obtained in this part of the present study clearly show that *E. fetida* did not seem to develop an increased tolerance or even an adaptation towards nickel (Table 10). In contrast, the *E. fetida* with a history of exposure to nickel showed an increased sensitivity to that metal when mortality was used as endpoint. This is in agreement with the findings in a previous part of this study (Chapter 4.3.4), where *E. fetida* pre-exposed to nickel has taken up higher amounts of nickel than specimens without a history of nickel exposure. The only explanation for the increased sensitivity of nickel pre-exposed worms is, that the concentrations of nickel in both the media to which the worms were exposed, were actually an add-on to the concentrations already present in the bodies of the worms and hence caused stronger toxic effects. To address that issue, further research needs to be conducted.

Also, no possible cross resistance or tolerance of manganese pre-exposed worms towards nickel was detected. The mortality rates were found to be similar to the mortality observed in the *E. fetida* without a history of previous exposure. Thus, it can be concluded that in terms of mortality, *E. fetida* pre-exposed to nickel did not develop an increased tolerance towards nickel.

As a conclusion one has to keep in mind that in terms of field relevance, such an approach based on artificial substrates is limited as its main purpose is the evaluation of a single endpoint of a single toxicant under controlled conditions, not the assessment of such a complex system as soil is. Even artificial soil systems such as the OECD standard soil for earthworm toxicity testings (OECD 2004) are often criticized as being not environmentally relevant (Davies et al. 2003a). Thus, such an exposure system has to be restricted to either the screening of single, potentially toxic substances or to complement a battery of different bioassays and biomarker which may use such a substrate to identify if a substance has an effect on a distinct level of organisation or not. Another field of application might lie in the estimation of the bioavailability, by comparing the uptake of a toxicant in an earthworm under the controlled conditions of the exclusion of all edaphic factors to draw conclusions of the bioavailable amount of the defined toxicant in soil.

7. Micro-distribution of metals in the tissue of *Eisenia fetida* exposed to ultramafic soils

7.1 Introduction

It is well documented by numerous publications that earthworms do accumulate high concentrations of several metals such as cadmium, chromium, copper, lead, mercury and zinc in their tissues (Gish and Christensen 1973, Beyer et al. 1982, Van Gestel et al. 1993, Vijver et al. 2005). Metals do not only affect the earthworm populations themselves by influencing the reproduction or growth inhibition as stated by a number of authors (Malecki et al. 1982, Neuhauser et al. 1984a, Van Gestel et al. 1993, Spurgeon et al. 1994, Reinecke and Reinecke 1996, Khalil et al. 1996b, Reinecke and Reinecke 1997b, Maboeta et al. 1999, Lock and Janssen 2002a, Homa et al. 2003, Davies et al. 2003a, Lukkari et al. 2005), but earthworms themselves can also pose a potential pollution hazard (Ireland 1983). Few predators feed exclusively on earthworms, but earthworms are a food source for different groups of animals ranging from insects such as larvae of the families Lampyridae (firefly) and Cantharidae, fishes, birds to large omnivore mammals such as pigs. Hence, earthworms are an important part of many food chains and are able to remobilise and redistribute soil-bound toxicants. Thus, by analysing earthworms one can gain information on the presence of potential toxic metals in the soil, which may set predators being on a higher trophic level at risk.

In ecotoxicology, dose response relationships and the use of sensitive endpoints addressing different levels of plant and animal organisation form the basis for risk assessment and hazard identification. To take effect, a noxious substance needs to be bioavailable. The bioavailable amount of a substance is defined by the amount taken up into an organism through the membranes and distributed, metabolised, sequestrated or excreted in the body; seen from a toxicological point of view, the toxicant also has to reach its specific site of action (Reinecke et al. 2004). The concentration of a specific substance can vary spatially and quantitatively during the course of an exposure to that specific substance.

For the understanding of the mechanisms of uptake and excretion as well as the identification of the sites of action involved, micro-Particle-induced X-ray emission (micro-PIXE) provides an accurate, fully quantitative method with a sensitive spatial resolution on organs and tissues (Hill et al. 2003). In bridging the gap between highly sensitive techniques for bulk analysis such as atomic absorption spectrometry (AA) or inductively coupled plasma atomic emission spectrometry (ICP-AES) (Stoeppler 1991) and morphological analysis of small structures such as organs and tissues down to cellular level by means of scanning electron microscopy (SEM) or transmission electron microscopy (TEM), micro-PIXE provides the possibility to perform quantitative elemental mapping (Ryan 2000, Przybylowicz et al. 2001). In general, simultaneous localisation and highly sensitive determination of a range of different elements can be considered as one of the main advantages of the PIXE method (Llabador and Moretto 1996). A wide variety of different biological samples can be analysed simultaneously on a wide range of elements at a minimum detection limit at or even

below the ppm (parts per million) level (Llabador and Moretto 1996). Also, micro-PIXE enables to discover previously not suspected elements. Although due to the fact that PIXE is suitable for non-destructive *in situ* microanalyses, it is most commonly used in the field of geoscience (Ryan et al. 2002), but it has also been proven useful in the field of biology. Besides different applications in plant science (Mesjasz-Przybylowicz et al. 1994, Przybylowicz et al. 1999, Mesjasz-Przybylowicz et al. 2001a, Mesjasz-Przybylowicz et al. 2001b), micro-PIXE has also found its application in animal science. Here, micro-PIXE was applied e.g. to investigate the accumulation and distribution of cadmium in the nephridia of earthworms (Prinsloo et al. 1999) or the nickel distribution in *Chrysolina pardalina*, a beetle feeding on nickel-hyperaccumulating plants (Przybylowicz et al. 2002, Przybylowicz et al. 2003).

7.1.1 Principles of Particle induced X-ray emission (PIXE)

A proton beam consisting of protons accelerated to energies between 0.5 and 4 MeV penetrates a target sample where the protons interact with the electrons in the inner shells of the target atom. If sufficient energy is transferred, the proton beam causes an electron to “jump” out of the inner shell, usually a K or L shell. In a rapid reordering process, the vacancy becomes filled by an electron from the outer shell and the vacancy moves outwards (Llabador and Moretto 1996). In this process, an excess of energy originates which is released by an X-ray emission (Figure 33).

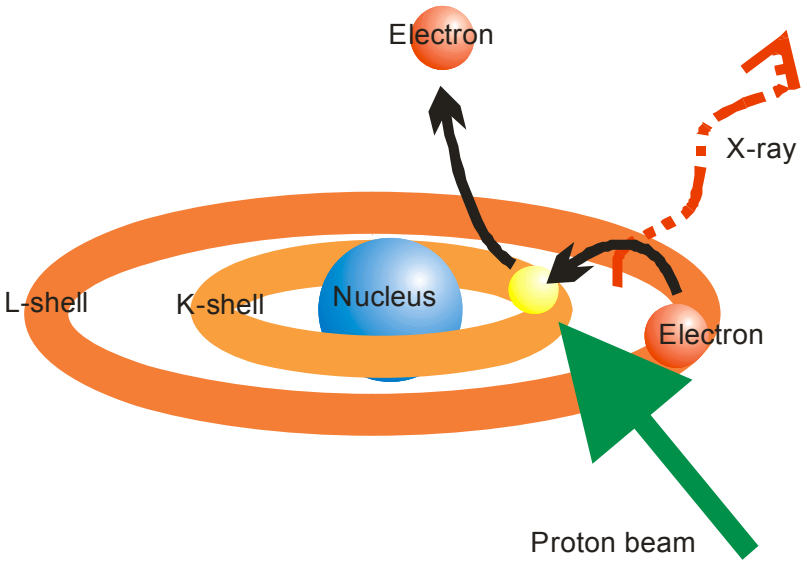

Figure 33: Principle of PIXE: accelerated protons interact with electron shells of the atom, producing characteristic X-ray emission.

Depending on the shell in which the vacancy is filled, the corresponding X-ray spectra are called K- or L-lines. Every element has a different energy gap between the electron shells and thus, every element can be characterized by the differences in the K- respectively L, M-… lines to every other element detectable using an energy dispersive detector (Si(Li) or HPGe) (Johansson and Campbell 1988, Campbell 1990).

Besides the above mentioned X-ray emissions, a number of different signals are emitted by the interaction of the particle beam with the specimen, by this the so called Auger electrons, backscattered particles (protons) detectable by RBS (Rutherford backscattering spectroscopy), secondary electrons, cathodoluminescence, gamma-rays and photons (Tapper and Malmqvist 1991).

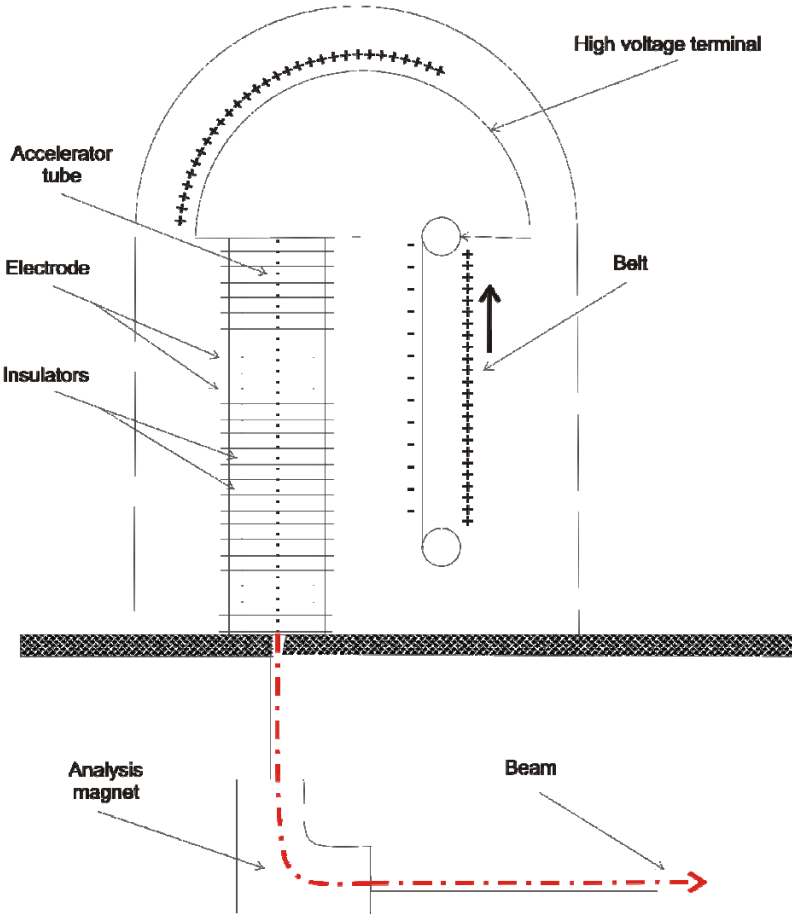

Figure 34: Schematic view of a Van de Graaff accelerator (adapted and simplified from Llabador and Moretto (1996)).

The proton beam is generated in an electrostatic accelerator based on the design invented by Lord Kelvin in 1890 and developed by Van de Graaff (1931). It is based on the principle that by an insulating rotating belt, charge is transferred to the equipotential surface of the terminal sphere (Figure 34). To avoid damages by discharges, the accelerator is placed in a high pressure tank filled with an insulating gas. The accelerator produces a MeV proton beam which can be directly focused to a spot with a diameter of about one micron or even smaller using a magnetic quadrupole lens system in the microbeam line (Ryan 2000). The beam spot can be raster-scanned over a sample providing information about the elemental composition of the sample as a geometrical function of its position (y,x) on the sample. The micro beam is directed into the experimental chamber fitted with various signal detectors able to acquire signals of X-ray, charged particles, electrons, γ-rays (Figure 35). In PIXE, for the detection of all elements down to sodium,

the Si(Li) or HPGe detector is fitted with an ultra-thin beryllium window (Llabador and Moretto 1996, Ryan 2000).

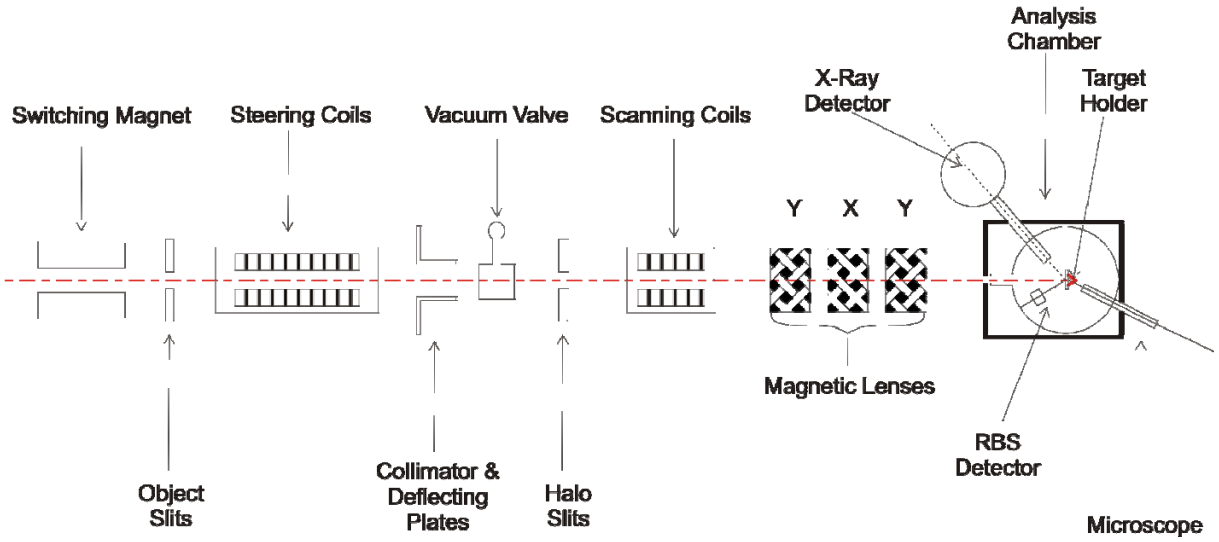

Figure 35: Schematic view of the different elements of the nuclear microprobe at iThemba LABS (modified and redrawn from Prozesky et al. (1995); not drawn to scale)

A complex computer data acquisition system is used to handle several complex tasks such as beam scanning and acquisition of signals from different electronic channels simultaneously. The most popular combination of techniques used in biological applications is PIXE and RBS used simultaneously (Przybylowicz et al. 1999).

7.1.2 Aims

The present study deals with the question how earthworms are able to survive in ultramafic soils which contain a range of different metals of natural origin at concentrations known to be toxic to soil living organisms (Robinson et al. 1935). Earthworms are considered as good indicators of the bioavailability of metals (Morgan et al. 1992). Thus earthworms were used to assess the toxic potential of ultramafic soils collected at the Barberton area, Mpumalanga, South Africa. However, very little is known of the micro distribution of metals in earthworms. This part of the study aimed at obtaining a picture of this distribution.

The aims of this part of the study were twofold. With regard to the metals available to earthworms in ultramafic soils, the first aim was to determine the tissue distribution of metals in earthworms. To determine in which tissue of the earthworms a particular metal is preferably concentrated, as the biological significance of a metal's toxicity is not necessarily predictable solely by the total amount of a given metal in the body, but also depends on the tissue where it is deposited (Rainbow 2002).

The second aim was to investigate if elements for which earthworms have not previously been analysed using bulk analysis technique, may be present in micro quantities.

7.2 Materials and methods

7.2.1 Earthworm exposure

The substrate preparation and exposure of earthworms is described in detail in Chapter 2.6. The OECD reference species (OECD 1984) for soil toxicity testing *Eisenia fetida* (Savigny 1826) was exposed to soils collected from known ultramafic areas (Anhaeusser 2001) close to Barberton, Mpumalanga, South Africa. One sampling location was located in the Barberton Nature Reserve, hereafter called Barberton, and two sampling locations were on grassland close to Kaapsehoop, therefore called Kaapsehoop 1 and 2. Three replicates per soil exposure were conducted and ten specimens of clitellated *E. fetida* from a synchronized culture bred at the laboratories of the Department of Botany and Zoology at the University of Stellenbosch, South Africa were exposed to the ultramafic soils and as a control to a soil collected from an unpolluted area close to the Stellenbosch sports grounds.

Prior to the cryopreservation and freeze drying required for the elemental microanalysis (Kirby and Legge 1991), worms were kept for two days on moist filter paper to empty their guts (Krontowsky and Rumiauzew 1922).

7.2.2 Specimen preparation

Sample preparation was performed at the facilities of the MRG (Materials Research Group) at iThemba LABS, Faure, South Africa. After immobilization over liquid nitrogen, cross sections of five earthworms of every exposure group and the control group were made by cross cutting discs as thin as possible with a sharp metal scalpel out of the three different body sections of every worm, from the posterior section before the clitellum, the middle section and the anterior section.

Additionally, from five other worms of every exposure, coelomic fluid was extracted. For technical reasons, only one cross cut of an earthworm exposed to the control soils and one batch of coelomic fluid was analyzed: One cross cut and two portions of coelomic fluid of the earthworms exposed to the soils collected at Barberton, three cross cuts of two worms and coelomic fluid from two further earthworms exposed to Kaapsehoop 1 and one cross cut and one portion of coelomic fluid of the *E. fetida* exposed to Kaapsehoop 2.

The dissected body parts were immediately preserved by impact cryofixation (Warley 1997). Cryofixation is done to cool the specimens down to less than -150°C as fast as possible to avoid the formation of ice crystals. For that purpose, the cross sections were mounted on a Kimfoil (polymer film) covered foam plate and punched against a metal mirror block (copper covered with gold) cooled by liquid nitrogen (-196°C) in the Leica EM CPC Cryoworkstation (Leica EM CPC, Leica, Austria). 20 µL of coelomic liquid was dripped on Formvar film (1.5%) and plunged directly in liquefied propane, cooled by liquid nitrogen. Subsequently, the samples were freeze-dried in the Leica EM CFD Cryosorption Freeze Dryer (Leica EM CFD, Leica, Austria). Freeze-drying process followed a 208 hours' programmed cycle starting at -80^{o} C and eventually reaching ambient temperature, in vacuum of the order of 10^{-3} mbar. The specimens, which were not immediately

freeze-dried after cryofixation, were stored in small plastic cryovials in liquid nitrogen in a Dewar flask.

Prior to the microelemental analysis, freeze-dried samples were fixed by means of a two-component glue (Araldite) on the Formvar film in the specimen holder and covered by the same size carbon coated holder to prevent charge build up during irradiation (Przybylowicz et al. 1999). Photographs of all samples were taken to facilitate orientation on the specimen before micro PIXE analysis.

7.2.3 PIXE analysis

The micro elemental analysis was performed using the nuclear microprobe at the MRG, iThemba LABS. A detailed description of the iThemba LABS nuclear microprobe facility has been reported by Prozesky et al. (1995). The microprobe uses a proton beam of 3.0 MeV energy and a current of about 400 pA using a triplet magnetic quadrupole lens system (LM 150; Oxford Microbeams, Oxford, U.K. (Grime et al. 1991)). Five specimens were mounted on a metal ladder and the metal ladder positioned in the analysis chamber of the microprobe (Oxford nuclear microprobe chamber). The proton beam was focused on a 3 μm^2 spot and scanned over an area of approximately 1.5 mm^2. PIXE and proton backscattering were used simultaneously. Between the specimen and the Si(Li) PIXE detector, a 175 µm Kapton absorber was positioned. As low scanning speed provides a more favourable ratio between time spent by the beam and on movement from pixel to pixel, a scanning speed of 10 ms per pixel was selected. Scanning regions were divided into 128 * 128 pixels to obtain reasonable statistics of counts per pixel. The XSYS data acquisition system running on a 4000/VLC VAXstation was used for the collection of list-mode files (Churms et al. 1993, Przybylowicz et al. 1999). The normalization of results was done by using the integrated beam charge, collected simultaneously from a Faraday cup located behind the specimen and from the insulated specimen holder. Further data analysis was performed using a PC based version of the GeoPIXE software package which included a Dynamic Analysis for quantitative image projection and tools for multi-layered elemental maps (Ryan et al. 2002). Quantitative microanalysis using GeoPIXE is standardless and has been described in detail elsewhere (Ryan et al. 1990a; Ryan et al. 1990b) The Dynamic Analysis (DA) uses a matrix transformation which relates the X-ray yields obtained from the PIXE spectra directly to elemental concentrations (Przybylowicz et al. 1999). The elemental images obtained from the GeoPIXE II software were overlap-resolved and background-subtracted. Final maps showed concentrations in mg/kg.

7.3 Results

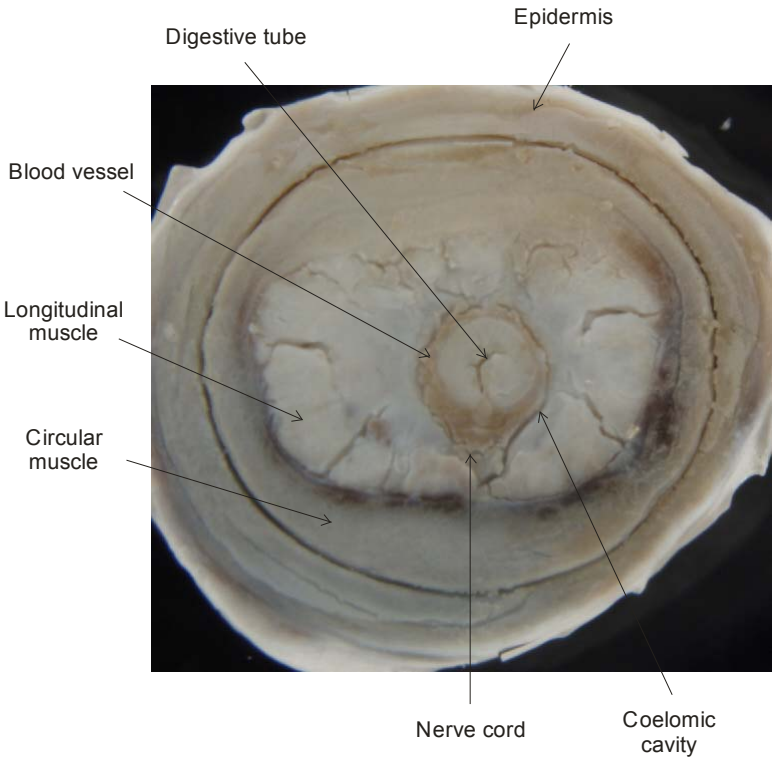

Figure 36: Micrograph of a cross section of *Eisenia fetida* exposed to control soil after freeze drying and prior to micro PIXE analysis

Figure 36 shows an overview of the different organs and tissues in the cross section of *E. fetida* after freeze drying of the specimen and before analysis. After freeze drying, specimens were covered with a white layer. The layer probably was a result of an interaction between propane and mucus surrounding the specimen (Prinsloo 1999). Different tissues such as muscles and epidermis and organs were easily identifiable. Elemental concentrations are given as average specimen concentration ± uncertainty calculated using the non-linear least-squares method by the GeoPIXE II. The metal concentration in the cross sections and the coelomic fluid of the earthworms are shown in Table 11, the corresponding detection limits in Table 12. In Appendix 5-1, elemental concentrations and detection limits are shown in detail. Cobalt, although found in the soil samples (Chapter 3), was detected in concentrations too low for elemental image mapping in any of the specimens.

Table 11: Concentrations of selected elements obtained in cross sections (CS) and coelomic fluid (FL) extracted from *Eisenia fetida* exposed for 20 weeks to different ultramafic soils and to a control soil from an area with a known history of no metal pollution at Stellenbosch by micro-PIXE technique (mg/kg ± 1σ uncertainty).

		K	As	Co	Cr	Cu	Fe	Mn	Ni	Ti	Zn
Ctr.	CS	10075 ±44	9.9 ±0.6	3 ±1	n.d.	9.5 ±0.7	147 ±5	3 ±1	3.3 ±0.7	n.d.	119 ±5
	FL	3592 ±36	4 ±4	3 ±2	<3 ±2	7 ±2	208 ±5	<3 ±1	<3 ±2	5 ±2	13 ±2
Barb.	CS	8249 ±64	5.1 ±0.5	6 ±3	n.d.	36 ±1	462 ±10	12 ±2	344 ±9	n.d.	174 ±4
	FL	7200 ±31	<4 ±2	13 ±6	259 ±5	<3 ±1	5305 ±84	85 ±6	221 ±6	23 ±2	28 ±2
Kh.1	CS	7834 ±47	31 ±1	5 ±1	n.d.	28 ±2	196 ±6	3.9 ±0.9	0.7 ±0.4	n.d.	91 ±4
	FL	4037 ±44	<3 ±2	5 ±2	54 ±2	5 ±2	1098 ±21	25 ±3	52 ±2	34 ±2	18 ±2
Kh.2	CS	7913 ±52	10.3 ±0.4	5 ±2	n.d.	12.9 ±0.6	373 ±10	4 ±1	125 ±3	n.d.	116 ±3
	FL	3170 ±28	<3 ±2	8 ±3	18 ±1	41 ±2	626 ±11	12 ±1	39 ±2	2 ±1	31 ±3

n.d.: not detected;

Ctr.: Stellenbosch control site

Barb.: Barberton Nature Reserve;

Kh.: Kaapsehoop

Table 12: Detection limits (99% confidence) of selected elements in cross sections (CS) and coelomic fluid (FL) extracted from *Eisenia fetida* exposed for 20 weeks to different ultramafic soils and to a control soil from an area with a known history of no metal pollution at Stellenbosch by micro-PIXE technique (mg/kg).

		K	As	Co	Cr	Cu	Fe	Mn	Ni	Ti	Zn
Ctr.	CS	1.1	0.43	0.5		0.63	0.51	0.56	0.65		0.53
	FL	8.3	4	2.5	3.1	3.5	2.7	2.9	3.4	4.5	3.6
Barb.	CS	1.3	0.65	0.61		0.79	0.61	0.65	0.79		0.72
	FL	6.6	3.8	2.9	2.6	3.3	2.8	2.8	3.5	3.5	3.4
Kh.1	CS	0.94	0.43	0.37		0.46	0.37	0.42	0.48		0.45
	FL	6.9	3.4	2.3	2.5	2.9	2.4	2.5	2.9	3.6	3.1
Kh.2	CS	0.62	0.29	0.25		0.3	0.24	0.27	0.3		0.29
	FL	4.2	3	1.6	1.8	2.4	1.7	1.7	2.3	2.3	2.6

Ctr.: Stellenbosch control site

Barb.: Barberton Nature Reserve;

Kh.: Kaapsehoop

7.3.1 *Eisenia fetida* exposed to control soils from Stellenbosch

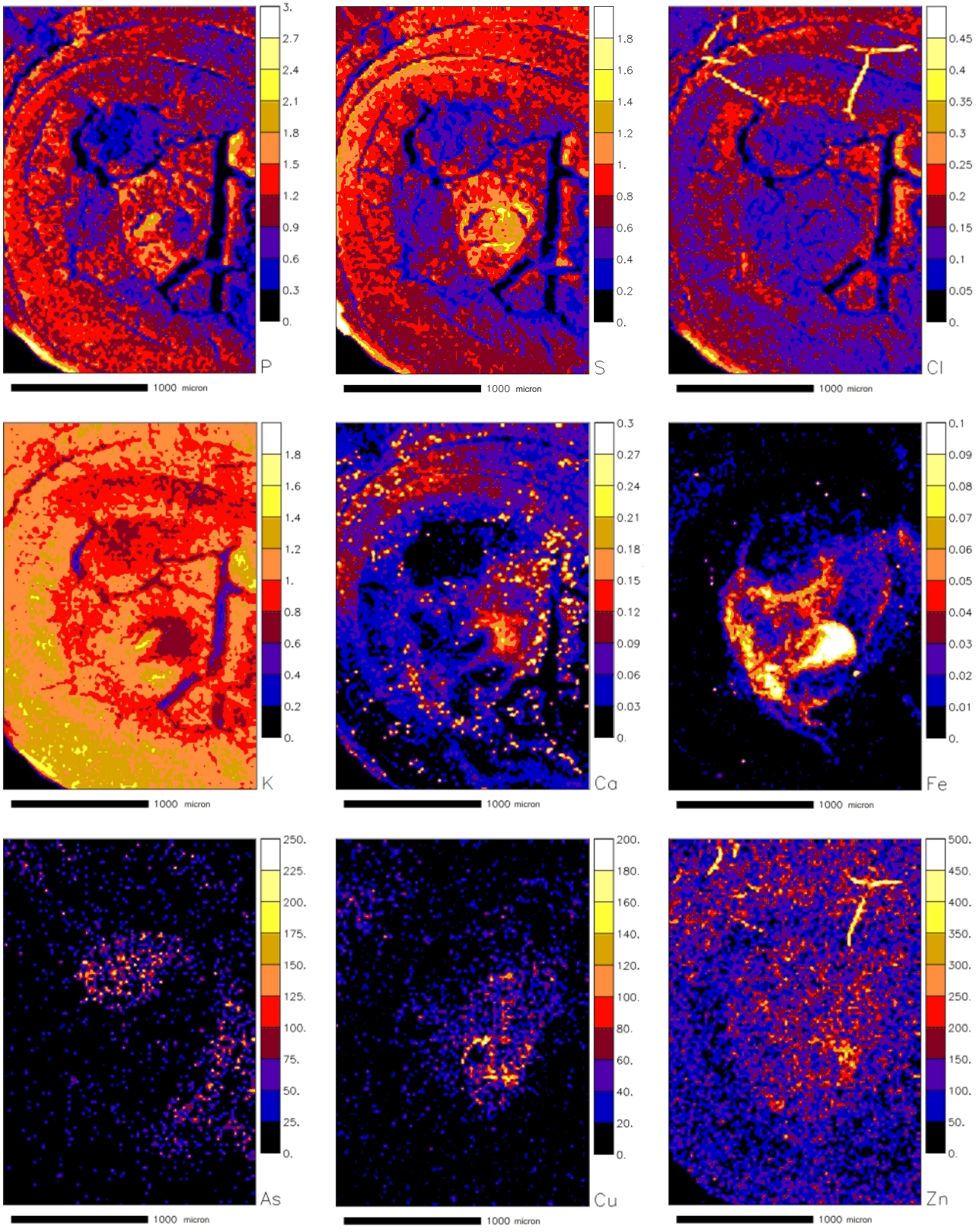

Figure 37: Quantitative elemental map of P, S, Cl, K, Ca, Fe, As, Cu and Zn in the cross section of Eisenia fetida exposed to control soil from Stellenbosch. Image intensity in weight percent (P, S, Cl, Ca, K and Fe) respectively in mg/kg (As, Cu and Zn). Indicated are blood vessel (BV) and setae (ST).

No chromium was detected in the cross section prepared from the earthworm exposed to the soils regarded as control from Stellenbosch, but arsenic was detected at a concentration of 9.9±0.6 mg/kg. The metal concentrations are shown in Table 11.

Zinc and chlorine seemed to show high accumulation in the setae. It was found that copper and iron showed a specific distribution pattern, the highest accumulation was in the blood vessels around the digestive tube (Figure 37). No distinct distribution pattern was observed for manganese and nickel.

7.3.2 *Eisenia fetida* exposed to ultramafic soils from the Barberton Nature Reserve

It was only possible to analyze one cross cut of the anterior section of the *E. fetida* exposed to the soil samples collected in the Barberton Nature Reserve (Figure 38). For copper (36±1 mg/kg), manganese (12±2 mg/kg) and zinc (174±4 mg/kg), no distinct accumulation pattern was detectable. Iron was accumulated mainly in the seminal vesicles. Nickel was detected in the coelomic fluid between the seminal vesicles and the longitudinal muscle tissue and in or on the epidermis at a mean concentration of 344±9 mg/kg. Also detected was arsenic in this specimen at a concentration of 5.1±0.5 mg/kg mainly accumulated in an unidentifiable area close to the seminal vesicles.

In the coelomic fluid of *E. fetida* exposed to the soils collected in the Barberton Nature reserve, manganese was detected at a concentration of 85±6 mg/kg and zinc at a concentration of 28±2 mg/kg, but the elemental maps did not show an accumulation pattern. Chromium (259±5 mg/kg), iron (5305±84) and nickel (221±6) showed a distinct accumulation pattern with high concentrations in a granular structure (Figure 39). The elemental maps also revealed that titanium was present in the coelomic fluid at a concentration of 23±2 mg/kg.

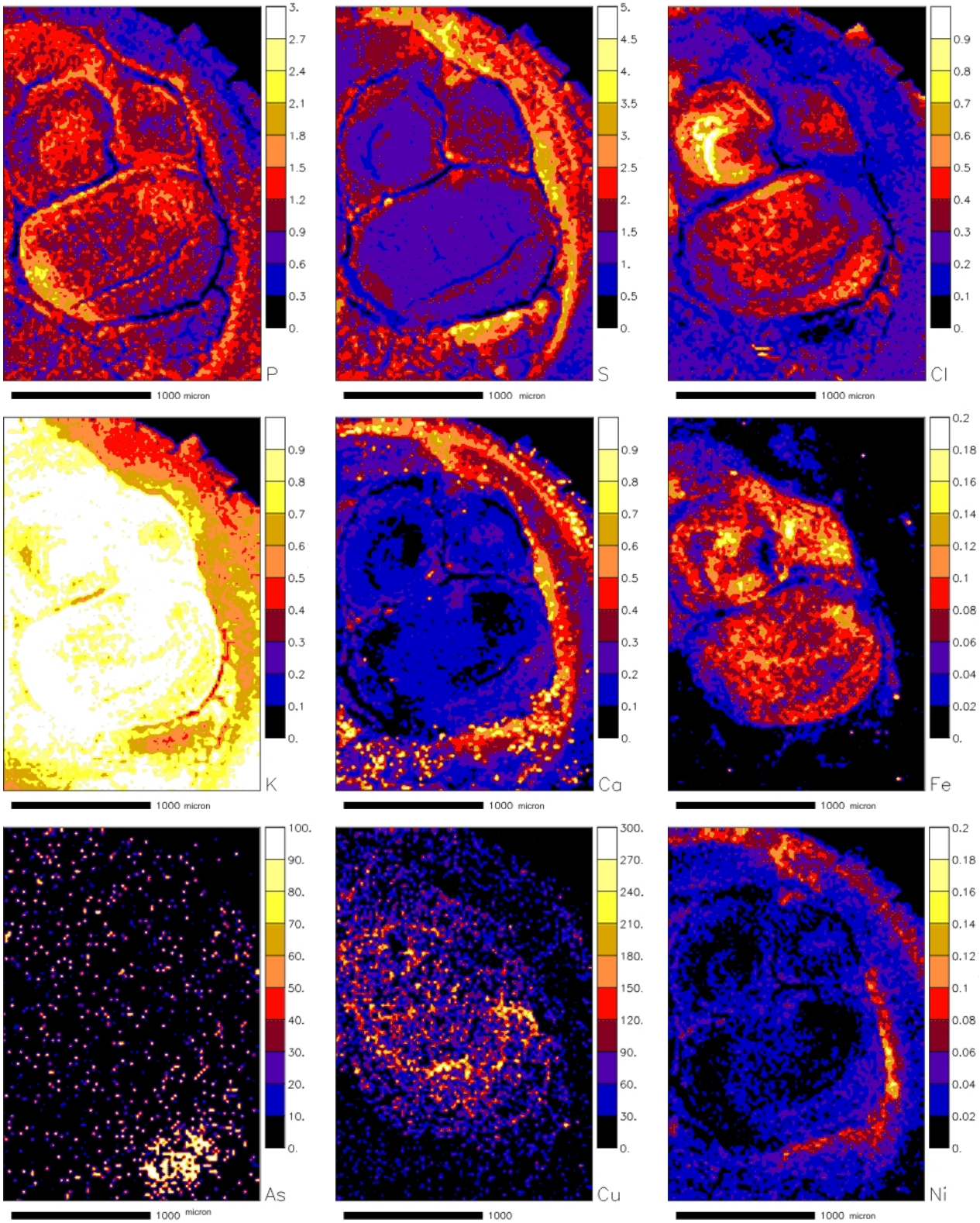

Figure 38: Quantitative elemental map of P, S, Cl, K, Ca, Fe, As, Cu and Ni in the cross section of *Eisenia fetida* exposed to soil collected in the Barberton Nature Reserve. Image intensity in weight percent (P, S, Cl, Ca, K, and Fe) respectively in mg/kg (As, Cu and Ni). Indicated are seminal vesicles (SV).

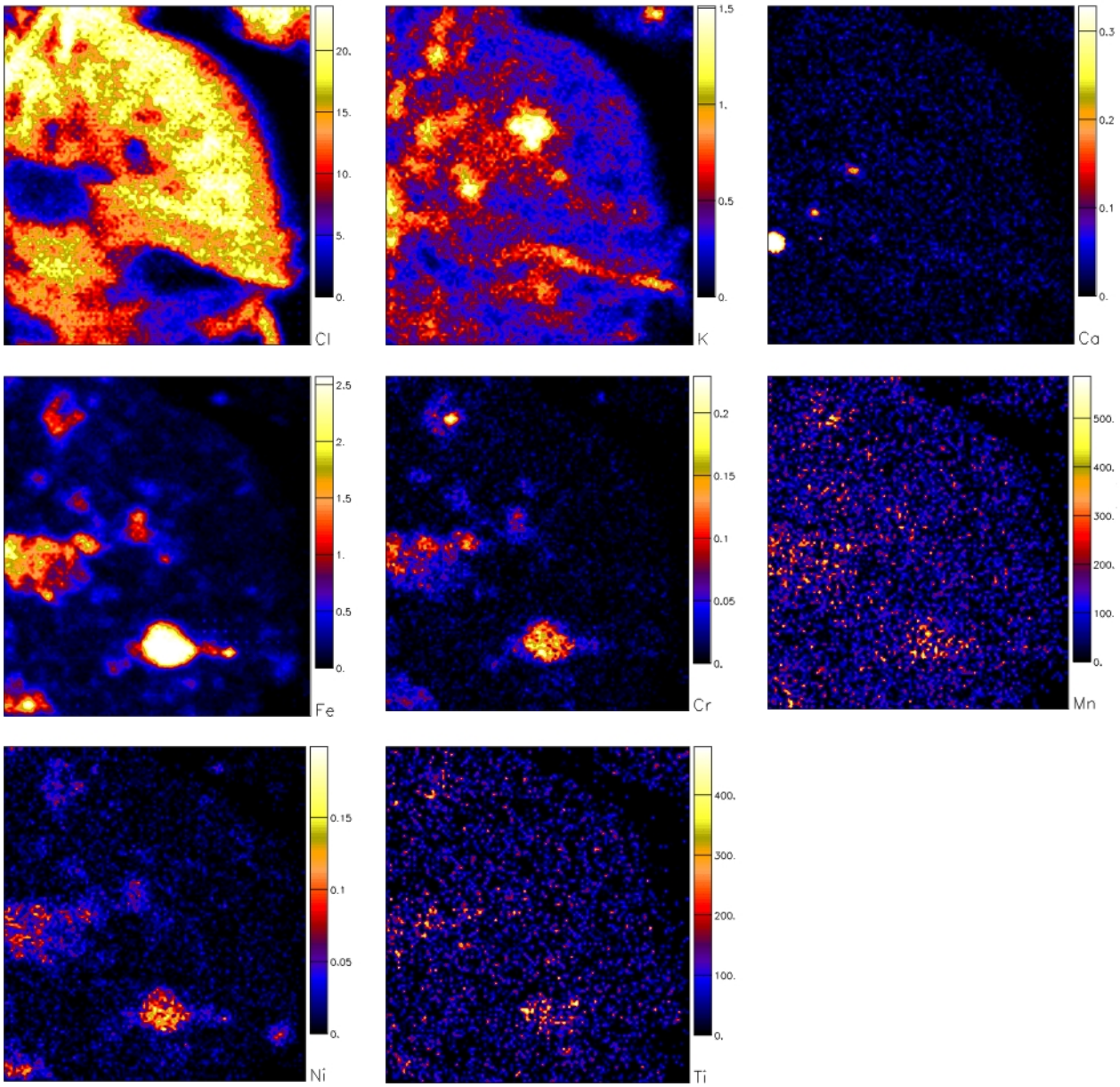

Figure 39: Quantitative elemental map of chlorine (Cl), potassium (K), calcium (Ca), iron (Fe), chromium (Cr), manganese (Mn), nickel (Ni) and titanium (Ti) in the coelomic fluid of *Eisenia fetida* exposed to soil collected in the Barberton Nature Reserve. Image intensity in weight percent (Cl, Ca, K, Fe, Cr and Ni) respectively in mg/kg (Mn and Ti).

7.3.3 *Eisenia fetida* exposed to ultramafic soil samples from Kaapsehoop 1

In none of the different sections of *E. fetida* specimens exposed to the soil sample collected at Kaapsehoop 1, mean manganese concentrations higher than 3.9±0.9 mg/kg and nickel concentrations higher than 0.7±0.3 were detected. Also, no chromium was found (Table 11). In all sections, copper and iron showed a clear distribution pattern around the digestive tube. Furthermore, arsenic was found in all specimens at concentrations of 31±1 mg/kg (Figure 40). It was not possible to assign the arsenic to a specific tissue or organ. Zinc was measured at a concentration of 91±4 mg/kg. No differences between the three body sections were observed.

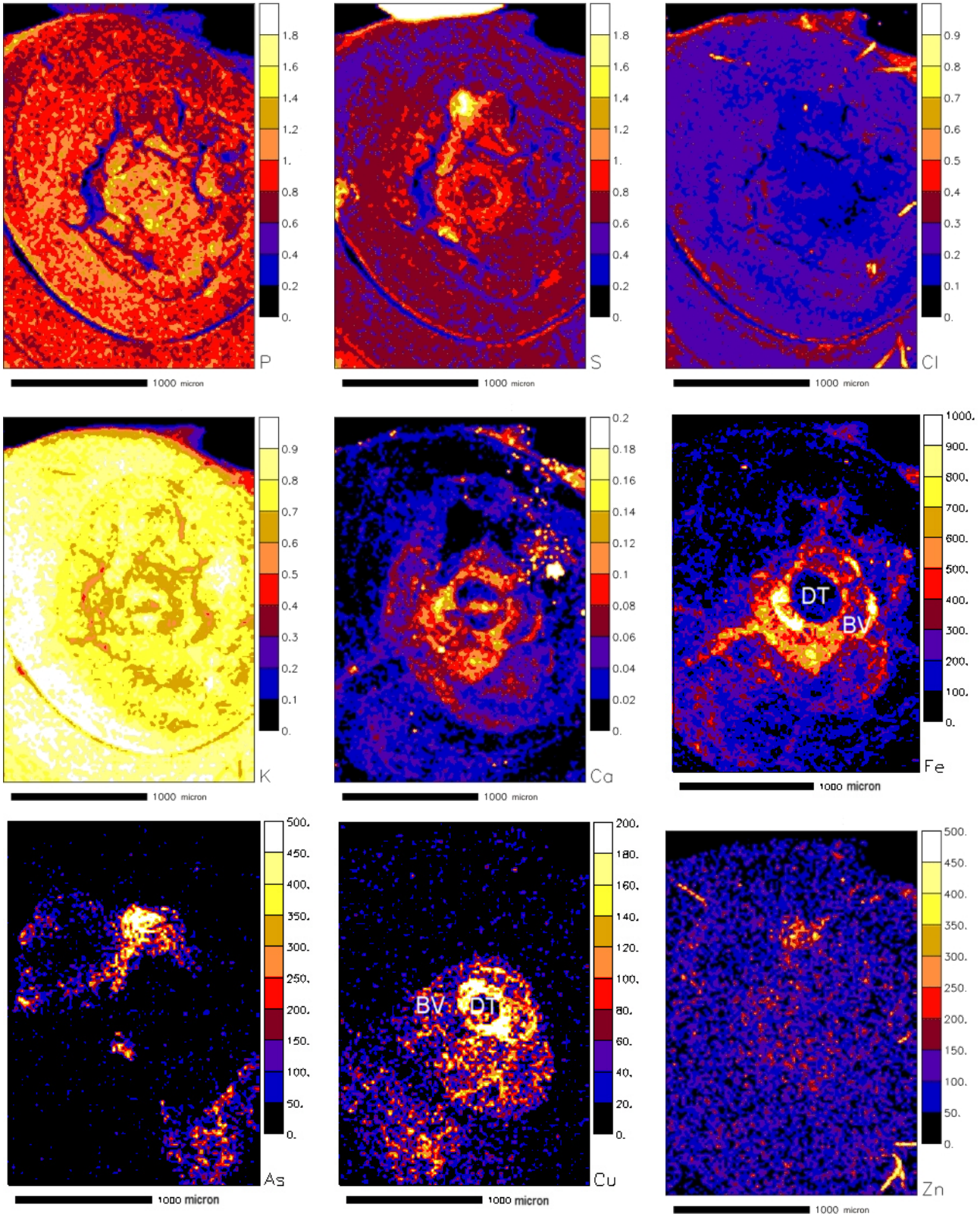

Figure 40: Quantitative elemental map of P, S, Cl, K, Ca, Fe, As, Cu and Zn in the cross section of *Eisenia fetida* exposed to soil samples from Kaapsehoop 1. Image intensity in weight percent (P, S, Cl, K, Ca) respectively in mg/kg (Fe, As, Cu, Zn). Indicated are blood vessels (BV) and digestive tube (DT).

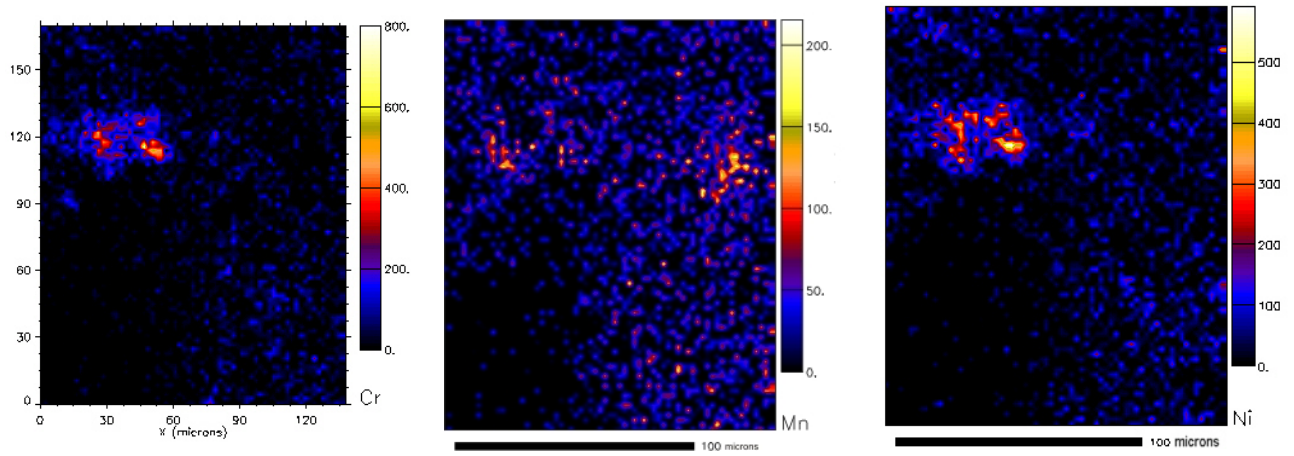

Figure 41: Quantitative elemental map of chromium (Cr), manganese (Mn) and nickel (Ni) in the coelomic fluid of *Eisenia fetida* exposed to soil collected at Kaapsehoop 1. Image intensity in mg/kg.

In the coelomic fluid extracted from *E. fetida* exposed for 20 weeks to the soil samples collected at Kaapsehoop 1, 54±2 mg/kg chromium, 1098±21 mg/kg iron, 25±3 mg/kg manganese, 52±2 mg/kg nickel, 34±2 mg/kg titanium and 18±2 mg/kg zinc was measured. Chromium, iron and nickel showed a congruent distribution in a granular structure (Figure 41) while the other elements detected were found evenly distributed over the whole measurement area. Maximum concentrations of 437 mg/kg Ni and 801 mg/kg of chromium were measured in the above mentioned granular structures.

7.3.4 *Eisenia fetida* exposed to ultramafic soil samples from Kaapsehoop 2

Also, in spite of being present at comparatively high concentrations in the soil (Chapter 3), comparably low concentrations of cobalt were detected in the specimens of earthworms exposed to Kaapsehoop 2 soils (Table 11). In the cross sections, also no chromium was detected. 10.3±0.4 mg/kg of arsenic, 12±0.8 mg/kg of copper, 125±3 mg/kg of nickel and 116±3 mg/kg of zinc were detected in a cross section cut from the middle of the worm. Copper and iron showed a congruent distribution in the blood vessels around the digestive tube and nickel and zinc were observed mainly in or outside the epidermis (Figure 42).

In the coelomic fluid extracted from *E. fetida* exposed to the Kaapsehoop 2 soil sample, 18±1.7 mg/kg of chromium, 626±11 mg/kg iron, 12±1 mg/kg manganese, 39±2 mg/kg nickel, 41±2 mg/kg copper and 31±3 mg/kg zinc were observed. A distinct accumulation pattern was observed only for iron and nickel. Both metals showed a congruent accumulation comparable to the findings in the coelomic fluid in the earthworms exposed to Kaapsehoop 1 (Figure 41)

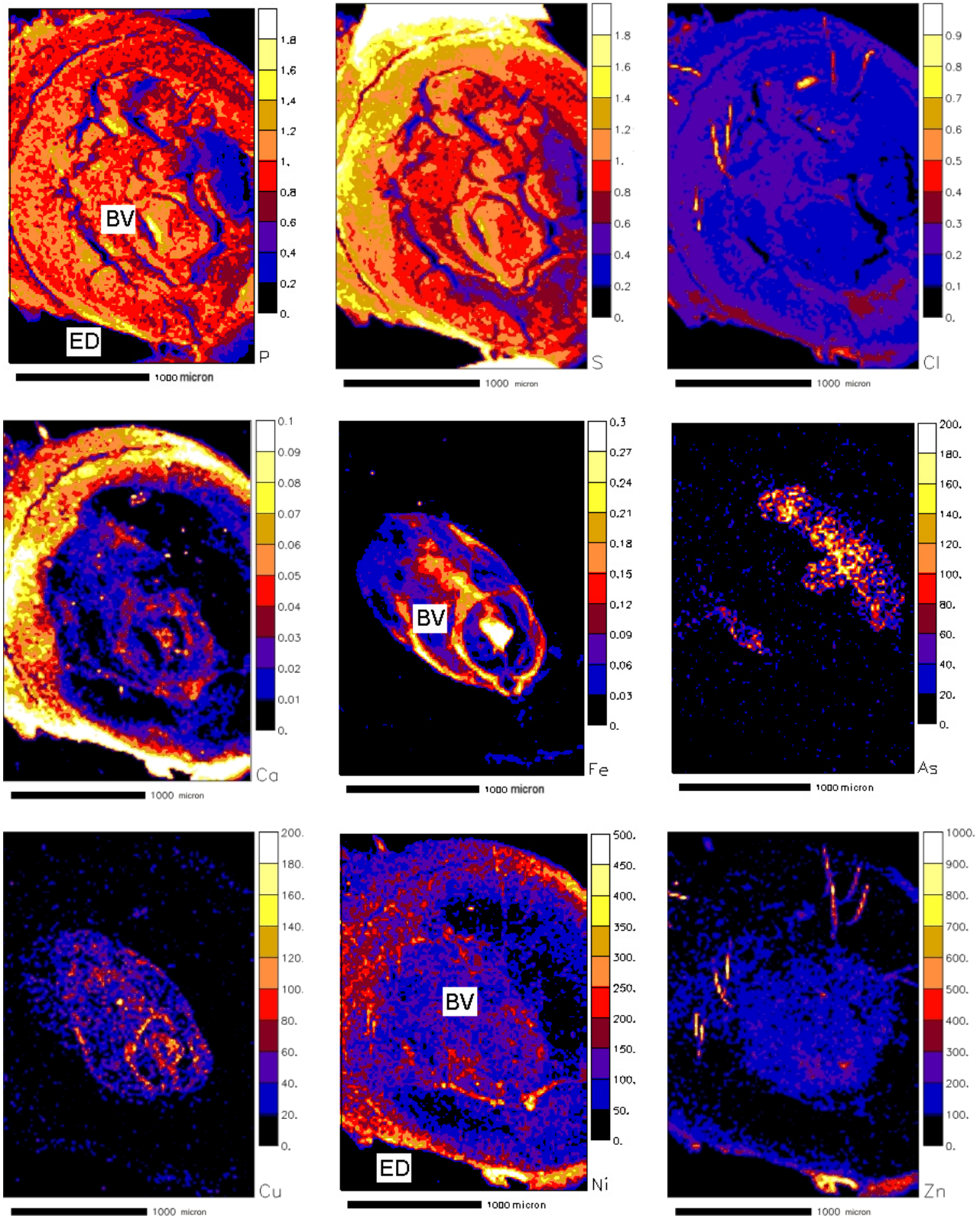

Figure 42: Quantitative elemental map of P, S, Cl, K, Ca, Fe, As, Cu, Ni and Zn in the cross section of *Eisenia fetida* exposed to soil samples from Kaapsehoop 2. Image intensity in weight percent (P, S, Cl, Ca, Fe) respectively in mg/kg (As, Cu, Ni and Zn). Indicated are blood vessels (BV) and epidermis (ED).

7.4 Discussion

On the elemental image maps, one can clearly identify a certain accumulation pattern, allowing one to distinguish between the distribution of non-essential metals and some of the essential metals (e.g. Figure 42 and Figure 39), keeping in mind that with regard to nickel and chromium, the question about its essentiality for earthworms is still not clear (Gauglhofer and Bianchi 1991, Conard 2003). Iron is an essential part of the haemoglobin, it is found in the cross sections mainly concentrated around the digestive tube in the blood vessels. In the coelomic fluid, it is found in granular structures and equally distributed in the fluid. Granules containing iron are described in terrestrial isopods as type C granules consisting of ferritin (Hopkin et al. 1989). Copper is also known as an essential trace element that is involved together with iron in the formation of haemoglobin (Liochev et al. 1996). The occurrence of copper in the coelomic fluid can be explained with the function of copper as a catalyst of glutathione activities and superoxide dismutase (Vijver et al. 2006). Zinc is a component of more than 3000 enzymes and is important in membrane stability and protein metabolism. Due to its many different functions, it is one of the most abundant trace metals in animal bodies and present in almost all tissues and body fluids (WHO 2001). With regard to the non essential metals, animals developed different strategies of response to these metals (Morgan et al. 1995):

- Regulation: tissue concentration remains constant over a broad range of exposure concentrations.
- Exclusion: metal is prevented from entering the body or excretion rate complies with excretion rate
- Sequestration: metal is accumulated in storage compartment and subsequently physiologically unavailable
- Intrusion: metal interferes with biochemical processes what is expressed in toxic effects at low concentrations.

According to Morgan et al. (1994), arsenic is distributed in the cells of the coelomic liquid, where it is accumulated according its sulphydryl-seeking trivalent form, in a distinct sulphur-bearing compartment within the chloragosomes (Morgan et al. 1995). The source of the arsenic found in the body of *E. fetida* (Table 11) is unknown, but in earthworms exposed in previous studies to uncontaminated soils, a mean concentration up to 96 mg/kg was found (Langdon et al. 1999, Langdon et al. 2001). As the concentration of arsenic is higher in the *E. fetida* specimens exposed to the control soil, one can conclude that the arsenic in the bodies of the *E. fetida* exposed to the ultramafic soils is not ultramafic-specific. Arsenic is biomagnified considerably in *E. fetida*. However, the accumulation rate decreases with increasing soil concentrations (Fischer and Koszorus 1992). Geißinger et al. (1998) found a significant higher accumulation factor in earthworms in uncontaminated soils than in earthworms exposed to arsenate contaminated soils. In this study, neither in the control soils nor in the ultramafic soils increased concentrations of arsenic were detected by atomic absorption spectrometry. Thus, one can only speculate why

higher concentrations of arsenic were observed in *E. fetida* in the control soils. One possibility could be that the small sample size failed to provide the full picture. Another possibility is that the arsenic uptake and consequently the bioaccumulation is influenced by other metals present in the ultramafic soils. This is supported by the fact that arsenic has a high binding affinity to metals such as aluminium, iron and manganese (Langdon et al. 2003b).

Chromium was only detected in the coelomic fluid of *E. fetida* exposed to the ultramafic soils (Figure 39 and Figure 41). As the speciation of chromium was not determined and the fact that *E. fetida* is able to actively reduce Cr(VI) to Cr(III) (Arillo and Melodia 1991), no conclusions about the toxicity of chromium can be drawn in this case. The accumulation pattern of chromium in the coelomic fluid was congruent with the pattern of nickel in all specimens where chromium was detected. In the coelomic fluids, nickel, iron and chromium were located in a granular structure (Figure 39). With regard to nickel, these findings are in agreement with previous studies. Vijver et al. (2006) found nickel predominantly accumulated in the granular fraction in the type B granules (Stürzenbaum et al. 1998b) of *A. caliginosa*. Type B granules have a short residence period and eliminate their mineralized metal content regularly (Morgan et al. 1995). Except for the specimen of the earthworms exposed to the soils from the Barberton Nature Reserve (Figure 38), the nickel concentrations in the tissue samples of the earthworms were low. One explanation for that might be, that in this worm some coelomic fluid with granulocytes remained behind after the dissection process, as it was also not possible to allocate the nickel accumulation on the elemental map to a specific tissue. Also, except for the earthworm exposed to the Barberton Nature Reserve soil, the nickel concentrations were below concentrations known to be toxic to earthworms (Scott-Fordsmand et al. 1998, Lock and Janssen 2002b). Why there were such comparably high concentrations of nickel in the tissue sample and in the coelomic fluid from two different earthworms exposed to the Barberton soils, remains unknown. To find an explanation for that, the analysis of a larger number of samples would have to be undertaken.

Concerning manganese, no specific distribution pattern was found. Titanium was also found in the earthworm bodies (Table 11). Nothing is known about the toxicity of titanium towards earthworms. Titanium dioxides are frequently used as white pigments, thus the toxicology of titanium was subject to a number of different studies, but no toxic effects were discovered (Nordman and Berlin 1986).

7.5 Conclusions

With regard to the distribution of chromium and nickel in *Eisenia fetida* one can conclude from the results of this part of the study that both metals show a clear accumulation pattern in the granules of the coelomocytes which are probably part of the sequestration system of *E. fetida*. Remarkable are the low concentrations of cobalt in the earthworms, which was found in considerable concentrations in the soil samples (Chapter 3). Due to the limitations of the experimental setup, especially the small number of earthworms, and the fact, that it was for technical reasons not

possible to synchronize the duration of the exposure with the other bioassays conducted in the frame of the present study, it is difficult to draw further conclusions from the quantitative assessment of the metals detected in the worms. Also, it was not possible to identify the cellular and subcellular components of the coelomic fluid extractants specifically with the techniques available. After freeze-drying, the whole specimens were covered with a white layer and cells and cell organs were not identifiable by light microscopy.

In general, the micro PIXE technology offers advantages that very few other analytical methods have. The simultaneous analyses of a broad range of elements with high sensitivity of the order of few mg/kg and the elemental mapping which reveals the distribution of elements within organs and tissues, can be considered as very valuable in understanding the mechanisms of uptake, storage and sequestration of metals.

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8. Cytotoxicity in earthworms exposed to ultramafic soils

8.1 Introduction

It has been shown by chemical analysis of ultramafic soils, that these soils do contain a mixture of unfavourable chemicals of natural origin, consisting of high concentrations of metals such as chromium, cobalt, manganese and nickel (Proctor 2003). Since especially soil physicochemical characteristics determine density and distribution of earthworms (Hubers et al. 2003), the question arises, whether and how soil inhabiting animals can cope with the biological risk originating from ultramafic soils. For the assessment of biological risk emerging from toxic threats in terrestrial environments, earthworms, as ecologically important macro invertebrates, are considered as an ideal tool (Callahan 1988). Earthworms are widely used as test organisms for environmental studies and they are one of the most important representative groups of invertebrates in soil toxicity studies (Diogène et al. 1997). For technical reasons it is often not possible to conduct a whole study or parts thereof under field conditions.

Various guidelines for the application of ecotoxicological standard procedures have been established (EEC 1985, ISO 1998, OECD 2004). An important problem in ecotoxicological research is the fact that field organisms could differ in sensitivity from laboratory bred organisms (Van Straalen and Denneman 1989, Van Gestel 1997). In spite of this, most studies were in the past conducted under laboratory conditions (Saint-Denis et al. 2001, Ribera et al. 2001, Reinecke and Reinecke 2004a, Reinecke and Reinecke 2004b).

To assess the heterogeneous, complex and often unique conditions found in the fields a number of biomarkers were established to close the gap between results obtained in field and laboratory studies. These biomarkers address a wide range of different endpoints applicable for the evaluation of the ecological impact of these soil conditions. To assess the environmental harm caused by toxic substances to such a complex system as soil, sub-organismal biomarkers are often applied within a battery of different tests addressing different ecotoxicological endpoints (Bierkens et al. 1998, Spurgeon et al. 2004). The investigation of sub-cellular alterations resulting from toxic exposure provides an early stage response to environmental pollutants, before a toxic effect becomes evident at a higher physiological level (Reinecke et al. 2002).

8.1.1 MTT colouring assay

One cytotoxicological assay used in the present study is the MTT (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl-tetrazoliumbromide) assay (Carmichael et al. 1987). Presently, in earthworm ecotoxicology, the MTT assay is applied mainly for the determination of the viability of coelomocytes after extraction and purification of coelomic fluid (Kauschke et al. 1997, Affar et al. 1998). Developed by Mosmann (1983a, 1983b), the MTT assay is widely established as an indicator of *in vitro* mitochondrial proliferation and metabolism, and is also applied in clinical analysis for drug screening and in chemosensitivity testing where it is called the “succinate dehydrogenase inhibition test (SDI)” (Tonn et al. 1994). It is based on the principle of the reduction

of the yellow MTT exclusively by metabolically active cells to a blue formazan salt. The tetrazolium salt is reduced by involvement of the pyridine nucleotide cofactors NADH and NADPH or along the mitochondrial transport chain (with Complex II, coenzyme Q and cytochrome C) using substrates such as succinate, malate, glutamate or pyruvate (Figure 43). As MTT is not permeable to lipid membranes, its cellular uptake takes place by endocytosis. After reduction the formazon salt is transported outside through exocytosis. The difference of the absorption of the yellow monotetrazolium salt and the blue formazan product can be measured photometrically at a wavelength of 570 nm. The response of the MTT cytotoxicity assay is also strongly dependent on the cell concentration (Twentyman and Luscombe 1987).

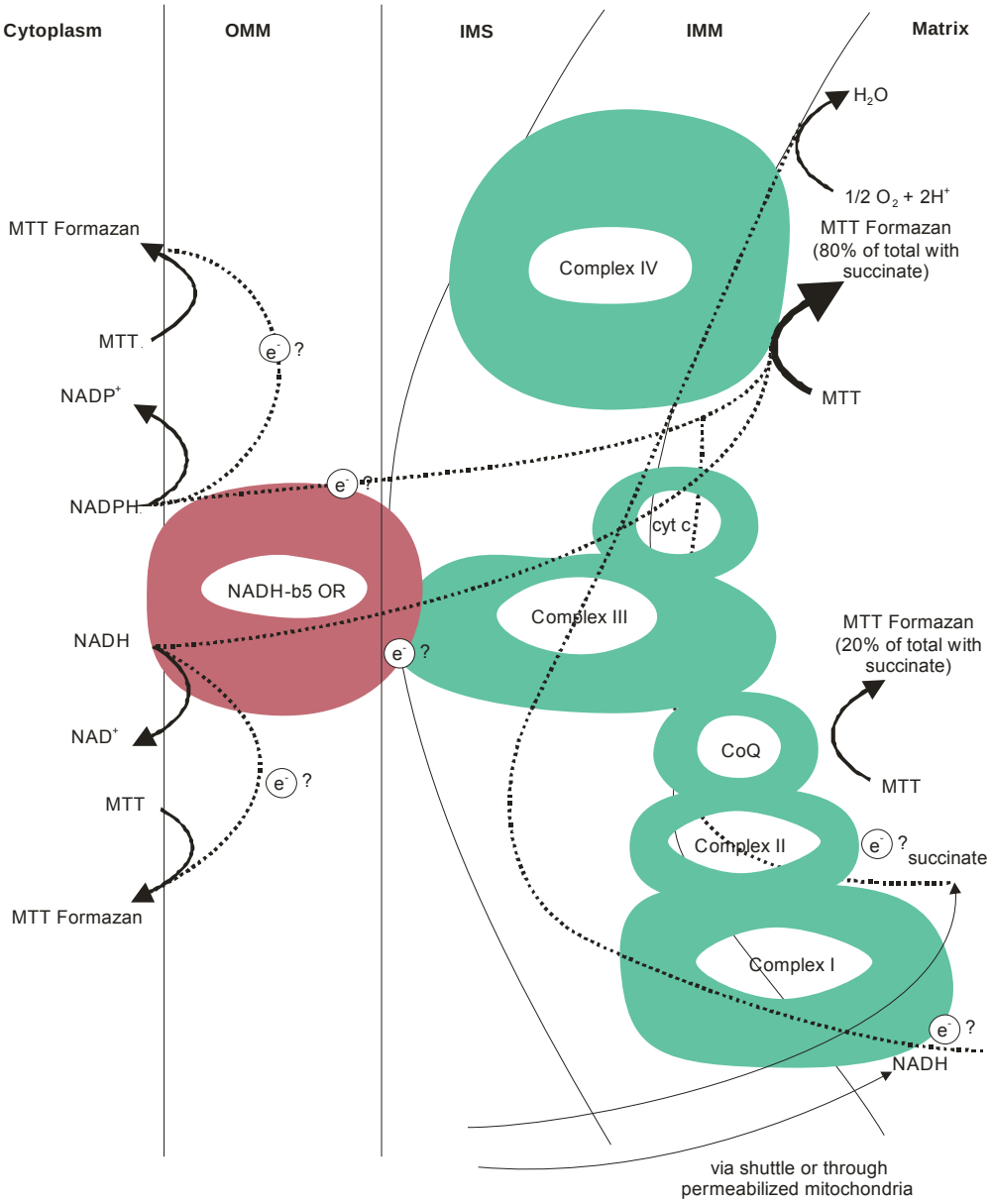

Figure 43: Schematic representation of the mitochondrial electron transport chain involved in MTT reduction in isolated 32D clone 23 cell (long-term bone marrow cultures of C3H/HeJ mice) mitochondria. OMM: outer mitochondrial membrane; IMS: inter membrane space; IMM: inner mitochondrial membrane; Matrix: mitochondrial Matrix; modified after Berridge and Tan (1993)

8.1.2 Neutral Red Retention Assay

As a biomarker for cellular stress, the Neutral Red Retention Time (NRRT) assay has been suggested by Weeks and Svendsen (1996) to evaluate the effects of metals on earthworms. It was established by different studies as a cytotoxicological biomarker for the evaluation of the toxicity of different noxious threats. For example Svendsen et al. (1996) used the NRRT Assay to determine the effects of an industrial plastics fire on the earthworm *Lumbricus castaneus*. Comparable studies, using also the NRRT Assay for the assessment of complex toxic threats as a sum parameter for cytotoxicological assessments were also conducted (Honsi et al. 2003, Hankard et al. 2004). The NRRT Assay has been utilised for the assessment of the toxicity of a range of metals. Weeks and Svendsen (1996, 1997b) conducted a NRRT Assay on coelomocytes of *Lumbricus rubellus* exposed in copper loaded forest soils. The NRRT Assay has as well been performed to evaluate copper toxicity with *Aporrectodea rosea, in situ* under field related conditions (Harreus et al. 1997), under field conditions in a vineyard with *A. rosea* and *Allolobophora thaleri* (Belotti 1999), and with *Eisenia fetida* (Svendsen and Weeks 1997a) under laboratory conditions.

Gupta (2000) found a dose related response in the earthworm *Metaphire posthuma* exposed to cadmium. For the evaluation of nickel toxicity, the NRRT Assay was conducted with the earthworm *E. veneta* exposed in a sandy loamy soil (Scott-Fordsmand et al. 1998). The toxicity of lead has also been assessed by different studies (Reinecke and Reinecke 2003, Booth et al. 2003). The fact that earthworm species do vary in their responses to different toxicants has been addressed by the simultaneous exposure of four earthworm species, amongst them *E. fetida*, to elevated concentrations of zinc (Spurgeon et al. 2000). The NRRT Assay has also been applied for the evaluation of the effects of fungicides (Maboeta et al. 2002, Reinecke et al. 2002) and of several organic toxicants, such as TNT (Robidoux et al. 2002) and organophosphorous insecticides (Booth and O'Halloran 2001).

The Neutral Red Retention Time Assay is based on the appraisal of changes in the lysosomal membrane stability caused by toxic stress (Scott-Fordsmand and Weeks 2000).

Lysosomes are a group of morphological heterogeneous membrane-bound cellular organelles. Amongst other substances, they contain acidic hydrolases which maintain a low internal pH to warrant the efficiency of the membrane bound proton pumps (Seglen 1983). Neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) is a lipophilic, slightly basic colorant that is taken up in uninjured cells and accumulated in the lysosomes. Over time, the colorant, neutral red, tends to leak out of the lysosomes into the cytosol (Harreus et al. 1997) which is then stained by the dye. With increasing membrane damage, the retention time, i.e. the time the colorant remains solely in the lysosomes, decreases in relation of the damage and the dose of the toxicant. The leaking out of the dye into the cytosol can be evaluated microscopically by counting the pinkish to slightly reddish coloured cells. Another approach to investigate the lysosomal membrane integrity is the photometrical evaluation of the amount of neutral red dye remaining in the lysosomes after washing the colorant leaked out into the cytosol. As in most of the modern cytotoxicity assays, this

assay has been optimized for the use of the 96 well microtiterplate readers (Weyermann et al. 2005). The photometrically conducted Neutral Red Retention Assay is widely used as a simple, rapid assay for the prediction of the acute toxicity of toxicants, conveniently carried out in 96-well microtiter plates (Borenfreund and Puerner 1985). In contrast to the NRRT, the NRR is linearly dependent on the cell density and is therefore applied preferably on cells in culture (Borenfreund and Puerner 1985).

8.1.3 Bradford Protein content determination

One of the main problems when conducting *ex vivo* earthworm cytotoxicological assays is to obtain a constant cell concentration from every cell extraction procedure. Different methods for the extraction of coelomocytes from earthworms are available (Eyambe et al. 1991, Hendawi et al. 2004), but with all methods proposed for cell extraction so far, a high variation in the concentration of cells in the coelomic fluid extracted is often experienced. Apparently, the number of cells extracted per individual is not only dependent of the dose of the toxicant to which the animal was exposed to (Harreus et al. 1997, Venkateswara Rao et al. 2003, Wieczorek-Olchawa et al. 2003), but also large individual differences within different species have been experienced (Diogène et al. 1997, Venkateswara Rao et al. 2003). As mentioned above, both assays proposed in this part of the study can also be conducted as microtiter plate assays. This allows a rapid and simultaneous analysis of many samples colourimetrically. One of the basic requirements of the microtiterplate use of both assays is either an identical cell titer in every well – that can be easily obtained when using cell cultures - or the knowledge of the number of cells in every well to be able to ascertain the response of the MTT or the NRR assay proportionally per cell. One way to determine the cell concentration of the coelomic fluid extracted from the earthworm is the time consuming manual count of the cells using a Neubauer haematocytometer (Kirik et al. 1975). Conducted prior to the microplate assays, the advantage of the simple and rapid determination of cytotoxicological effects of many samples simultaneously is nullified. Implying that cells do consist, except of water, mainly of proteins and implying that the protein content of the coelomic extracts of two different specimens which contain equal cell numbers are also equal, the cell concentration must be assessable by a protein assay such as the protein assay developed by Bradford (1976). The Bradford assay is based on the photometrical evaluation of an absorbance shift of the cationic unbound form of the dye Coomassie (470 nm) to the arginine and hydrophobic amino acid residues bound form of the dye (595 nm).The increase of absorbance is proportional to the amount of anionic (bound) form of the dye and consequently the concentration (amount) of protein in the solution tested (Bradford 1976).

8.1.4 Aims

The aim of this part of the study was to determine if the potential threat that metals in ultramafic soils pose for earthworms can be reliably assessed at the sub-organismal level with the use of

biomarkers. Primarily to assess the cytotoxic effects of the so called “ultramafic challenge” (Proctor 1999) to soil living organism such as earthworms. Secondarily, to evaluate if in combination with the Bradford protein assay, the NRR Assay as a biomarker for the determination of lysosomal membrane stability and the MTT assay as a biomarker for cellular proliferation are suited for the rapid and simple automated photometrical assessment of cytotoxic effects that may be emerging from these very unique soils.

8.2 Materials and methods

The cytotoxicity assays were performed with the OECD reference species *Eisenia fetida* (Savigny 1826) because in previous studies conducted with earthworm species, considered to be more field relevant, a high individual variability was observed compared to “laboratory species” (Spurgeon et al. 2000). All earthworms used throughout this study were bred in the ecotoxicology laboratories of the Department of Botany and Zoology at the University of Stellenbosch under controlled temperature and moisture conditions.

8.2.1 Preliminary tests

In a preliminary test, the Bradford, MTT and the NRR assay were conducted on coelomic cells extracted from *E. fetida* exposed in OECD soil to 10 mg/kg and 50 mg/kg of cadmium. Cadmium was selected because this metal is known to affect the lysosomal membrane stability of *E. fetida* (Reinecke and Reinecke 1999). Further, eight specimens of *E. fetida* were exposed to 100 mg/kg of zinc as $ZnSO_4$ and eight specimens of pre clitellate *E. fetida* long-term (for more than ten generations) exposed to cadmium were used. The substrate for the preliminary test was prepared according to the OECD guideline 222 (OECD 2004); consisting of 70% sieved sand, 10% peat moss and 20% clay. The pH was adapted with calcium carbonate ($CaCO_3$) to a pH of 6.0±0.5 and the soil moistened. After an incubation time of 48 hours, the soil was spiked with 10 mg/kg and 50 mg/kg of cadmium as $CdCl_2$ and 100 mg/kg Zn as $ZnSO_4$ respectively, dissolved in 100 mL of distilled water to obtain a total moisture content of 60% to 65% of the water holding capacity.

In one replicate consisting of 400 g of OECD soil, eight pre clitellate specimens of *E. fetida* were exposed for six weeks in a climate controlled chamber at a constant temperature of 20°C in darkness. Two replicates per exposure concentration and four replicates for the controls were used. The worms were fed every second week with approximately 5g of fresh, urine-free cattle manure. The *E. fetida* specimens exposed over the long-term to cadmium were directly taken out of the laboratory cultures.

8.2.2 Exposure of *Eisenia fetida* to ultramafic soils

The exposure setup is the same as that shown in detail in Chapter 2.6. Ten specimens per replicate of synchronized clitellate *E. fetida* were exposed for nine weeks to 500 g of soil samples collected at three different ultramafic sites in the Barberton area, Mpumalanga Province, South

Africa (see chapter 2.2), labelled as Barberton, Kaapsehoop 1 and 2. As a control soil, soil from an uncultivated area close to the sports grounds of Stellenbosch with a history of no pesticide usage was used (Maboeta et al. 2002). Three replicates per exposure or per control were exposed.

8.2.3 Cell extraction

Prior to each cell extraction, the earthworms were kept on moist filter paper for 48 hours to clean their guts (Krontowsky and Rumiauzew 1922). For the collection of coelomic fluid, the worms were washed in distilled water, dried on tissue paper and the hind parts were massaged carefully to remove remnants of soil in the hind part of the gut. Thereafter, the worms were rinsed in ice cold PBS (phosphate buffered saline; Merck Kg Aa, Darmstadt, Germany). Coelomic fluid was collected by non-invasive alcoholic extrusion (Eyambe et al. 1991). The earthworms were incubated separately for 3 minutes at room temperature in a 1.5 mL Eppendorf micro centrifuge tube containing 1 mL of extrusion solution. The extrusion solution for 60 worms consisted of 800 mg GCE (Guaiacol Gliserol Ether; Sigma-Aldrich Inc. St. Louis, MO, USA), 200 mg EDTA (Saarchem, Wadeville, South Africa); and 4 mL of ethanol (99%) in 19 mL of PBS. After 3 minutes of incubation the earthworms were removed from the Eppendorf vials and returned to the culture. The coelomic fluid was washed three times for five, three and one minutes at 2000 U/min at 4°C (Biofuge fresco, Heraeus Instruments, Osterode, Germany). After each centrifugation, the supernatant was removed and the vials were refilled with cold PBS and thoroughly mixed. After extrusion, the coelomocytes were kept on ice until used.

8.2.4 Cytotoxicity assays

The three different assays applied, the Bradford Protein Assay, the MTT assay and the Neutral Red Retention assay were performed simultaneously to obtain comparable results. 50 µL of coelomic liquid for the MTT and the NRR assay was pipetted in each well on 96-well microtiterplates (Greiner Bio-one GmbH, Frickenhausen, Germany), and 25µL of coelomic liquid for the Bradford Protein solution. One well was used per coelomic fluid of every worm and one eight well lane for each exposure. The first and the last lane of every microtiterplate were filled with medium (PBS) instead of coelomic fluid. The microplates were read photometrically at the corresponding wavelengths (Multiskan EX; Thermo Electron Corporation Vantaa, Finland). All readings were corrected by the subtraction of the median of the absorption of the PBS blank. The values obtained from the MTT and the NRR assays were divided by the readings of the Bradford Protein Assay and the values obtained were calculated as a percentage of the control.

8.2.4.1 Bradford Protein Assay

Prior to the Bradford assay performed on coelomocytes of exposed specimens, range finding tests were carried out by creating a standard curve for the Bradford Protein Assay. That was conducted by filling concentrations of BSA (bovine serum albumin, Sigma-Aldrich Inc. St.Louis, MO, USA)

ranging from 0 to 25µL in the first ten lanes of a 96 well microtiterplate and coelomic liquid extracted from *E. fetida* exposed to the unpolluted OECD soils in lane 11 and 12.

With the standard curve obtained from these range finding tests, no linear relation between absorption and protein content was determinable at concentrations below 1 µg/L. The corresponding absorption to a protein concentration 1 µg/L was 0.2. Thus, absorptions of the Bradford Protein assay smaller than 0.2 were considered as not valid. In the experimental setup, the Bradford Protein Assay was conducted by adding cold 250µL of undiluted Bradford Reagent (B6916; Sigma-Aldrich Inc. St.Louis, MO, USA) to 25µL of sample consisting of coelomic fluid. For the blanks, PBS substrate was filled into the first and the last lane of the 96 well microtiterplate. After five minutes of incubation at room temperature, the absorption of the protein dye was read photometrically at a wave length of 595 nm.

8.2.4.2 MTT assay

The MTT assay was conducted according to Carmichael et al. (1987). 50 µL of coelomic fluid per sample was pipetted into the wells of 96 wells plates. As a blank, 50 µL of PBS was used and applied into the two outer rows on the 96 wells plate, one sample per worm and eight samples per replicate/exposure concentration per lane were pipetted into the wells. 25 µL of microfiltered (0.45 µm cellulose nitrate filter, Sartorius, Göttingen, Germany) colouring solution, consisting of 10 mg/mL MTT (3-(4,5dimethyldiazol-2-yl)-2,5-dipenyl; Sigma-Aldrich, St.Louis, MO, USA) dissolved in 20 mL PBS was added. After an incubation time of four hours, 100 µL of MTT extraction buffer was added into every well. The extraction buffer was made up with 0.1 N HCl in isopropanol (Sigma-Aldrich), 10% Triton-X and the pH adjusted with acetic acid to 4.7. After an incubation time of three more hours, the absorption was read photometrically at 570 nm. The results were extrapolated from the absorption of the controls after linear regression analysis. Tests were considered valid only if the right PBS blank did not differ more than 20% from the left blank and if the absorption of the Bradford assay in the corresponding wells was higher than 0.2.

8.2.4.3 Neutral Red Retention Assay

For the NRR assay, 1mL of stem solution (4 mg/mL 0,4% microfiltered NRR in distilled water) was added to 80mL cold PBS. 50 µL of the extracted cell solution was applied into the 96 well microtiterplates in the same manner as described above for the Bradford- and the MTT assay. Subsequently, 50µL of the colouring solution was added into every well. After an incubation time of three hours, the colouring solution was decanted and the wells washed for one minute in 100µL of cold PBS. Then, 100µL of extraction buffer (50% ethanol, 1% acetic acid in distilled water) was added into the wells. After resolving the dye by shaking for 30 minutes, the plates were incubated for further 15 minutes and then photometrically read at a wavelength of 540nm. Corresponding to the MTT assay, the results of the exposures were extrapolated from the absorption of the controls consisting of coelomic liquid of specimens exposed to unpolluted substrate. Tests were considered

valid if the right PBS blank did not differ more than 20% from the left blank and if the absorptions of the Bradford assay in the corresponding wells were higher than 0.2.

8.2.5 Statistics

The protein content was calculated after the linear regression equation obtained from the corresponding standard curve (Microsoft Excel, Redmond, VA. USA).

As a test for normality, Lilliefors test for normality was performed. If the data were found normally distributed, a One-Way-ANOVA on Ranks was applied. As a post-hoc test, Fischers LSD test was performed. Non parametric data were log-transformed. Differences were considered as statistically significant at $p<0.05$.

8.3 Results

8.3.1 Protein content

The protein content of the coelomic fluid of the *E. fetida* ranged between 0 mg/L and 7.9 mg/L. The One-Way-ANOVA did reveal neither a dose dependent protein concentration in the OECD soil exposures, nor significant differences between the ultramafic exposures. Although not significant, rather high individual variations within the exposures and the control were observed in all tests. In none of the specimens exposed to the 100 mg/kg of zinc, absorption values higher than 0.2 were measured. Thus this test was considered as not valid. Hand counting of these extracts using a haematocytometer also revealed a cell concentration of less than 50 cells per mL.

8.3.2 Exposure of *Eisenia fetida* to cadmium in OECD soil

The results of the MTT assay on *E. fetida* exposed in OECD soil to different concentrations of cadmium are shown in Figure 44. Fishers' LSD test, conducted after an One-Way-ANOVA ($F=5.41$; $p<0.05$) revealed significant differences ($p<0.05$) between the *E. fetida* exposed to the control soils and those exposed to cadmium. Although not significant, a dose response relationship was observed. In relation to the control, in the cells of the worms exposed to 10 mg/kg Cd a MTT reduction of 64.59±29.08% and in the cells of the worms exposed to 50 mg/kg Cd a reduction of 48.51±40.92% was observed. The percentage of the MTT reduction in the worms exposed for a long-term to cadmium was 90.88±32.97% of the cells extracted from worms exposed to the unloaded control soil. The reduction of MTT in the control worms, set at a percentage of 100% showed a standard deviation of 33.89%.

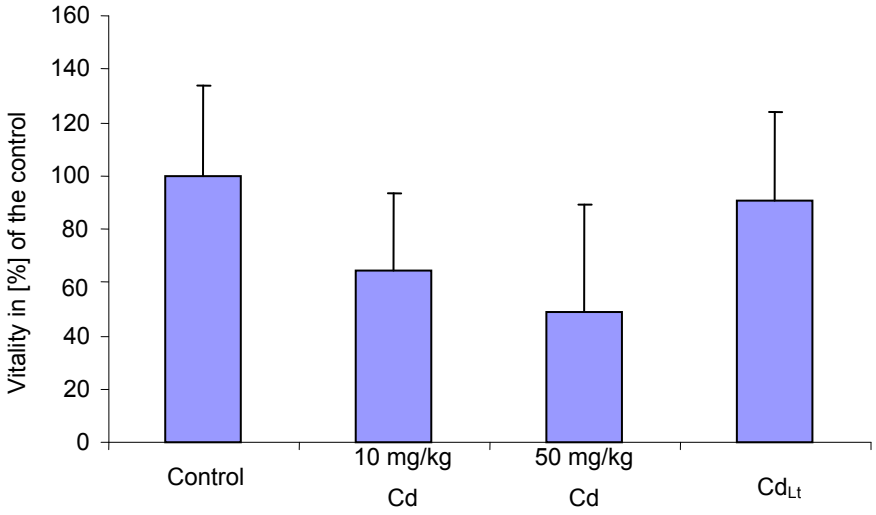

Figure 44: Reduction of the colorant MTT by coelomic cells of *Eisenia fetida* after exposure for six weeks to different concentrations of cadmium; metabolism was determined of the median of the absorption at 570 nm and expressed as the percentage + standard deviation of the control; n=8. CdLt: cells extracted from worms long-term exposed to cadmium

The results of the NRR assay conducted preliminarily on *E. fetida* exposed to different concentrations of cadmium in OECD soil are shown in Figure 45.
The standard deviation of the control was 39.27% and the percentage of Neutral Red retention in the worms exposed to 10 mg/kg Cd was 62.56±36.63% of the control. In the worms exposed to 50 mg/kg Cd Neutral Red retention was 18.45±46.25% of the control. In the worms long-term exposed to cadmium, a retention of 60.68±51.35% comparing to the control worms was observed. The LSD post-hoc test revealed a significant difference ($F=7.64$; $p<0.05$) between the control and the cadmium exposures and the long-term exposed worms.

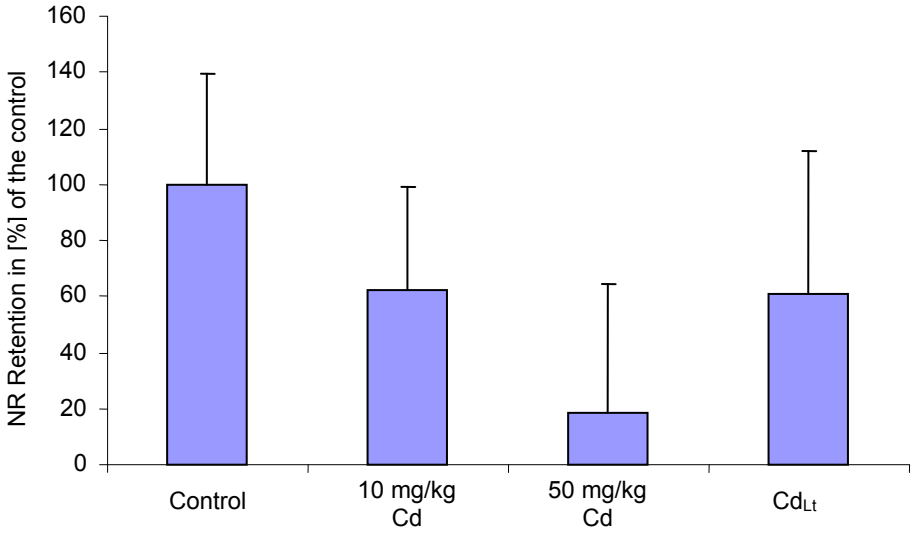

Figure 45: Retention of Neutral Red after three hours in the lysosomes of *Eisenia fetida* after exposure for six weeks to different concentrations of cadmium; retention was determined of the median of the absorption at 540 nm and expressed as the percentage + standard deviation of the control; n=8. CdLt: cells extracted from worms long-term exposed to cadmium

8.3.3 Exposure of *Eisenia fetida* to different ultramafic soils

The results of the MTT assay conducted on *E. fetida* exposed for nine weeks to ultramafic soils collected at the Barberton area are shown in Figure 46.

After nine weeks of exposure, the MTT reduction in the worms exposed to the soils collected at the Barberton Nature reserve was 48.22±4.27% of the control, the reduction of the worms exposed to Kaapsehoop 1 was at 36.77±59.55% of the control and in the worms exposed to Kaapsehoop 2 it was 59.11±21.53% of the control. The standard deviation of the control was 22.58. Statistically, the One-Way Analysis of Variance (ANOVA) returned an F-value of 6.42 ($p<0.05$). The LSD post-hoc test showed significant differences only between the control and all of the ultramafic exposures ($p<0.05$).

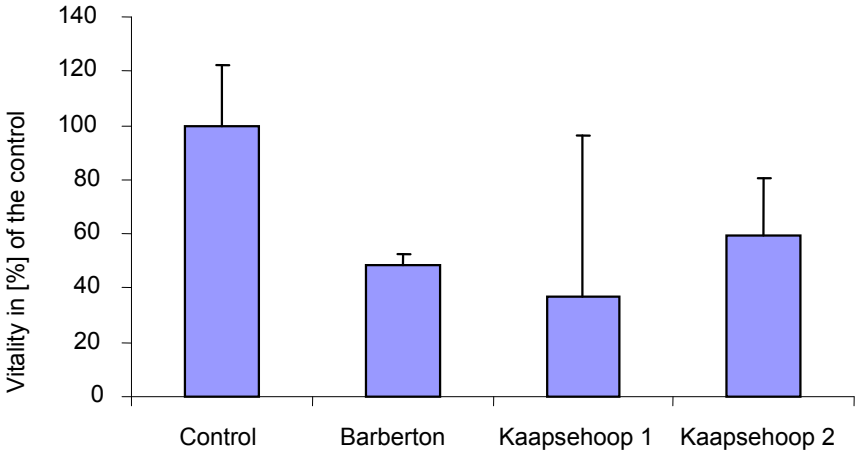

Figure 46: Reduction of MTT by the metabolism of coelomic cells of *Eisenia fetida* after exposure for nine weeks ultramafic soils collected at the Barberton area and a control soil from Stellenbosch; metabolism was determined of the median of the absorption at 570 nm and expressed as the percentage + standard deviation of the control; n=8.

The results of the Neutral Red Retention assay conducted on *E. fetida* after an exposure of nine weeks to the ultramafic soils are shown in Figure 47.

In the *E. fetida* exposed for nine weeks to the Barberton soil, a retention of 44.26±21.16% of the control of the colorant Neutral Red was observed in the lysosomes. In the Kaapsehoop 1 exposure, 48.53±27.78% of the control retention, and in the Kaapsehoop 2, 33.56±16.13% of the control colorant retention was measured. The One-Way ANOVA on ranks revealed significant differences between the different treatments ($F=13.48$, $p<0.05$), the LSD post-hoc analysis showed significant differences between control and all of the ultramafic exposures.

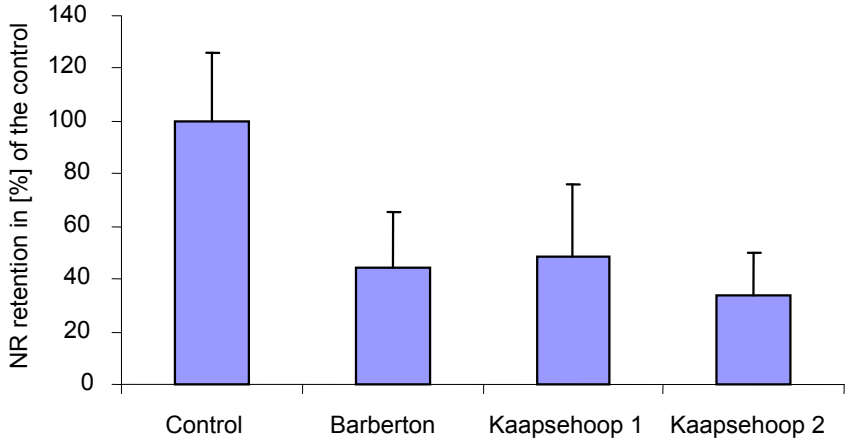

Figure 47: Retention after three hours of the Neutral Red dye in the lysosomes of *Eisenia fetida* after exposure for nine weeks to different ultramafic soils and a control soil collected at Stellenbosch; retention was determined of the median of the absorption at 540 nm and expressed as the percentage + standard deviation of the control; n=8.

8.4 Discussion

8.4.1 Exposure of *Eisenia fetida* to cadmium in OECD soil

As a preliminary test, the Neutral Red Retention assay and the MTT colouring assay were for the first time conducted photometrically *ex vivo* to evaluate the cytotoxicity of cadmium, an important environmental pollutant (Cikutovic et al. 1993).

The results demonstrate that the MTT and the NRR assay, conveniently performed photometrically on 96-wells microtiterplates, were able to detect cadmium induced cytotoxicity in earthworm coelomocytes (Figure 44 and Figure 45). Significantly less of the tetrazolium dye was reduced by the coelomocytes of the *E. fetida* exposed to Cadmium (Figure 44), with a decrease in the reduction at increasing doses of cadmium. Also, the NRR assay revealed a decrease in the neutral red colorant retention after a washing step (Chapter 8.2.4.3) in the lysosomes at increasing concentrations of cadmium. Although not statistically significant, in both assays, the MTT and the NRR assay showed a tendency of a dose relationship. Furthermore, a statistically significantly lower neutral red retention between *E. fetida* exposed for a long term to cadmium and the 'clean' control worms was observed.

However, as these tests were aimed primarily at the calibration of the two assays in terms of incubation time and concentrations of the colorants, no accompanying endpoints such as body load, survival, mass change and fertility were recorded. Thus, no comparisons with regard to responses of other established biomarkers or bioassays to these tests are available. As it is recommended that biomarkers should be linkable to higher levels such as physiological responses, live cycle parameters and population responses (Van Gestel and Weeks 2004), these assays conducted with cadmium have to be considered as preliminary tests and are strongly limited in their expressiveness regarding the subcellular toxicity of cadmium to *E. fetida*.

In terms of the 'conventional' NRRT assay, it is known that cadmium affects the stability of the lysosomal membranes of earthworm coelomocytes negatively (Gupta 2000, Spurgeon et al. 2004). With regard to the lower lysosomal membrane stability in *E. fetida,* long-term exposed to cadmium, similar observations were made by Reinecke and Reinecke (2003). They conducted the NRRT assay on *E. fetida* exposed to lead and discovered a considerably lower retention time in earthworms that have been long-term exposed, i.e. more than ten generations, than in specimens without an ancestral history of exposure. Also by the comparison of *E. fetida* long-term exposed to cadmium to *E. fetida* without a history of exposure, Reinecke & Reinecke observed a diminished retention time in the long-term exposed specimens (unpublished data).

The MTT assay has not previously been used for the assessment of metal toxicity in earthworms and no comparable data were available from previous studies. It has been previously used for the evaluation of the effects of cadmium on the cellular metabolism *in vitro* cell lines. Husoy et al. (1993) compared the MTT and NRR assay exposing the BHK-21 cell line (Syrian hamster kidney cells) to nine different chemicals, amongst them cadmium chloride and copper chloride, and found the results of both assays in good agreement. Olabarrieta (2001) exposed proximal tubule renal

cells (LLC-PK1) to different concentrations of cadmium chloride and found a dose dependent response with the MTT and the NRR assay with LC50s of 5.6 µg/kg for the MTT assay and 4.5 µg/kg for the NRR assay.

8.4.2 Exposure of *Eisenia fetida* to ultramafic soils

Also, as in the OECD soil exposures, the results of the MTT and the NRR assay showed a significant response of the *E. fetida* exposed to the metal loaded ultramafic soils (Figure 46 and Figure 47) compared to the specimens exposed to the control soils.

With *E. fetida* exposed for six weeks to the ultramafic soil samples and to the uncultivated soil collected at the Stellenbosch sports grounds, a range of different bioassays and biomarkers were performed simultaneously with the MTT and NRR assay. Thus, the results of the MTT and the NRR assay are supported by a significant mass loss after six weeks of exposure in the *E. fetida* specimens exposed to the ultramafic soils (Figure 24). Also a lower fecundity of *E. fetida* in the ultramafic soil exposures after six weeks of exposure was supporting the MTT and NRR results (Appendix 3-3). Further, after six weeks of exposure, elevated concentrations of chromium (Figure 13) and nickel (Figure 18) have been measured in the *E. fetida* exposed to the ultramafic soil samples compared to the control soils.

Although no significant difference in the results of the MTT and the NRR assay were found between the different ultramafic exposures of *E. fetida*, one can conclude that the responses on sub cellular-level revealed by the MTT and the NRR assay are related to the adverse ultramafic conditions. With regard to the elevated concentrations of chromium and nickel in the bodies of the earthworms, one can also speculate that these adverse effects revealed by the MTT and the NRR assay may be related to the presence of these two metals. In support of that, it was shown in the preliminary test (previous paragraph), that the MTT and NRR assays might be suited for the detection of metal caused sub-cellular damages.

However, only a small sample size was allocated for this part of the present study. Hence, some aspects related to the methodology require further investigation as some questions could not be addressed in this study:

1) As a basic prerequisite, it has to be validated if the protein content as determined by the Bradford Protein Assay (Bradford 1976) is in fact linearly related to the number of the coelomocytes in the coelomic fluid. It is known that the coelomic fluid often contains not only different types of coelomocytes (Stein et al. 1977, Stein and Cooper 1978), but also very often bacteria, fungi, protozoa and nematoda (Cameron 1932), organisms obviously also consisting of proteins which could interfere with the assay. Recent research have also shown that the bacterial content in earthworms may increase with increasing metal pollution, whereas at least in some cases, the number of coelomocytes decreases (Wieczorek-Olchawa et al. 2003). So far, one can only speculate that most of the bacterial and fungal contamination in this study was either washed off during the centrifugation steps

after the extrusion, or the amount of non-cellular bound proteins was small enough not to influence the return of dose dependent responses.

2) In this study, it was also planned to conduct the MTT and the NRR assay on earthworms collected in the fields. In context to that, the performance of the MTT assay on specimens collected in the field raises the question to what extent the metabolism is influenced by abiotic factors such as temperature and moisture. It is generally accepted that the metabolic rate of ectothermic animals such as earthworms, increases at increasing temperatures (Janssen et al. 1996). Also, the MTT assay might be influenced by the presence of enzyme inhibitors, as it is dependent on different enzymatic reactions (Figure 43; (Weyermann et al. 2005)).
3) In the case of the Neutral Red Retention Assay, the incubation time of the colouring solution that was added to the cells in the 96 well microtiterplates was crucial. This became evident in a series of previously done exposures where cells extracted from *E. fetida* were incubated for different time spans. In an overnight incubation after washing off the excess dye in the cytosol and the solution, no neutral red dye remained in the lysosomes and no absorption was measured. Further, the neutral red retention times, even of worms kept under controlled conditions, differed substantially from species to species (Spurgeon et al. 2000). Therefore, the optimum incubation time for different earthworm species might differ from the three hours experienced as most suited for *E. fetida* in this study. Thus, although the NRR assay was not conducted in this study with *A. caliginosa*, probably a different incubation time could have been expected for this species.

8.4.3 Conclusions

The MTT and the NRR assay are well established techniques for the assessment of sub lethal effects of a range of different toxicants on cell cultures (Seth et al. 2004, Fernandez et al. 2005). Also in this part of the present study, the MTT and the NRR assay were shown to be rapid, objective and easily conductible tools. Both assays, the MTT and the NRR assay, demonstrated a significant response related to two sub-cellular endpoints in the specimens exposed to toxic stress. Thus, the adaptation of the two techniques used, examining the cellular metabolism (MTT) and the lysosomal membrane integrity (NRR) can be considered as promising.

It is obvious that any single biomarker cannot assess the toxic potency of chemicals in such a complex system as soil, unless it is done within a series of complementary assays addressing different hierarchical levels of organisation (Spurgeon et al. 2005). As part of such an integrative assay, the Neutral Red Retention assay and the MTT assay provided a useful support to the bioassays employed to evaluate the toxic stress on earthworms in ultramafic soils.

9. Application of the single cell gel electrophoresis technique for the detection of genotoxic damage in *Eisenia fetida* long-term exposed to ultramafic soils

9.1 Introduction

Because of its chemical structure and its size, the DNA as an inherent unstable molecule is subjected to numerous spontaneous, metabolically and chemically induced changes (Vonarx et al. 1998). One of the main effects of these changes is the mispairing and loss of bases during replication and damage repair during DNA-synthesis. Consequently, the stability of the genetic information is provided largely by repair systems (Friedberg et al. 2005). Apart from oxidative stress caused by reactive oxygen species (ROS) accruing during cellular metabolism, a vast number of noxious effects can interact with the DNA, also causing numerous primary effects such as strand breaks, adducts, base losses or modifications, e.g. at the 8-hydroxyguanine and apurinic sites (Lindahl 1993). To some extent, the above mentioned repair mechanisms themselves also produce an error rate, related to noxious effects, resulting in mutation and recombination. The mutagenic potency of noxious substances and effects is usually evaluated in bacterial tests such as the Ames test (Ames 1973) or the *Salmonella* mutagenicity assay (Knize et al. 1987). In fact, according to a literature review by Claxton et al. (1998), 82% of all studies evaluating the genotoxic potency of industrial effluents used the *Salmonella* assay and other gene mutation assays, about 7% used DNA damage assays and only about 2.5% used *in vivo* animal assays. Arguably, mutation is a sounder and more expressive endpoint for the assessment of genotoxicity, than the evaluation of primary effects. Alas, due to long generation times and the complexity of the eukaryotic genome, mutagenicity assays are not applicable to cells of eukaryotic animals. Also, the direct transfer of the results from mutagenic assays, to e.g. eukaryotic organisms, is so far considered not possible (Schnurstein 2001). A major drawback of most bacterial genotoxicity assays is the need of the substrates to be fractionated and consequently chemically changed, as for technical reasons it is, in contrast to bacterial cytotoxicity tests, not possible to expose the bacterial strains directly to e.g. native soils or sediments, complicating the use of Ames- or *Salmonella* assay for environmental screening (Knize et al. 1987). For the *in situ* evaluation of genotoxicity in individual eukaryotic cells of organisms, a range of different test systems for different fields of application are available (Table 13).

Table 13: Selection of tests for eukaryotic genotoxicity established or applied in environmental monitoring

Genotoxicity Assay	Endpoint/principle
Alkaline elution	Correlation between filtration flow rate and amount of strand breaks (Bihari et al. 1992).
Chromosomal aberration assays	Detection of chromosomal aberrations in metaphase (also see micronucleus assay) by light microscopy (Ray et al. 1973).
Detection of DNA adducts	Identification of DNA disrupting molecules (e.g. 8-hydroxyguanine) (Lindahl 1993, Walsh et al. 1997)
Micronucleus Assay	Formation of a micronucleus from either a whole chromosome (aneugenic event) or a chromosome fragment (clastogenic event) during cell division (metaphase/anaphase transition of mitosis) (Evans et al. 1959).
PCR (polymerase chain reaction)/ DNA sequence analysis	Detection of genetically induced changes by means of gene technology (Wirgin and Waldman 1998).
SCGE (single cell gel electrophoresis technique)	Investigation of migration of DNA fragments after electrophoresis (Singh et al. 1988).
Sister chromatid exchange formation	Detection of symmetrical or asymmetrical exchange between the sister chromatids of a single chromosome (Taylor 1958, Latt and Allen 1977).
Unscheduled DNA synthesis (UDS assay)	Identification of repair synthesis induced by DNA damages (Walton et al. 1983)

Especially the evaluation of genotoxic effects originating from soils is considerably difficult. One approach often conducted is the chemical identification of genotoxic compounds from different sources such as industry (metals and PAHs), agriculture (pesticides, fertilizers and herbicides) and of natural origin (metals), but due to the complexity and interaction between different soil compartments and contents inhibiting or expediting toxicity, the evaluation of the genotoxic potency is difficult (Monarca et al. 2002). Another approach for the capture of the genotoxic potency as a sum parameter is the application of bacterial mutagenic tests such as the Ames-test (Ames 1973) or the umu test (Oda et al. 1985). But as mentioned above, bacterial tests are restricted to the use of extractants of soils, thus can never capture the 'real' genotoxic potential. A third approach – the approach used in this study - is the application of tests monitoring genotoxicity *in situ*. *In situ* genotoxicity tests such as the SCGE assay or the micronucleus assay (Table 13) are usually focussed on DNA strand breaks as endpoint.

9.1.1 Single cell gel electrophoresis

Rydberg and Johanson (1978) first developed a method for the visualization and quantification of primary DNA damage by lysing and embedding individual cells under mild alkaline conditions in agarose. Following a neutralization step, the cells were stained with acridine orange to evaluate the ratio between double stranded DNA (green) and single stranded DNA (red) photometrically.

Ostling and Johanson (1984) refined the principle of Rydberg and Johanson by micro-electrophoresing the lysed DNA under near neutral conditions (pH 9.5), providing a higher sensitivity. The major deficiency of the method by Ostling and Johanson was the fact, that only double stranded DNA breaks can be detected. Single stranded DNA breaks (SSB) are by far more frequent than double stranded DNA breaks (DSB) which do occur in usually less than 0.5% of all strand breaks (Friedberg et al. 2005) except in the case of radiation damage. That limits the method to the detection of damage caused by radiation and radiomimetic chemicals (Rojas et al. 1999). Consequently, a method suited for the detection of SSBs was established by Singh et al. (1988), the so called 'single cell gel electrophoresis technique' (SCGE), or more commonly named the 'Comet Assay'. Strictly seen the name 'Comet Assay' is erroneously used for the SCGE as the assay developed and named Comet Assay by Olive (1989) is a slightly different assay were the electrophoresis is to be conducted under neutral or slightly alkaline conditions unlike the assay developed by Singh et al. which is performed under strong alkaline conditions. Initially developed for the quantification of genotoxic effects on mammalian cell cultures and tissues, the comet assay found its application as a biomonitoring tool for the evaluation of genotoxic effects on prokaryotic cells and eukaryotic cells of organisms of such a broad range of different clades as fungi (Bhanoori and Venkateswerlu 1998), algae (Erbes and Weßler 1997), higher plants (Navarrete et al. 1997), molluscs (Wilson et al. 1998), annelids (De Boeck and Kirsch-Volders 1997), amphibians (Ralph and Petras 1998), fish (Lee and Steinert 2003) and cetaceans (Taddei et al. 2001).

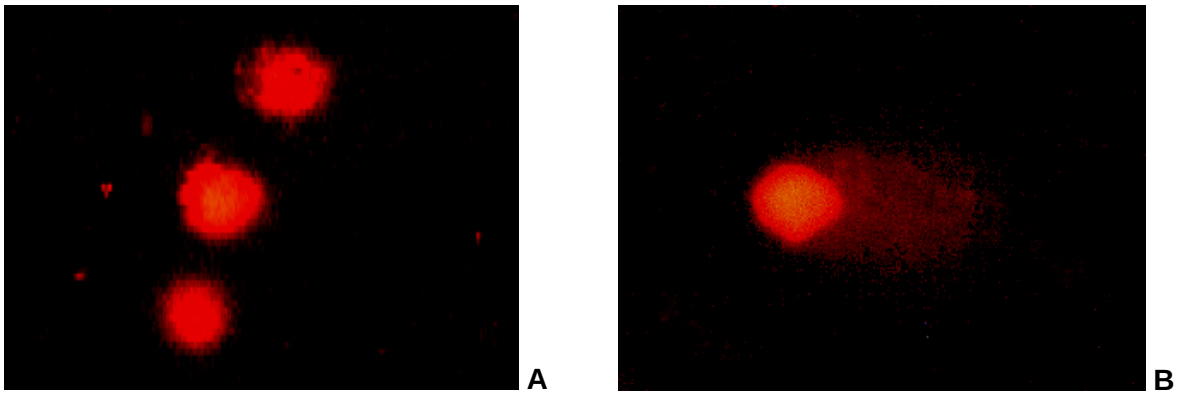

Figure 48: Fluorescence micrograph (400x) of earthworm neutrophilic coelomocytes (*Eisenia fetida*) after 12 min. of single cell gel electrophoresis under alkaline conditions.
A: undamaged cells taken from a control population of *Eisenia fetida*
B: highly damaged cell from an *Eisenia fetida* specimen exposed for 96 hours to 80 mg/L $NiCl_2$

For the SCGE assay, cells are embedded in a single layer of agarose and treated with a highly concentrated salt solution and a detergent to convert them to nucleotides (Collins et al. 1995). As breaks in the DNA molecule induce a release of the complex supercoiling structure of the DNA double helices, during alkaline (pH >12) electrophoresis, free DNA is set free and migrates to the anode (Duthie and Collins 1996), forming a so called 'comet'. The proportion of DNA migrating to the anode consequently correlates with the degree of DNA damage in an individual cell. Stained

with a fluorescent dye such as ethidium bromide, the proportion of the liberated DNA is directly visible and quantifiable under a fluorescence microscope (Singh et al. 1988). Therefore, the degree of damage can be expressed in size and intensity of the comet-tail (Figure 48).

9.1.2 Micronucleus test

Evans et al. (1959) first described production of micronuclei in root cells of *Vicia faba* threatened with single doses of neutrons and gamma-rays, but only early in the seventies of the previous century the micronucleus assay was established as a routine test procedure for irreversible DNA damage in eukaryotic cells (Heddle 1973, Schmid 1973). Since then, the formation of micronuclei caused by aneugenic or clastogenic events was used as a biomarker for DNA damaging effects, mainly in vertebrates such as marine (Arkhipchuk and Garanko 2005) and freshwater fishes (Belpaeme et al. 1996, Rodriguez-Cea et al. 2003, Cavas and Ergene-Gozukara 2005), amphibians (Bekaert et al. 1999) and mammals (Meehan et al. 2004, Knopper et al. 2005) and on freshwater (Klobucar et al. 2003) and marine invertebrates (see review by Dixon et al. (2002)). In terms of invertebrates, the Micronucleus Assay was successfully applied in different studies, mainly dealing with marine invertebrates such as crustaceans and marine molluscs (Galdies 1999, Dixon et al. 2002, de la Sienra et al. 2003, Dailianis et al. 2003, Siu et al. 2004, Koukouzika and Dimitriadis 2005) and also on freshwater invertebrates (Klobucar et al. 2003) but to the best of my knowledge, it was not performed yet on soil living invertebrates.

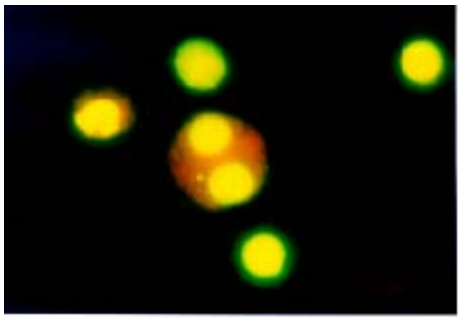

Figure 49: Micronuclei of V79 cells (Chinese hamster) exposed to an unknown clastogenic substance (www.genpharmtox.com)

Micronuclei (Figure 49) develop either by acentric fragmentation of the nucleus caused by a lack of a kinetochore during cell division (clastogenic) or by a lag of a whole chromosome due to a damaged kinetochore or spindle apparatus during mitosis, or by complex chromosomal rearrangements during anaphase by either spindle inhibitors, as e.g. colchicines or clastogens as e.g. radiation (Walker et al. 1996). In contrast to the stand break, the formation of a micronucleus is an irreversible event, potentially causing detrimental damage for somatic cells as well as for germ cell development.

9.1.3 Genotoxicity assays on earthworms

This part of the present study focussed on the evaluation of genotoxic effects of ultramafic soils on earthworms. Besides being characterized by some other disadvantageous conditions for organisms living in and on these soils, ultramafic soils have a relatively high content of metals known or suspected to be genotoxic, e.g. nickel, chromium, cobalt and manganese (Krause 1958). Several studies have shown that metals do have genotoxic effects on a wide range of animals (Talmage and Walton 1991, Hartwig et al. 1994, Fairbairn et al. 1995, Levinton et al. 2003, Lee and Steinert 2003, Levina and Lay 2005). Little is however known about genotoxic effects of metals in ultramafic soils on earthworms.

As it is known that earthworms do occur in these soils, the genotoxic potential emerging from these ultramafic soils was evaluated using the SCGE-assay. For the testing of irreversible DNA damage, the micronucleus assay was conducted to find out whether the exposure of earthworms to ultramafic soils results in DNA strand breaks respectively the formation of micronuclei. Of course one has always to keep in mind that, when conducting a genotoxicity assay with strand break as endpoint, that a number of primary DNA damages are translated exclusively by the technical conditions of the genotoxicity assay to strand breaks. Thus, with such a test only an interaction between noxious substrate and DNA can be proven - commonly expressed as a rate of strand breaks - and no conclusions about the lesion itself can be drawn. Also, the lesion not necessarily has to be induced by primary strand breaks (Schnurstein 2001).

Since Verschaeve et al. (1993) first conducted the SCGE assay on earthworms, only few studies have dealt with the SCGE assay on earthworms, the majority still uses bacterial mutagenicity or genotoxicity assays (Robidoux et al. 2004). Salagovic et al. (1996) conducted the SCGE assay on the earthworm *E. fetida* exposed to soil samples collected next to a coke oven, Bierkens et al. (1998) incorporated the SCGE into a test battery of 20 different biomarkers included the Comet Assay on *E. fetida* exposed to four different toxicants in OECD soils. Zang et al. (2000) applied the Comet Assay on the earthworm *E. fetida* exposed to two pesticides, Delgado-Sureda (2001) applied the SCGE assay on the earthworm *Aporrectodea caliginosa* exposed to different soil samples containing mixtures of toxicants. Bustos Obregon and Goicochea (2002) evaluated the effect of the pesticide parathion on male germ cells of *E. fetida*. Rajaguru et al. (2003) exposed *E. fetida* to sediment samples collected at a river polluted by industrial effluents, Reinecke & Reinecke (Reinecke and Reinecke 2004b) exposed the earthworm *E. fetida* to different concentrations of nickel chloride. Only recently, Di Marzio et al. (2005) proposed some improvements for the application of the SGCE assay on the reference species for earthworm toxicity tests (OECD 2004) *E. fetida* and Martin et al. (2005) executed the SCGE assay on different tissues of *Aporrectodea longa*. As far as it could be established, the micronucleus assay has not been conducted on soil invertebrates.

9.1.4 Aims

In the broader frame of this study, aiming for a comprehensive assessment of the toxicity emerging from ultramafic soils affecting earthworms on different levels of organization, the aims of this part of the study were the evaluation of the genotoxic potential of ultramafic soils on earthworms. Thus, the ubiquitous occurring earthworm *Eisenia fetida* (Savigny 1826) was long-term exposed to ultramafic soils and the SCGE and the micronucleus assay were applied to find out whether a long-term exposure of earthworms to ultramafic soils resulted in DNA damage.

9.2 Materials and methods

9.2.1 Experimental animals and exposure

The exposure setup is shown in detail in Chapter 2.6 (page 19). In short, *E. fetida* was exposed to an uncultivated control soil from Stellenbosch with a known history of no pesticide usage (Maboeta et al. 2002) and soil samples collected at three different ultramafic sites in the Barberton area, Mpumalanga, South Africa (see chapter 2.2). 12 specimens per replicate of synchronized and clitellated *E. fetida* were exposed to soil samples collected at the Barberton Nature Reserve and at two sites in Kaapsehoop, hereafter called Kaapsehoop 1 and 2. For the SCGE assay, coelomic fluid of four worms per sample exposed to the same soils was extracted after two weeks, six weeks, nine weeks and after four months of exposure.

For the Micronucleus assay, coelomic fluid from four worms per sample exposed for four weeks to the control soil and the three ultramafic soils were used. Cells were obtained by the collection of earthworm coelomocytes by non-invasive alcoholic extrusion ((Eyambe et al. 1991), Chapter 2.7.5.1.1).

9.2.2 Single Cell Gel Electrophoresis

During the present study, the procedure of the SCGE assay followed the description of Singh *et al.* (1988) and was slightly modified by Reinecke & Reinecke (2004b).

An overview of the different steps of the SCGE is shown in Figure 50. The assay was conducted on coelomocytes of *E. fetida* extracted after two, four and six weeks of exposure to the ultramafic soils from the Barberton area and to the unpolluted soil samples from Stellenbosch.

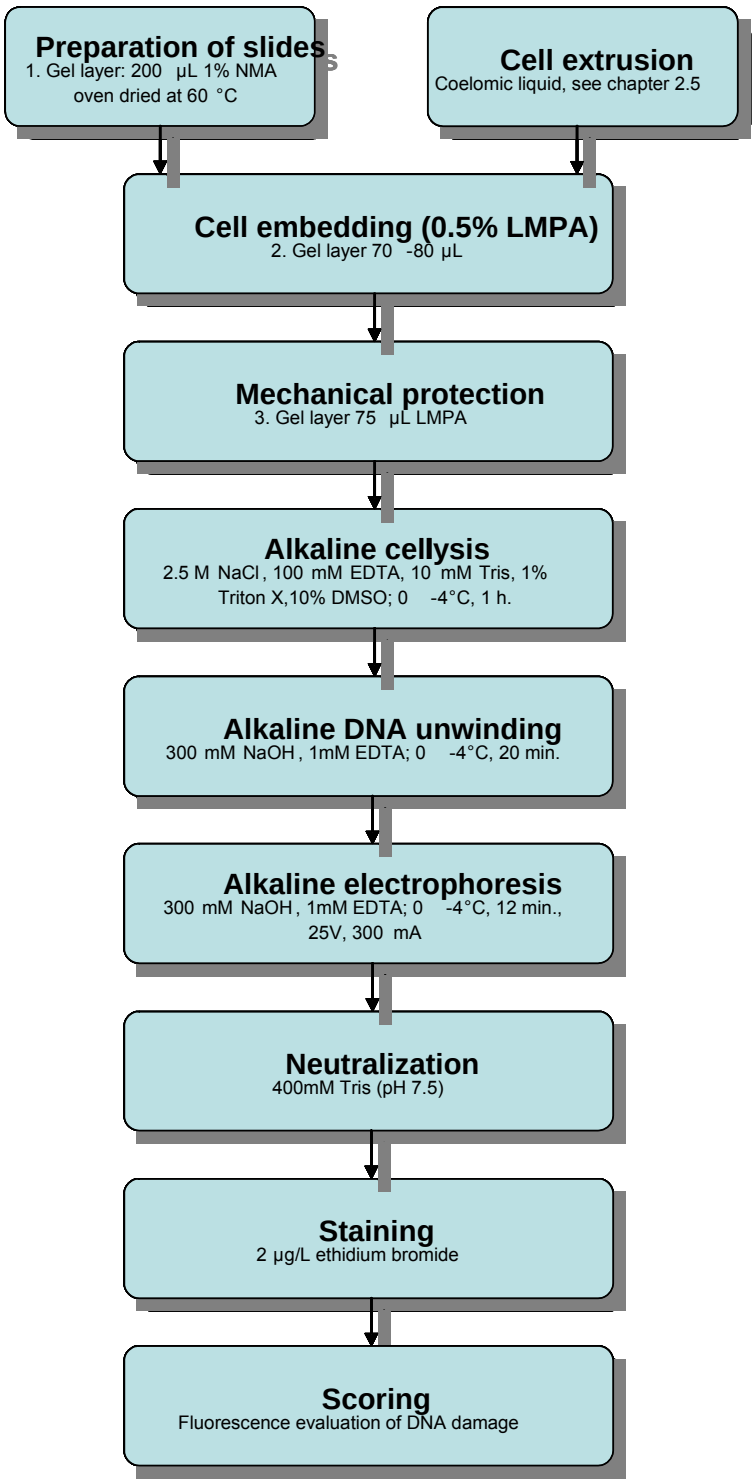

Figure 50: Procedure of the Single Cell Gel Electrophoresis assay on coelomocytes of *Eisenia fetida* according to the method proposed by Reinecke & Reinecke (2004b)(slightly modified)

As the adhesion of the gels is of crucial importance for the SCGE assay, slides (76 x 26 mm, B & C, Germany) were degreased in 99% ethanol prior to the application of the first gel layer. The first gel layer was prepared mixing 500 mg of NMA (normal melting agarose D1 LE, Whitehead Scientific (Pty) Ltd. Brackenfeld, South Africa) in 50 mL of PBS (phosphate buffer saline, calcium and magnesium free, MP Biomedicals LLC, Ohio, USA) to a 1% NMA solution. The NMA solution

was heated carefully in a microwave oven (MS-1907C, LC Electronics, Korea) close to the boiling point. While hot, 700 µL of 1% NMA was spread on the microscope slides. The gel layer was covered with a cover slide (No.1; 50 x 24 mm, Paul Marienfeld GmbH & co. KG, Lauda-Königshofen, Germany) to spread agarose evenly over the surface of the gel. Any remains of the agarose were wiped from the underside of the slides. Then, the slides were labelled and put on ice until hardened. After hardening properly, the cover slips were removed and the second gel layer was prepared under dimmed (yellow) light to prevent UV-damage of the cell suspension. For the second layer, 125 mg of LMPA (low melting point agarose, gel point 24°C – 28°C, LM2, Whitehead Scientific (Pty) Ltd) was dissolved in 25 mL of PBS to obtain a 0.5 % agar solution and placed in a water bath at 25°C until further use. 5 to 10 µL of cell suspension obtained by alcoholic extrusion was mixed with 70 µL of LMPA in an Eppendorf vial (1.5 mL micro centrifuge tube, Elkay Products Inc., Eircann, Galway, Ireland) and embedded onto prepared slides. Cover slips were replaced and the slides were placed on ice until hardening of the second gel layer (3-5 min.). After solidifying, cover slips were removed and a third gel layer for mechanical protection consisting of 90 µL of LMPA was spread on the slides and the cover slips replaced; slides were again put for 3-5 minutes on ice for hardening. Then, cover slips were carefully removed and the slides were transferred for lyses into Coplin jars containing cold, freshly made lysing solution, protected in lightproof tin foil and stored for at least one hour at 4°C in a refrigerator. 1 L of lysing solution stock was made up with 146.1 g of NaCl (2.5 M, Merck aA KG, Darmstadt, Germany), 37.2 g 100mM EDTA (2.5 M ethylenediaminetetraaceticacid di-sodium salt dehydrate, Merck aA KG) and 1.2 g Tris (10 mM Hydroxymethyl aminomethane, Merck aA KG) dissolved in $\pm$ 700 mL distilled water. After stirring the mixture, 8 g of pelletized NaOH (10 M Merck aA KG) was added and the stock solution stirred for about 20 minutes. The pH was adjusted to 10 and the solution filled up to 890 mL with distilled water.

For the final lysing solution, 10 mL (5%) of Triton X-100 (iso-Octylphenoxypolyethanol, Merck aA KG) and 100 mL (10%) of DMSO (Dimethylsulphoxide, Merck aA KG) was added and the solution was refrigerated for about 30 to 60 min. prior to use.

After at least an exposure of one hour to lysing solution (slides can be kept in lysing solution for about four weeks), slides were removed and the surfaces carefully rinsed with Milli-Q water. Electrophoresis buffer was freshly prepared by mixing 3% (30 mL) of NaOH with 0.5% (5 mL) of EDTA filled up to 1 L of aqua dest. from the following stock solutions: 10 M NaOH (200g per 500 mL aqua dest.) and 200 mM EDTA (14.89 g per 200 mL aqua dest. buffered at pH 10). The electrophoresis box (Cigen ltd.) was put horizontally on ice and the slides placed in the electrophoresis box close to the anode (+). Then the electrophoresis box was filled bubble-free with freshly made electrophoresis buffer until slides were covered with liquid. As the DNA is unprotected after lyses, it is essential to work in the dark. Before electrophoresis, slides were left for 20 min. in the electrophoresis box for unwinding of DNA.

The electrophoresis was conducted at 25 volts and 300 milliamperes (power supply by J.D. Instruments, Noordhoek, South Africa) for 12 minutes. In preliminary tests, he optimum electrophoresis time was ascertained as being 12 minutes. For the “calibration” of the electrophoresis time a migration amongst the control cells without being excessive has to be obtained, which is dependent on the cell type. After electrophoresis, slides were removed, rinsed with Milli-Q water and placed on a staining rack for neutralization. For neutralization, slides were rinsed and drained three times with neutralization buffer (48.5 g Tris (0.4 M) in 1 L aqua dest., pH 7.5). Under neutral conditions, the DNA reforms the helical structure, which is a prerequisite for the successful ethidium bromide staining. Prior to the scoring of the comets, slides were stained under dimmed light with 75 µL of ethidium bromide solution (2µM in aqua dest.) and the cover slides replaced. The relative fluorescence intensity of head and tail, expressed as the percentage of DNA in the tail and the tail moment were taken into account as parameters for the evaluation of genotoxicity.

The visualization of the DNA damage was conducted by scoring the comets of neutrophilic coelomocytes on the ethidium bromide stained slides using a fluorescent microscope (Autoplan 2, Diaplan, Leitz AG, Wetzlar, Germany) equipped with an excitation filter of 515 – 560 nm (green filter) and a barrier filter of 950 nm. The scoring was performed by gathering 50 cells per slide at a magnification of 400x. Pictures of the fluorescence stained comets were taken with a Leica DC-300 digital camera system (Leica Microsystems GmbH, Wetzlar, Germany), saved and converted into TIF format. The analysis of the comet images was conducted with aid of an open source public domain program for the image analysis of comets, CASP version 1.2.2, developed by Dr. Maria Wojewodzka, Institute of Theoretical Physics, University of Wroclaw, Poland (Konca et al. 2003).

For the analysis, the pictures were loaded into CASP, where comets where marked and “assayed” in the “image view” window (Figure 51) by drawing a frame around the comets to adjust for measurement. Comets already scored were marked as “assayed”. On a second window, a fluorescence profile for each comet was displayed (Figure 52), showing parameters such as head length and radius, tail length, head and tail DNA and percentage of DNA in head and in tail.

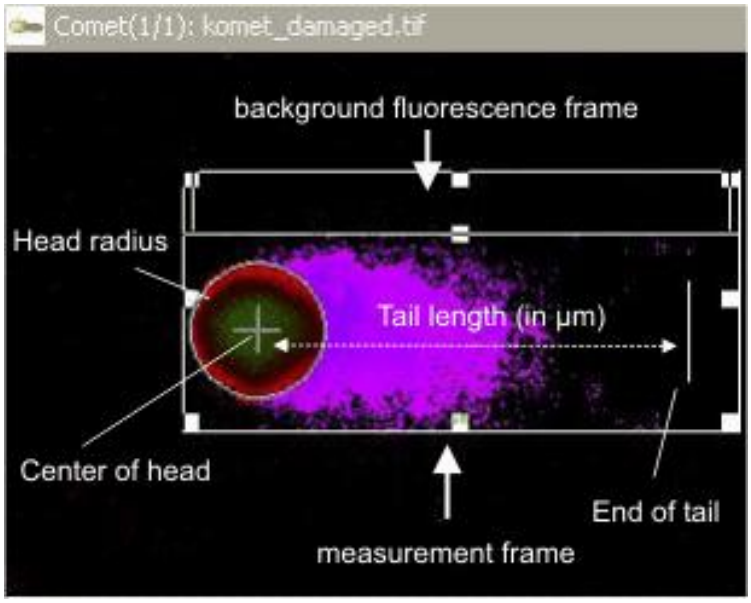

Figure 51: Comet-measurement with the digital image analysis program CASP. The measurement frame is divided in two frames. In the lower frame, tail and head of the comet are marked. In the upper frame, background fluorescence is determined to evaluate the borders of the comet.

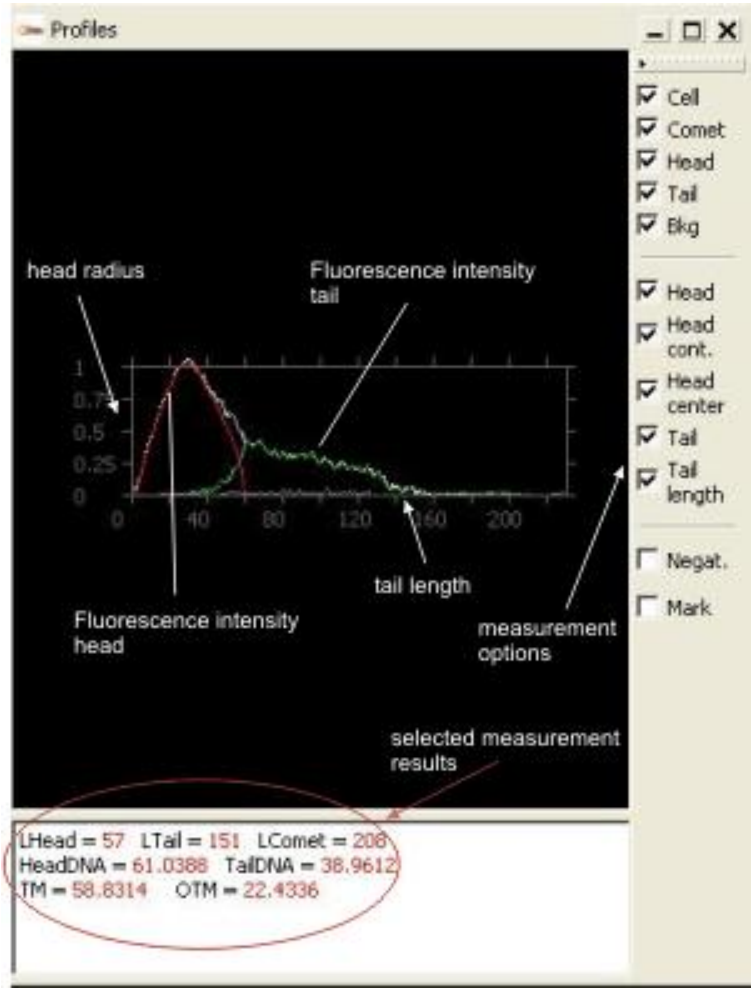

Figure 52: Fluorescence intensity profiles generated by the image analysis software CASP.

The percentage tail DNA is calculated by the division of the intensity of tail DNA by the intensity of the total DNA and the tail moment is a product of length and fluorescence intensity of tail:

$$\text{Percentage Tail DNA} = \frac{\text{intensity tail DNA}}{\text{intensity total DNA}} \bullet 100$$

$$\text{Tail Moment (TM)} = \frac{\text{Tail length} \bullet \text{Tail DNA}}{100}$$

Although often used, mean tail length is not considered to be a suitable parameter, as at increasing doses the tail usually increases in intensity, not necessarily in length and is also susceptible to background or threshold settings of the image analysis program (Collins 2004).

9.2.3 Micronucleus Assay

The Micronucleus assay (MN) was performed at the facilities of the Radiation Biophysics Division of iThemba LABS, Fauvre, South Africa, kindly provided by Dr. J.P. Slabbert according to a method adapted from Meehan et al. (2004), who slightly modified the method proposed by Hayashi et al. (1983). Instead of acridine orange as proposed by Meehan et al. (2004), Giemsa solution was used for staining (Diekmann et al. 2004, Cavas and Ergene-Gozukara 2005). The Micronucleus assay was conducted in a preliminary experiment with specimens of the species *E. fetida* exposed for four weeks to OECD soil (OECD 2004) spiked with 225 mg/kg Ni, a metal known to known to have genotoxic effects on *E. fetida* at this concentration (Reinecke and Reinecke 2004b), and with cells extracted from specimens of *E. fetida* exposed for four weeks to the ultramafic soils and the control soil from Stellenbosch. Two slides per worm and four worms per exposure/control were prepared. The MN assay was conducted with cells obtained by alcoholic extrusion (Eyambe et al. 1991) (see chapter 2.8). Cytochalasin B (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the coelomic fluid to stop uncontrolled cell division in the Eppendorf vials to obtain a final concentration of 3 µg/mL and cells were incubated for 24 hours. Cytochalasin B effectuates the karyokinesis, but prevents cytokinesis to control the amount of cell divisions since extraction (Schmid 1973, Fenech and Morley 1985). After centrifugation for 15 min. at 3000 rpm and 4°C (Biofuge fresco, Heraeus Instruments, Thermo Electron Corporation, Waltham, MA, USA) the supernatant was removed from the vials containing the coelomic liquid and the cells were spread under sterile conditions on clean, fat-free microscope slides. Then, the cells were fixed with a methanol – acetic acid solution (3:1). After drying for about one hour on air, the slides were covered for 1 min. with 10% Giemsa solution (Gurr, BDH Laboratory Supplies, Poole, UK), rinsed with Milli-Q water and mounted in Canada balsam (Galdies 1999). The scoring of cells took place under a fluorescence microscope at a magnification of 1200 x under oil immersion (Zeiss Aristoplan, Zeiss AG, Wetzlar). For the evaluation of the Micronucleus Assay, the

relation between cells with and without micronuclei is determined. The criteria for the scoring of a micronucleus are:

- Sharply bordered micronuclei
- Complete segregation from the macronucleus (main core), no overlapping
- Same fluorescence intensity, same depth of field
- No trinucleated, quadranucleated or multinucleated cells
- Both nuclei are of the same size
- Frequencies of micronuclei are expressed per 1000 cells (‰) (Heddle 1973)

9.2.4 Statistical analyses

All parameters obtained from the SCGE assay were automatically exported from the CASP software into a text file. Tail DNA percentage data and tail moment data were transferred version 7.0 of Sigma Stat™ (Jandel Scientific) for Windows. As a probability level for statistical significance, $p<0.05$ was chosen. Significant differences between the exposures and the control were determined using an one-way-ANOVA on ranks (Kruskal-Wallis-ANOVA). In case of significance, a multiple comparison of mean ranks for all groups, a test analogous to parametric post-hoc tests such as Dunnetts, Fishers LSD or Tukeys HSD test, was conducted as a post-hoc test. Two results for each dependent variable were obtained from the multiple comparisons of mean ranks for all groups:

$$z'_{uv} = \frac{R_u - R_v}{\sqrt{\frac{N \bullet (N+1)}{12 \bullet (\frac{1}{n_u} + \frac{1}{n_v})}}} \text{ and } p = p(z') \bullet k \bullet (k-1)$$

The z'-values correspond to the different significance levels (p values) and the p values are computed as two-sided significance levels for each comparison. Correlations between percentage DNA in tail and tail moment were computed with the Spearman rank correlation coefficient (Siegel and Castellan 1988). As for data with a skewed distribution preferably the median instead of the mean is used (Altman and Bland 1994), sets of measurements were summarized with the median and the standard deviations.

For the Micronucleus assay, no statistical test was applicable (see results).

9.3 Results

9.3.1 SCGE Assay

The scoring of the SCGE Assay conducted after *E. fetida* was initially exposed for two weeks to the ultramafic soils was aborted as too few cells were found on the slides prepared from worms exposed to the Stellenbosch control soils.

The relative intensity of the DNA (expressed as percent tail DNA) in the comet tail, compared to the head intensity of the coelomocytes of the worms exposed for six weeks, is shown in Figure 53

and the tail moment in Figure 54. The data of the tail moments correlated with the percentage of DNA in the tail (R=0.14).

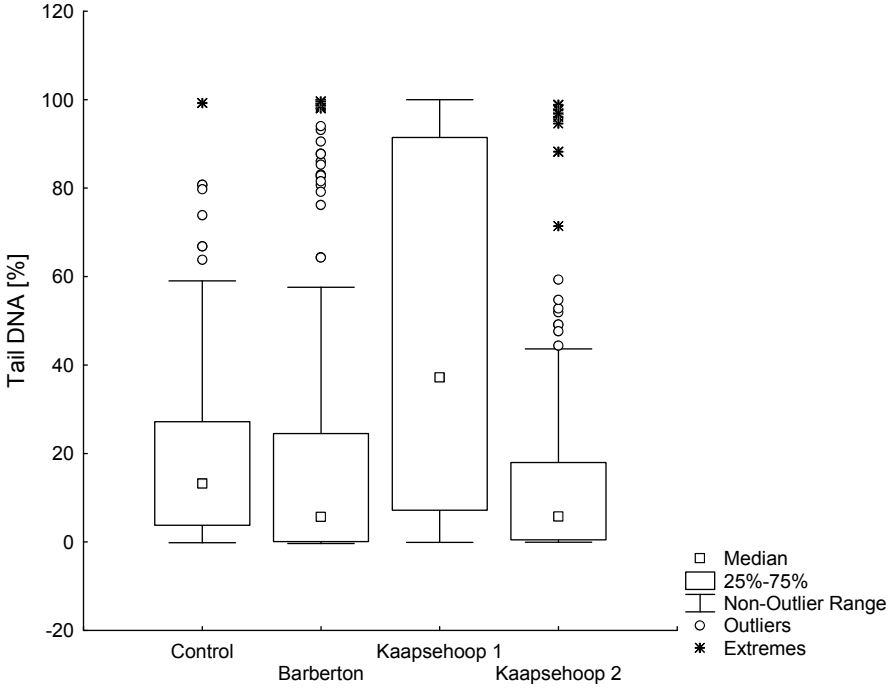

Figure 53: Percentage tail DNA determined with the SCGE assay in isolated coelomocytes of *Eisenia fetida* exposed for six weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n>200; H=130.07; p<0.05).

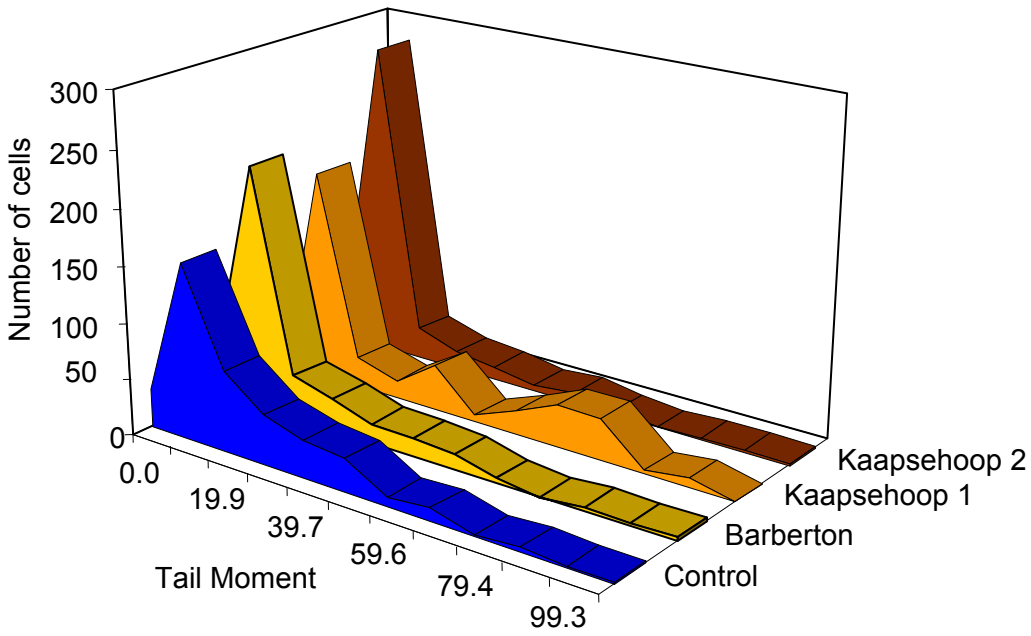

Figure 54: Tail moment of isolated coelomocytes of *Eisenia fetida* exposed for six weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n= 400; H=107.14; p<0.05) determined with the SCGE assay.

The median percentage of tail DNA (Figure 53) for the exposure to the control was 13.22±16.75% of DNA in the tail, for the Barberton Nature Reserve 5.65±25.94% and for the Kaapsehoop 2 site (5.74%±19.7%). For the Kaapsehoop 1 sample it was 37.23±39%, the over-all median being 10.86±27.5%. The tail moment showed similar results; comparatively higher damage in the control and in the specimens of *E. fetida* exposed to the ultramafic soil collected at Kaapsehoop 1 than the specimens exposed to the soils from the Barberton Nature Reserve and Kaapsehoop 2.

The Kruskal-Wallis one way ANOVA on ranks indicated a significant difference in tail DNA percentage ($H=130.7$; $p<0.05$) and tail moment ($H=107.14$; $p<0.05$) between the exposures. The multiple comparison of the two-tailed p-values showed a significant difference between control and all ultramafic exposures (for Barberton $z'=3.47$, for Kaapsehoop 1 $z'=7.38$ and for Kaapsehoop 2 $z'=4.06$), and a correlation between the DNA damage in coelomocytes of *E. fetida* exposed to soils from the Barberton Nature Reserve and Kaapsehoop 2 for the tail DNA percentage. Using the tail moment as a parameter for DNA damage (Figure 54; Table 14), the multiple comparisons of p-values (two tailed) and z'-values showed that the control was significantly different only from Barberton ($z=5.15$) and Kaapsehoop 2 ($z'=7.12$), not from Kaapsehoop 1.

Table 14: Tail moment determined with the SCGE assay in isolated coelomocytes of *Eisenia fetida* exposed for six weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n= 400).

	Control	Barberton	Kaapsehoop 1	Kaapsehoop 2
Median:	10.81	1.75	10.67	0.89
SD:	23.72	20.93	29.43	16.03
Minimum:	0.00	-0.01	0.00	-10.77
Maximum:	177.00	110.15	92.81	124.00
Distribution of tail Moments:				
t < 5*:	156	242	183	285
t < 15**:	80	45	29	58
t < 40***:	105	68	72	34
t > 40****:	59	45	116	23

* = no damage to less damage; ** = moderate damage; *** = strong damage
**** = extreme damage ("head" often completely dissolved); apoptotic.

The degree of DNA damage in worms exposed to Barberton soil was significantly lower compared to the worms exposed to Kaapsehoop 1 (% tail DNA $z'=9.91$; tail moment $z'=7.01$) and in Kaapsehoop 1 also the DNA damage was significantly higher than in Kaapsehoop 2 (% tail DNA $z'=10.4$; tail moment $z'=0.98$).

After nine weeks of exposure significantly less DNA damage was observed in the cells of the *E. fetida* specimens exposed to the Stellenbosch control soils compared to the damage three weeks earlier (% tail DNA H=296.23, z'=14.79; tail moment 325.05, z'=16.6; p<0.05). More DNA damage was measured in the cells of *E. fetida* exposed to the three ultramafic soils (Barberton % tail DNA H=22.07, z'=4.18, tail moment H=164.8, z'=10.94; Kaapsehoop 1 % tail DNA H=117.97, z'=6.92, tail moment H=180.17, z'=3.69; Kaapsehoop 2 % tail DNA H=123.08, z'=7.89, tail moment H=578.25, z'=18.11; p<0.05). But for the percentage tail DNA, in the Kaapsehoop 1 soil, a decrease from 37.2±39% tail DNA to 11.99±11.19% was observed. In the coelomocytes of *E. fetida* exposed in the Stellenbosch control soils 0.51±4.79% of the DNA was found in the comet tails (Figure 55), and this corresponded to a median tail moment of 0.02 (H=1082.75). It was also substantially lower in the controls than in the ultramafic exposures (Barberton Nature Reserve z'= 24.33, Kaapsehoop 1 z'=27.89 and Kaapsehoop 2 z'=28.54; p<0.05) (Figure 56; Table 15).

In the soils from the Barberton Nature Reserve, 9.41±12.54% of DNA was measured in the tail. In the worms exposed to the soil samples from Kaapsehoop 1 11.99±11.19% and from Kaapsehoop 2 11.46±11.01% of DNA was measured in the tail. In the control worms, 0.51±4.79% of the DNA was found in the tails. Statistically, a significant difference (p<0.05) was found between all samples except the samples from Kaapsehoop 1 and 2. For the tail moment, within the groups exposed to the ultramafic soils, a significant difference was found only between Barberton and Kaapsehoop 2 (z'=3.16) with a lower tail moment in the worms exposed to the Barberton Nature Reserve soils (Table 15). The DNA percentage in the tail also correlated (R=0.47) with the tail moment.

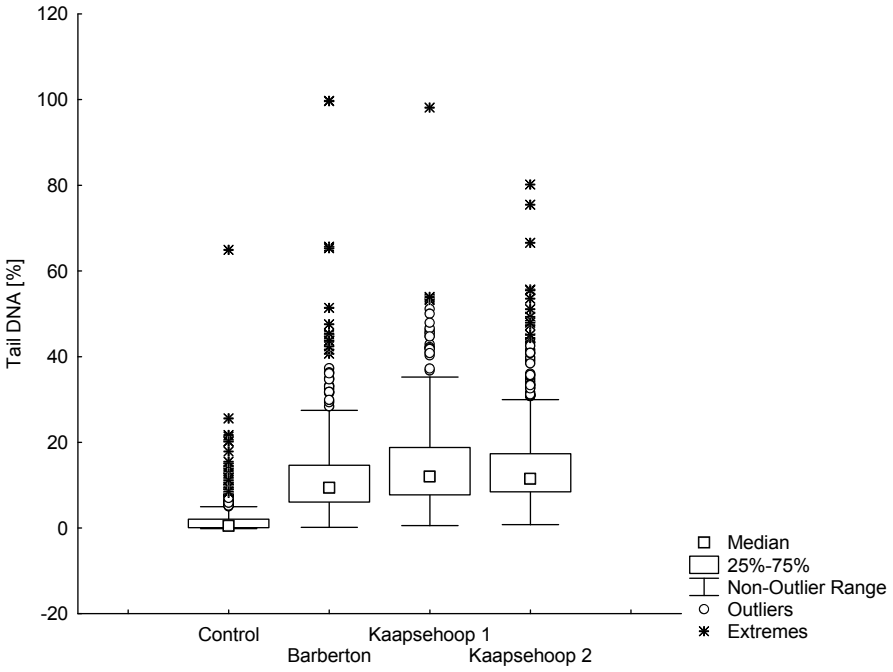

Figure 55: Percentage tail DNA determined with the SCGE assay in isolated coelomocytes of *Eisenia fetida* exposed for nine weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n=400; H=705.67; p<0.05).

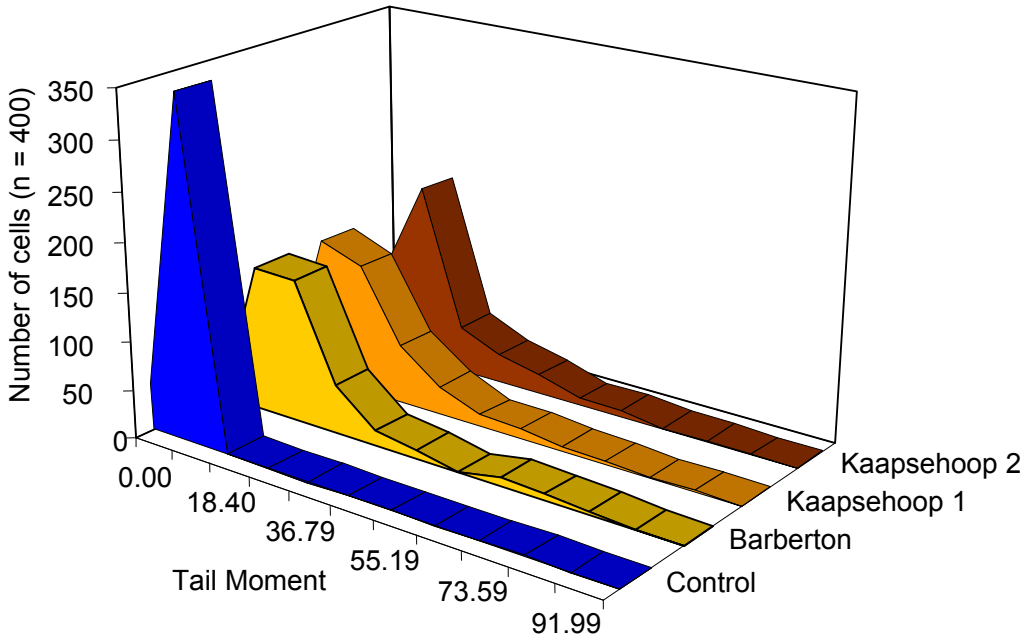

Figure 56: Tail moment of isolated coelomocytes of *Eisenia fetida* exposed for nine weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area determined with the SCGE assay. (n> 400; H=1082.75; p<0.05).

Table 15: Tail moment as a parameter for DNA damage in coelomocytes of *Eisenia fetida* exposed for nine weeks to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n= 400).

	Control	Barberton	Kaapsehoop 1	Kaapsehoop 2
Median:	0.02	12.12	13.06	13.51
SD:	3.53	16.06	13.51	12.98
Minimum:	0.00	0.00	0.13	0.20
Maximum:	75.51	136.00	99.69	89.76
Distribution of Tail Moments:				
t < 5*:	397	67	80	21
t < 15**:	2	187	164	210
t < 40***:	0	118	131	140
t > 40****:	1	28	25	29

* = no damage to less damage; ** = moderate damage; *** = strong damage
**** = extreme damage ("head" often completely dissolved), apoptotic.

Compared to the SCGE assay conducted after nine weeks of exposure, no significant difference was observed in the control (p>0.05), but after four month of exposure, in all ultramafic soils a

significant decrease of DNA damage in cells from exposed *E. fetida* specimens was found ($p<0.05$). The median of the percentage of tail-DNA (Figure 57) after four months of exposure was 0.35±10.56% in the control, significantly ($p<0.05$) lower than in the cells of worms exposed to the Barberton Nature Reserve soil (7.16±123.12%) and in the soils from Kaapsehoop 1 (7.99±16.42%) and 2 (4.48±12.14%). No significant difference was observed between the exposures to the Barberton Nature Reserve soils and the Kaapsehoop 1 soils ($z=0.59$).

Similar to the parameter DNA-percentage in the tail, the parameter tail moment revealed a lower DNA damage in the cells of worms exposed to all ultramafic soils compared to the cells extracted after nine weeks of exposure (Figure 58, Table 16). Significant differences ($H=319.27$) were observed between cells from worms exposed to the control, the Barberton soil ($z=14.91$) and the Kaapsehoop 1 ($z=15.64$) and 2 ($z=7.69$) soils, also between those from Barberton and Kaapsehoop 1 ($z=7.01$) and between those from Kaapsehoop 1 and 2 ($z=8.97$). Also similar to all of the previous exposures, the Spearman rank test revealed a correlation ($R=0.21$) between the parameter DNA-percentage in the tail and tail moment.

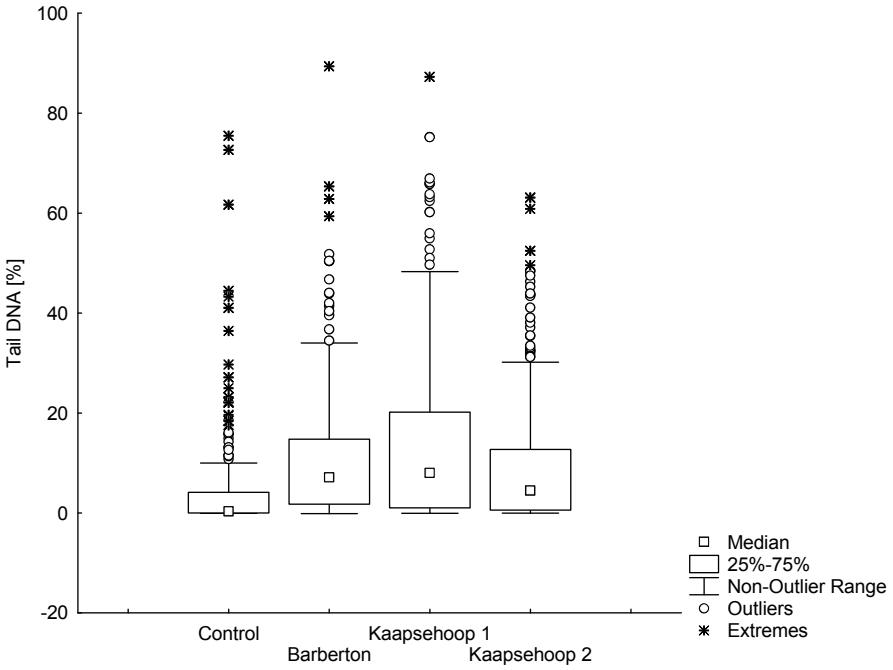

Figure 57: Percentage tail DNA determined with the SCGE assay in isolated coelomocytes of *Eisenia fetida* exposed for four month to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area ($n=408$; $H=222.19$; $p<0.05$).

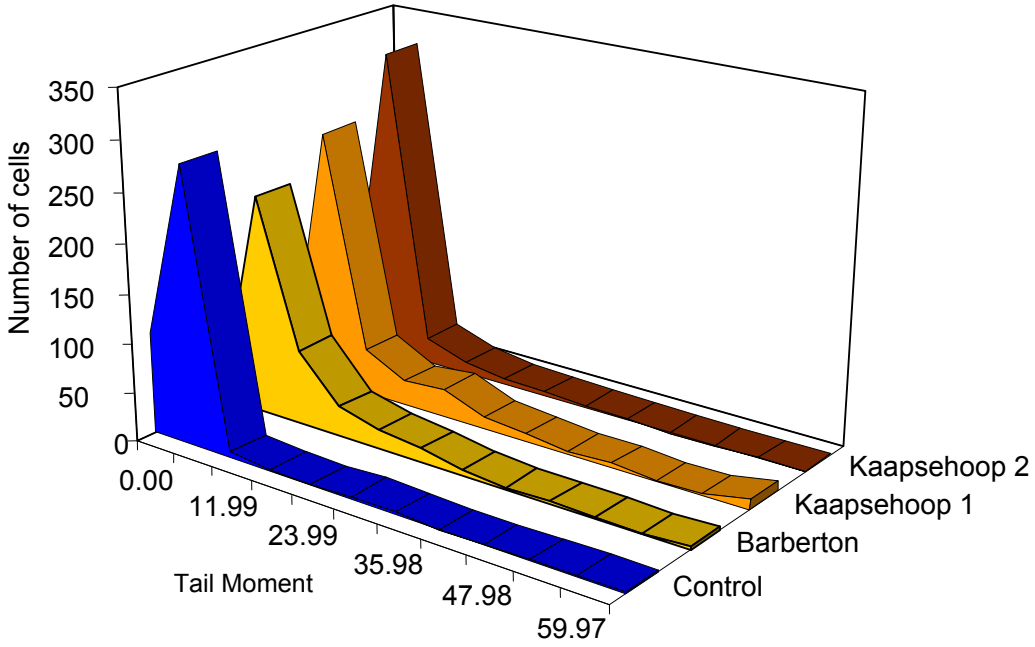

Figure 58: Tail moment of isolated coelomocytes of *Eisenia fetida* exposed for four months to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area determined with the SCGE assay. (n= 400; H=319.27; p<0.05).

Table 16: Tail moment as a parameter for DNA damage in coelomocytes of *Eisenia fetida* exposed for four months to an unpolluted control soil and to ultramafic soils collected at different sites in the Barberton area (n= 400).

	Control	Barberton	Kaapsehoop 1	Kaapsehoop 2
Median:	0.03	3.05	2.58	0.39
SD:	8.28	12.35	14.89	6.27
Minimum:	0.00	0.00	0.00	0.00
Maximum:	127.82	126.21	99.25	55.52
Distribution of Tail Moments:				
t < 5*:	382	227	260	341
t < 15**:	9	107	63	41
t < 40***:	6	56	60	15
t > 40****:	3	10	17	3

* = no damage to less damage; ** = moderate damage; *** = strong damage
**** = extreme damage ("head" often completely dissolved), apoptotic.

For the parameter 'tail moment', in almost all cases the mean values of the tail moments, taking the outliers and the extreme values into account, were substantially higher than the median values.

9.3.2 Micronucleus Assay

As neither the preliminary experiment nor the Micronucleus assay with cells from worms of the species *E. fetida* exposed for four weeks to ultramafic soils returned any results except one single micronucleus, the application of the Micronucleus as an assay for irreversible DNA damage was finally aborted. As with the Giemsa staining the cell cores were stained substantially more intense than the surrounding cytoplasm, the nucleus was easily identifiable. In total, only two cells were observed having undergone cell division (Figure 58).

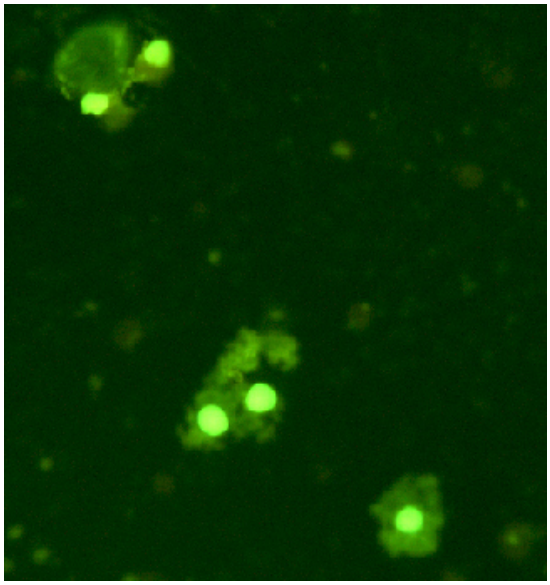

Figure 59: Nuclei of coelomocytes of *Eisenia fetida* stained with Giemsa for the Micronucleus assay at a magnification of 1000 x

9.4 Discussion

9.4.1 Single Cell Gel Electrophoresis Assay

After six weeks of exposure the DNA damage, evaluated with the parameter tail DNA percentage and tail moment in the cells of *E. fetida* exposed to the Kaapsehoop 1 soil correlated with the $CaCl_2$ (mobile) amounts of nickel and to the highest DTPA-extractable amounts of nickel and manganese, found in this sample (Chapter 3.4). No correlation between the total amounts of the metals tested in the soil samples and the responses of the SCGE assay was found. These findings clearly indicate a connection between accessible or rather available metal content and highest DNA damage in the coelomocytes of *E. fetida*. Also, mass change, mortality and the percentage of cocoons hatched in the ultramafic samples (Barberton 68.3±7.7, Kaapsehoop 1 64.3±13.3 and Kaapsehoop 2 81.7±8.3) seems to be related to the DNA damage after six weeks in these exposures (Table 8). In the worms exposed to the unpolluted soil collected at the Stellenbosch site, a high degree of DNA damage was observed after six weeks (% Tail DNA 13.2±16.7%; tail moment 10.8±23.7 Table 14). Other authors (Verschaeve and Gilles 1995, Ralph and Petras 1998, da Silva et al. 2000) also found comparatively high degrees of DNA damages in samples from control areas supposed not to be mutagenic. One has to consider that DNA damage usually is

comparatively higher in control groups of invertebrate cells than in vertebrate cells or cell cultures, most probably due to differences in the manner invertebrate DNA is packed (Mitchelmore et al. 1998) or caused by endogenous or artificial strand breaks arising while sample processing (Siu et al. 2004). Schnurstein (personal communication) relates these "control damages" to mechanical damage occurring during the procedure of the SCGE assay. It is also known that these 'background damages' depend on the cell type itself (Rojas et al. 1999). In our case, also in the cells of earthworms exposed to the control soils, a higher damage than in controls consisting of cells from cell cultures (e.g. in the work of Vock et al. (1999)) was found. After the exposure period of six weeks, the damage in the cells from the worms exposed to the control soils were even with regard to probably occurring 'background damage' very high. There apparently also seems to be a relation to the mass loss observed in the same soils after six weeks (Chapter 3.2), but also, no satisfactory explanation was found, as in these soils, neither elevated metal contents were measured, nor were any metals found in the specimens of *E. fetida* exposed to the control soils (Chapter 6.3). One can only suspect that this may reflect a short-time event of toxic pollution.

After an exposure time of nine weeks, no correlation or relation to the mobile or mobilisable metal content was detected, but, though not significant, the genotoxicity seems to be related to the total, acid extractable content of the metals analyzed, especially in the case of the Kaapsehoop 1 and 2 soils where similar concentrations of manganese and nickel were measured. However, bioavailability of metals can increase by the degradation of organic phases if the metals are bound to these organic phases (McBride 1995, McLaughlin et al. 2000). Thus, one suggestion might be that either the earthworms themselves or microbial activity in the earthworms' gut do remobilise metals bound to the soils. Most studies at present only investigated the remobilization or immobilization of pesticides in soils. Usually, over time, bioavailability of organic toxicants decreases in soils (ageing) (Alexander 1995). Other studies also found that earthworm activities decrease the mobility or degrade certain pesticides such as atrazine in soil but others showed an ability to remobilize pesticides (Calderbank 1989, Gevao et al. 2001). Concerning metals, little is known about the ability of soil living organisms to remobilise or immobilize metals. Some indications that earthworms do mobilise certain metals have been found (Naftel et al. 2002), but with no clear evidence. As another reason for the damage, independent of the bioavailable amount of metals, the presence of toxicants other than metals or metals present in the soil, but not analyzed, cannot be excluded. Asbestos, for example, is often found in soils deriving from ultramafic rocks (Robinson et al. 1996), the ultramafic rocks of the Barberton Greenstone Belt also contain asbestos (Ward 1995, Anhaeusser 2001). Even though no asbestos was found (Chapter 5.3) in the soils samples, the possible occurrence of asbestos in these soils cannot be excluded completely. Asbestos causes strand breaks (Okayasu et al. 1999) and tests on earthworms have shown a long lag time until toxic effects were observed (Schreier and Timmenga 1986).

After four months of exposure the highest damage was shown, using both the tail moment and tail DNA percentage, in the Kaapsehoop 1 soils, correlating with the results on mass and reproduction

(Chapter 3.3). Although significantly higher than in the control groups, the DNA damage was, compared to other studies conducted on earthworms (Verschaeve and Gilles 1995, Delgado-Sureda 2001, Reinecke and Reinecke 2004b), still on a comparatively low level. This might be an indication of a recovery of the earthworms or even the development of resistance. Another probability might be related to the experimental setup and the degree of individual variation. Long-term experiments have often shown an exponential decline in bioavailability caused by increased binding of chemicals to soil colloids during ageing of soils (Naidu et al. 2003). This could also have played a role in the present study.

9.4.2 Micronucleus Assay

The Micronucleus Assay is considered to be a fast and sensitive test for the detection of persistent genomic damage due to clastogenic events and damage of the mitotic spindle apparatus (Borras and Nadal 2004), and is frequently used in many vertebrate species. In invertebrate species, it is used mainly in marine invertebrates. However, it can also be found frequently in scientific papers dealing with genotoxicity, that the comet or SCGE assay was conducted on invertebrates such as earthworms, but the Micronucleus assay in the same publication was performed on vertebrates such as mice or human cell lines (Delgado-Sureda 2001) or plants (Zang et al. 2000). In some cases even extractants of invertebrates were infused into vertebrates to examine the formation of micronuclei (Ahn et al. 2005). For this assay, actively dividing cells are an essential prerequisite (Kirsch-Volders and Fenech 2001). As only two actively dividing cells were found in about 1000 coelomocytes in the whole assay, an interpretation of the results in terms of irreversible DNA damage was not possible. Obviously, without cell division no induction of micronuclei could be detected. Reports on the successful cell division of earthworm coelomocytes *in vitro* are scarce. On the contrary, Toupin et al. (1977) reported no increase in cell numbers. They also did not observe any mitotic phases in cultures of coelomic cells of *Lumbricus terrestris* maintained viable and uncontaminated over ten days. For that, they delivered three possible explanations:

- Free coelomocytes are terminally differentiated and incapable of further division,
- Tetracycline or Fungizone, added to the culture medium, inhibits division.
- The medium lacked a nutrient essential for cell division,

whereas they state that a terminal differentiation is the most likely explanation. Several authors also report the culture of cells extracted from different species of Oligochaeta such as *L. terrestris* and *E. fetida*, but no observation about cell division or the occurrence of mitotic phases is stated (Roch et al. 1975, Hrzenjak et al. 1992, Bilej et al. 1993, Kauschke et al. 1997, Battaglia and Davoli 1997, Mitsuhashi 2002). Thus one can speculate that the Micronucleus assay was unsuccessful because the basic prerequisite, the division of cells, was not fulfilled. The rate of cell division of coelomocytes is also very slow as indicated by Eyambe et al. (1991), reporting that the recovery after collection of coelomic leukocytes in the earthworm *L. terrestris* takes about six weeks. Bilej et al. (1992) suggested an antigenic stimulation to increase proliferation. Another suggestion might be

to induce or accelerate cell division by the addition of a defined mitogen (Rozengurt 1986), such as phytohemagglutinin (PHA), pokeweed (Needleman and Weiler 1981), or 4-acetylaminofluoren (Schmid 1973, Braithwaite and Ashby 1988).

10. General Discussion

10.1 Metal background concentrations and bioavailability of metals in ultramafic soils estimated by chemical extraction

The first aim of the study was to determine the living conditions of earthworms in ultramafic soils in Mpumalanga and their hazardous potential in terms of the metals known to occur in abundance in these soils.

The chemical analysis of the metals in the ultramafic soils revealed exceptionally high concentrations of cobalt, chromium, iron, manganese and nickel in all ultramafic soil samples (Table 3), compared to the control soils collected at a sampling location with a known history (Maboeta et al. 2002) of no anthropogenic pollution in Stellenbosch. The total metal contents extracted from the soils by acid digestion are well within the range reported by a number of previous studies conducted on ultramafic soils elsewhere in the world (Nemec 1954, Vergnano 1958, Gasser and Dahlgren 1994, Marino et al. 1995), and thus can be considered as typical for ultramafic soils. Also, no cadmium and lead were detected at elevated concentrations in these soils, giving an indication that comparably few anthropogenic influences in terms of these metals are present.

For all metals analysed, a total metal content significantly higher in the ultramafic soils than in the reference or control soils was measured. The proportion of the mobile and mobilisable metal fraction measured by chemical extraction methods resulted in a proportionally smaller estimated amount of biologically available metals in these soils than the mobile and mobilisable amounts chemically extracted from the unpolluted, non-mafic soil collected at Stellenbosch (Table 2 and 3). Further, an indication is given by Echevarria et al. (2006) that this seems also to be the case for anthropogenically polluted soils. They found a far lower availability of nickel in ultramafic soils than in anthropogenic contaminated soils. For nickel, it was shown that especially the DTPA extraction is a fairly good indicator for nickel's bioavailability in ultramafic soils.

The $CaCl_2$ and the DTPA extraction gave an indication why in spite of these extremely high total concentrations of metals, earthworms can live in these soils by showing that the amount of metals potentially available to earthworms are far lower than the amounts present in the substrate. In support of that, it is widely accepted that total metal concentrations of soils alone are of secondary importance when estimating the environmental risk to soil-living organisms. The total metal concentrations in such a complex system as soil are usually not directly related to the environmentally available concentrations (Reinecke et al. 2004).

The $CaCl_2$ and the DTPA extraction are widely accepted as indices of the bioavailable amounts of metals in soils (Sims 2000) since they provide a physico-chemical compartment defined by its solubility in the different extractants. Thus, *stricto sensu*, the expression "bioavailable" used in context with these indirect chemical measures is misleading, as by definition, only organisms can determine bioavailability (Lanno et al. 2004). Nevertheless, the principle of the extraction of mobile or mobilisable (i.e. complexed, adsorbed and carbonated metals), have been adopted successfully

for a preliminary assessment or estimation of the amounts of metals in soil supposed to be available for the uptake by earthworms (Peijnenburg et al. 1997, Weltje 1998, Conder et al. 2001).

In most cases the total metal burden of the worms was higher than the amount of metals extracted by the $CaCl_2$ solvent and at about the same magnitude as the amount extracted by the DTPA extraction (Chapter 4). In proportion to the metal content measured in the earthworms exposed to the ultramafic soils, as well as to the metal content in the worms collected by J. Mesjasz-Przybylowicz in ultramafic soils in the Barberton region, the DTPA extraction results, representing complexed, adsorbed and carbonated metals, suggest that the earthworms might be able to assimilate chromium, manganese and nickel.

By comparing the DTPA extraction with actual earthworm body burdens, there seems no clear correspondence. Dai et al. (2004) found the metal concentration measured after DTPA extraction at the same order of magnitude as the amount measured in the earthworms (as it was the case in the present study), although they also did not always find a direct correlation. Rida and Bouché (1995) reported that the concentrations of the metals cadmium and lead in earthworms exposed to natural soils are not predictable by analysing the total metal content, acetic acid extraction or DTPA extraction of the soil substrates. Arnold et al. (2003) found a strong positive correlation between DTPA extractable copper and mortality in *E. fetida* exposed to cupric nitrate, but no correlation between the DTPA extracted copper and mortality in *E. fetida* exposed to other copper salts, and therefore claimed that both extraction methods used as indicators of copper availability, were not advisable. Arnold et al. (2003) though, used OECD soil which has frequently been shown to overestimate the effects of metals compared to field tests, probably because of a higher metal availability in these artificial soils (Spurgeon et al. 1994, Spurgeon and Hopkin 1995, Davies et al. 2003b, Fountain and Hopkin 2004, Reinecke et al. 2004, Schaefer 2005). Furthermore, a negative correlation between pH and DTPA extracted metals has been found (Mitsios et al. 2005). So far, there is no final evidence that the pH not only influences the metal availability in soils – something that is widely accepted (Theis and Richter 1979, Beyer and Cromartie 1987, Morgan and Morgan 1988) - but also the efficacy of the extraction method itself.

In a number of publications, the analysis of pore water is recommended for the assessment of the environmentally available fraction of metals in soils (Van Gestel and Ma 1988, Belfroid et al. 1994, Lanno et al. 2004). From a methodological-analytical point of view, the pore water gathers only the metals in the liquid phase of the soils and probably underestimates the easily Ca^{2+}-exchangeable metal ions also available for earthworms. Consequently, although not measured in this study, the metal content of pore water in these soils would be even lower than the $CaCl_2$ extractable content, and thus underestimating the environmentally available concentrations of especially chromium, manganese and nickel even more.

10.2 Body burden of metals in earthworms

In agreement with previous studies, stating that the bioavailability can be assessed only by involving organisms (Peijnenburg et al. 1999, Peijnenburg and Jager 2003, Lanno et al. 2004, Reinecke et al. 2004, Van Straalen et al. 2005), from the present findings one can conclude that the DTPA extraction might be a good indicator for the presence of environmentally available metals such as chromium, manganese and nickel. However, for the evaluation of potentially bioavailable metals the DTPA extraction can only provide a fair estimation. Nevertheless, depending on the metal analysed for, especially the DTPA extractable concentration might be preferable to the total concentration. The values were found to be closer to the amounts measured in the earthworms. In general, one has to keep in mind that no chemical extraction alone can be a suitable indicator for the bioavailability of all metals to earthworms in general (Basta et al. 2005). Also, from the findings in this study it can be concluded that the $CaCl_2$ extraction clearly underestimates the concentration of the metals bioavailable to the two earthworm species.

Disregarding the concept of soil ageing (Lock and Janssen 2003), the amount of environmentally available metals in a specific field soil usually is static, while the content of metals in soil dwelling organisms often fluctuates over time (Reinecke et al. 2004) and can therefore be considered as dynamic. Also, different factors such as the ability of earthworms to sequestrate certain metals, the remobilisation of metals by microbial activity either in the soil or in the earthworm gut, and a range of abiotic factors have to be considered (Edwards and Bohlen 1996, Kersanté 2004).

For the evaluation of the direct bioavailability, the earthworm species *Aporrectodea caliginosa* and *Eisenia fetida* as well as two specimens collected on-site were exposed to the ultramafic soils collected at the Barberton Region. As no background information was available for the unidentifiable earthworm specimens collected on-site, the only statement that can be made in regard to these two earthworms is that they had elevated concentrations of chromium, cobalt, manganese and nickel in their bodies in comparison to earthworms living in soils not loaded with these metals. They did, however, provide evidence that earthworms may be able to survive in these ultramafic soils with body burdens at these levels.

10.3 Uptake of metals in earthworms

The ecophysiologically different species *A. caliginosa* and *E. fetida* showed fundamental differences in the uptake of chromium, manganese and nickel in their body (Chapter 4). *A. caliginosa* seemed to absorb the metals over the whole body length, but had a tendency to concentrate them in the tail section, which is probably linked to the ability of autotomization as means of getting rid of these metals (Chapter 4.3.3.1). At the same time, regeneration, most probably following autotomization, was observed in these specimens. Therefore, for the assessment of the bioavailable concentration of metals, the epigeic species *A. caliginosa* showed a distinctly higher individual variability than *E. fetida*, at a level where no estimation or even predictions towards the bioavailable amount of metals were possible. Furthermore, some of the

specimens of *A. caliginosa* underwent a quiescence during the exposure period, which most probably had an influence on the metal uptake. The specific reasons for the quiescence remain unknown, but it could involve a quiescence as response to adverse conditions (Lee 1985) or an obligatory quiescence (Satchell 1967, Morgan and Winters 1991).

To verify the results of worms exposed to the ultramafic soils and to test the predictability of metal accumulation in earthworms, especially with respect to manganese and nickel, *E. fetida* was directly exposed to different concentrations of these metals in an aqueous medium. The results clearly showed that both metals were not biomagnified (Chapter 4.3.5). In terms of manganese, this result is supported by different previous studies (Ireland 1979, Reinecke and Reinecke 1997b, Dai et al. 2004, Maddocks et al. 2005). For nickel, there seems to be no general agreement in the literature (Chapter 4.4.6). The bioaccumulation rate was furthermore dependent on the concentration of these metals in the medium (Chapter 4.3.5). With increasing concentrations of manganese or nickel, the uptake decreased proportionally to the medium concentration. As a consequence, the bioaccumulation factor cannot be considered as a suitable indicator for the bioavailability of manganese and nickel, as the measurement of the body burden of earthworms exposed to high concentrations might return a too low value.

For chromium, an essential metal (Gauglhofer and Bianchi 1991, Crossley et al. 1995), the results of this study indicate that this metal was regulated actively by the earthworms, what has also been observed in different other studies (Oste et al. 2001, Van Straalen 2002, Van Straalen et al. 2005) (Chapter 4).

In conclusion, together with the fact that in the ultramafic exposures no equilibrium in the body loads of manganese and nickel in *E. fetida* was reached after at least eight weeks of exposure (Figure 8), the analysis of earthworms did not return an indication of the bioavailable amount of these two metals. The findings in this study conducted with *A. caliginosa* and *E. fetida* are supported by different findings of previous studies (Spurgeon and Hopkin 1996b, Crommentuijn et al. 2000, Oste et al. 2001, Van Straalen 2002), stating that the bioavailability estimated by measurements of the body burden of the animals provides only a poor predictability for metals as this concentrations might not be constant. Similar results were obtained by Sandoval et al. (2001) who exposed *E. fetida* to highly contaminated soils of mining effluents.

10.4 Tissue distribution of metals in earthworms

The distribution of metals in the body of *A. caliginosa* was, as indicated above, evaluated only with respect to the longitudinal distribution in the body. The results indicate that *A. caliginosa* transports metals such as nickel, manganese and chromium into the posterior section and subsequently autotomizes this section. *A. caliginosa* accumulates different metals primarily in the posterior alimentary canal (PAC) (Morgan and Morgan 1998). This phenomenon of accumulation in the chloragog in the PAC has been observed for different earthworm species and metals and is suspected to be a detoxification strategy of accumulative immobilisation (Morgan and Morgan

1990, Gruber et al. 2000, Morgan et al. 2002). One can speculate that the autotomization is the next step following the accumulative immobilisation.

The more detailed distribution of metals in *Eisenia fetida* was evaluated by elemental image mapping (Chapter 7). The results, although for technical reasons only few samples could be analyzed, indicated, that *E. fetida* sequestrates chromium and nickel in granular-like bodies in the coelomic fluid. With regard to nickel, these results are supported by the findings of Vijver et al. (2006), stating that nickel, together with the non-essential lead, was found mainly in the granular fraction of the coelomic fluid. Manganese did not show a specific distribution pattern, whereas cobalt seemed to be completely absent. Metal containing chloragosomal granules have been described by Morgan (1984) who conducted microprobe studies on different terrestrial invertebrates. Morgan also experienced differences in the elemental distributions of metals between different earthworm species. Therefore, these results give no indication about the mechanism underlying the metal distribution and elimination of *A. caliginosa*. With regard to *E. fetida*, one can conclude that the metals probably sequestered in these granulocytes are not biological reactive within the earthworms as granular fractions are known to be responsible for metal immobilisation (Vijver et al. 2006).

10.5 Bioassays

10.5.1 Mortality

On the population level, the highest mortality was observed in the *A. caliginosa* exposed to the ultramafic soils from Kaapsehoop 3 containing the highest concentrations of chromium, manganese and nickel measured in all soil phases. Also, the highest body burdens were found in the specimens of *A. caliginosa* exposed to the Kaapsehoop 3 soils. Only in the control groups was no mortality observed (Table 7).

For the two ultramafic soils collected at Agnes Mine and Songimvelo, no relation was found between the endpoint mortality and metal concentrations in the worms, DTPA extracted metals or total amounts of metals. Although after eight weeks of exposure chromium, manganese and nickel concentrations in the worms, DTPA extractable phase and total concentration in the soils were higher in the soils from Agnes Mine than in those from Songimvelo, the mortality in the worms exposed to Songimvelo was higher. Furthermore, after the whole exposure period of 24 weeks, no worms survived in the Songimvelo soils (Chapter 4.3.2.1). As the soils did not significantly differ in edaphic factors such as pH, moisture and organic matter, one can only speculate that a further, unidentified metal or other toxicant caused the high mortality in all replicates of the Songimvelo exposures. An indication might be given by the fact that, in comparison to the Kaapsehoop 3 exposures, the worms died at a later stage of the exposure. Similar observations were made on earthworms exposed to asbestos. *Lumbricus rubellus* survived high concentrations of asbestos for a short time (30 days), but not for longer (42 weeks) (Schreier and Timmenga 1986). It is known that ultramafic soils of the Barberton Greenstone Belt sometimes contain high concentrations of

asbestos (Ward 1995, Anhaeusser 2001), although no asbestos fibres longer than 5µm were detected in this study (Chapter 4).

Mortality of *E. fetida* was observed only in the exposures to two different ultramafic soils, the soils from Kaapsehoop 1 and 2 (Chapter 5.3.2). A comparison of the mortality of *E. fetida* observed in the two different artificial media containing different concentrations of nickel, with the mortality of *E. fetida* observed in the ultramafic exposures, is difficult. In Kaapsehoop 1 the mortality was 18.75±7.98%, in Kaapsehoop 2 it was 10.42±12.5% and in the Barberton Nature Reserve soils no mortality was observed. One might speculate now, that the LC values obtained in this part of the study in these artificial media do reflect the actual available amount of nickel as all soil related parameters influencing nickel uptake and toxicity were excluded. Thus, one might estimate the bioavailable amount of nickel in these soils by equating the mortality observed from the soil exposures to the corresponding LC values in the artificial media exposures and obtain an estimate of the amount of nickel actually bioavailable. In this case, the mortality of 18.75±7.98% in the Kaapsehoop 1 soil would relate to an average nickel concentration of 85.42 mg/kg and the mortality of 10.42±12.5% at Kaapsehoop 2 to an average concentration of 67 mg/kg bioavailable nickel. Probably, in terms of such a comparably lesser sensitive parameter as mortality (Van Gestel et al. 1991, Spurgeon et al. 2005) one might gain a realistic estimation, but in fact, one has to keep in mind that in terms of field relevance, such an approach is limited, especially because it does not take the effects of mixture toxicity into account and neglects all edaphic factors influencing toxicity (Khalil et al. 1996a).

10.5.2 Mass change

For *A. caliginosa*, the parameter mass change showed significant differences between the ultramafic exposures and the control soil (Table 8). These results indicate adverse effects originating from the ultramafic soils. Also, in the specimens exposed to the control soils, a loss of mass of 13.4± 3.9% was observed. The mass loss in the control queries the validity of the exposure of *A. caliginosa* as this can be considered as an indication of suboptimal conditions for the experimental setup. Metal toxicity effects increase if the organism is exposed under suboptimal conditions (RIVM 1998). It is recommended that if more than 15% to 20% mass loss occurs in the control, the test should be considered as invalid (Spurgeon et al. 2003).

For *E. fetida*, a significantly lower final mass was observed in the specimens exposed to the Kaapsehoop 1 and 2 soils (Table 8). In the exposures to the Barberton Nature Reserve soils, a retarded growth was observed although the worms had a similar final mass compared to the controls after eight weeks of exposure (Figure 24). From that finding, it can be concluded that *E. fetida* needs a certain time span to accommodate to the ultramafic soil conditions, as *E. fetida* is not a soil dwelling species but prefers organic rich compost or manure (Van Gestel and Van Straalen 1994). It also took a time span of at least four weeks before *E. fetida* showed a response

in mass to the ultramafic conditions. Thus one can conclude that the parameter mass change is mainly affected over the long-term.

10.5.3 Reproduction

Over the whole observation period, *A. caliginosa* reproduced poorly. This can be related to the slower reproduction rate of *A. caliginosa* when compared with *E. fetida* (Holmstrup et al. 1991, Nair and Bennour 1998, Lowe and Butt 2005), and most probably in combination with the suboptimal experimental conditions.

A retarding effect was observed in the cocoon production of *E. fetida*, in the ultramafic soil samples collected at the Barberton Nature Reserve and Kaapsehoop 1, with a significantly lower production at the beginning of the exposure (Chapter 5.3.3). A significant difference in cocoon production was found only between the Kaapsehoop 1 sample with the highest DTPA extractable manganese and nickel and the control. Furthermore, it was observed that the fecundity, expressed as hatchlings per cocoon of *E. fetida*, was highest in the group of worms exposed to the soils containing the highest amounts of DTPA- $CaCl_2$ and acid-extractable metals and negatively correlated to growth and mortality (Chapter 5). One explanation for that finding is, that *E. fetida* responded to such adverse conditions by increasing the production of offspring, probably at the costs of growth and mortality. An “at-cost” strategy was observed previously in connection with an increased tolerance towards adverse environmental conditions (Reinecke et al. 1999). It is incontestable that for the maintenance and increase of a population the hatching success is more important than the number of cocoons or individual growth.

10.5.4 Subcellular biomarkers

As subcellular biomarkers, the MTT colouring assay and the Neutral Red Retention assay were performed on coelomic fluid of *E. fetida*.

As the responses of the MTT and the NRR assay are directly related to the cell concentration, and it was assumed that the number of cells is directly related to the protein content of the coelomic liquid, the results were presented proportional to the protein content measured by the Bradford assay. The results of the MTT and the NRR assays (Chapter 8.3), performed on coelomocytes of *E. fetida* exposed for six weeks to the different ultramafic soils and the control soil clearly demonstrated a reduction in the mitochondrial proliferation measured by the MTT assay and an increased lysosomal membrane damage (NRR) in the ultramafic exposures compared to the controls. Both assays have not before been performed in the way they were conducted in this study, thus, no comparable results were available. Nevertheless the results of the MTT and the NRR assay are supported by a lower fecundity of *E. fetida* after six weeks of exposure (Appendix 3-3) and by elevated concentrations of chromium and nickel measured in the specimens after the same time of exposure. Furthermore, preliminary tests, exposing *E. fetida* to different concentrations of cadmium, have shown that the MTT and the NRR assay are suited for the

assessment of metal toxicity in *E. fetida* on sub-cellular level (Chapter 8.4.1). The MTT assay showed a reduction in mitochondrial metabolism related to increasing concentrations of metals such as chromium and nickel in the ultramafic exposures and cadmium in the preliminary tests. The NRR assay showed a decrease in the retention of neutral red. That revealed an increased lysosomal membrane damage at increasing concentrations of these metals. Both assays were rapidly and objectively conducted *ex vivo* on 96 well microplates. The MTT and the NRR assay are clinically established techniques for the screening of toxicological endpoints in cell cultures (Tonn et al. 1994, Seth et al. 2004).

10.5.5 Genotoxicity

For the assessment of genotoxicity, two assays were conducted; the Single Cell Gel Electrophoresis (SCGE) or Comet assay, and the micronucleus assay (MN) (Chapter 9).

The comet assay is a genotoxicity assay for the detection of DNA single strand breaks (Singh et al. 1988) in single cells. In contrast to that, the micronucleus evaluates the splitting off of chromosomes or parts thereof during the cell division, consequently gathering permanent DNA damage (Walker et al. 1996).

Especially in the first six weeks of the exposure, the comet assay showed a clear correlation between DNA damage of *E. fetida* in the ultramafic exposures and the DTPA extractable amounts of manganese and nickel, but no correlation between DNA damage and total metal content (Chapter 9.4.1). Also, mass change, mortality and fecundity seemed to be related to the DNA damage. After nine weeks of exposure, the damage observed on DNA level seemed to be related more to the total concentrations of metals, indicating that earthworms are probably able to remobilise certain metals from the soils. This has also been suspected by Naftel et al. (2002). After four month of exposure, a decrease in DNA damage in all ultramafic exposures was observed, indicating a recovery of the earthworms, probably caused by an increased tolerance to these unfavourable conditions.

For the micronucleus assay, actively dividing cells are required (Meehan et al. 2004). No dividing cells were found in the coelomocytes, neither in the control nor the ultramafic exposures thus, the micronucleus assay did not return any results.

10.5.6 Comparison of *Aporrectodea caliginosa* and *Eisenia fetida*

One critical characteristic of a good biological indicator endpoint is the low natural variability (Venables et al. 1992). *A. caliginosa* was showing extremely high variations of metal body burden, in the case of chromium even when exposed to the control soils. For that reason, in this study *A. caliginosa* could not be considered as a good biological indicator for metal body burden in spite of the fact that this species is supposed to be better suited for the experimental, field related conditions than *E. fetida*. In terms of fertility and fecundity, *A. caliginosa* showed slow reproduction (Table 9). Whether the quiescence observed at *A. caliginosa* was a response to the

disadvantageous effects of the ultramafic soils or to an unsuitable experimental setup, could also not be revealed.

10.6 Field sampling in the Barberton area

The field trip to the Barberton area was undertaken at the middle of April following three months of intensive precipitation in this area (according to the Pretoria Central Forecasting Office). No earthworms were found during this specific sampling period, neither by handsorting nor by the application of the two chemical extraction methods (mustard solution extraction method and the formalin method (chapter 2)). An indication of the former presence of earthworms was however found, at the sampling location situated in the Barberton Nature Reserve, about a dozen of cocoons of an indeterminable earthworm species were collected. The cocoons showed distinct signs of dehydration and, in the light of a microscopic examination, did not contain any living embryos. It is known that earthworms do occur in the ultramafic soils of the Barberton Greenstone Belt, as two specimens of an unidentifiable species collected by J. Mesjasz-Przybylowicz in this area were analyzed within the present study (see Chapter 4). Also, it is known from ultramafic soils in other areas that earthworms do occur in these soils (Marino et al. 1995, Hubers et al. 2003).

The success of the chemical extraction methods is strongly dependent on different factors varies with species, such as soil temperature and moisture (Satchell 1969, Bouché and Gardner 1984). Handsorting is considered as more reliable, providing a mean recovery rate of about 80% of earthworms present and about 90% of the biomass present (Schmidt 2001). About five blocks per sampling location and three sampling locations were investigated in the Barberton area. No worms were found and therefore one can deduct that at the specific sampling period no worms were present in these soils. The probable reasons being the very dry period of many months preceding the rainy season when the sampling took place. The winter in 2003 was exceptionally dry, even by the standards of a dry season in this region (Figure 5). No rain was recorded for the months July until September in 2003 and the first precipitation was recorded only at the 18. of October, 2003 (South African Weather Service 2005). Before that, in May 0.4 mm and in June (2003) 0.2 mm of precipitation were recorded at the Nelspruit Weather Station which is in the vicinity of Barberton. Although one can assume that native earthworms are able to withstand a certain drought period due to the prevalent climatic conditions in this area, it could be possible that these five months without any notable precipitation might have led to a breakdown of the earthworm population. Some earthworm species are able to face longer periods of drought by entering quiescence, and cocoons can resist desiccation over a certain time span (Edwards and Bohlen 1996). In that context, one might assume that the drought period was too long to endure in quiescence or that cocoons might have survived. Whether and how the earthworm populations can recover after such a climatic cataclysm remains an open question in need of further investigation. Furthermore, because of the absence of native earthworms, no evaluation of the mechanisms of survival under the ultramafic condition was performable.

11. Conclusions

Ultramafic soils, and, for comparison purpose, a control soil from Stellenbosch, were fractionated in a mobile ($CaCl_2$-extractable), mobilisable (DTPA-extractable) and total metal content phase (acid-extractable). The fractionation revealed that in proportion, a far lower amount of metals such as chromium, iron, manganese and nickel is environmentally available in ultramafic soils when compared with non-ultramafic soils and anthropogenic contaminated soils. Other metals such as cobalt, although present in abundance in these ultramafic soils, were found to be environmentally available only in traces. This explains the presence of earthworms in general in these soils rather than an increased tolerance or even a genetically based adaptation.

Two ecophysiologically different species of earthworms, *Aporrectodea caliginosa* and *Eisenia fetida* were exposed to the ultramafic soils collected at the Barberton Greenstone Belt and a control soil from Stellenbosch. Both species differed substantially in their metal elimination behaviour. *A. caliginosa* transported metals such as chromium, manganese and nickel to the posterior section to discard this section. *E. fetida* sequestrated metals such as chromium and nickel in compartments in the coelomic fluid distributed over the whole length of the body.

Comparisons of mass changes revealed, that environmental stress originating from ultramafic soils is only partially responsible for mass loss of *A. caliginosa*. Also, unfavourable experimental conditions and the possibility of quiescence played a role. In terms of reproduction and fecundity, *A. caliginosa,* even when exposed to control soils, showed an extremely slow reproduction. From these findings, it was concluded that *E. fetida*, although not a real soil dwelling species, was better suited for the assessment of ultramafic soils.

Responses to the ultramafic conditions were found on all levels of organisation tested, with the lowest sensitivity at the mortality endpoint. In *E. fetida* exposed to the ultramafic soils containing the lowest concentrations of metals, extractable as well as total concentrations (Barberton), no mortality was observed. Also, after an exposure of eight weeks, no significant differences were found in the number of cocoons produced per worm. Although resulting in comparable end mass and total cocoon production over the whole exposure period, during the period of exposure a retarded growth and cocoon production was observed in the ultramafic exposures. Therefore, it was concluded that *E. fetida* needed a certain time span to accommodate to these soils.

In the soil sample with the highest DTPA-extractable concentrations of nickel and manganese, the number of hatchlings produced per *E. fetida* over the exposure period showed an increase. From that finding, it was concluded that *E. fetida* may be able to respond to these disadvantageous conditions by increasing the production of offspring to offset mortality. Also, it was indicated that fecundity as a single endpoint contributing to the population growth rate was not as severely affected as biomass change. With regard to population development, reproduction seems to be a more important endpoint than individual mass change. It can thus be speculated that reproduction is maintained at the cost of individual mass.

On sub-organismal level, the MTT and the NRR assay revealed significant damages of the cellular metabolism and the lysosomal membranes of *E. fetida* exposed for six weeks to ultramafic soils. Also, the responses of both sub-cellular biomarkers were in agreement with the responses of the fecundity, biomass change and Comet assay endpoints, indicating that MTT and NRR can be valuable tools for the assessment of cytotoxicology *ex vivo*.

After four month of exposure, no significantly genotoxic effects were found by means of the Comet assay. In contrast to the micronucleus assay, the responses of this assay are not necessarily translated into permanent genetic damages. Thus, from the decrease in DNA damage, it can be concluded also that *E. fetida* accommodated to the ultramafic conditions.

E. fetida pre-exposed to nickel showed a higher nickel body concentration when exposed to the ultramafic soils, than specimens without a history of previous exposure. At the endpoint mortality of *E. fetida* exposed in an artificial medium to different concentrations of nickel, it was observed that a long-term pre-exposure to nickel induces a higher sensitivity. From these findings it was concluded, that the long-term (over several generation) exposed worms did not develop an increased tolerance to nickel. In contrast, nickel in the exposure soils may be accumulated in excess of the concentrations already present in the body, and increase the toxic effects of nickel loaded soils. Also, no cross-resistance or cross-tolerance of manganese- or cadmium pre-exposed worms towards nickel was detected.

In general, body burdens as well as responses of the different endpoints of *E. fetida* showed dynamic fluctuations during the course of the exposures. No equilibrium of any of the metals tested was found in the body burden. Therefore, it can be concluded that the earthworms are able to regulate uptake and/or regulation of metals actively.

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13. Appendix

13.1 Soil analysis

Appendix 1-1: Metal contents [mg/kg] after $CaCl_2$ extraction of soil samples from different ultramafic sites as measured by atomic absorption spectrometry; n.d.= not detected; arsenic, cadmium, copper and lead not mentioned as not detected.

Soil sample	Aluminium				Chromium			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Agnes Mine	n.d.	n.d.	n.d.	n.d.	0.07	0.12	n.d.	0.20
Barberton	0.37	0.40	n.d.	0.80	0.07	0.12	n.d.	0.20
Songimvelo	n.d.	n.d.	n.d.	n.d.	0.13	0.06	0.10	0.20
Kaapseh. 1	n.d.	n.d.	n.d.	n.d.	0.07	0.12	n.d.	0.20
Kaapseh. 2	n.d.	n.d.	n.d.	n.d.	0.10	0.17	n.d.	0.30
Kaapseh. 3	n.d.	n.d.	n.d.	n.d.	0.03	0.06	n.d.	0.10
All groups	0.05	0.18	n.d.	0.80	0.07	0.10	n.d.	0.30

	Cobalt				Iron			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	0.20	0.17	0.10	0.40	0.53	0.15	0.40	0.70
Agnes Mine	0.50	0.10	0.40	0.60	2.63	0.87	1.90	3.60
Barberton	0.27	0.31	n.d.	0.60	0.70	0.17	0.60	0.90
Songimvelo	0.37	0.06	0.30	0.40	0.80	0.52	0.50	1.40
Kaapseh. 1	0.17	0.21	n.d.	0.40	7.60	9.36	1.90	18.40
Kaapseh. 2	0.20	n.d.	0.20	0.20	0.23	0.06	0.20	0.30
Kaapseh. 3	0.27	0.06	0.20	0.30	1.03	1.35	0.10	2.58
All groups	0.28	0.17	n.d.	0.60	1.93	3.90	0.10	18.40

	Manganese				Nickel			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	2.73	1.85	0.60	3.90	0.27	0.46	n.d.	0.80
Agnes Mine	14.73	1.78	12.80	16.30	3.13	0.21	2.90	3.30
Barberton	1.73	0.32	1.50	2.10	1.37	0.06	1.30	1.40
Songimvelo	11.40	1.25	10.10	12.60	5.23	0.23	5.10	5.50
Kaapsehoop 1	0.60	0.10	0.50	0.70	5.57	0.06	5.50	5.60
Kaapsehoop 2	0.73	0.67	0.30	1.50	1.10	0.10	1.00	1.20
Kaapsehoop 3	0.73	0.42	0.40	1.20	12.20	0.79	11.30	12.80
All groups	4.67	5.64	0.30	16.30	4.12	3.91	n.d.	12.80

Appendix 1-2: Metal contents of DTPA - extract of soil samples [mg/kg] from different ultramafic sites as measured by atomic absorption spectrometry; no arsenic, cadmium and lead were detected.

Soil sample	Aluminium				Cobalt			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	20.17	0.64	19.80	20.90	0.22	0.19	0.11	0.44
Agnes Mine	4.29	5.53	1.10	10.67	1.58	0.06	1.54	1.65
Barberton	0.44	0.40	0.11	0.88	2.32	3.26	0	9.47
Kaapseh. 1	n.d.	-	-	-	6.86	2.89	3.52	9.66
Kaapseh. 2	n.d.	-	-	-	3.69	2.89	0.66	8.10
Kaapseh. 3	0.30	0.24	0.11	0.66	6.22	0.39	5.83	6.60
Songimvelo	8.80	3.25	6.16	12.43	1.50	0.06	1.43	1.54

	Chromium				Copper			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	n.d.	-	-	-	5.15	8.82	0	15.33
Agnes Mine	0.07	0.13	0	0.22				
Barberton	0.18	0.32	0	0.55	4.56	5.26	0	9.11
Kaapseh. 1	0.15	0.13	0	0.22	4.33	5.01	0	9.11
Kaapseh. 2	0.11	0.19	0	0.33	2.28	3.91	0	8.10
Kaapseh. 3	0.36	0.64	0	1.32				
Songimvelo	0.51	0.06	0.44	0.55				

	Iron				Manganese			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	190.40	55.50	126.50	242.70	10.06	3.43	6.16	15.53
Agnes Mine	57.90	13.53	49.17	73.48	37.69	2.54	34.76	39.27
Barberton	261.73	162.72	61.60	433.72	73.52	43.25	31.35	135.90
Kaapseh. 1	94.45	44.16	41.25	155.94	82.87	53.18	32.12	164.33
Kaapseh. 2	101.27	103.23	25.52	317.20	35.29	13.10	20.24	53.43
Kaapseh. 3	53.21	7.09	44.22	60.94	74.22	15.10	53.68	86.57
Songimvelo	23.54	5.36	18.48	29.15	30.51	1.78	29.37	32.56

	Nickel			
	Means	Std.Dev.	Minimum	Maximum
Control	2.48	1.45	1.10	4.18
Agnes Mine	14.48	1.10	13.42	15.62
Barberton	74.68	32.68	40.26	116.80
Kaapseh. 1	336.16	163.57	218.90	654.50
Kaapseh. 2	90.68	37.82	49.83	141.30
Kaapseh. 3	30.72	1.35	29.37	32.56
Songimvelo	14.52	0.96	13.86	15.62

Appendix 1-3: Total metal contents of soil samples from different ultramafic sites as measured by atomic absorption spectrometry; n.d. = not detected, n.m. = not measured; arsenic and lead not scheduled as not detected

Soil sample	Cobalt				Chromium			
	Means	Std.Dev	Minimu	Maximu	Means	Std.Dev.	Minimum	Maximum
Control	0.67	0.15	0.56	0.77	n.d.	n.d.	n.d.	n.d.
Agnes	83.05	20.07	54.60	101.60	742.25	306.46	446.00	1057.20
Barberton	95.00	4.24	92.00	98.00	996.00	28.28	976.00	1016.00
Kaapseh. 1	345.00	4.24	342.00	348.00	1635.00	24.04	1618.00	1652.00
Kaapseh. 2	498.00	39.60	470.00	526.00	2894.00	0	2894.00	2894.00
Kaapseh. 3	196.65	41.32	159.40	234.20	1549.25	87.21	1442.00	1641.40
Songimvel	84.20	8.20	78.40	90.00	1251.80	32.81	1228.60	1275.00

	Copper				Iron			
	Means	Std.Dev	Minimu	Maximu	Means	Std.Dev.	Minimum	Maximum
Control	8.15	10.55	2.00	20.33	6671.90	209.36	6434.70	6830.90
Agnes	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Barberton	16.06	5.89	10.00	21.11	40640.36	4221.37	36913.72	46674.20
Kaapseh. 1	12.53	6.44	6.00	18.21	83719.83	728.90	82674.60	84243.94
Kaapseh. 2	10.18	4.01	7.60	16.10	113045.6	12284.3	101482.2	128317.2
Kaapseh. 3	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Songimvel	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.

	Manganese				Nickel			
	Means	Std.Dev	Minimu	Maximu	Means	Std.Dev.	Minimum	Maximum
Control	49.94	7.16	41.70	54.60	8.45	2.33	7.10	11.14
Agnes	1297.5	105.20	1196.00	1398.00	572.80	182.51	382.20	814.00
Barberton	458.00	62.23	414.00	502.00	4408.00	195.16	4270.00	4546.00
Kaapseh. 1	1814.0	16.97	1802.00	1826.00	13281.00	151.32	13174.00	13388.00
Kaapseh. 2	2048.3	189.82	1808.00	2249.07	13926.00	33.94	13902.00	13950.00
Kaapseh. 3	1993.5	13.00	1974.00	2000.00	6647.15	713.26	5806.40	7436.20
Songimvel	1915.0	120.21	1830.00	2000.00	1139.20	3.96	1136.40	1142.00

	Zinc			
	Means	Std.Dev	Minimu	Maximu
Control	48.40	0.00	48.40	48.40
Agnes	n.m.	n.m.	n.m.	n.m.
Barberton	36.30	2.69	34.40	38.20
Kaapseh. 1	72.75	2.19	71.20	74.30
Kaapseh. 2	107.25	20.01	93.10	121.40
Kaapseh. 3	n.m.	n.m.	n.m.	n.m.
Songimvel	n.m.	n.m.	n.m.	n.m.

13.2 Metal analysis of earthworms

Appendix 2-1: Average, minimum, maximum and median of the cadmium concentrations [mg/kg] in *Aporrectodea caliginosa* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Cadmium		Means	Std.Dev.	Minimum	Maximum	Median
Control	4 weeks	2.06	0.41	1.36	2.62	2.16
	8 weeks	n.d.	-	-	-	-
Agnes Mine	4 weeks	0.90	0.81	0	2.18	0.78
	8 weeks	0.03	0.07	0	0.21	0
Kaapseh. 3	4 weeks	0.065	0.18	0	0.52	0
	8 weeks	0.05	0.14	0	0.40	0
Songimvelo	4 weeks	2.44	1.42	0.74	4.77	2.12
	8 weeks	0.02	0.07	0	0.18	0
All Groups		0.70	1.11	0	4.77	0

Appendix 2-2: Average, minimum, maximum and median of the chromium concentrations [mg/kg] in *Aporrectodea caliginosa* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Chromium		Means	Std.Dev.	Minimum	Maximum	Median
Control	4 weeks	4.25	0.93	2.64	5.70	4.15
	8 weeks	1.41	1.53	0	3.40	1.00
	24 weeks	1.21	2.97	0	7.27	0
Agnes Mine	4 weeks	74.95	102.38	6.19	316.60	52.05
	8 weeks	98.39	117.07	0	279.23	47.95
	24 weeks	98.52	106.88	2.72	264.35	77.83
Kaapseh. 3	4 weeks	35.52	37.33	14.73	123.32	19.26
	8 weeks	101.78	91.92	15.75	224.00	77.77
	24 weeks	n.d.	-	-	-	-
Songimvelo	4 weeks	54.02	69.09	2.09	183.56	14.71
	8 weeks	69.07	71.40	0	171.91	57.79
All Groups		50.17	78.05	0	316.61	10.72

Appendix 2-3: Average, minimum, maximum and median of the chromium concentrations [mg/kg] in *Eisenia fetida* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Chromium		Means	Std.Dev.	Minimum	Maximum	Median
Control	2 weeks	5.52	5.08	0.00	12.00	5.69
	4 weeks	3.46	2.99	0.52	6.49	3.38
	6 weeks	2.24	2.27	0.00	4.55	2.22
	8 weeks	4.20	1.96	2.34	7.19	3.42
	14 weeks	n.m.	n.m.	n.m.	n.m.	n.m.
Barberton	2 weeks	13.11	4.47	9.90	21.75	11.22
	4 weeks	8.20	3.38	4.38	10.81	9.42
	6 weeks	13.21	3.69	8.58	17.05	13.61
	8 weeks	12.57	7.49	4.53	22.07	12.37
	14 weeks	n.m.	n.m.	n.m.	n.m.	n.m.
Kaapsehoop 1	2 weeks	11.75	2.04	8.84	14.57	11.92
	4 weeks	8.99	0.91	8.23	10.00	8.75
	6 weeks	10.16	1.74	8.06	12.08	10.24
	8 weeks	13.67	10.00	5.92	32.33	10.85
	14 weeks	n.m.	n.m.	n.m.	n.m.	n.m.
Kaapsehoop 2	2 weeks	7.75	4.55	0.00	11.76	9.61
	4 weeks	13.56	4.89	8.70	18.48	13.50
	6 weeks	12.26	1.51	10.19	13.68	12.58
	8 weeks	25.10	21.45	5.70	52.79	19.65
	14 weeks	n.m.	n.m.	n.m.	n.m.	n.m.
All Groups		10.74	8.93	0.00	52.79	9.64

Appendix 2-4: Average, minimum, maximum and median of the cobalt concentrations [mg/kg] in *Aporrectodea caliginosa* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Cobalt		Means	Std.Dev.	Minimum	Maximum	Median
Control	4 weeks	3.98	1.33	2.31	6.03	3.91
	8 weeks	0.09	0.13	0.00	0.26	0
Agnes Mine	4 weeks	9.75	3.98	5.00	14.06	10.12
	8 weeks	0.86	0.95	0	2.36	0.59
Kaapseh. 3	4 weeks	10.25	3.61	6.35	17.56	9.33
	8 weeks	0.69	0.38	0.00	1.20	0.67
Songimvelo	4 weeks	9.88	4.87	3.94	17.78	8.62
	8 weeks	0.34	0.39	0.00	0.92	0.19
All Groups		4.48	5.08	0.00	17.78	2.33

Appendix 2-5: Average, minimum, maximum and median of the manganese concentrations [mg/kg] in *Aporrectodea caliginosa* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Manganese		Means	Std.Dev.	Minimum	Maximum	Median
Control	4 weeks	10.26	6.99	1.78	20.57	7.77
	8 weeks	3.87	3.11	0	8.77	3.32
	24 weeks	14.10	5.55	6.55	21.19	13.04
Agnes Mine	4 weeks	39.88	27.98	2.45	84.67	46.65
	8 weeks	117.83	145.16	0	354.27	48.65
	24 weeks	154.52	164.58	19.14	453.57	109.19
Kaapseh. 3	4 weeks	81.11	126.59	1.50	384.60	51.38
	8 weeks	195.43	151.01	0	413.79	203.29
	24 weeks	15.99	7.91	6.56	25.95	14.21
Songimvelo	4 weeks	41.20	42.53	5.32	98.70	18.44
	8 weeks	97.29	107.98	0	329.45	78.03
All Groups		70.76	109.03	0	453.57	19.67

Appendix 2-6: Fisher's LSD post-hoc test; body load of manganese in *Aporrectodea caliginosa* exposed to ultramafic soils; differences marked in red are significant at $p<0.05$

Exposure/ time	week	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}
Control	4		0.25	0.50	0.09	0.00	0.00	0.10	0.00	0.43	0.16	0.02
	8	0.25		0.09	0.01	0.00	0.00	0.01	0.00	0.07	0.01	0.00
	24	0.50	0.09		0.36	0.02	0.02	0.38	0.00	0.91	0.53	0.13
Agnes Mine	4	0.09	0.01	0.36		0.09	0.10	0.96	0.02	0.42	0.75	0.49
	8	0.00	0.00	0.02	0.09		0.97	0.08	0.61	0.02	0.05	0.31
	24	0.00	0.00	0.02	0.10	0.97		0.09	0.58	0.02	0.05	0.33
Kaapseh. 3	4	0.10	0.01	0.38	0.96	0.08	0.09		0.02	0.45	0.79	0.46
	8	0.00	0.00	0.00	0.02	0.61	0.58	0.02		0.00	0.01	0.11
	24	0.43	0.07	0.91	0.42	0.02	0.02	0.45	0.00		0.61	0.16
Songimvelo	4	0.16	0.01	0.53	0.75	0.05	0.05	0.79	0.01	0.61		0.32
	8	0.02	0.00	0.13	0.49	0.31	0.33	0.46	0.11	0.16	0.32	

Appendix 2-7: Average, minimum, maximum and median of the manganese concentrations [mg/kg] in *Eisenia fetida* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Manganese		Means	Std.Dev.	Minimum	Maximum	Median
Control	2 weeks	3.34	2.67	0	7.02	3.76
	4 weeks	7.93	1.69	6.76	9.87	7.14
	6 weeks	7.60	1.23	6.07	9.09	7.63
	8 weeks	6.47	3.52	1.84	10.57	6.60
	14 weeks	1.73	1.20	0	2.60	2.16
Barberton	2 weeks	4.31	3.74	1.55	11.70	2.97
	4 weeks	2.18	1.92	0	3.62	2.92
	6 weeks	8.51	3.57	4.53	13.11	8.20
	8 weeks	5.43	2.54	2.42	8.24	5.53
	14 weeks	8.31	4.23	4.67	14.22	7.18
Kaapsehoop 1	2 weeks	11.42	4.98	3.36	17.68	11.31
	4 weeks	10.49	0.43	10.00	10.77	10.70
	6 weeks	6.19	5.23	0	10.96	6.89
	8 weeks	11.38	8.04	5.38	26.33	8.15
	14 weeks	27.98	6.09	23.06	36.70	26.08
Kaapsehoop 2	2 weeks	1.84	2.86	0	7.06	0.34
	4 weeks	6.41	3.43	2.45	8.58	8.18
	6 weeks	4.82	2.33	2.74	8.15	4.19
	8 weeks	10.63	7.05	3.73	20.86	9.91
	14 weeks	21.26	0.73	20.41	21.98	21.32
All Groups		8.21	7.11	0	36.70	6.58

Appendix 2-8: Average, minimum, maximum and median of the nickel concentrations [mg/kg] in *Aporrectodea caliginosa* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Nickel		Means	Std.Dev.	Minimum	Maximum	Median
Control	4 weeks	5.6	3.93	2.04	14.55	4.32
	8 weeks	0.49	0.42	0	1.11	0.32
	24 weeks	n.d.	-	-	-	-
Agnes Mine	4 weeks	40.74	43.27	4.13	133.52	33.34
	8 weeks	30.53	25.79	10.32	88.28	22.53
	24 weeks	26.72	35.06	0	85.70	12.52
Kaapseh. 3	4 weeks	55.07	63.38	8.00	191.61	27.03
	8 weeks	85.02	84.63	6.86	219.00	56.53
	24 weeks	6.58	16.12	0	39.48	0
Songimvelo	4 weeks	35.71	38.27	4.09	94.53	13.22
	8 weeks	26.61	33.01	0	93.33	14.10
All groups		30.48	46.48	0	219.00	10.72

Appendix 2-9: Fisher's LSD post-hoc test; tissue concentration of nickel in *Aporrectodea caliginosa* exposed to ultramafic soils; for completion, for control after 24 weeks p-values from Mann-Whitney-U test are added, differences marked in red are significant at $p<0.05$

Exposure/ time	week	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}
Control	4		0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.06	0.01	0.00
	8	0.00		0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	24	0.01	0.02		0.01	0.01	0.20	0.01	0.01	0.67	0.01	0.09
Agnes Mine	4	0.01	0.00	0.01		0.84	0.27	0.50	0.20	0.59	0.88	0.48
	8	0.00	0.00	0.01	0.84		0.34	0.64	0.29	0.65	0.72	0.60
	24	0.00	0.00	0.20	0.27	0.34		0.54	0.87	0.88	0.22	0.63
Kaapseh. 3	4	0.00	0.00	0.01	0.50	0.64	0.54		0.54	0.82	0.41	0.91
	8	0.00	0.00	0.01	0.20	0.29	0.87	0.54		0.95	0.16	0.67
	24	0.06	0.00	0.67	0.59	0.65	0.88	0.82	0.95		0.54	0.87
Songimvelo	4	0.01	0.00	0.01	0.88	0.72	0.22	0.41	0.16	0.54		0.41
	8	0.00	0.00	0.09	0.48	0.60	0.63	0.91	0.67	0.87	0.41	

Appendix 2-10: Average, minimum, maximum and median of the nickel concentrations [mg/kg] in *Eisenia fetida* long-term exposed to ultramafic soils collected at the Barberton area and at Stellenbosch (Control).

Nickel		Means	Std.Dev.	Minimum	Maximum	Median
Control	2 weeks	3.93	4.03	0	10.00	3.15
	4 weeks	n.d.	-	-	-	-
	6 weeks	3.07	1.55	0.77	4.09	3.72
	8 weeks	0.60	0.93	0	1.82	0
	14 weeks	1.69	1.17	0	2.60	2.07
Barberton	2 weeks	8.95	2.48	5.42	11.70	9.39
	4 weeks	1.03	1.78	0	3.09	0
	6 weeks	30.08	19.52	8.89	47.21	32.11
	8 weeks	7.35	5.97	0.68	13.66	7.65
	14 weeks	8.87	0.64	8.23	9.48	8.87
Kaapsehoop 1	2 weeks	12.16	2.36	9.35	15.90	12.18
	4 weeks	3.06	2.01	0.82	4.71	3.64
	6 weeks	24.55	14.48	7.44	40.95	24.90
	8 weeks	6.48	4.06	1.60	11.99	6.19
	14 weeks	54.22	12.98	44.66	73.39	49.42
Kaapsehoop 2	2 weeks	4.16	2.20	1.58	7.84	3.81
	4 weeks	6.91	2.11	5.12	9.24	6.38
	6 weeks	11.84	3.15	8.65	15.29	11.71
	8 weeks	17.96	16.62	1.40	41.10	16.08
	14 weeks	45.95	9.22	34.84	57.07	45.94
All Groups		12.21	15.59	0	73.39	7.37

Appendix 2-11: Means, standard deviation, minima, maxima and median of the concentrations of chromium (Cr), manganese (Mn) and nickel (Ni) in *Aporrectodea caliginosa* and *Eisenia fetida* after four and 24 weeks of exposure to soil from Stellenbosch (Control) and Kaapsehoop 3 in mg/kg.

				Means	Std.Dev.	Minimum	Maximum	Median
Control	Ni	4 weeks	*A. caliginosa*	5.61	3.93	2.04	14.55	4.32
			E. fetida	2.00	0.67	1.07	2.67	2.13
		24 weeks	*A. caliginosa*	n.d.	-	-	-	-
			E. fetida	n.d.	-	-	-	-
	Cr	4 weeks	*A. caliginosa*	4.25	0.93	2.64	5.70	4.15
			E. fetida	0.27	0.53	0	1.07	0
		24 weeks	*A. caliginosa*	1.21	2.97	0	7.27	0
			E. fetida	0.00	n.d.	-	-	-
	Mn	4 weeks	*A. caliginosa*	10.26	6.99	1.78	20.57	7.77
			E. fetida	1.47	2.93	0	5.87	0
		24 weeks	*A. caliginosa*	14.10	5.55	6.55	21.19	13.04
			E. fetida	11.11	1.98	9.74	13.37	10.21
Kaapseh. 3	Ni	4 weeks	*A. caliginosa*	55.07	63.38	8.00	191.61	27.03
			E. fetida	91.47	38.31	18.60	152.73	93.83
		24 weeks	*A. caliginosa*	6.58	16.12	0	39.48	0
			E. fetida	99.70	53.85	56.95	182.65	72.12
	Cr	4 weeks	*A. caliginosa*	35.52	37.33	14.73	123.32	19.26
			E. fetida	10.50	6.95	1.81	24.36	9.68
		24 weeks	*A. caliginosa*	0.00	n.d.	-	-	-
			E. fetida	49.80	39.57	23.73	119.82	37.04
	Mn	4 weeks	*A. caliginosa*	81.11	126.59	1.50	384.60	51.38
			E. fetida	9.92	2.43	5.58	13.82	10.37
		24 weeks	*A. caliginosa*	15.99	7.91	6.56	25.95	14.21
			E. fetida	46.42	15.54	28.54	67.95	48.16
All Groups				26.83	47.45	0	384.60	9.87

Appendix 2-12: Mean, median, maximum, minimum in [mg/kg] and standard deviation of different metals in different body sections of *Aporrectodea caliginosa* exposed to soils from different ultramafic sites and a control site from Stellenbosch (Control)

Exposure	Body section	Metal	Means	Std.Dev.	Minimum	Maximum	Median
Control	anterior	Cr	0.16	0.36	0	0.80	0
		Mn	6.15	0.82	4.80	7.00	6.22
		Ni	0.34	0.46	0	0.89	0
	mid	Cr	3.25	3.74	0	7.27	1.68
		Mn	4.09	1.96	1.71	6.23	3.37
		Ni	0.34	0.75	0	1.68	0
	posterior	Cr	0.51	0.76	0	1.68	0
		Mn	7.87	2.60	4.21	11.08	8.62
		Ni	0.34	0.75	0	1.68	0
Kaapseh. 3	anterior	Cr	27.19	8.75	15.69	38.00	24.44
		Mn	25.33	21.70	10.00	64.86	14.51
		Ni	8.65	16.06	0	36.78	0
	mid	Cr	32.20	5.71	19.94	37.38	34.74
		Mn	45.40	21.99	13.46	80.00	39.43
		Ni	15.00	27.78	0	60.80	0
	posterior	Cr	36.84	8.62	26.67	56.00	35.56
		Mn	49.10	23.39	0	72.73	54.00
		Ni	25.86	28.34	0	62.50	19.74
Agnes Mine	anterior	Cr	26.79	7.12	13.33	35.56	27.25
		Mn	38.76	14.71	19.14	71.11	37.23
		Ni	10.91	15.36	0	35.56	0
	mid	Cr	40.03	7.09	32.00	49.66	39.56
		Mn	47.84	19.30	19.51	74.67	42.86
		Ni	23.54	32.64	0	66.21	0
	posterior	Cr	37.68	9.21	23.30	48.00	40.87
		Mn	62.79	36.53	24.85	137.14	52.83
		Ni	44.55	47.94	0	100.00	40.00
All Groups			25.94	25.78	0	137.14	25.04

Appendix 2-13: Mean, median, maximum, minimum in [mg/kg] and standard deviation of different metals in different body sections of *Eisenia fetida* exposed to soils from different ultramafic sites and a control site from Stellenbosch (Control)

Exposure	Body part	Metal	Means	Std.Dev.	Minimum	Maximum	Median
Control	anterior	Ni	1.18	0.86	0	1.89	1.41
		Mn	4.65	2.67	1.09	7.55	4.99
		Cr	4.16	1.11	3.17	5.66	3.91
	mid	Ni	1.79	0.29	1.51	2.11	1.78
		Mn	2.94	1.19	1.96	4.52	2.64
		Cr	2.08	1.46	0	3.18	2.56
	posterior	Ni	1.76	1.72	0	3.85	1.60
		Mn	2.73	3.46	0	7.69	1.60
		Cr	3.50	1.44	2.32	5.38	3.14
Barberton	anterior	Ni	7.01	2.26	4.49	9.76	6.90
		Mn	8.08	1.25	6.74	9.76	7.91
		Cr	8.56	1.45	6.74	9.76	8.88
	mid	Ni	6.15	3.31	3.23	9.52	5.92
		Mn	7.80	1.44	6.45	9.52	7.62
		Cr	7.80	1.44	6.45	9.52	7.62
	posterior	Ni	7.68	4.60	3.85	13.33	6.77
		Mn	5.33	1.70	3.81	7.69	4.90
		Cr	6.62	2.29	3.85	8.89	6.88
Kaapsehoop1	anterior	Ni	10.34	2.35	8.33	13.33	9.86
		Mn	10.02	2.59	6.45	12.50	10.56
		Cr	7.01	2.41	4.17	10.00	6.93
	mid	Ni	9.70	1.48	8.16	11.11	9.75
		Mn	7.96	1.49	5.80	9.01	8.53
		Cr	7.96	1.49	5.80	9.01	8.53
	posterior	Ni	10.96	1.86	9.43	13.33	10.53
		Mn	10.60	2.55	7.55	13.33	10.76
		Cr	8.81	1.43	6.67	9.62	9.48
Kaapsehoop2	anterior	Ni	13.75	1.09	12.20	14.66	14.07
		Mn	12.75	1.29	11.88	14.66	12.22
		Cr	9.66	0.61	8.80	10.20	9.83
	mid	Ni	13.93	4.51	7.41	17.78	15.26
		Mn	10.17	2.39	7.66	13.04	10.00
		Cr	10.59	1.16	8.89	11.48	10.99
	posterior	Ni	13.06	4.87	7.69	19.51	12.51
		Mn	12.32	2.08	9.62	14.63	12.51
		Cr	9.65	4.09	3.85	12.82	10.98
All Groups			7.75	4.09	0.00	19.51	8.16

Appendix 2-14: Mean, median, maximum, minimum in [mg/kg] and standard deviation of different metals in *Eisenia fetida* with a different history of previous exposure to ultramafic soils collected at Kaapsehoop 3.Cd-pre: *E. fetida* long-term exposed (>10 generations) to $CdSO_4 * 7H_2O$; Ni-pre: *E. fetida* long-term exposed (>10 generations) to $NiCl_2 * 6H_2O$

exposure	metal	weeks	body conc. [mg/kg]				
			Means	Std.Dev.	Minimum	Maximum	Median
Control	Ni	4 weeks	2.00	0.67	1.07	2.67	2.13
		24 weeks	n.d.	-	-	-	-
	Cd	4 weeks	n.d.	-	-	-	-
		24 weeks	n.d.	-	-	-	-
	Cr	4 weeks	0.27	0.53	0	1.07	0
		24 weeks	n.d.	-	-	-	-
	Mn	4 weeks	1.47	2.93	0	5.87	0
		24 weeks	11.11	1.98	9.74	13.38	10.21
Kaapseh. 3	Ni	4 weeks	91.47	38.31	18.60	152.73	93.83
		24 weeks	99.70	53.85	56.95	182.65	72.12
	Cd	4 weeks	n.d.	-	-	-	-
		24 weeks	n.d.	-	-	-	-
	Cr	4 weeks	10.50	6.95	1.81	24.36	9.68
		24 weeks	49.80	39.57	23.73	119.82	37.04
	Mn	4 weeks	9.92	2.43	5.58	13.82	10.37
		24 weeks	46.42	15.54	28.54	67.95	48.16
Cd-pre exposed	Ni	4 weeks	77.22	29.20	18.11	108.98	88.63
		24 weeks	98.87	18.58	80.71	125.75	94.15
	Cd	4 weeks	90.36	27.81	47.70	139.27	84.36
		24 weeks	77.79	57.50	28.85	166.32	49.93
	Cr	4 weeks	8.95	4.15	0.73	15.09	10.38
		24 weeks	41.24	11.10	26.85	57.69	40.16
	Mn	4 weeks	15.67	8.51	4.36	30.18	13.64
		24 weeks	49.30	15.53	34.25	73.01	50.53
Ni-pre exposed	Ni	4 weeks	105.45	51.79	25.26	199.25	111.18
		24 weeks	148.37	34.52	115.50	190.93	142.78
	Cd	4 weeks	n.d.	-	-	-	-
		24 weeks	n.d.	-	-	-	-
	Cr	4 weeks	11.50	5.84	3.65	23.30	10.48
		24 weeks	33.98	22.70	5.46	62.93	29.35
	Mn	4 weeks	13.10	8.57	6.04	35.92	9.96
		24 weeks	56.64	29.59	31.69	104.53	49.48
All Groups			37.80	46.46	0	199.25	13.15

Appendix 2-15: Mean, median, maximum, minimum in [mg/kg] and standard deviation of different metals in different body sections of *Eisenia fetida* with histories of previous exposure exposed to ultramafic soils collected at Kaapsehoop 3 after an exposure period of four weeks; Cd-pre: *E. fetida* long-term exposed (>10 generations) to $CdSO_4 * 7H_2O$; Ni-pre: *E. fetida* long-term exposed (>10 generations) to $NiCl_2 * 6H_2O$

Exposure	Body	Metal	Means	Std.Dev.	Minimum	Maximum	Median
Control	anterior	Ni	n.d.	-	-	-	-
		Mn	0.64	1.10	0	1.91	0
		Cr	n.d.	-	-	-	-
		Cd	n.d.	-	-	-	-
	mid	Ni	1.09	1.89	0	3.27	0
		Mn	2.09	1.10	1.09	3.27	1.90
		Cr	n.d.	-	-	-	-
		Cd	n.d.	-	-	-	-
	posterior	Ni	2.50	2.46	0	4.92	2.58
		Mn	0.66	1.14	0	1.97	0
		Cr	1.64	2.84	0	4.92	0
		Cd	n.d.	-	-	-	-
Cd-pre	anterior	Ni	94.31	11.19	84.85	106.67	91.43
		Mn	62.72	11.09	50.79	72.73	64.65
		Cr	6.54	2.74	4.85	9.70	5.08
		Cd	0.05	0.09	0	0.16	0
	mid	Ni	103.79	11.64	93.39	116.36	101.62
		Mn	69.25	7.21	64.86	77.58	65.31
		Cr	6.65	2.83	4.32	9.80	5.82
		Cd	n.d.	-	-	-	-
	posterior	Ni	86.74	14.55	75.68	103.23	81.31
		Mn	64.91	12.48	52.46	77.42	64.86
		Cr	5.19	5.16	0	10.32	5.25
		Cd	n.d.	-	-	-	-
Ni-pre	anterior	Ni	65.15	57.32	0	107.83	87.62
		Mn	56.40	16.35	38.10	69.57	61.54
		Cr	4.47	4.15	0	8.21	5.22
		Cd	97.30	6.28	92.31	104.35	95.24
	mid	Ni	79.52	26.35	53.33	106.02	79.19
		Mn	62.01	21.51	38.55	80.81	66.67
		Cr	1.72	1.63	0	3.23	1.93
		Cd	82.76	16.35	67.47	100.00	80.81
	posterior	Ni	84.11	4.96	78.55	88.07	85.71
		Mn	53.08	21.97	29.36	72.73	57.14
		Cr	2.93	2.94	0	5.87	2.91
		Cd	96.76	9.58	85.71	102.75	101.82
NiMp7	anterior	Ni	103.18	7.48	95.69	110.64	103.23
		Mn	35.64	10.72	25.81	47.06	34.04
		Cr	4.19	3.16	1.70	7.74	3.14
		Cd	0.52	0.91	0	1.57	0
	mid	Ni	93.82	9.95	82.96	102.50	96.00
		Mn	54.45	17.60	39.51	73.85	50.00
		Cr	3.62	3.17	0	5.93	4.92

Exposure	Body	Metal	Means	Std.Dev.	Minimum	Maximum	Median
		Cd	n.d.	-	-	-	-
	posterior	Ni	106.57	10.82	94.12	113.60	112.00
		Mn	53.46	6.47	47.06	60.00	53.33
		Cr	3.57	3.16	0	6.00	4.71
		Cd	n.d.	-	-	-	-
All			34.46	40.78	0	116.36	5.23

Appendix 2-16: Mass, body concentration [mg/kg] and accumulation factor of nickel in *Eisenia fetida* exposed for 48 h to different concentrations of nickel chloride in artificial ground water

	mass [g]					Ni conc.			Acc.F.		
conc.	Means	Std.Dev.	Minimum	Maximum	Median	Means	Std.Dev.	Median	Means	Std.Dev.	Median
0	0.33	0.06	0.25	0.39	0.35	0	0	0	0	0	0
8.5	0.32	0.08	0.25	0.46	0.29	3.91	0.42	3.87	0.46	0.05	0.46
16.98	0.35	0.11	0.29	0.57	0.32	3.56	0.82	3.70	0.21	0.05	0.22
34.07	0.48	0.17	0.34	0.76	0.40	3.83	0.84	3.64	0.11	0.02	0.11
68.14	0.26	0.06	0.19	0.36	0.26	7.11	1.08	7.40	0.10	0.02	0.11
136.29	0.25	0.05	0.20	0.35	0.24	12.02	1.03	12.07	0.09	0.01	0.09
All Grps	0.33	0.12	0.19	0.76	0.30	5.63	3.84	4.24	0.16	0.15	0.10

Appendix 2-17: Mass, body concentration [mg/kg] and accumulation factor of manganese in *Eisenia fetida* exposed for 48 h to different concentrations of manganese sulphide in artificial ground water

	mass [g]					Mn conc.			Acc.F.		
Conc.	Means	Std.Dev.	Minimum	Maximum	Median	Means	Std.Dev.	Median	Means	Std.Dev.	Median
0	0.33	0.06	0.25	0.39	0.345	0	0	0	0	0	0
8.51	0.35	0.11	0.28	0.57	0.309	3.08	0.43	3.05	0.36	0.05	0.36
17.03	0.29	0.11	0.18	0.49	0.259	3.92	1.19	4.06	0.23	0.07	0.24
34.06	0.37	0.06	0.29	0.43	0.373	7.71	1.82	8.38	0.23	0.05	0.25
68.11	0.29	0.09	0.20	0.41	0.259	5.88	2.88	6.64	0.09	0.04	0.10
136.22	0.37	0.10	0.21	0.53	0.360	12.06	5.20	11.35	0.09	0.04	0.08
All Grps	0.34	0.09	0.18	0.57	0.330	6.08	4.50	5.06	0.16	0.13	0.13

Appendix 2-18: Mean, median, maximum, minimum in [mg/kg] and standard deviation of manganese and nickel concentrations in different body sections of *Eisenia fetida* exposed for 48 hours in artificial ground water (n=4).

metal	body part	body load				
		Means	Std.Dev.	Minimum	Maximum	Median
Ni	anterior	4.61	0.56	4.00	5.33	4.55
	mid	5.51	0.90	4.36	6.40	5.65
	posterior	5.21	2.28	1.89	7.11	5.91
Mn	anterior	5.87	2.91	2.00	8.49	6.50
	mid	5.77	2.20	4.00	8.97	5.05
	posterior	6.39	1.38	5.33	8.42	5.91
All Groups		5.56	1.77	1.89	8.97	5.33

Appendix 2-19: Mean, median, maximum, minimum in [mg/kg] and standard deviation of the accumulation factor of manganese and nickel in different body sections of *Eisenia fetida* exposed for 48 hours in artificial ground water (n=4).

metal	body part	Accumulation factor				
		Means	Std.Dev.	Minimum	Maximum	Median
Ni	anterior	0.14	0.02	0.12	0.16	0.13
	mid	0.16	0.03	0.13	0.19	0.17
	posterior	0.15	0.07	0.06	0.21	0.17
Mn	anterior	0.17	0.09	0.06	0.25	0.19
	mid	0.17	0.06	0.12	0.26	0.15
	posterior	0.19	0.04	0.16	0.25	0.17
All Groups		0.16	0.05	0.06	0.26	0.16

13.3 Growth and reproduction

Appendix 3-1: mass [mg] of *Eisenia fetida* long-term exposed to ultramafic soils from the Barberton area and to unpolluted Stellenbosch soils (Control) (n=12).

Exposure	Week 0					Week 2				
	Means	Median	SD	Min.	Max.	Means	Median	SD	Min.	Max.
Control	133.79	131.67	22.05	109	163	396.79	356.75	107.95	321	553
Barberton	155.69	172.50	35.66	102	175	317.60	323.71	26.84	280	343
Kaapseh. 1	133.64	137.67	26.65	98	162	271.79	274.38	23.99	240	298
Kaapseh. 2	126.67	126.83	7.04	120	133	277.98	278.75	21.51	257	298
All Grps	137.45	132.71	25.11	98	175	316.04	298.12	73.01	240	553

Exposure	Week 4					Week 6				
	Means	Median	SD	Min.	Max.	Means	Median	SD	Min.	Max.
Control	501.53	517.28	74.73	402	570	370.39	399.17	54.08	308	404
Barberton	423.64	420.78	61.92	369	484	427.56	378.83	87.73	375	529
Kaapseh. 1	295.37	300.28	22.19	264	317	254.13	247.60	26.61	231	283
Kaapseh. 2	359.50	347.22	54.12	315	429	319.86	304.33	28.94	302	353
All Grps	395.01	373.89	93.79	264	570	342.98	330.63	81.65	231	529

Exposure	Week 8				
	Means	Median	SD	Min.	Max.
Control	426.66	401.16	76.03	367	537
Barberton	430.27	443.54	64.99	342	492
Kaapseh. 1	263.68	212.00	100.09	200	379
Kaapseh. 2	276.70	301.67	78.26	189	339
All Grps	360.63	373.19	106.87	189	537

Appendix 3-2: mass [mg] of *Eisenia fetida* with a history of pre-exposure long-term exposed to an ultramafic soil from the Barberton area (Kaapsehoop 3) and to unpolluted Stellenbosch soils (Control) (n=12); Kh3: *E. fetida* without a history of pre-exposure exposed to Kaapsehoop 3 soils; Kh3 Cd_{pre}: *E. fetida* long-term (more than 10 generations) exposed to cadmium; Kh3 Ni_{pre}: *E. fetida* long-term (more than 10 generations) exposed to nickel.

Exposure	Week 0				Week 4			
	Means	Std.Dev.	Minimum	Maximum	Means	Std.Dev.	Minimum	Maximum
Control	302	7.64	295	310	513	42.93	482	562
Kh3	411	44.29	360	442	411	35.91	384	452
Kh3 Cd_{pre}	394	70.87	315	452	465	83.92	386	553
Kh3 Ni_{pre}	470	59.08	402	511	480	28.62	462	513
All Grps	394	76.66	295	511	467	58.89	384	562

	Week 6				Week 8			
	Means	Means	Means	Means	Means	Std.Dev.	Minimum	Maximum
Control	Means	Std.Dev.	Minimum	Maximum	564	42.33	308	388
Kh3	530	31.88	501	564	472	8.49	309	325
Kh3 Cd_{pre}	459	69.18	398	534	485	105.28	187	387
Kh3 Ni_{pre}	483	45.18	443	532	538	5.51	219	230
All Grps	517	51.80	479	576	515	67.11	187	388

	Week 24			
	Means	Std.Dev.	Minimum	Maximum
Control	340	42.33	308	388
Kh3	315	8.49	309	325
Kh3 Cd_{pre}	267	105.28	187	387
Kh3 Ni_{pre}	225	5.51	219	230
All Grps	287	67.11	187	388

Appendix 3-3: Reproduction of *Eisenia fetida* exposed to ultramafic soils and to an unpolluted soil from Stellenbosch (Control).

Total	# of cocoons	mean coc./worm	Hatchlings	Hatchlings/Cocoon	H/C SD	Mean % Hatch
Control	226	8.06	519	2.21	0.37	91.57
Barberton	132	5.31	180	1.34	0.11	68.35
Kaapseh. 1	128	5.41	218	1.67	0.40	64.29
Kaapseh. 2	94	4.58	190	2.16	0.79	81.72

4 weeks	# of cocoons	mean coc./worm	Hatchlings	Hatchl./Cocoon
Control	17	0.35	41	2.35
Barberton	1	0.02	4	4.00
Kaapseh. 1	4	0.08	13	3.25
Kaapseh. 2	2	0.04	6	3.00

6 weeks	# of cocoons	mean coc./worm	Hatchlings	Hatchl./Cocoon
Control	72	2.00	223	3.05
Barberton	12	0.33	27	2.58
Kaapseh. 1	55	1.53	107	1.52
Kaapseh. 2	11	0.32	22	2.45

8 weeks	# of cocoons	mean coc./worm	Hatchlings	Hatchl./Cocoon
Control	137	5.71	255	1.84
Barberton	119	4.96	149	1.26
Kaapseh. 1	69	3.63	98	1.49
Kaapseh. 2	81	4.26	162	2.15

Appendix 3-4: Reproduction of *E. fetida* exposed to unpolluted soil (Control) and ultramafic soil samples collected at Kaapsehoop 3 with different histories of exposure; Kh3: *E. fetida* without a history of pre-exposure exposed to Kaapsehoop 3 soils; Kh3 Cd_{pre}: *E. fetida* long-term (more than 10 generations) exposed to cadmium; Kh3 Ni_{pre}: *E. fetida* long-term (more than 10 generations) exposed to nickel.

Total	# of cocoons	mean coc./worm	Hatchlings	Hatchlings/Cocoon	H/C SD	Mean % Hatch
Control	181	6.03	475	2.62	0.29	85.91
Kaapseh. 3	15	0.50	33	2.20	0.71	88.46
Kh3 Cd_{pre}	10	0.33	17	1.70	0.35	83.33
Kh3 Ni_{pre}	12	0.40	21	1.75	0.58	90.91

4 weeks	# of cocoons	mean coc./worm	Hatchlings	Hatchl./Cocoon	Mean % Hatched
Control	11	0.37	18	1.64	81.82
Kaapseh. 3	2	0.07	5	2.5	100
Kh3 Cd_{pre}	1	0.03	3	3	100
Kh3 Ni_{pre}	1	0.03	2	2	100

6 weeks	# of cocoons	mean coc./worm	Hatchlings	Hatchl./Cocoon	Mean % Hatched
Control	170	5.67	457	2.69	90
Kaapsehoop3	13	0.43	28	2.15	76.92
Kh3 Cd_{pre}	9	0.30	14	1.56	66.67
Kh3 Ni_{pre}	11	0.37	19	1.73	81.82

13.4 Acute toxicity

Appendix 4-1: Raw data of *Eisenia fetida* exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Proportion Responding Adjusted for Controls	Predicted Proportion Responding
13.63	18	0	0	0	0.0002
16.98	18	0	0	0	0.0006
27.26	18	0	0	0	0.0053
34.07	18	0	0	0	0.0126
54.52	18	0	0	0	0.0582
68.14	18	5	0.28	0.28	0.1052
136.29	18	6	0.33	0.33	0.3953
272.58	18	12	0.67	0.67	0.7646
545.16	18	18	1	1	0.9562
1090.31	18	18	1	1	0.9965
2180.62	18	18	1	1	0.9999

Appendix 4-2: *Eisenia fetida* exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); general statistics

LC/EC50	185	Positive-control:	none
LC/EC50 (Probit):	164.24	Correlation coefficient	0.85
LC/EC99 (Probit):	841.64	axis intercept.	-2.26243
LC/EC90 (Probit):	404.05	slope	3.278058

Estimated LC/EC Values and confidence

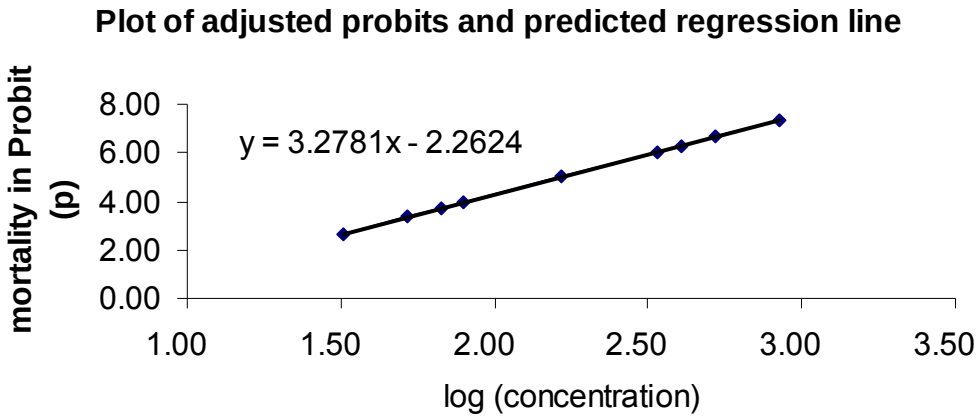
	Exposure Conc.	95% Confidence	
		Lower	Upper
Control	0	0	0
LC/EC1	32.05	17.80	46.24
LC/EC5	51.72	33.36	69.08
LC/EC10	66.76	46.27	86.23
LC/EC15	79.31	57.42	100.66
LC/EC50	164.24	131.39	210.58
LC/EC85	340.12	257.92	513.53
LC/EC90	404.05	298.88	641.86
LC/EC95	521.50	370.32	896.92
LC/EC99	841.64	549.11	1693.59

Chi-square for heterogeneity (calculated) = 9.286
Chi-square (tabular value at 0.05 level) = 16.92

Table of inhibition values:

Conc.	Mortality	Probits
0	0	n.d.
13.63	0	1.46
16.98	0	1.77
27.26	0	2.44
34.07	0	2.76
54.52	0	3.43
68.14	27.78	3.75
136.29	33.33	4.73
272.58	76.46	5.72
545.16	95.62	6.71
1090.3	99.65	7.69
2180.6	99.99	8.68

Appendix 4-3: *Eisenia fetida* exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); probits plotted against logarithms of concentration.



Appendix 4-4: Raw data of *Eisenia fetida* exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Proportion Responding Adjusted for Controls	Predicted Proportion Responding
13.63	18	0	0	0.00	0.002
16.98	21	0	0	0.00	0.0045
27.26	22	0	0	0.00	0.0203
34.07	21	3	0.14	0.14	0.0374
54.52	22	2	0.09	0.09	0.1111
68.14	21	3	0.14	0.14	0.17
136.29	23	7	0.30	0.30	0.4498
272.58	26	22	0.85	0.85	0.7585
545.16	26	24	0.92	0.92	0.9369
1090.31	21	21	1.00	1.00	0.9908
2180.62	18	18	1.00	1.00	0.9993

Appendix 4-5: *Eisenia fetida* exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); general statistics

LC/EC50 (graphically):	185
LC/EC50 (Probit):	151.49
LC/EC99 (Probit):	1062.39
LC/EC90 (Probit):	442.99

Correlation coefficient: 0.85
axis intercept.: -0.9961
Slope: 2.750041

Chi-square for heterogeneity (calculated) = 10.63

Chi-square (tabular value at 0.05 level) = 16.92

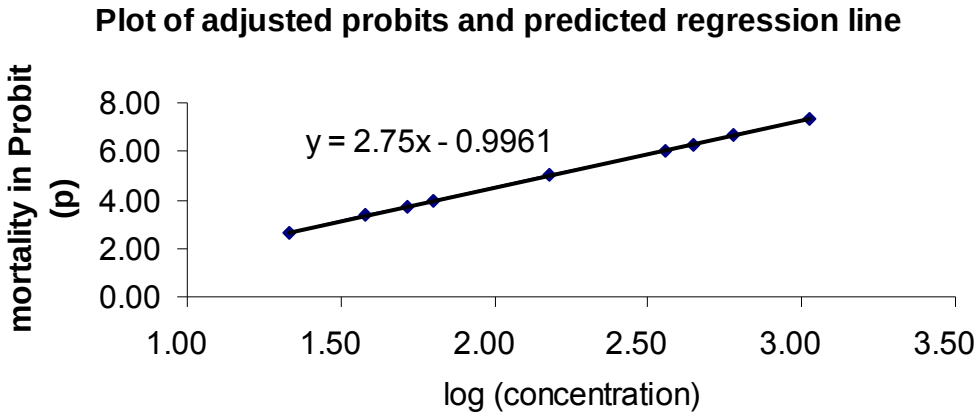
Estimated LC/EC Values and confidence limits

	Exposure Conc.	95% Confidence Limits	
		Lower	Upper
Control	0	0	0
LC/EC1	21.6	12.54	31.25
LC/EC5	38.22	25.45	50.99
LC/EC10	51.80	36.85	66.64
LC/EC15	63.61	47.10	80.20
LC/EC50	151.49	122.95	189.72
LC/EC85	360.77	276.38	521.08
LC/EC90	442.99	330.75	669.82
LC/EC95	600.49	429.87	975.58
LC/EC99	1062.39	697.35	1990.37

Table of inhibition values:

Conc. [mg/L]	Mortality %	Probits
0	0	0.00
13.63	0	2.12
16.98	0	2.39
27.26	0	2.95
34.07	14.29	3.22
54.52	9.09	3.78
68.14	14.29	4.05
136.29	30.43	4.87
272.58	75.85	5.70
545.16	93.69	6.53
1090.3	99.08	7.36
2180.6	99.93	8.19

Appendix 4-6: *Eisenia fetida* exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); probits plotted against logarithms of concentration.



Appendix 4-7: Raw data of *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Predicted Proportion Responding
13.63	18	0	0	0.0008
16.98	18	0	0	0.002
27.26	18	0	0	0.0115
34.07	18	0	0	0.0234
54.52	18	1	0.06	0.0827
68.14	18	6	0.33	0.1353
136.29	18	6	0.33	0.415
272.58	18	11	0.61	0.7492
545.16	18	18	1.00	0.9405
1090.31	18	18	1.00	0.9928
2180.62	18	18	1.00	0.9996

Appendix 4-8: *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); general statistics

LC/EC50 (graphically):	182
LC/EC50 (Probit):	161.20
LC/EC99 (Probit):	993.00
LC/EC90 (Probit):	438.89

Correlation coefficient: 0.85
axis intercept.: -1.50337
Slope: 2.946221
Chi-square for heterogeneity (calculated) = 10.5
Chi-square (tabular value at 0.05 level) =16.92

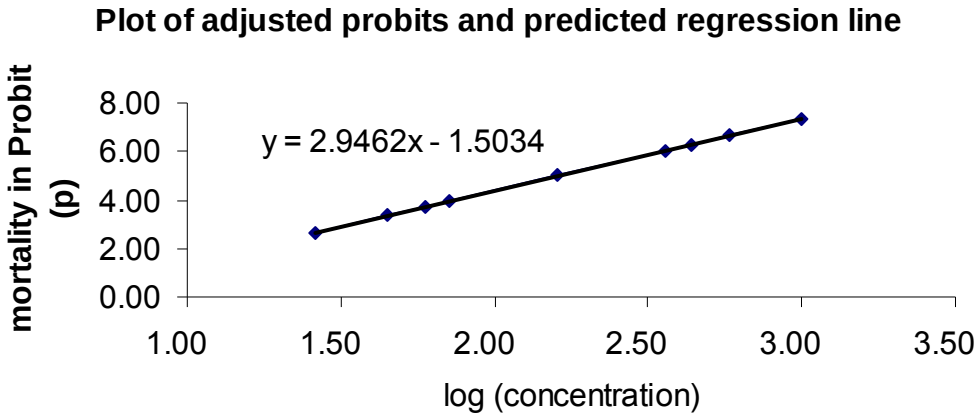
Estimated LC/EC Values and confidence limits

	Exposure Conc.	95% Confidence	
		Lower	Upper
Control	0	0	0
LC/EC1	26.17	14.33	38.48
LC/EC5	44.57	28.46	60.35
LC/EC10	59.21	40.71	77.34
LC/EC15	71.71	51.56	91.94
LC/EC50	161.20	127.86	208.84
LC/EC85	362.34	270.17	556.76
LC/EC90	438.89	318.52	710.89
LC/EC95	583.00	404.82	1025.30
LC/EC99	9921.00	629.53	2054.48

Table of inhibition values:

Conc. [mg/L]	Mortality %	Probits
0	0	0.00
13.63	0	1.84
16.98	0	2.12
27.26	0	2.73
34.07	0	3.01
54.52	5.56	3.61
68.14	33.33	3.90
136.29	33.33	4.79
272.58	74.92	5.67
545.16	94.05	6.56
1090.3	99.28	7.45
2180.6	99.96	8.33

Appendix 4-9: *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); probits plotted against logarithms of concentration.



Appendix 4-10: Raw data of *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Predicted Proportion Responding
13.63	18	0	0	0.007
16.98	21	0	0	0.004
27.26	22	0	0	0.02
34.07	21	0	0	0.0383
54.52	22	4	0.18	0.1197
68.14	22	6	0.27	0.1856
136.29	18	6	0.33	0.493
272.58	18	17	0.94	0.8049
545.16	18	16	0.89	0.9587
1090.31	18	18	1.00	0.9955
2180.62	18	18	1.00	0.9998

Appendix 4-11: *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); general statistics

LC/EC50 (graphically):	178
LC/EC50 (Probit):	138.19
LC/EC99 (Probit):	869.51
LC/EC90 (Probit):	380.67

Correlation coefficient: 0.85
axis intercept.: -1.2336
Slope: 2.91225

Chi-square for heterogeneity (calculated) = 9.68 Chi-square (tabular value at 0.05 level) = 16.92

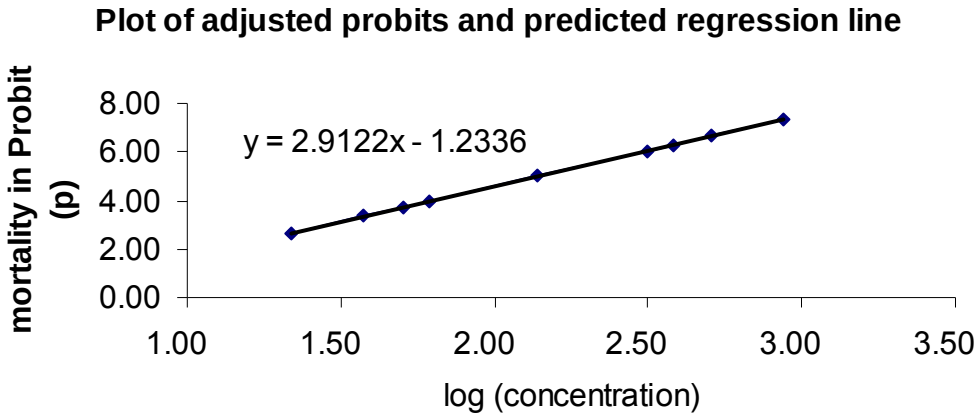
Estimated LC/EC Values and confidence limits

	Exposure Conc.	95% Confidence	
		Lower	Upper
Control	0	0	0
LC/EC1	21.96	12.72	31.42
LC/EC5	37.64	25.20	49.74
LC/EC10	50.17	35.96	64.17
LC/EC15	60.90	45.45	76.54
LC/EC50	138.19	111.12	178.12
LC/EC85	313.58	233.94	481.43
LC/EC90	380.67	276.14	615.40
LC/EC95	507.34	351.87	888.39
LC/EC99	869.51	550.69	1780.53

Table of inhibition values:

Conc. [mg/L]	Mortality %	Probits
0	0	n.d.
13.63	0	2.07
16.98	0	2.35
27.26	0	2.95
34.07	0	3.23
54.52	18.18	3.82
68.14	27.27	4.11
136.29	33.33	4.98
272.58	80.49	5.86
545.16	95.87	6.74
1090.3	99.55	7.61
2180.6	99.98	8.49

Appendix 4-12: *Eisenia fetida* previously exposed to $MnSO_4$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); probits plotted against logarithms of concentration.



Appendix 4-13: Raw data of *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Predicted Proportion Responding
13.63	18	0	0	0.22
16.98	18	0	0	0.01
27.26	18	2	0.11	0.12
34.07	18	6	0.33	0.26
54.52	18	12	0.67	0.69
68.14	18	15	0.83	0.85
136.29	18	18	1.00	0.996
272.58	18	18	1.00	1.00
545.16	18	18	1.00	1.00
1090.31	18	18	1.00	1.00
2180.62	18	18	1.00	1.00

Appendix 4-14: *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); general statistics

LC/EC50 (graphically):	45
LC/EC50 (Probit):	44.28
LC/EC99 (Probit):	115.85
LC/EC90 (Probit):	75.21

Correlation coefficient: 0.85
axis intercept.: -4.1671
Slope: 5.56878
Chi-square for heterogeneity (calculated) = 0.86
Chi-square (tabular value at 0.05 level) = 6.92

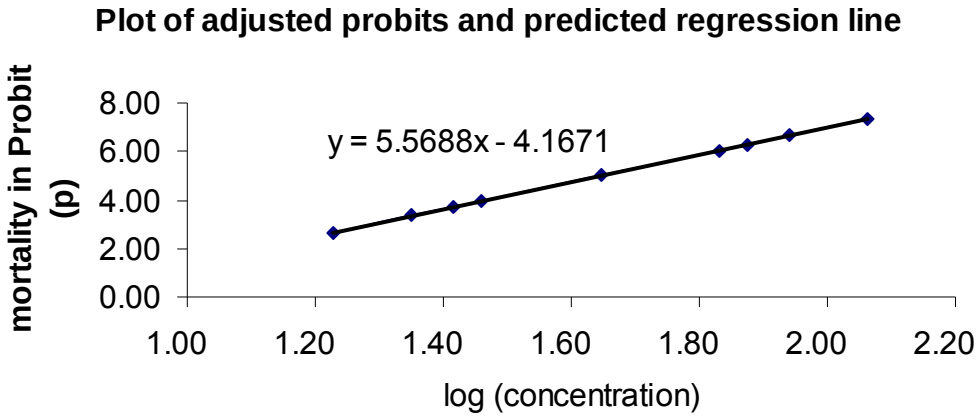
Estimated LC/EC Values and confidence limits

	Exposure Conc.	95% Confidence	
		Lower	Upper
Control	0	0	0
LC/EC1	16.92	10.72	21.74
LC/EC5	22.43	16.02	27.23
LC/EC10	26.06	19.76	30.82
LC/EC15	28.84	22.71	33.59
LC/EC50	44.28	38.63	51.23
LC/EC85	67.96	57.64	89.01
LC/EC90	75.21	62.72	102.50
LC/EC95	87.41	70.85	126.71
LC/EC99	115.85	88.58	189.64

Table of inhibition values:

Conc. [mg/L]	Mortality %	Probits
0	0	0.00
13.63	0	2.15
16.98	0	2.68
27.26	11.11	3.83
34.07	33.33	4.37
54.52	66.67	5.50
68.14	83.33	6.04
136.29	100	7.72
272.58	100	9.40
545.16	100	11.07
1090.3	100	12.75
2180.6	100	14.42

Appendix 4-15: *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 48 hours in artificial ground water to different concentrations of $NiCl_2$ (n=18); probits plotted against logarithms of concentration.



Appendix 4-16: Raw data of *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); no response was observed in control groups (n=18)

Conc.	Number exposed	Number responding	Observed proportion responding	Predicted Proportion Responding
13.63	18	0	0	0.0245
16.98	21	0	0	0.0505
27.26	22	4	0.18	0.176
34.07	22	8	0.36	0.2754
54.52	22	13	0.59	0.5429
68.14	21	15	0.71	0.6708
136.29	18	15	0.83	0.9307
272.58	18	18	1.00	0.9941
545.16	18	18	1.00	0.9998
1090.31	20	20	1.00	1.00
2180.62	18	18	1.00	1.00

Appendix 4-17: *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); general statistics

LC/EC50 (graphically):	45
LC/EC50 (Probit):	50.73
LC/EC99 (Probit):	239.64
LC/EC90 (Probit):	119.33

Correlation coefficient:: 0.85
axis intercept.: -0.88342
Slope: 3.450097

Chi-square for heterogeneity (calculated) = 5.57 Chi-square (tabular value at 0.05 level) = 16.92

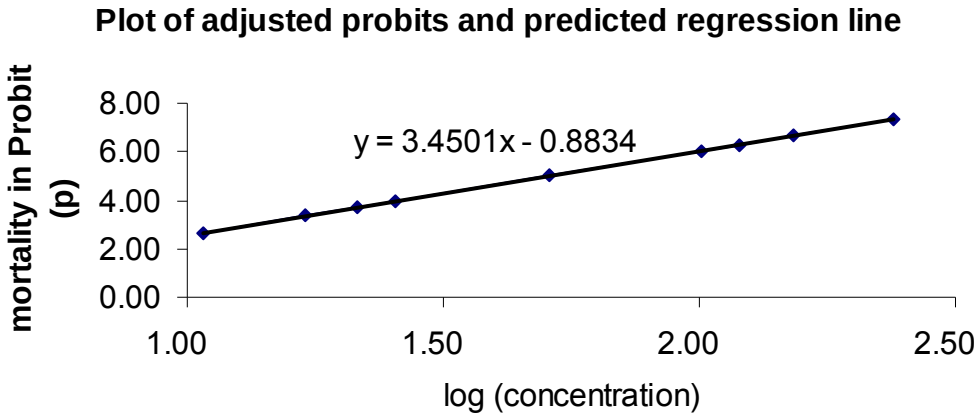
Estimated LC/EC Values and confidence limits

	Exposure Conc.	95% Confidence Limits	
		Lower	Upper
Control	0	0	0
LC/EC1	10.74	6.14	15.03
LC/EC5	16.93	11.29	21.81
LC/EC10	21.57	15.53	26.74
LC/EC15	25.40	19.19	30.81
LC/EC50	50.73	42.96	61.24
LC/EC85	101.32	80.35	145.64
LC/EC90	119.33	92.11	180.87
LC/EC95	152.08	112.40	250.10
LC/EC99	239.64	162.37	461.97

Table of inhibition values:

Conc. [mg/L]	Mortality	Probits
0	0	0.00
13.63	0	3.03
16.98	0	3.36
27.26	18.18	4.07
34.07	36.36	4.40
54.52	59.09	5.11
68.14	71.43	5.44
136.29	83.33	6.48
272.58	99.41	7.52
545.16	99.98	8.56
1090.3	100	9.60
2180.6	100	10.64

Appendix 4-18: *Eisenia fetida* previously exposed to $NiCl_2$ exposed for 96 hours in artificial ground water + gel to different concentrations of $NiCl_2$ (n>18); probits plotted against logarithms of concentration.



13.5 Micro-PIXE

Appendix 5-1: Concentrations of elements obtained in cross sections (CS) and coelomic fluid (FL) extracted from *Eisenia fetida* exposed for 20 weeks to different ultramafic soils and to a control soil from an area with a known history of no metal pollution at Stellenbosch by micro-PIXE technique (mg/kg ± 1σ uncertainty).

	Control		Barberton		Kaapsehoop 1		Kaapsehoop 2	
	CS	FL	CS	FL	CS	FL	CS	FL
P	10326±146	<6093±4685	10405±188	5944±5239	8851±130	<4904±4255	8743±138	<3329±3259
S	7797±92	<396±219	16925±178	<285±294	6994±79	<321±232	10054±119	<216±165
Cl	2038±44	245732±3076	3632±54	198231±4162	2915±48	251503±2877	2624±34	206905±2477
K	10075±44	3592±36	8249±64	7200±31	7834±47	4037±44	7913±52	3170±28
Ca	1337±11	210±12	3297±21	498±13	812±8	181±7	716±8	41±8
Ti	n.d.	5±2	n.d.	23±2	n.d.	34±2	n.d.	<2±1
Cr	n.d.	<3±2	n.d.	259±5	n.d.	54±2	n.d.	18±1
Mn	3±1	<3±1	12±2	85±6	3.9±0.9	25±3	4±1	12±1
Fe	147±5	208±5	462±10	5305±84	196±6	1098±21	373±10	626±11
Co	3±1	3±2	6±3	13±6	5±1	5±2	5±2	8±3
Ni	3.3±0.7	<3±2	344±9	221±6	0.7±0.4	52±2	125±3	39±2
Cu	9.5±0.7	7±2	36±1	<3±1	28±2	5±2	12.9±0.6	41±2
Zn	119±5	13±2	174±4	28±2	91±4	18±2	116±3	31±3
Ge	n.d.	6±2	n.d.	<4±2	n.d.	<3±1	n.d.	<3±1
As	9.9±0.6	<4±4	5.1±0.5	<4±2	31±1	<3±2	10.3±0.4	<3±2
Se	0.6±0.2	<5±2	2.2±0.3	<4±2	1.5±0.2	<4±2	0.7±0.1	<3±1
Br	7.3±0.6	74±5	9.3±0.5	86±5	8±0.6	85±5	7.5±0.5	80±3
Rb	18±1	15±5	22.9±1	10±4	15±1	10±4	19±0.9	6±3
Sr	2.3±0.4	12±5	6.1±0.6	<9±4	2.1±0.4	8±4	1.6±0.2	11±4
Mo	6±1	28±10	6±2	29±9	6±1	43±11	2.5±0.5	<16±7
Pb L	1.3±0.6	12±9	<1.4±0.8	13±6	<1±0.8	11±5	<0.7±0.4	13±5

n.d.: not detected

Appendix 5-1: Detection limits (99% confidence) of elements obtained in cross sections (CS) and coelomic fluid (FL) extracted from *Eisenia fetida* exposed for 20 weeks to different ultramafic soils and to a control soil from an area with a known history of no metal pollution at Stellenbosch by micro-PIXE technique (mg/kg).

Element	Control		Barberton		Kaapsehoop 1		Kaapsehoop 2	
	CS	FL	CS	FL	CS	FL	CS	FL
P	8.6	6093	10	4582	7.4	4904	5.3	3329
S	3.6	396	4.2	285	3.1	321	2.2	216
Cl	2.1	48	2.4	36	1.8	38	1.2	25
K	1.1	8.3	1.3	6.6	0.94	6.9	0.62	4.2
Ca	0.88	5.5	1	4.4	0.77	4.5	0.5	2.8
Ti		4.5		3.5		3.6		2.3
Cr		3.1		2.6		2.5		1.8
Mn	0.56	2.9	0.65	2.8	0.42	2.5	0.27	1.7
Fe	0.51	2.7	0.61	2.8	0.37	2.4	0.24	1.7
Co	0.5	2.5	0.61	2.9	0.37	2.3	0.25	1.6
Ni	0.65	3.4	0.79	3.5	0.48	2.9	0.3	2.3
Cu	0.63	3.5	0.79	3.3	0.46	2.9	0.3	2.4
Zn	0.53	3.6	0.72	3.4	0.45	3.1	0.29	2.6
Ge		3.9		3.7		3.3		2.7
As	0.43	4	0.65	3.8	0.43	3.4	0.29	3
Se	0.46	4.5	0.7	4	0.46	3.8	0.31	3.5
Br	0.47	5.2	0.69	4.8	0.47	4.1	0.32	3.9
Rb	0.57	7.7	0.94	8.8	0.66	6.9	0.44	6.1
Sr	0.66	9.1	0.98	9.4	0.7	8.2	0.46	6.6
Mo	1	18	1.4	17	1.1	15	0.85	16
Pb L	0.99	8.8	1.4	8.3	1	7.6	0.67	6.5