Comparing the sensitivity of five earthworm species to cadmium exposure using the comet assay

Frana Fourie
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Supervisor: Prof. S.A. Reinecke
Co-Supervisor: Prof A.J. Reinecke

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis 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.

Signature: Date: 09/03/2006

Abstract

It is known that species differ in their sensitivities to toxicants. This has been exploited to aid in environmental toxicity testing and environmental management. "Sensitivity" in this sense is usually seen as a function of lethality; assessed by determining the toxicant concentration where 50% of the test cohort die. The processes at the sub-organismal or sublethal level are however ignored. Little has been done to combine such sublethal sensitivities with the concept of species sensitivity differences. The present study therefore focused on the potential use of a cellular biomarker to compare the sensitivities of species.

The heavy metal cadmium, which bio-accumulates, is teratogenic, mutagenic and carcinogenic was chosen as toxicant. Earthworms were chosen as experimental animals and species were selected to represent various ecological types that may occur in soils. The species studied were representative of three ecological types: epigeic (*Amynthas diffringens*, *Dendrodrilus rubidus* and *Eisenia fetida*), endogeic (*Aporrectodea caliginosa*) and anecic (*Microchaetus benhami*). The alkaline single cell gel electrophoresis assay (SCGE or comet assay), which measures DNA integrity in individual cells, was used as biomarker.

Earthworms were exposed to a range of Cd concentrations (2.5, 5, 10 and 20 mg/l Cd) in the form of $CdSO_4$, in artificial soil water. A negative control (uncontaminated soil water) and a positive control, nickel (20 mg/l Ni) in the form of $NiSO_4$ were used.

Exposure to cadmium induced significantly higher levels of DNA damage in the exposed worms than in those exposed to negative controls. Species differed from each other in their sensitivity to Cd. The most sensitive species was *E. fetida* followed by *D. rubidus*, *A. caliginosa*, *A. diffringens* and *M. benhami*. Ecological type did not predict sensitivity, and it is concluded that physiology and possibly relatedness may provide a possible explanation.

All species exhibited a pattern where DNA damage was inhibited at low Cd exposure concentrations, and was increased again at high Cd concentrations. This corresponds with the hormetic dose-response, where a compensatory response is stimulated by low levels of a toxicant, but inhibited at high levels. Two possible compensatory mechanisms are proposed. Firstly, DNA repair could have been upregulated at low Cd concentrations, and inhibited by high Cd concentrations. Secondly, the production of metal-binding metallothioneins, which sequestrate Cd and renders it unavailable to cause toxic responses, could have been increased with low Cd concentrations. At high Cd concentrations, the rate of metallothionein production would not have been high enough to sequestrate Cd before it could cause damage.

The exposed earthworms accumulated Cd, but there was no definite relationship between Cd body loads and DNA damage. It is possible that a fraction of the measured Cd in the body was sequestrated, therefore not being available to cause genotoxic effects.

It is concluded that the comet assay is a useful biomarker to demonstrate DNA damage and species sensitivity differences in earthworms exposed to cadmium.

Opsomming

Dit is bekend dat spesies van mekaar verskil ten opsigte van hul sensitiwiteit vir toksiese stowwe. Hierdie eienskap word gebruik in toksisiteitstoetse en die bestuur van die vrystelling van moontlike toksiese stowwe in die omgewing. Toetse wat die sensitiwiteit van spesies vergelyk word meestal gedoen deur die konsentrasie van 'n toksiese stof te bepaal waar 50% van die toetsorganismes doodgaan. Die prosesse wat op die sub-organismiese vlak inwerk word egter geïgnoreer. Baie min studies is gedoen waar hierdie sub-letale sensitiwiteit gekombineer word met spesies-sensitiwiteitsverskille.

Kadmium is tydens die huidige studie as toksikant gebruik. Kadmium kan in organismes akkumuleer, dit is karsinogenies, veroorsaak mutasies en beïnvloed ontwikkeling en groei. Erdwurms was die gekose toetsorganismes, en spesies wat verskillende ekologiese groepe verteenwoordig is geselekteer. Hierdie spesies het drie ekologiese groepe verteenwoordig: Epigeïs (*Amynthas diffringens*, *Dendrodrilus rubidus* en *Eisenia fetida*), endogeïs, (*Aporrectodea caliginosa*) en anesies (*Microchaetus benhami*). Die komeet-toets (alkaliese-enkelsel-gel-elektroforese-toets), waarmee die DNS-integriteit in indiwiduele selle bepaal kan word, is as biomerker gekies.

Erdwurms is blootgestel aan 'n reeks kadmiumkonsentrasies (2.5, 5 10 en 20 mg/l Cd) in die vorm van $CdSO_4$ in kunsmatige grondwater. 'n Negatiewe kontrole (kunsmatige grondwater sonder kadmium) is gebruik, asook 'n positiewe kontrole, nikkel (20 mg/l Ni) in die vorm van $NiSO_4$.

Erdwurms wat aan kadmium blootgestel is, het hoër vlakke van DNS-skade getoon as die erdwurms in die negatiewe kontroles. Spesies het verskil van mekaar ten opsigte van hulle sensitiwiteit vir kadmium. Die mees sensitiewe spesie was *E. fetida*, gevolg deur *D. rubidus*, *A. caliginosa*, *A. diffringens* en *M. benhami*. Die ekologiese groepering het nie 'n invloed gehad op die spesie-sensitiwiteitsverskille nie, maar die moontlikheid bestaan dat fisiologiese verskille en selfs filogenetiese verwantskappe dalk 'n rol kan speel om hierdie verskille te verklaar.

'n Patroon waar DNS-skade onderdruk is by lae kadmium-konsentrasies, en weer hoër was by hoë konsentrasies, kon waargeneem word in al die spesies. Hierdie verskynsel kan moontlik verklaar word deur die teenwoordigheid van 'n kompenseringsreaksie, soos beskryf kan word deur die hormesiese dosis-respons. In die geval van hormesis word 'n kompenseringsreaksie gestimuleer deur lae konsentrasies van 'n toksikant. By hoë konsenstrasies word hierdie reaksie weer geïnhibeer. In die geval van die huidige studie word twee moontlike kompenseringsmeganismes voorgestel: eerstens bestaan die moontlikheid dat die herstel van

DNS-skade toegeneem het met lae kadmiumkonsentrasies. By hoë konsentrasies is hierdie herstelproses geïnhibeer. Tweedens kon lae kadmiumkonsentrasies die verhoogde produksie van metaalbindende metallothioneïene stimuleer. Met hoë konsentrasies sou die koers van metallothioneïen-produksie nie hoog genoeg gewees het om al, of meeste van, die kadmium te bind voordat dit skade kon veroorsaak nie.

Die blootgestelde erdwurms het kadmium geakkumuleer, maar geen besliste verwantskap het voorgekom tussen vlakke van kadmium in die erdwurms en DNS-skade nie. Dit is egter moontlik dat die gemete kadmium in die erdwurms gesekwestreer is en dus nie beskikbaar was om DNS-skade te veroorsaak nie.

Die gevolgtrekking kan gemaak word dat die komeettoets 'n bruikbare biomerker is om DNS-skade, asook spesies-sensitiwiteitsverskille te toon in erdwurms wat aan kadmium blootgestel is.

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1 Introduction

1.1 Sensitivity differences in organisms

The environment is under constant threat of being irreversibly damaged by excessively high levels of contaminants through human activities. It is thus necessary to have some measures of protection and legislation in place to control the input of these contaminants into the environment. To achieve this, it is essential to have a detailed knowledge of their fate and effects on the environment and species represented. Originating from this necessity, the science of ecotoxicology is concerned with the impacts of pollutants on the structure and functioning of ecological systems (Landis & Yu 1995) by integrating the conventional disciplines of toxicology and ecology (Forbes & Forbes 1994) and recently also genomics (Neumann & Galvez 2002).

In ecotoxicology, there are mainly two types of investigations concerned with the effects of contaminants on ecosystems. Retrospective investigations are conducted when a contaminant has reached the environment and has caused considerable effect. Alternatively, prospective ecotoxicology aims to predict the adverse effects of contaminants in the environment. It is desirable that the adverse effects of contaminants are known before they reach the environment, therefore it is necessary to test these contaminants in the laboratory prior to its potential release into the environment (Connell *et al.* 1999).

Mostly, such laboratory-based studies have in the past concentrated on single species toxicity tests, where the endpoints mostly consisted of whole animal responses. For example LC_{50}s were used, which are the concentrations of test substances where 50% of the test animals die during a specified time period (Suter 2002). However, testing the effect of a chemical on a few representatives of one species, in isolation, has been criticised as not reflecting the adverse effects of pollutants on populations and ecosystems in the field (Moriarty 1999). Indeed, some problems are evident from using single species toxicity tests, such as the choice of appropriate representative species (Beeby 2001) and the extrapolation from a single species to a community or ecosystem (Moriarty 1999).

It is well known that species differ inherently in their sensitivity to toxicants and these differences have been recognised as useful tools for determining environmental quality criteria and for use in ecological risk assessment. The way these sensitivity differences are addressed is through statistical (frequency) distributions called species sensitivity distributions (SSDs), using

available toxicity data such as LC_{50} values (Posthuma *et al.* 2002). Such a distribution is based on the assumption that there is a specific relationship between these sensitivity differences, and that it will fit a certain distribution curve. Information obtained from this distribution would allow for the derivation of environmental quality criteria (EQC). These are the concentrations of toxicants that can be allowed in the environment over a defined period of time without causing excessive, irreversible damage (Posthuma *et al.* 2002). Usually the value for determining EQCs is the concentration where only the most sensitive 5% of species are affected, also termed the HC5 (hazardous concentration where 5% of species are affected) value. SSDs originated in Europe and the United States in the 1970s and 1980s (Posthuma *et al.* 2002), and EQCs using SSDs for various substances, mostly for aquatic species, have been established in these countries (Suter 2002; Van Straalen & Van Leeuwen 2002). The SSD approach has also been applied to South African aquatic ecosystems (Roux *et al.* 1996; Palmer *et al.* 2004).

Seemingly a straightforward concept, it is however difficult to attain such a species sensitivity distribution, for there are many different models and ways of constructing SSDs, and many criteria and pitfalls encountered when using this approach (Forbes & Calow 2002). For example, Wheeler *et al.* (2002) have illustrated that data quality, quantity and different methods for constructing SSDs have an influence on the outcome and derivation of HC5 values and therefore regulatory guidelines. Nevertheless, SSDs still allow ecotoxicologists to obtain more accurate environmental criteria than they would have done using single species toxicity tests (Posthuma *et al.* 2002, Wheeler *et al.* 2002).

In SSDs, endpoints relating to whole organismal responses (lethality) such as LC_{50}s for acute tests, and NOECs (no observed effect concentration) for chronic tests are used (Posthuma *et al.* 2002). The processes at the sub-organismal or sublethal level are however not taken into consideration. If damage on the sub-individual level occurs, even before organisms die, it may have a substantial impact on the species or even the ecosystem. For example, a pollutant may wipe out half a population, but perhaps would not have much ecological significance, because the population may recover afterwards. Considering effects on the sublethal level, a pollutant may not kill organisms, but could cause e.g. development or reproduction to be impeded, resulting in the slow demise of the population, which could in turn cause substantial ecological impact (Moriarty 1999). It is therefore important to be able to assess these sublethal effects.

The effects of sublethal concentrations of toxicants can be assessed with the use of biomarkers, which detect effects on the sub-organismal level and could augment whole-organism tests. A biomarker can be defined as a biological response to an environmental chemical below the individual level, or at the level of biochemical or physiological processes (Van Gestel & Van

Brummelen 1996). This could be seen as an early warning system of exposure of very low concentrations of toxicants, because it focuses on the effects of sublethal concentrations of toxicants long before the effects on the whole-organism level emerge. Biomarkers are used with increasing frequency and success in environmental risk assessment (Adams *et al.* 2001; Eason & O'Halloran 2002) and constitute an array of cellular and biochemical endpoints to determine the sensitivity of species to toxicants (Schlenk 1999). Biomarkers are considered to be more sensitive than some tests at higher levels of biological organisation such as at the level of organisms or populations (McCarthy & Shugart 1990; Eason & O'Halloran 2002). However, some (Hyne & Maher 2003) suggest that biomarkers should not replace e.g. conventional biomonitoring techniques, but that biomarkers should rather be used in a supplementary way.

It would be useful if species sensitivity differences could be revealed on grounds of sublethal data. Effects of toxicants can be detected at earlier stages, resulting in more refined EQCs. A number of experiments have already been conducted to compare the sensitivity of species for sublethal effects. For example, Spurgeon *et al.* (2000) conducted a study that compared the relative sensitivities of earthworm species using biomarker responses. They found that neutral red retention times (measuring lysosomal membrane stability) differed between four ecologically different earthworm species exposed to zinc and that there are clear species sensitivity differences. In another example, three marine invertebrate species (the limpet *Patella vulgata*, the shore crab *Carcinus maenas* and the blue mussel *Mytilus edulis*) were compared for their sensitivity to copper using an array of biomarkers (Brown *et al.* 2004). One species (*P. vulgata*) was consistently the most sensitive, with a clear distinction between biomarker responses between the species. For DNA damage (as measured with the alkaline filter elution technique), differences were found for sublethal levels of benzo[*a*]pyrene between five marine invertebrates (Bihari & Fafanđel 2004). It is therefore possible to use biomarker endpoints to compare the sensitivity of species.

Studies on the comparison of species differences of sublehtal effects are becoming more frequent (e.g. Capowiez *et al.* 2005; Langdon *et al.* 2005; Reinecke *et al.* 2001; Suavé *et al.* 2002). Usually these studies use different species assemblages and different endpoints to test for different sets of chemicals, with little or no similarity between studies (Edwards & Coulson 1992). It is clear that the need exist for a research programme to be established to investigate the effects of various toxicants to a fixed set of species under similar test conditions. Therefore the aim of the present study was to use sublethal toxicity data obtained from a biomarker test to compare the sensitivities of species. The biomarker, toxicant and species will be introduced in the next sections.

1.2 DNA damage and the comet assay

Deoxyribonucleic acid (DNA) is an integral part of all living cells, and can normally be found in a supercoiled state (coiled up very tightly) in cell nuclei and other cellular organelles such as mitochondria. DNA is an unstable molecule, with natural disintegration (without any exogenous damage) occurring on a large scale on a daily basis. These lesions are however quickly repaired (Lindahl 1993; Shugart 2000). This is important to note, because there are only a few agents (one of them ionizing radiation) that directly cause breakages in the DNA molecule. For the most part, DNA damage may occur as a result of the interruption of normal cell processes such as repair (Eastman & Barry 1992). Damage to DNA may be caused by e.g. interference with DNA repair, formation of free radicals and subsequent breakage of phospodiester linkages, and chemical modification of existing bases. These may lead to e.g. mutations, strand breaks and altered bases (Shugart 2000). Eventually, carcinogenesis, teratogenesis and health disorders such as the genotoxic disease syndrome (Kurelec 1993) may occur. It is therefore clear that when DNA is affected, it can result in severe consequences for the individual, species and even the stability of ecosystems (Klobucar *et al.* 2003). The assessment of DNA damage is thus considered important for toxicity testing.

Assessing DNA damage may be done in two ways, where different aspects of DNA damage are assessed. Firstly, structural damage such as DNA strand breaks that may be induced by a genotoxicant can be viewed as a biomarker of exposure. Secondly, the biological events following DNA strand breakage (as mentioned above) may be viewed as biomarkers of effects (Shugart 2000). DNA strand breakage may be assessed by using, for example, agarose gel electrophoresis (Theodorakis *et al.* 1994) and the comet assay (Östling & Johanson 1984). The use of the comet assay in particular has increased during the last few years (Rojas *et al.* 1999). This can be ascribed to various advantages such as sensitivity for detecting low levels of DNA damage, the small numbers of cells required per sample, and ease of application (Tice *et al.* 2000).

For the comet assay, potentially damaged cells are embedded in agarose on microscope slides and subjected to an electrical field. The damaged nuclear DNA will migrate towards the anode. After electrophoresis, the bulk of the (undamaged) nuclear DNA will remain in the nucleus, and a tail of damaged strands and fragments will be present (Östling and Johanson 1984). When the slide is stained with a fluorescent dye such as ethidium bromide, and visulized with a fluorecent microscope, these cells are seen to resemble comets (Fig 1.1), which are characteristic of the

assay, hence the name comet assay. These comets reflect the amount of DNA damage in the cells, where longer and brighter tails and less distinct heads indicate higher levels of damage (Östling & Johanson 1984; Fairbairn *et al.* 1995).

Comet formation was traditionally believed to be dependent on two principles. First, at low damage levels, the free broken ends of large pieces of DNA will migrate to the tail end. Secondly, as damage increases, fragmentation of DNA will increase, and these free DNA fragments will migrate to the tail of the comet (Fairbairn *et al.* 1995). Recently, however, it has become known that single strand breaks and double strand breaks cause the supercoiled structure of DNA to relax. Loops may subsequently form, and these migrate into the tail, and not DNA fragments which are too large (Collins 2004). Initially, tail length will increase with damage, but will eventually reach a maximum (Fairbairn *et al.* 1995), but the amount of DNA in the tail may still increase (Olive & Durand 2005).

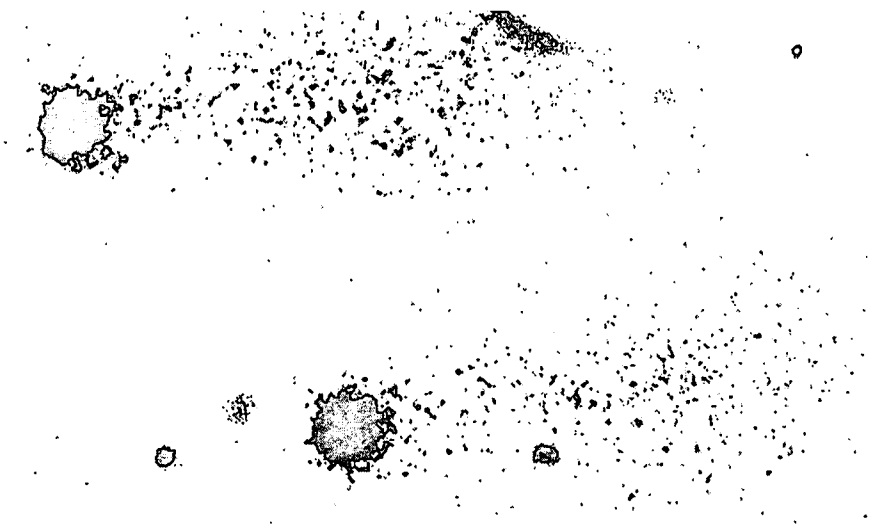

Figure 1.1: Coelomocytes from *Eisenia fetida* subjected to the comet assay, showing damage (here the image was converted to a black and white negative for illustrative purposes, but usually the images have a black background with the ethidium bromide-stained DNA fluorescing red).

The comet assay, also known as the single cell gel electrophoresis assay (SCGE) or microgel electrophoresis (MGE) had its origins in 1978, when Rydberg & Johanson described a technique for quantification of DNA damage using a mammalian single cell suspension (Fairbairn *et al.* 1995). The cells were embedded in agarose on slides, lysed to allow the DNA to unwind partially, neutralized and subsequently stained with acridine orange. They assessed DNA damage

by measuring the ratio of green to red fluorescence, where green indicated double-stranded DNA and red single-stranded DNA (Fairbairn *et al.* 1995).

Östling and Johanson (1984) developed the comet assay from this method. The cells were lysed under neutral conditions in the presence of high salt levels and detergents, which prevented separation of DNA strands, allowing for the detection of double-stranded breaks. The next step was electrophoresis under neutral conditions. The relaxed and broken DNA strands migrated further than the bulk of the DNA from the nucleus of damaged cells. Subsequently the slides were stained with ethidium bromide and visualised under fluorescent conditions. The resulting images had a characteristic appearance leading to the name "comet" assay (Östling & Johanson 1984; Fairbairn *et al.* 1995).

Since then, the comet assay has evolved into a rapid, sensitive and repeatable means to detect DNA damage in individual cells (Fairbairn *et al.* 1995; Rojas *et al.* 1999; De Boeck *et al.* 2000). Utilised mainly in the medical field, the method was developed for mammalian cells (Östling & Johanson 1984). However, because this assay operates on the DNA level, it can be applied to virtually any eukariotic cell (Rojas *et al.* 1999), provided that single cells in suspension are obtained from fresh, living tissue (Reinecke & Reinecke 2004b). Thus far, the comet assay has been successfully used to detect genotoxicant-induced damage in unicellular animals and plants (e.g. Watanabe & Suzuki 2002), vertebrates such as fish (e.g. Bombail *et al.* 2001) and mammals (e.g. Betti & Nigro 1996), vascular plants (e.g. Koppen & Verschaeve 1996) and invertebrates (e.g. Pruski & Dixon 2002; Lee & Steinert 2003; Reinecke & Reinecke 2004b).

Various reviews and efforts to produce consistent guidelines exist for the comet assay (Fairbairn *et al.* 1995; Rojas *et al.* 1999; Cotelle & Ferard 1999; Tice *et al.* 2000; Collins 2004), as well as suggestions for experimental setup, measurement and quantification of comets and appropriate statistical analyses (Duez *et al.* 2003; Wiklund & Agurell 2003; Duez *et al.* 2004). There are various methods for conducting the comet assay (Fairbairn *et al.* 1995), but the most widely used is the alkaline method, as developed by Singh *et al.* (1988), which allows for fast and effective detection of single strand breaks and alkali labile sites. Different versions of the alkaline comet assay exist, but consensus has recently been reached to produce a universal protocol (Tice *et al.* 2000; Hartmann *et al.* 2003; Wiklund & Agurell 2003). Such a protocol is necessary because variations between different protocols may lead to different results (Fairbairn *et al.* 1995, Rojas *et al.* 1999). For example, small differences in voltage during electrophoresis, as well as electrophoresis time, can lead to a variation in results such as tail length, especially because the DNA only migrates distances in the order of micrometers (Fairbairn *et al.* 1995).

In contrast to the original neutral method (Östling & Johanson 1984), the alkaline version (Singh *et al.* 1988) is more sensitive and has been preferred by researchers. This is because neutral lysing and electrophoresis detects double strand breaks, whereas the alkaline version allows for the detection of single strand breaks, which may be 5-2000 fold more than double strand breaks (Fairbairn *et al.* 1995; Rojas *et al.* 1999). Normally DNA remains supercoiled, but when placed in an alkaline lysing and unwinding conditions, the DNA will start unwinding at sites of strand breakage (Rojas *et al.* 1999) thus allowing for the detection of single strand breaks. In addition, this method will lead to the expression of alkali labile sites, which are previously induced DNA breaks that only become apparent after alkali treatment (Fairbairn *et al.* 1995). Furthermore, cellular RNA will be degraded under alkaline conditions, which will result in a reduction of background fluorescence from these nucleic acids that could hamper the scoring of comets.

One of the more relevant problems of using an assay to detect DNA damage is the possibility that the toxicant does not necessarily induce DNA damage, but instead causes cell death (Shugart 2000). Apoptosis, also termed programmed cell death, is an active and deliberate mechanism of eliminating old or damaged cells. During the process of cell death, DNA is degraded, a result that may easily be confused with genotoxicant-induced damage. Apoptotic cells may however be detected with a modified comet assay (Chandna 2004), which also gives this assay the advantage above other assays such as the agarose gel electrophoresis assay. For example, during electrophoresis most DNA from apoptotic cells migrate from the head to the tail, over a much longer distance than that of "real" comets (Fairbairn *et al.* 1995; Rojas *et al.* 1999). Roser *et al.* (2001) found that apoptosis does not necessarily correlate with DNA damage, and will therefore not confound comet assay results.

1.3 Cadmium

Genotoxicants are defined by Shugart (2000) as environmental chemicals and/or physical agents that have the capacity to interact with and modify (damage) DNA structure, and by Fairbairn *et al.* (1995) as chemicals that have the ability to alter DNA replication and genetic transmission.

One of the groups of environmental contaminants that has received attention as having the potential to act as genotoxicants and also as carcinogens, is the heavy metals. Making heavy metals particularly dangerous is the fact that they may be accumulated by many species, posing either a threat to the organism itself or to higher levels on the trophic scale (Croteau *et al.* 2005).

Therefore, the steady increase of anthropogenic release of heavy metals in the environment is becoming a matter of great concern (Stoeppler 1992).

One contaminant of great concern worldwide, especially in the release of sewage sludge or sludge-amended soils into the environment, is the heavy metal cadmium (Water Research Commision of South Africa 1997). Cadmium is a nonessential element that can accumulate over time in organisms (especially in renal tissue) and has a long biological half-life in humans of 16 to 33 years (Richardson 1993). It may accumulate in soil invertebrates such as isopods and earthworms, which will result in availability of Cd to organisms on higher trophic levels that utilise these invertebrates as prey (Hopkin 1989).

Cadmium is classified as a Group I human carcinogen by the International Agency for Research on Cancer and has also been shown to be carcinogenic to experimental animals and wildlife (IARC 1994; Hartwig 1998; Waisberg *et al.* 2003). This heavy metal may cause e.g. aneuploidy in humans (Guerci *et al.* 2000) and induce either apoptosis or necrosis in cultured rat neurons depending on the exposure concentration (López *et al.* 2003). In soil, high concentrations of Cd may even cause soil microbe metabolism to be less efficient (Renella *et al.* 2005). Various adverse effects of cadmium on humans and wildlife are reviewed in Richardson (1993).

In addition to being problematic in contaminated sewage sludge, cadmium is released into the environment, for example, as part of phosphate fertilizers, through leaching from NiCd batteries and coal combustion (Irwin *et al.* 1997). The maximum allowed level of Cd in fertilizers in South Africa is 100 mg/kg (Regulation Gazette no. 9715, 2004). In sewage sludge, Cd may not exceed 15.7 mg/kg (as an available fraction, measured with the Toxic Characteristic Leaching Procedure) or a total amount of 100 mg/kg (Regulation Gazette no. 9715, 2004). In South African soil, the maximum permissible metal content in soil for Cd is 2 mg/kg; the bioavailable fraction should not be more than 1 mg/kg; and the total load may not exceed 3140 g/h in 25 years (Water Research Commission of South Africa 1997). In South African water, Cd concentrations have, for example, been measured as 0.01 to 0.08 mg/l (Awofolu *et al.* 2005), which is higher than the permissible level of 0.005 mg/l (DWAF 1996; DWAF 1998).

Mechanisms of cadmium toxicity are manifold and complicated. In mammals, Cd influences gene regulation and signal transduction (e.g. by interfering with transcription and translation factors), inhibition of DNA repair and generation of free radicals (Waisberg *et al.* 2003). Because Cd is not a Fenton metal, the generation of reactive oxygen species occurs through an indirect process (Waisberg *et al.* 2003). The Fenton reaction causes hydroxyl radical (·OH) and hydroxide ions (OH^-) to be formed after oxidation of a metal such as Fe (Cohen 1985). These OH groups

subsequently cause oxidative stress, which will result in various metabolic disfunctions, including DNA damage (Halliwell & Aruoma 1991).

Concerning DNA, the mechanisms of Cd toxicity and mutagenicity are still poorly understood, but recently it has become clear that Cd has a high mutagenic activity (it may produce large deletion mutations in e.g. human-hamster hybrid A_L cells, (Filipic & Hei 2004)) and it can interfere with DNA repair systems. It can interfere with two types of DNA repair systems, both at a low effect concentration of 0.5 µM Cd (Hartwig 1998). Firstly, it interferes with nucleotide excision repair of DNA damaged by UVC by competing with Zn^{2+} in a DNA damage recognition step. Secondly, it can affect base excision repair by interfering with the excision of a damaged base when oxidative DNA base modifications (damage) have occurred. Cadmium may target especially zinc finger structures in DNA transcription factors and DNA repair enzymes. It has been argued that Cd is only weakly genotoxic in mammals and bivalves (Pruski & Dixon 2002). Conversely, it has been found to induce dose-response relationships for the comet assay in human lung fibroblasts (Mourón *et al.* 2001).

Because DNA integrity in all living cells is constantly changing as a result of natural processes (Shugart 2000), some baseline level of structural modifications is to be expected. Therefore, when using a biomarker of genotoxicity, such as the comet assay, some level of "background" damage will be detected. The degree of damage induced by toxicants will be detected when these structural modifications are found to exceed normal levels such as those found in negative controls in the experimental setup (Shugart 2000). Because Cd is known to prevent DNA repair, rather than directly causing damage (Pruski & Dixon 2002, Waisberg *et al.* 2003), the amount of irreparable damage will actually be measured during this study.

Cadmium poisoning causes various biological and biochemical effects. A few effects on humans and other mammals are mentioned in the following paragraph, as reviewed by Vallee & Ulmer (1972). Cadmium can compete with Zn to bind with various biological complexes, such as sulfhydryl or imidazole groups in alubumin, dithiols in enzymes and carboxypeptidases, which in effect alters the activities of various enzymes important to normal biological function. Cadmium can also bind with phospholipids to expand them, and is possibly involved in toxicity of mitochondria, kidney tubules and nerve membranes. It can interfere with oxidative phophorylation and cause teratogenic effects, e.g. it has been found to induce developmental abnormalities in hamster embryos. Prolonged exposure to Cd may also result in kidney disfunctioning and hypertention.

In earthworms, the effects of cadmium have been investigated extensively, and is regarded by some (Khalil *et al.* 1996) to be one of the most toxic heavy metals. For example, Cd can affect the population density and individual growth, sexual development and reproduction of earthworms (Reinecke & Reinecke 1996; Siekierska & Urbanska-Jasik 2002). It may also affect neurosecretory processes (Siekierska 2003), impair immunity (Homa *et al.* 2003) and affect osmoregulation (Reinecke *et al.* 1999) of earthworms. In earthworm bodies, Cd is accumulated in granules in the chloragogenous tissue surrounding the digestive tract (Morgan & Morris 1982) as well as the nephridia (Prinsloo *et al.* 1999).

Cd is known to induce the formation of metal-binding proteins, called metallothioneins that bind the heavy metal and allow it to be sequestrated (Dabrio *et al.* 2002). In addition to the binding of cadmium to metallothioneins, another general mechanism for cadmium detoxification by eukariotes such as yeast and mammals is the chelation of the metal by glutathione (GSH: L-γ-glutamyl-L-cysteinylglycine) and the subsequent compartmentalization of this GSH–metal complex (Perego & Howell 1997).

1.4 Earthworms

Earthworms have numerous roles in terrestrial ecosystems (Edwards & Bohlen 1996). They act as decomposers and remove decaying organic material from the soil surface and incorporate it into the soil. They may also improve soil structure and increase aeration by their burrowing activities. They form a major component of soil faunal biomass and are therefore an important food source for many organisms at higher trophic levels (Edwards & Bohlen 1996). Because earthworms are known to accumulate toxicants such as heavy metals and some insecticides, organisms feeding upon them may be affected detrimentally (Reinecke 1992). Earthworms are in close contact with the soil substrate, specifically the pore water and are vulnerable to physical and chemical changes to soils (Reinecke & Reinecke 2004a). For these reasons, and because earthworms are readily available, easy to handle and to use in toxicity tests, and some also suitable to culture in the laboratory (Reinecke & Reinecke 2004a), these macroinvertebrates are deemed suitable test organisms for ecological risk assessment in terrestrial ecosystems (Eijsackers 2004). Indeed, the earthworm species *Eisenia fetida* has been prescribed as a terrestrial invertebrate test species by the Organization for Economic Cooperation and Development in Europe (OECD 1984) as well as the Environmental Protection Agency in the USA (EPA 1996).

Earthworms occur globally in terrestrial and often also in aquatic systems, except in areas with extreme conditions of, for example, humidity and temperature, such as deserts or areas under constant snow and ice (Edwards 2004). Some species are ubiquitous and termed "peregrine", for they are introduced species occurring worldwide that have become dominant over indigenous species (Edwards 2004). For example, surveys for this study conducted in the beginning of 2004 in the Stellenbosch area, South Africa, showed the most common occurring species to be the peregrine *Aporrectodea caliginosa*, originating from the western Palearctic and eastern Nearctic regions (Sims & Gerard 1985).

South African endemic earthworms belong to the family Microchaetidae, which is divided into four genera (*Microchaetus*, *Proandricus*, *Tritogenia*, *Michalakus*) containing 137 currently known species (Plisko 1998; Plisko 2003). South African earthworms have been described since the mid 1800s (Beddard 1895). More than half of the currently known earthworm species are described from KwaZulu-Natal (where the most intensive surveys have been done for the past 15 years), but it is speculated that there are probably more species elsewhere awaiting discovery (Plisko 2003).

Earthworm species tend to be associated with specific soil types, and species diversity is thought to vary between different habitats and, according to Edwards (2004) will be fairly low in most cases. Being detrivores, they feed on decomposing plant material and animal dung (Edwards & Bohlen 1996). They also consume seeds, algae, fungi and protozoa, although they are known to be discriminate consumers (Morgan *et al.* 1993). Some species are characterised by a discrete diapause state during unfavourable climatic conditions, while other species can be seasonally quiescent (Morgan *et al.* 1993).

Although having an apparent uniform morphology, and being virtually indistinguishable from each other, earthworm species differ considerably in terms of physiology, morphology and behaviour. Three earthworm ecological types (sometimes also referred to as ecophysiological types) are recognised (Bouché 1972, 1992):

Epigeic species are usually relatively small (from a few millimetres), litter and topsoil inhabiting species, and are usually fairly darkly pigmented, either red-brown or green. They are subject to high predation pressure as a result of their habitat, but compensate by having an r-selected reproductive strategy (high numbers of small hatchlings) (Bouché 1992).

Endogeics live in the upper soil layer, in horizontal burrows, and may be either small or large. They lack skin pigmentation. This is a very diverse group, e.g. some may feed on relatively

organic rich food (such as humus) or organic poor food (mineral soil). Their ecophysiological regulation of activity is closely linked to soil conditions, e.g. hibernation/aestivation are mediated by soil temperature and moisture (Bouché 1992).

Anecic species are generally large worms (7 cm or more in length) living in deep vertical burrows (1 to 6 m deep) and which may feed occasionally on the soil surface. They may be pigmented dorsally (Bouché 1992).

Species belonging to these different ecotypes are known to differ with regard to characteristics such as their gut morphology, rate of transport of food through the alimentary canal and calcium gland activity as well as differ biochemically such as in the formation of metal binding metallothioneins (Morgan & Morgan 1992). They may also differ with regard to uptake, accumulation, excretion of and sensitivity to numerous environmental chemicals. Up to the present, a plethora of data exists on these topics for many different earthworm species (Spurgeon *et al.* 2003; Eijsackers 2004; Reinecke & Reinecke 2004a).

In organisms such as earthworms, bioaccumulation (and therefore the manner of uptake) of a metal is often seen as an indicator of exposure, particularly as metals are not metabolised and it may reach the target unchanged (Luoma & Rainbow 2005). Bioaccumulation may be complex, and may differ between earthworm species (Morgan & Morgan 1999), therefore it is important to consider the different modes and routes of uptake between the different species or types of species investigated, especially since this will affect dosage. When the uptake abilities of species for a specific chemical differ, the bioavailability and eventually the toxicity of that chemical will differ between the species and could result in species sensitivity differences. As an example, there are indications that, when organisms have smaller body sizes, chemicals absorbed through the skin may be more toxic (and these species would seem to be more sensitive) than for larger organisms. This could be due to the fact that smaller species have greater body surface areas relative to their volumes, which would result in higher uptake of toxicants (Rozman & Klaassen 2001).

In earthworms, unlike most other soil invertebrates, two pathways of chemical or toxicant uptake should be considered: dermal and oral uptake. Because earthworms are directly in contact with soil pore water, and because the earthworm body walls are highly vascularised (to accommodate respiratory gas exchange (Weber 1978)), considerable exchange of water and other molecules occurs across the body wall (Morgan *et al.* 1993). Chemicals in the dissolved state, such as free metal ions, can enter the body via this route. The other route to be considered is

ingestion. Pollutants such as metals are adsorbed to soil and organic particles that serve as earthworm food, or the soil water itself may be ingested. The conditions in the gut renders the amount of metal binding sites in the food in such a way that more metal ions than would have otherwise have been available, enter the gut wall (Morgan *et al.* 1993).

It is has been proposed that, for dermal uptake, the concentration of chemicals in tissues should in theory be directly related to the pore water concentration (Allen 1997) which would lead to the conclusion that bioavailability of chemicals to earthworms may be determined by the concentration in the soil water itself (Kiewiet & Ma 1991). When ingestion is considered however, it is difficult to explain uptake of chemicals from pore water concentration or soil properties. This is because not only the concentration of metals in soil may affect the uptake of heavy metals by earthworms, but also several physical and chemical properties of soil, such as pH, organic content, clay content, calcium content and adsorption of metals to soil particles (Kiewiet & Ma 1991).

The uptake of toxicants is a complex issue, and is dependent on the type of toxicant and the medium in which it occurs. For example, the uptake of cadmium by earthworms (*Lumbricus rubellus*) in reconstituted ground water is reduced by calcium, but is not influenced by pH (Kiewiet & Ma 1991). However, it has been shown that in soil, pH influences Cd uptake and accumulation by earthworms by means of changing the degree of soil adsorption (Van Gestel 1992). Acidification of soil will decrease adsorption of heavy metals to soil particles (Kiewiet & Ma 1991), leading to an increased concentration of these metals in the soil water and thus increased availability for uptake.

It is known that earthworm species differ in uptake ability of metals (Morgan & Morgan 1999; Dai *et al.* 2004). It has been proposed that dietary intake (selective feeding) plays a major role in these uptake differences, along with other factors such as niche separation (Morgan & Morgan 1992) and behaviour (Eijsackers 2004).

Earthworms have been shown to accumulate metals to a greater extent than higher trophic level organisms as well as other terrestrial macro-invertebrates such as geophilid centipedes and slugs (Morgan *et al.* 1993). Species differences in metal accumulation have been observed in earthworms inhabiting the same soil (Morgan & Morgan 1992; Morgan & Morgan 1999; Aziz *et al.* 1999; Dai *et al.* 2004), but these differences may not be consistent between different sites. Also, the amount of accumulation for each metal is different. Morgan & Morgan (1992) found that for Cd, endogeic species accumulated significantly higher concentrations than either anecic or epigeic species. However, these were animals taken from the environment where they occur

naturally, where soil would be heterogeneous in terms of metal concentration. Therefore, habitat preference could be accounted for as a factor determining differences in accumulation.

Earthworm species also differ in their sensitivity to heavy metals, as previously mentioned (Spurgeon *et al.* 2000). The reason for the species differences in sensitivity and accumulation is complicated and still under debate, but clues may be found in the uptake routes (as discussed above), differences in physiological utilisation, sequestration and excretion ability. It is known that the detoxification and sequestration ability of earthworm species differ, such as calcium metabolism (involved in sequestration and elimination of metals), and other physiological processes such as metal-binding proteins called metallothioneins (Spurgeon & Hopkin 1996a).

1.5 Aims

The present study aims to contribute to a better understanding of species sensitivity differences to sublethal levels of contaminants, and to determine whether a biomarker, and specifically a biomarker of genotoxicity, can be used to compare the sensitivity of different species (earthworms) to a heavy metal (cadmium).

The specific aims were to determine

1. whether DNA damage, as measured with the comet assay, is a successful biomarker to elucidate earthworm species differences to cadmium;
2. whether there will be an increase in DNA damage with increasing Cd exposure concentration for each species (is there a dose-response relationship?);
3. whether the chosen earthworm species differ from each other in sensitivity to cadmium as measured with the comet assay;
4. if species sensitivity differences are found, whether *Eisenia fetida*, which is an acknowledged test species (OECD 1984), is sensitive enough to be used as a representative species in toxicity testing, compared to other species;
5. whether DNA damage is affected by the amount of Cd that an earthworm has accumulated.

2 Materials and Methods

2.1 Earthworms

For a study such as the present one, the choice of species should allow for comparison between (and within) all three ecological groups, the epigeic, endogeic and anecic species. Surveys in the immediate Stellenbosch area (33°55'58.33" S, 18°51'53.49" E), South Africa, have been conducted from February to June 2004 and yielded a variety of species, representing all three ecological types. Surveys were done by digging at sites where earthworm casts were present, hand sorting and identification with the aid of Sims & Gerard (1985). The most common occurring earthworm species found in Stellenbosch soils was *Aporrectodea caliginosa*. Other species include *Lumbricus rubellus*, *Octolasion cyaneum* and species from the megascolecid *Pheretima* complex. Compost-dwelling species included *Dendrodrilus rubidus*, *Eisenia fetida* as well as *Pheretima spp.* and *Amynthas spp.* These are all exotic species, and have seemingly almost entirely replaced the indigenous earthworms in the immediate Stellenbosch area where human settlement has taken place. According to Ljungström (1972), apparently no competition exists between introduced and indigenous species, and the decline in indigenous species numbers could be due to anthropogenic influences such as habitat destruction. Indigenous microchaetid worms (*Microchaetus benhami*) have however been found during this study on the farm Middelvlei near Stellenbosch. Sufficient numbers of individuals were available and were used for the study. The following species were used: *Eisenia fetida* (epigeic) *Dendrodrilus rubidus* (epigeic), *Aporrectodea caliginosa* (endogeic), *Amynthas diffringens* (epigeic) and the indigenous *Microchaetus benhami* (anecic).

Belonging to the phylum Annelida, earthworms comprise the suborder Lumbricina in the class Oligochaeta (which includes over 7000 species from more or less 739 genera, (Reynolds & Cook 1993)). The taxonomy of earthworms is still an enigmatic issue (Edwards & Bohlen 1996; Edwards 2004). A complete molecular phylogeny is lacking, but recent attempts have been made to establish phylogenetic relationships among various earthworm groups (Jamieson *et al.* 2002; Pop *et al.* 2003).

The taxonomic positions of the species used in this study are as follows (Sims & Gerard 1985; Reynolds & Cook 1993):

PHYLUM ANNELIDA

Subphylum Clitellata

Class Oligochaeta

Order Haplotaxida

Suborder Lumbricina

Superfamily Lumbricoidea

Family Lumbricidae (Rafinesque-Schmaltz 1815)

Subfamily Lumbricinae (Rafinesque-Schmaltz 1815)

Aporrectodea caliginosa (Savigny 1826)

Dendrodrilus rubidus (Savigny 1826)

Eisenia fetida (Savigny 1826)

Superfamily Glossoscolecoidea

Family Microchaetidae (Michaelsen 1900)

Microchaetus benhami (Rosa 1891)

Superfamily Megascolecoidea

Family Megascolecidae (Rosa 1891)

Amynthas diffringens (Baird 1869)

Amynthas diffringens (Baird 1869)

Previously known as *Pheretima diffringens*, or even part of the *Amynthas corticis* complex (Reynolds & Cook 1976; Blakemore 2003). Members of this species grow to 49 – 95 mm (Ljungström 1972). Individuals may be light reddish brown to dark brown and inhabit top-soil and places with high organic content such as compost heaps. This species possibly originates from China (Ljungström 1972). *A. diffringens* found in South Africa may reproduce parthenogenetically (Ljungström 1972), but specimens collected for this study reproduce sexually (J.D. Plisko *Pers. comm.*). No toxicity data have been found in the present literature survey for this species.

Aporrectodea caliginosa (Savigny 1826)

Aporrectodea caliginosa is believed to contain four morphs differing phenotypically (in e.g. pigmentation), previously described as separate species (Sims & Gerard 1985). These four "species" were *A. caliginosa* (Savigny 1826), *A. tuberculata* (Eisen 1874), *A. nocturna* (Evans 1946) and *A. trapezoides* (Dugès 1829). Presently *A. caliginosa* is recognised as a heterogeneous species, containing these four morphs (Sims & Gerard 1985). The earthworms sampled for this study belongs to the *trapezoides* morph (J.D. Plisko *Pers. comm.*). *A. caliginosa* is dominant in gardens and cultivated land. Smaller individuals live in the topsoil in temporary horizontal burrows, whilst larger individuals may be deep-burrowing and produce large surface casts (Sims & Gerard 1985). Bouché (1992) considers *A. caliginosa* to be endogeic. *A. caliginosa* originate from the western Palaearctic and eastern Nearctic and was introduced into the temperate regions of the world (Sims & Gerard 1985). This species has however been reported to survive soil moisture conditions as low as 10% soil moisture (Buckerfield 1992). Individuals may reach 80-140 mm (Sims & Gerard 1985). The colour is variable, ranging from pale pink to almost purplish brown (depending on the morph). Members of this species are obligatory biparental, where sexual reproduction is necessary (Sims & Gerard 1985). *A. caliginosa* is a regularly used species for toxicity testing, although not as extensively as *Eisenia fetida* (e.g. Khalil *et al.* 1996; Morgan & Morgan 1998; Maboeta *et al.* 2003; Friis *et al.* 2004; Booth *et al.* 2005).

Dendrodrilus rubidus (Savigny 1826)

Dendrodrilus rubidus is a fairly small species, reaching 20 – 100 mm in length. These worms range from a dark red colour to pale red or pink with a distinct yellow or orange caudal region. The yellow colouration is often caused by the accumulation of metabolic waste products (not toxicants) in the last three to eight segments (Sims & Gerard 1985). *D. rubidus* is able to reproduce parthenogenetically (Sims & Gerard 1985), but specimens collected for this study reproduces sexually (J.D. Plisko *Pers. comm.*). This is a species with epigeic characteristics; it occurs in places with high organic content such as under moss and loose bark, in moist litter, wet habitats such as marshes, compost, manure heaps, under dung in grasslands and even caves. It originates from the Holarctic and has been introduced to various parts of the world (Sims & Gerard 1985). *D. rubidus* has also been used for toxicity testing, although not as extensively as *Eisenia fetida* (Morgan & Morgan 1991; Morgan & Morgan 1993; Terhivuo *et al.* 1994; Spurgeon & Hopkin 1996; Holmstrup & Simonsen 1996; Rundgren & Nilsson 1997; Langdon *et al.* 2001a; Langdon *et al.* 2001b; Morgan *et al.* 2002). Four morphs for this species exist, which have previously been described as separate species. These include *rubidus*, *subrubicundus*, *tenuis* and *norvegicus* (Holmstrup & Simonsen 1996). It is not clear as to which morph the specimens used in the present study belong.

Eisenia fetida (Savigny 1826)

For soil toxicity testing, the epigeic worm *Eisenia fetida* is the most widely used earthworm species (Reinecke & Reinecke 2004a). The life cycle is well documented (Venter & Reinecke 1988), the physiology well known, and the mode of uptake, sequestration and toxicity of an array of environmental chemicals have been investigated (Spurgeon *et al.* 2003). It is the recommended species for OECD and EPA guidelines (OECD 1984; EPA 1996), a fairly robust species, and easily cultured. This robustness has raised concern and considerable debate whether *E. fetida* is really the best or most sensitive species to use for toxicity testing (Edwards & Coulson 1992; Reinecke & Reinecke 2004a). Indeed, several studies have shown that *E. fetida* is not as sensitive as other species for a range of toxicants (e.g. Edwards & Coulson 1992; Spurgeon & Hopkin 1996a; Lukkari *et al.* 2005; Langdon *et al.* 2005). *E. fetida* reaches 60 – 120 mm in length. Their colour ranges from light pink to purplish red or brown. Characteristic for this species is the unpigmented intersegmental areas, forming light coloured rings around the body (hence the name "tiger worm"). It occurs under damp rotting vegetation such as compost heaps and manure piles. *E. fetida* has a Palearctic (Europe, North America and Russia) origin and are currently

widespread around the world's temperate regions. It is also an economically important species, and is widely used for e.g. vermicomposting and angling (Sims & Gerard 1985). *E. fetida* also has a somewhat enigmatic taxonomic status, because a subspecies has been recorded from cultures with a low pH, known as *andrei*. Recent DNA analyses suggest that *Eisenia fetida* and *Eisenia andrei* are separate phylogenetic species (Perez-Losada *et al.* 2005).

Microchaetus benhami (Rosa 1891)

This species indigenous to Southern Africa has been described from specimens collected from the Stellenbosch area as well as from sites in the Cape peninsula from moist soil on riverbanks, near streams and in gardens (Michaelsen 1913, Pickford 1975). It is speculated that the species has been introduced to agricultural soils or alternatively that it has survived after cultivation of its habitat (Pickford 1975, Plisko 2003). Individuals collected in December and April (the dry season) by Pickford (1975) showed signs of estivation or diapause, which could probably be a mechanism to survive the dry local summer conditions. Individuals collected for the present study also showed signs of estivation (J.D. Plisko *Pers. comm.*). Interestingly, individuals of *M. benhami* tend to autotomize their caudal region (own observations, subsequent to sampling, Fig. 2.1). A closely related species, *M. mkuzi* also exhibits autotomy (J.D. Plisko, *Pers. comm.*), as well as do other *Microchaetus* species (Ljungström & Reinecke 1969). The reason for this phenomenon in these and other *Microchaetus* species is unclear, but it could possibly be a tactic to evade predators such as moles (Ljungström & Reinecke 1969). No exotoxicological data exist for *M. benhami*, and the only ecotoxicological study on other indigenous earthworm species has been published by Maboeta *et al.* (2002). In that study, the neutral red retention assay (a cellular biomarker) was employed to determine the effects of the fungicide copper oxychloride on an unidentified *Microchaetus sp.* in the Namakwaland region of the Western Cape. These earthworms were used successfully for toxicity testing and showed significant responses in sensitivity to copper oxychloride in both biomass changes, population numbers as well as neutral red retention times. In the light of these findings it is possible to use indigenous South African earthworm species in ecotoxicological-type studies and biomonitoring.

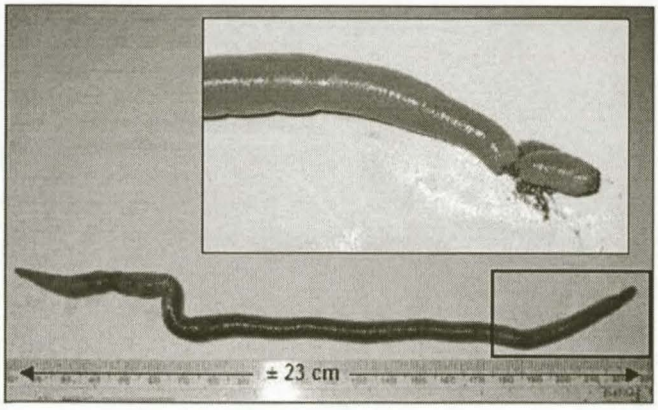

Figure 2.1: A specimen of *Microchaetus benhami*. The insert depicts caudal region autotomy.

2.2 Cadmium

The element cadmium (Cd^{48}_{112}, CAS Registry no. 7440-43-9) is a soft, silver-white transition metal. In its natural state, Cd occurs bound to sulphides (especially zinc sulphide) and is obtained as a by-product from smelting of ores, most importantly zinc (Richardson 1993). It is an economically important metal, and the main uses of Cd include production of nickel-cadmium batteries, pigments, plastic stabiliser, and to a lesser extent, alloys, solders and electroplating (Irwin 1997). Cd occurs naturally as well as resulting from anthropogenic activities in soils, sediments, air and water. Natural dispersal mechanisms include volcanism, forest fires and wind transport of soil (Irwin *et al.* 1997). The main anthropogenic sources are zinc, copper and nickel smelting, fossil fuel combustion, the application of contaminated sewage sludge as a soil amendment, as ingredient in pesticides and as an impurity in phosphate fertilisers (Richardson 1993, Irwin 1997).

2.3 Experimental methods

2.3.1 Culture and collection of animals

Cultures of *E. fetida*, originating from Europe, are maintained in our laboratory. These cultured animals are fed on urine-free cattle manure once a week and kept in darkness at 20°C and 60% humidity in a climate controlled room. The other species were collected from the field by digging and hand sorting. They were either kept in the climate controlled room at 20°C and 60% humidity for a few days in their original substrate or directly used in experiments. Identifications of earthworm species were either done with the aid of the key from Sims & Gerard

(1985), or specimens were sent for identification to Dr. J.D. Plisko (earthworm taxonomist, Natal Museum, Pietermaritzburg, South Africa).

M. benhami was collected from waterlogged soil on the farm Middelvlei near Stellenbosch. *A. caliginosa* was collected from a regularly used and pollution-free collecting site on the farm Vergelegen belonging to the University of Stellenbosch. *A. diffringens* individuals originated from the Stellenbosch University botanical garden compost heaps. *D. rubidus* was collected near the cattle pen (in soil covered with cattle manure) at the Stellenbosch University experimental farm Welgevallen.

2.3.2 Exposures in artificial soil water

All exposures were done in an artificial soil water medium (Kiewiet & Ma 1991)(Appendix A). Soil water exposures allow for some control in the experimental set-up, where possible differences in feeding strategies, habitat preferences or behaviour such as avoidance responses (Morgan & Morgan 1992; Yeardly Jr. *et al.* 1996; Morgan & Morgan 1999; Eijsackers 2004) are excluded. Such a set-up could therefore aid in the assessment of potential differences in physiology, species-specific metal bioavailability and inherent sensitivity in different species.

Exposures were done for two days in a climate-controlled room at 20°C and 60% humidity. Animals were exposed in 600 ml glass beakers to 300 ml of soil water. The artificial soil water was aerated during exposures.

Mature (clitellate) worms were used in all exposures, except for *M. benhami*, where juveniles were also used. For this species, sampling proved difficult because the method of sampling (digging) caused injuries in adults because they are large (up to 30 cm in length) and were thus harmed by the spade.

2.3.2.1 Range finding tests

Range finding tests, to determine the appropriate concentrations of Cd to be used for the testing of sublethal effects, were conducted by exposing *E. fetida* in artificial soil water. Only *E. fetida* was used for these tests on the assumption that if species differences are to be found, effect concentrations may not differ in orders of magnitude (within a factor of 10) for closely related species. In addition, this species has been recommended for the initial screening of chemicals (Edwards & Coulson 1992). Ten adult (clitellate) animals of roughly similar weight were exposed

per concentration of Cd in the form of $CdSO_4$ (CAS No.: 10124-36-4) dissolved in artificial soil water.

The concentrations used for the first set of tests were 0 mg/l (negative control), 0.1 mg/l, 1 mg/l and 4 mg/l Cd. After three days, all animals survived the test conditions. The second set of range finding tests included concentrations of 6.25 mg/l, 12.5 mg/l, 25 mg/l and 50 mg/l Cd. Over a two-day period, animals had a 100% survival rate at 6.25 mg/l and 12.5 mg/l Cd. However, at 25 mg/l Cd, 14% of animals died, and at 50 mg/l Cd, 50% of animals died. The third set of range finding tests included concentrations of 5 mg/l, 10 mg/l and 20 mg/l Cd, where all animals survived. Preliminary visual inspections showed that comet tail lengths increased with increasing concentration of Cd.

2.3.2.2 Experimental exposures

The final exposure range was: 0 mg/l (negative control), 2.5 mg/l, 5 mg/l, 10 mg/l and 20 mg/l Cd in artificial soil water. Animals were exposed for two days after the earthworm gut contents were removed (by keeping the earthworms on damp filter paper for two days). Such short exposure times have been illustrated to be sufficient for accumulation of Cd in earthworms, as well as to induce responses in coelomocytes (Homa *et al.* 2005).

Two exposure experiments (henceforth referred to as "replicates") were performed for each species (i.e. each experiment was repeated for every species) in order to increase the amount of data). Data from these two experiments were pooled together. Six treatments were used per replicate and consisted of a negative control (uncontaminated artificial soil water), a positive control (Ni, 20 mg/l, in the form of $NiSO_4$) and the chosen range of Cd concentrations. Therefore, for each of the six treatments, five animals were exposed (per beaker) per species. These exposures were then repeated with five animals per treatment per species, therefore the total number of animals per species per treatment was ten. All individual animals were treated as independent individuals in data analyses.

The positive control (Ni) was chosen to belong to the same chemical class as the test compound (Tice *et al.* 2000). The heavy metal nickel is known to induce DNA damage (in a dose-response relationship) in earthworms (*E. fetida*) as measured with the comet assay (Reinecke & Reinecke 2004b).

2.3.3 Collection of coelomocytes

Coelomocytes were chosen as the cell type used in this study. These cells occur within the coelomic fluid of earthworms (Jamieson 1981); they are therefore already in suspension for direct use in the comet assay. Coelomocytes are actively involved in immune responses (Stephenson 1930), making them an ideal target for both xenobiotics as well as for toxicity testing.

A non-invasive technique (Eyambe *et al.* 1991) was used to extract coelomocytes from four species (except for *M. benhami*), following a modified protocol (Reinecke & Reinecke 2004b) with a few alterations for the present study. Each animal was exposed for 3 minutes in an Eppendorf tube (or a suitably sized tube to accommodate the larger species, e.g. *A. diffringens* and *A. caliginosa*) to 1 ml (1.5 ml for larger species) of an extrusion fluid (Appendix A) to irritate and induce the animal to expel coelomic fluid through its dorsal pores. For *M. benhami*, a syringe filled with 20 µl PBS (phosphate buffer saline) was used to extract cells by piercing the body wall and withdrawing approximately 20 µl coelomic fluid. This method was used for cell extraction because *Microchaetus* species do not have dorsal pores through which the coelomic fluid may be extruded (Stephenson 1930).

The cell suspension (as obtained from either of the two methods) was centrifuged at 4000*rpm* for 5 min in a Biofuge fresco centrifuge (Heraeus Instruments). The supernatant was removed by drawing it from the tube with a plasic pipette. The pellet was then suspended in PBS by filling the tube up to 1.5 ml, and centrifuged again at 4000*rpm* for 3 min. The supernatant was removed and the cell suspension, which has thus been washed and concentrated, was stored on ice until further use. Animals were individually labelled and frozen immediately after extrusion until used for analyses of metal body loads.

2.3.4 Analysis of metal content in earthworms and soil samples

Soil samples from the following collecting sites were prepared and screened for metal (Cd, Ni, Cu, Mn, Pb and Zn) contents: Vergenoegd farm for *A. caliginosa*; Middelvlei farm for *M. benhami*, Welgevallen farm for *D. rubidus* and the Stellenbosch University botanical garden compost heap for *A. diffringens*. The cattle manure substrate on which the laboratory culture of *E. fetida* is kept is assumed to be without additional contaminants and were thus not analysed for metal contents.

Fundamental to the critical body residue (CBR) approach is the comparison of the residues of toxicants in organisms to their responses (Conder *et al.* 2002). Using this approach, it is presumed

that, for example, metal concentrations in earthworms are a measure of bioavailability (Conder *et al.* 2002), and that this should be comparable to toxicological endpoints. Therefore, the cadmium (or nickel, when applicable) content for all exposed earthworms was determined.

All exposed earthworms used in experiments, as well as soil samples from collecting sites, were analysed for cadmium content after nitric/perchloric acid digestion (Katz & Jennis 1983). The heavy metal concentration analyses were done using a flame atomic absorption spectrophotometer (Varian AA – 1275) at the department of Physics, University of Stellenbosch. The detection limits for the metals were: 0.01 mg/l for Cd, 0.08 mg/l for Ni, 0.04mg/l for Cu, 0.3 mg/l for Mn , 0.11mg/l for Pb and 0.01 mg/l for Zn.

Prior to acid digestions, the guts of field-collected animals were allowed to be voided (depurating the guts on damp filter paper for two days), as not to influence the analysis by overestimating the metal content by measuring the metal content in the gut contents. Frozen earthworms were thawed prior to acid digestion and subsequently weighed. Soil samples were dried for 48 hours and subsequently ground to a uniform particle size of which 1 g per sample was subjected to acid digestion.

Acid digestions were done as follows: after weighing, animals or soil samples were digested with 10 ml nitric acid (55%) for at least 8 hours. Then the samples were heated to 40-60°C for 2 hours and subsequently to 110-120°C until it was boiling and emitting brown fumes. After cooling, perchloric acid (70%) was added, 1 ml for soft tissue and 5 ml for soil, and the samples heated to 110-120°C until boiling and emitting white fumes. After cooling, 5 ml of distilled water was added to each sample, which was heated to 110-120°C until boiling and emitting white fumes. After allowing for the samples to cool completely, they were filtered with Whatman no. 6 filter paper. The filtrate was totalled to 20 ml with distilled water and filtered through 0.45 µl cellulose nitrate filters. The samples were stored in polyvinil containers (metals do not adsorb to their surface) at room temperature until further analysis.

Results from atomic absorption spectrophotometer (AA) readings (in mg/l) were converted to mg/kg with the following formula:

$$\frac{A \times V}{M},$$

where A is the AA reading in mg/l; V is the volume of the sample (20 ml); and M is the mass of the sample digested (1 g in the case of soil samples).

2.3.5 Comet assay

2.3.5.1 Preparation of slides and electrophoresis

From each animal, two microgel slides were prepared. The protocol of Singh *et al.* (1988) was followed, with minor modifications (Reinecke & Reinecke 2004b). Conventional microscope slides were coated with a layer of 1% normal melting point agarose (NMA, Appendix A) and dried completely at 60°C to allow for the adherence of the gel layer to the slides. On this first layer, a layer of the cell suspension (20 µl), mixed with 0.5% low melting point agarose (LMA, 60 µl, Appendix A) at 25°C was placed. A cover slip was placed on top and the gel allowed to solidify on ice after which the coverslip was removed. A third layer of agarose (75 µl LMA) was added and allowed to solidify with the coverslip replaced.

The coverslips were removed and the slides immersed in cold lysing solution (Appendix A) in Coplin jars and incubated at 4°C overnight. After lysing, the slides were washed with distilled water and immersed in the electrophoresis buffer (pH > 13, Appendix A) for 20 minutes to allow for unwinding. This period of incubation also allows for the lysing solution (containing a high salt concentration, which may compete with DNA as an electrolyte) to diffuse out of the agarose on the slide (Rojas *et al.* 1999). Electrophoresis was done in the same buffer for 10 minutes at 25 V and 300 mA. The slides were washed once with distilled water and three times with neutralization buffer (Appendix A) where the slides were flooded twice with buffer for 8 minutes and a third time for 5 minutes. The slides were dried and stored in darkness until staining and analysis.

2.3.5.2 Scoring of comets

Slides were stained with approximately 100 µl ethidium bromide (20 µg/ml, Appendix A) and visualised under a Leitz Diaplan fluorescent microscope with Ploemopak 2.3 (excitation filter 515-650 nm, barrier filter 580 nm). At least fifty cells per slide were randomly selected and scored.

There exist various methods of evaluating and quantifying comets. A simple method is scoring the comets visually by classing them as damaged or undamaged (Fairbairn *et al.* 1995). It is also possible to classify degrees of damage by measuring the tail lengths and shapes with the aid of an eyepiece micrometer (Reinecke & Reinecke 2004b). Visual scoring of comets have been shown to be an accurate method of determining the levels of DNA damage in cells and is deemed by some to be comparable and equally reliable to using image analysis (Dr. J.P. Slabbert, *Pers.*

comm.; Rojas *et al.* 1999; Garcia *et al.* 2004). However, more sophisticated measurements can be done with image analysing software. For example, by using a software package it is possible to assess the fluorescent intensity of the head or tail quantitatively, which is a measure of the amount of DNA present where higher fluorescence indicates to higher levels of DNA present. Using such a software package allows for increased sensitivity and discriminate power between different treatments that may have subtle variations in sensitivity (Fairbairn *et al.* 1995, Rojas *et al.* 1999). For the present study, digital images (e.g. Fig 2.2) of comets were obtained with a digital camera (Leica DC 300). The images were analysed with CASP (Konca 2005). For this study, the comet parameters Tail DNA % and Olive tail moment were used. Tail DNA % is the percentage of DNA in the tail, measured as the amount of fluorescence in the tail and converted to a percentage of the fluorescence in the entire comet (head and tail included). Olive tail moment (Fig 2.2) is the product of Tail DNA % and the distance between the centre of gravity of DNA in the tail and the centre of gravity of DNA in the head (Duez *et al.* 2004). Higher Tail DNA% and Olive tail values indicate to higher DNA damage levels, because these two parameters measure the amount of DNA in the tail, and the presence of a tail indicates to DNA damage.

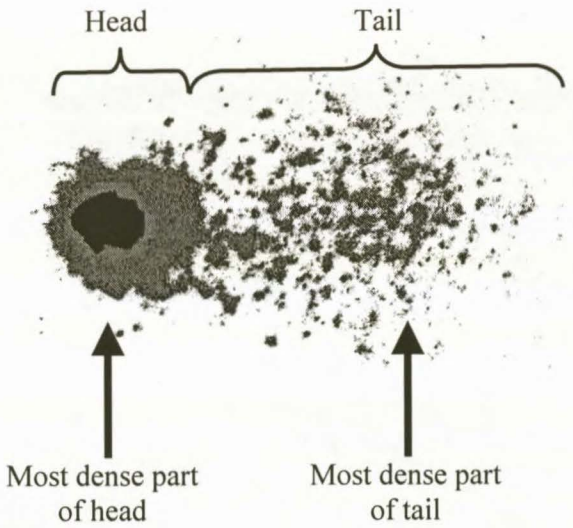

Figure 2.2: An example of a comet of an earthworm coelomocyte when viewed under fluorescent conditions (this image was converted to a black and white negative for illustrative purposes). The comet parameter Tail DNA % is the percentage of DNA in the tail, measured as the percentage of fluorescence of the tail. Olive tail moment is the product of Tail DNA % and the distance between the most dense part (or the centre of gravity) of the head and the most dense part of the tail.

2.4 Statistical analyses

Statistical analyses were conducted using Statistica 7 (StatSoft 2004). All Tail DNA % and Olive tail moment data were tested for normality with the Kolmorogov-Smirnov test, and were found to deviate significantly from the normal Gaussian distribution ($P < 0.01$). To assess whether comparisons between treatments could nonetheless be made with an analysis of variance test (ANOVA), homogeneity of variances were tested with the Brown-Forsythe test. These were not found to be homogenous. Therefore the non-parametric Kruskal-Wallis ANOVA by ranks was used to assess whether there are differences between treatments within a species and also to test for differences between species. When treatments differed significantly, pairwise comparisons were done with the Mann-Whitney U test.

As the earthworm mass and metal body load data were also found to deviate from normality, Kruskal-Wallis ANOVA was used to compare metal body loads between treatments, and the non-parametric Spearman rank order correlation was used to infer relationships between these and DNA damage. The level of significance was chosen to be $P < 0.05$.

For each species, a mean value for Tail DNA % and Olive tail moment for the negative control was calculated. This value was then taken as the "threshold" value that, when it is passed, a negative effect on DNA is inferred. Subsequently, the "net" levels of DNA damage in each exposure concentration for each species were calculated by subtracting the mean value in the negative control from the mean value in each exposure concentration, for each species separately.

In order to calculate an EC 50, the mean values of Tail DNA % and Olive tail moment in the negative control in each species were subtracted from the Tail DNA % and Olive tail moment value in each individual within each exposure concentration in each species separately. EC 50 concentrations were then calculated with Probit version 1.5 (EPA 2005) where the number of animals per treatment showing a response (i.e. a Tail DNA % or Olive tail moment value higher than the mean of the negative control) were used.

Head areas from comets without tails were measured in comets without tails in the negative controls for each species. These are considered as the "normal" state where no DNA damage has taken place, and were therefore used as a surrogate of nucleus size and therefore DNA amount in coelomocytes. These nucleus areas were compared between species, and were compared with DNA damage.

3 Results

Not all worms from all species yielded sufficient cells for data analyses (Appendix B Table 1), but all exposed worms survived all treatments. As the experimental unit for the comet assay is seen as the animal and not the cell (Lee & Steinert 2003; Collins 2004), means (and standard deviations) for Tail DNA % and Olive tail moment were calculated per animal and used in data analyses. Data obtained from this study are summarized in Appendix B. All statistical results are summarized in Appendix C.

Please note the following:

1. Whenever there is referred to a significant difference in the following text, it is a statistically significant difference ($P < 0.05$).
2. Earthworm mass is used as a surrogate for earthworm size.
3. Tail DNA % is the percentage tail fluorescence of the total fluorescence of a comet (stained with ethidium bromide and visualized with a fluorescent microscope). Olive tail moment is the product of the Tail DNA % and the length between the most dense part (also termed the centre of gravity) of the comet head and the most dense part of the comet tail (Fig 2.2).
4. Higher Tail DNA % and Olive tail moment values equate higher levels of DNA damage.
5. For *Amynthas diffringens*, animals labeled P1 and P3 from the 2.5 mg/l Cd exposure concentration (see Appendix B, Table 3) were removed from the data set as their much higher weight suggested that they might be a different species as a result of misidentification.
6. For *Eisenia fetida*, 5 animals from the 20 mg/l Cd exposure concentration were not weighed and analyzed for Cd body loads due to loss of samples.
7. Earthworms were exposed to $CdSO_4$ and $NiSO_4$, but in the following text and figure legends, reference is made only to Cd and Ni respectively, because the concentrations used are for the Cd and Ni contents and not for the salts $CdSO_4$ and $NiSO_4$.

8. The term "treatments" is used to describe all exposures collectively; the negative control (uncontaminated artificial soil water), positive control (20 mg/l Ni) and the four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd).
9. The number (*n*) of comets analyzed per animal is given in Appendix B (Tables 3 to 7) and will therefore not be indicated in figure legends.
10. Illustrative figures for correlations are mostly only given when significant correlations were found, due to the large number of correlations done. Tables giving statistical results can be found in Appendix C.

3.1 DNA damage and metal accumulation patterns within species

3.1.1 DNA damage patterns within species

3.1.1.1 Effect of exposure concentration

For *A. diffringens*, DNA damage as Tail DNA % (Fig 3.1a) differed significantly between exposure concentration 2.5 mg/l Cd and the three exposure concentrations 5, 10 and 20 mg/l Cd ($P < 0.05$, Appendix C Tables 1 & 2). For Olive tail moment (Fig 3.1b), exposure concentration 2.5 mg/l differed significantly from the three exposure concentrations 5, 10 and 20 mg/l Cd ($P < 0.05$, Appendix C Tables 1 & 3). No significant correlation was found between exposure concentration (mg/l Cd) and either mean Tail DNA % (Spearman R = 0.5, $P > 0.05$, Appendix C Table 4) or mean Olive tail moment (Spearman R = 0.5, $P > 0.05$, Appendix C Table 4) (Fig 3.2).

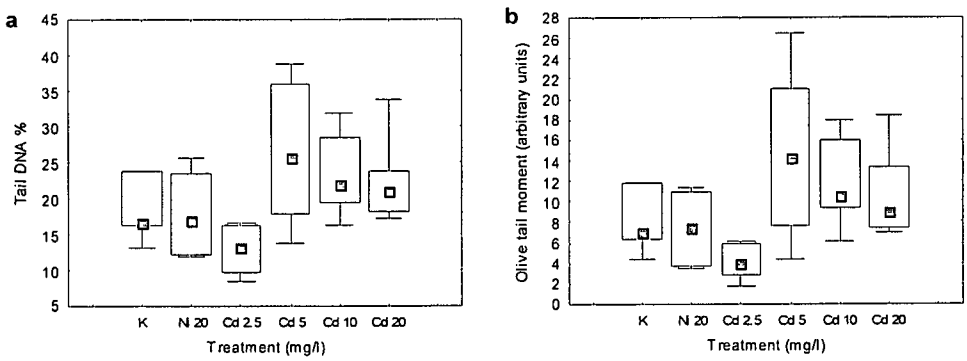

Figure 3.1: Results of the comet assay done on coelomocytes of *A. diffringens* exposed to Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. K = negative control; Ni 20 = positive control (20 mg/l Ni). The numbers of animals used were: $n = 5$ for K, $n = 4$ for Ni 20, $n = 6$ for Cd 2.5, $n = 8$ for Cd 5, $n = 7$ for Cd 10 and $n = 8$ for Cd 20.

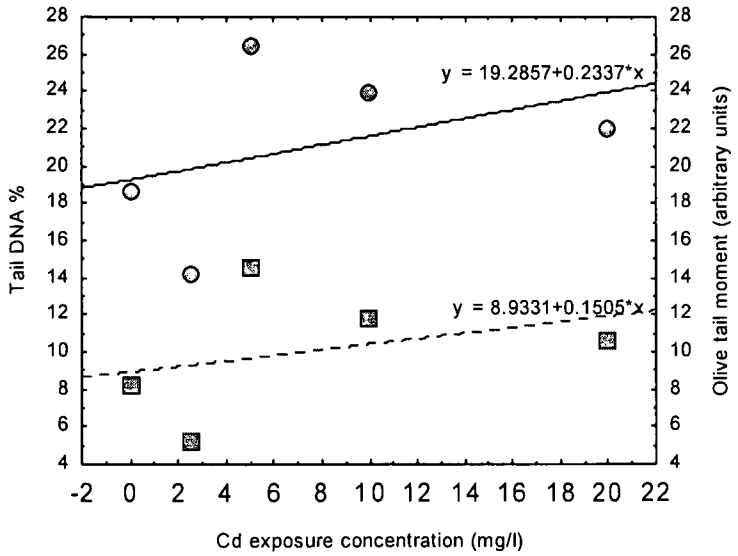

Figure 3.2: DNA damage as measured with the comet assay in *A. diffringens* exposed to Cd in artificial soil water. Mean values are for Tail DNA % (closed circles ○, solid line) and Olive tail moment (closed squares □, dashed line) for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). The numbers of animals used were: $n = 5$ for the negative control, $n = 6$ for 2.5 mg/l Cd, $n = 8$ for 5 mg/l Cd, $n = 7$ for 10 mg/l Cd and $n = 8$ for 20 mg/l Cd.

For *Aporrectodea caliginosa*, DNA damage as Tail DNA % (Fig 3.3a) did not differ significantly between treatments ($P > 0.05$, Appendix C Table 1), nor did Olive tail moment (Fig 3.3b) differ significantly between treatments ($P > 0.05$, Appendix C Table 1). A significant correlation was found between exposure concentration (mg/l Cd) and mean Tail DNA % (Spearman R = 0.9, $P < 0.05$, Appendix C Table 4). No significant correlation was found between Olive tail moment and exposure concentration (Spearman R = 0.7, $P > 0.05$, Appendix C Table 4), although the correlation coefficient is close to 1 (Spearman R = 0.7) (Fig 3.4).

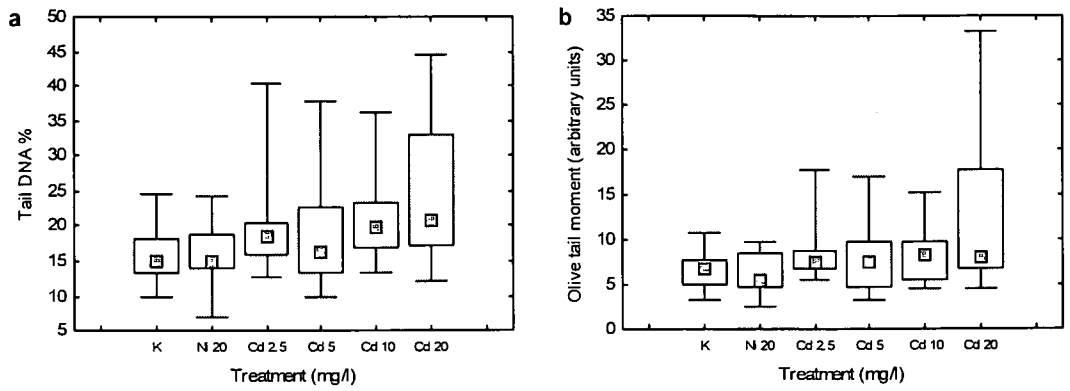

Figure 3.3: Results of the comet assay done on coelomocytes of *A. caliginosa* exposed to Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. K = negative control; Ni 20 = positive control (20 mg/l Ni). The number of animals used (*n*) was 10 for each treatment.

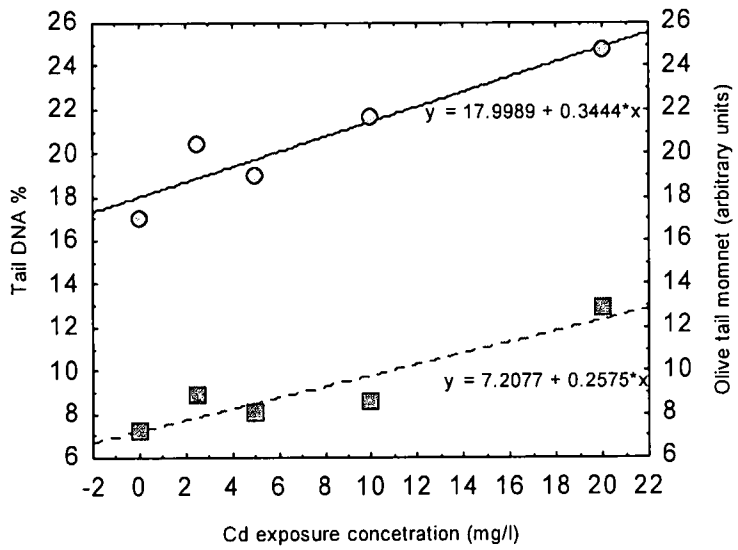

Figure 3.4: DNA damage as measured with the comet assay in *A. caliginosa* exposed to Cd in artificial soil water. Mean values are for Tail DNA % (closed circles ●, solid line) and Olive tail moment (closed squares ■, dashed line) for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). The number of animals used (*n*) was 10 for each treatment.

For *Dendrodrilus rubidus*, DNA damage as Tail DNA % (Fig 3.5a) differed significantly between the negative control and all the other treatments ($P < 0.05$, Appendix C Tables 1 & 2). Exposure concentration 10 mg/l Cd differed significantly from exposure concentrations 5 and 20 mg/l Cd ($P < 0.05$, Appendix C Table 2). For Olive tail moment (Fig 3.5b), the negative control differed significantly from all other treatments ($P < 0.05$, Appendix C Tables 1 & 3). Exposure

concentrations 10 and 5 mg/l Cd differed significantly from each other ($P < 0.05$, Appendix C Table 3). No significant correlation was found between exposure concentration (mg/l Cd) and mean Tail DNA % (Spearman R = 0.8, $P > 0.05$, Appendix C Table 4), although the correlation coefficient is close to 1 (Spearman R = 0.8). A significant correlation was found between Olive tail moment and exposure concentration (Spearman R = 0.9, $P < 0.05$, Appendix C Table 4) (Fig 3.6).

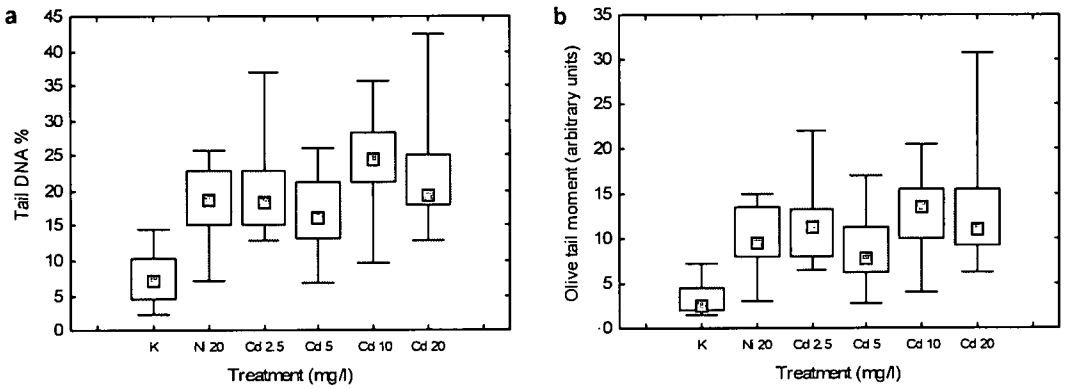

Figure 3.5: Results of the comet assay done on coelomocytes of *D. rubidus* exposed to Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. K = negative control; Ni 20 = positive control (20 mg/l Ni). The number of animals used (*n*) was 10 for each treatment.

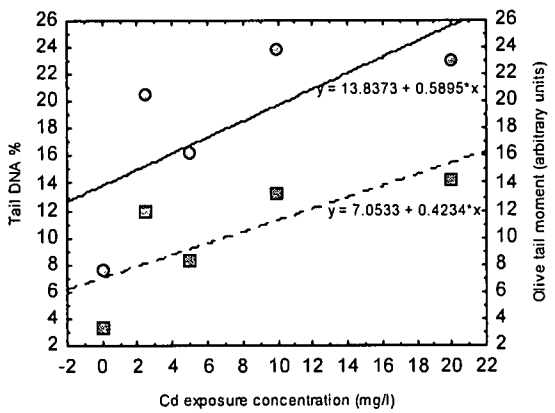

Figure 3.6: DNA damage as measured with the comet assay in *D. rubidus* exposed to Cd in artificial soil water. Mean values are for Tail DNA % (closed circles ●, solid line) and Olive tail moment (closed squares ■, dashed line) for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). The number of animals used (*n*) was 10 for each treatment.

For *E. fetida*, DNA damage as Tail DNA % (Fig 3.7a) differed significantly between the negative control and all the other treatments ($P < 0.05$, Appendix C Tables 1 & 2), except for exposure concentration 2.5 mg/l Cd ($P > 0.05$, Appendix C Table 2). Exposure concentration 20 mg/l Cd differed significantly from all other treatments ($P < 0.05$, Appendix C Table 2). For Olive tail moment (Fig 3.7b), the negative control differed significantly from all other treatments ($P < 0.05$, Appendix C Tables 1 & 3), except for exposure concentration 2.5 mg/l ($P > 0.05$, Appendix C Table 3). Exposure concentration 2.5 mg/l Cd differed significantly from all other treatments ($P < 0.05$, Appendix C Table 3), except for the negative control. Exposure concentration 20 mg/l Cd differed significantly from all other treatments ($P < 0.05$, Appendix C Table 3). A significant correlation was found between exposure concentration (mg/l Cd) and mean Tail DNA % (Spearman R = 0.9, $P < 0.05$, Appendix C Table 4). A significant correlation was found between Olive tail moment and exposure concentration (Spearman R = 1, Appendix C Table 4) (Fig 3.8).

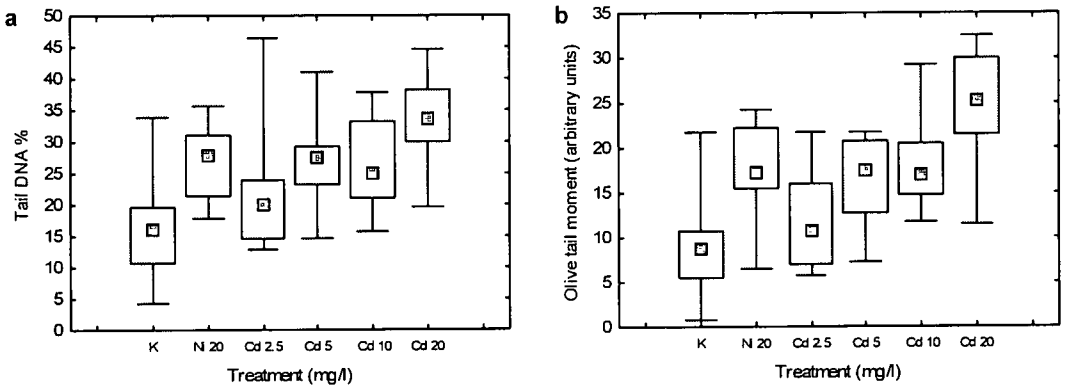

Figure 3.7: Results of the comet assay done on coelomocytes of *E. fetida* exposed to Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. K = negative control; Ni 20 = positive control (20 mg/l Ni). The number of animals used (n) was 10 for each treatment.

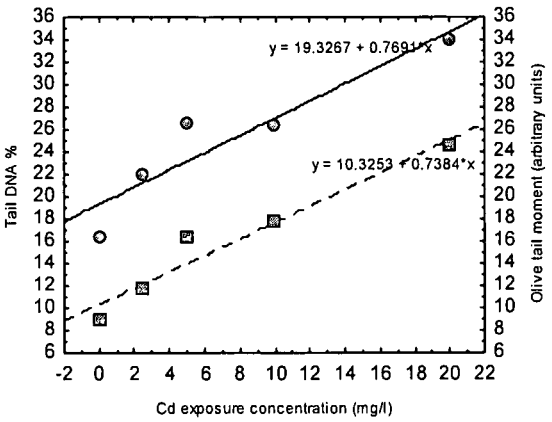

Figure 3.8: DNA damage as measured with the comet assay in *E. fetida* exposed to Cd in artificial soil water. Mean values are for Tail DNA % (closed circles ○, solid line) and Olive tail moment (closed squares □, dashed line) for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). The number of animals used (*n*) was 10 for each treatment.

For *Microchaetus benhami*, DNA damage as Tail DNA % (Fig 3.9a) did not differ significantly between treatments ($P > 0.05$, Appendix C Table 1), nor did Olive tail moment (Fig 3.9b) differ significantly between treatments ($P > 0.05$, Appendix C Table 1). No significant correlation was found between exposure concentration (mg/l Cd) and mean Tail DNA % (Spearman R = 0.1, $P > 0.05$, Appendix C Table 4). A significant correlation was found between Olive tail moment and exposure concentration (Spearman R = 0.9, $P < 0.05$, Appendix C Table 4) (Fig 3.10).

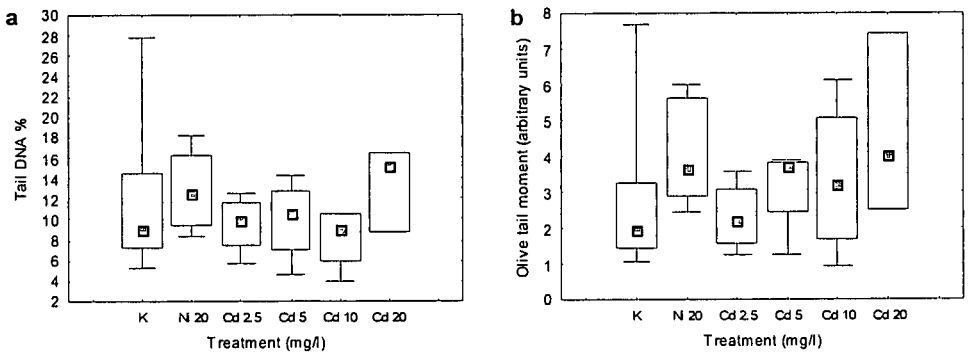

Figure 3.9: Results of the comet assay done on coelomocytes of *M. benhami* exposed to Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. K = negative control; Ni 20 = positive control (20 mg/l Ni). The numbers of animals used were: $n = 5$ for K, $n = 7$ for Ni 20, $n = 4$ for Cd 2.5, $n = 4$ for Cd 5, $n = 4$ for Cd 10 and $n = 3$ for Cd 20.

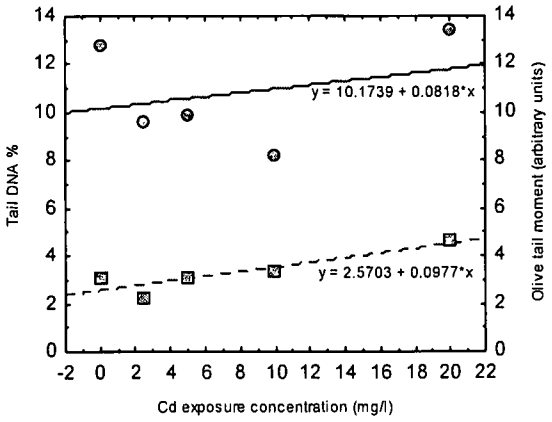

Figure 3.10: DNA damage as measured with the comet assay in *M. benhami* exposed to Cd in artificial soil water. Mean values are for Tail DNA % (closed circles ●, solid line) and Olive tail moment (closed squares ■, dashed line) for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). The numbers of animals used were: $n = 5$ for the negative control, $n = 4$ for 2.5 mg/l Cd, $n = 4$ for 5 mg/l Cd, $n = 4$ for Cd 10 and $n = 3$ for 20 mg/l Cd.

3.1.1.2 Effect of earthworm body size

For *A. diffringens*, no significant correlation was found in any of the treatments for earthworm mass and either Tail DNA % ($P > 0.05$, Appendix C Table 9) or Olive tail moment ($P > 0.05$, Appendix C Table 9). In *A. caliginosa*, no significant correlation was found in any of the treatments for earthworm mass and either Tail DNA % ($P > 0.05$, Appendix C Table 9) or Olive tail moment ($P > 0.05$, Appendix C Table 9). For *D. rubidus*, a significant correlation was found in exposure concentration 10 mg/l Cd between earthworm mass and Tail DNA % (Spearman R = 0.7455 $P < 0.05$, Appendix C Table 9) and Olive tail moment (Spearman R = 0.8424 $P < 0.05$, Appendix C Table 9) Figure 3.11 illustrates, as an example, the relationship between mass and DNA damage for exposure concentration 10 mg/l Cd for *D. rubidus*. In *E. fetida*, a significant correlation was found between earthworm mass and Tail DNA % in the negative control (Spearman R = -0.6606 $P < 0.05$, Appendix C Table 9) and in exposure concentration 10 mg/l Cd (Spearman R = -0.6848 $P < 0.05$, Appendix C Table 9). Figure 3.12 illustrates the relationship between mass and Tail DNA % for the negative control and exposure concentration 10 mg/l Cd for *E. fetida*. For *M. benhami*, a significant correlation was found between earthworm mass and Tail DNA % in exposure concentration 20 mg/l Cd. (Spearman R = -1, Appendix C Table 9). A significant correlation was found between earthworm mass and Olive tail moment in exposure concentration 2.5 mg/l Cd (Spearman R = -1, Appendix C Table 9). Although not significant, the

correlation coefficient for earthworm mass and Tail DNA % in exposure concentration 2.5 mg/l Cd is close to -1 (Spearman R = -0.8, Appendix C Table 9). Figure 3.13 illustrates the relationship between earthworm mass and Tail DNA % in exposure concentrations 2.5 and 20 mg/l Cd and Olive tail moment in exposure concentration 2.5 and 20 mg/l Cd.

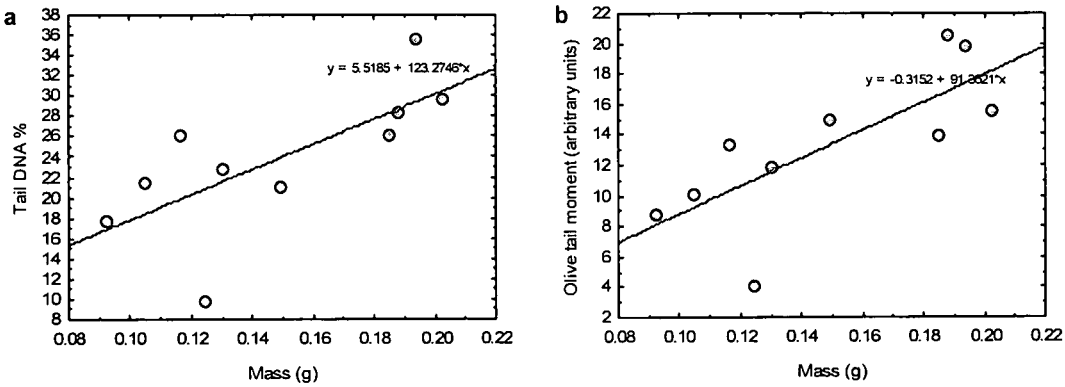

Figure 3.11: DNA damage as measured with the comet assay in *D. rubidus* exposed to 10 mg/l Cd in artificial soil water. Mean values for each individual for (a) Tail DNA % and (b) Olive tail moment plotted against body mass. The number of animals used (*n*) was 10.

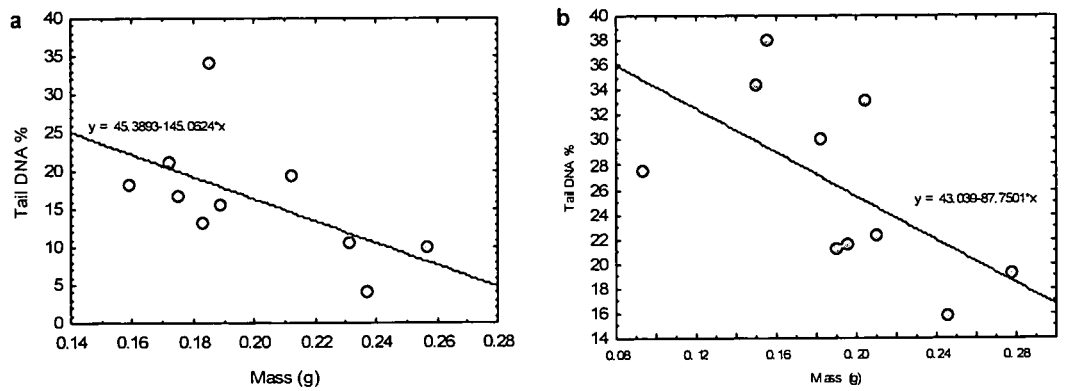

Figure 3.12: DNA damage (mean Tail DNA %) as measured with the comet assay in *E. fetida* plotted against body mass for each individual in (a) the negative control (0 mg/l Cd) and (b) exposed to 10 mg/l Cd in artificial soil water. The number of animals used (*n*) was 10.

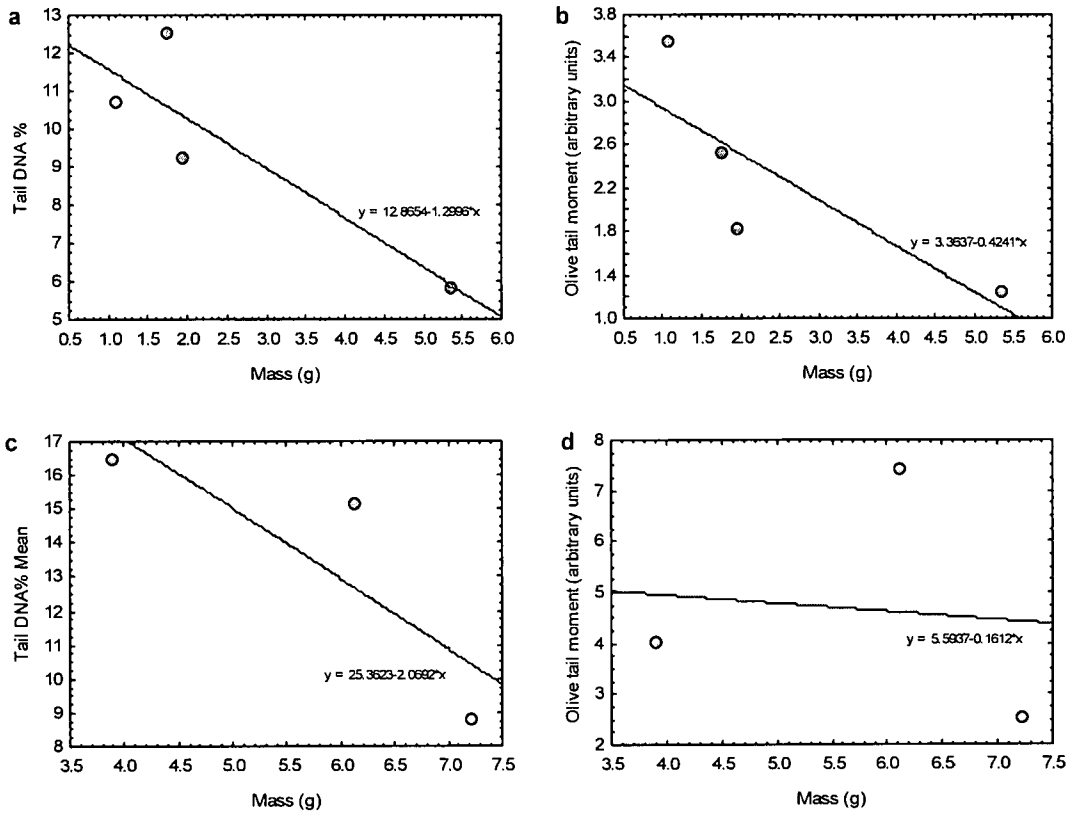

Figure 3.13: DNA damage as measured with the comet assay in *M. benhami* exposed to Cd in artificial soil water. Mean values per individual plotted against body mass for (a) Tail DNA % in exposure concentration 2.5 mg/l Cd, (b) Olive tail moment in exposure concentration 2.5 mg/l Cd, (c) Tail DNA % in exposure concentration 20 mg/l Cd, and (d) Olive tail moment in exposure concentration 20 mg/l Cd. The number of animals used were: $n = 4$ for 2.5 mg/l Cd and $n = 3$ for 20 mg/l Cd.

3.1.2 Metal accumulation patterns within species

In *A. diffringens*, all earthworms from the negative control and the Cd exposure concentrations contained Cd (Fig 3.14a, Appendix B Table 3). The negative control differed significantly from all the Cd exposures ($P < 0.05$, Appendix C Tables 5 & 6). Exposure concentration 20 mg/l Cd differed significantly from exposure concentrations 5 and 10 mg/l Cd ($P < 0.05$, Appendix C Table 6). No significant correlation was found between the mean Cd body load per treatment and the Cd exposure concentration (Spearman R = 0.6 $P > 0.05$, Appendix C Table 7, Fig 3.14b). No significant correlation was found between earthworm mass and Cd body load in any of the treatments ($P > 0.05$, Appendix C Table 8). No significant correlation was found between Cd body load and DNA damage for either Tail DNA % or Olive tail moment ($P > 0.05$, Appendix C Table 10) in any of the treatments.

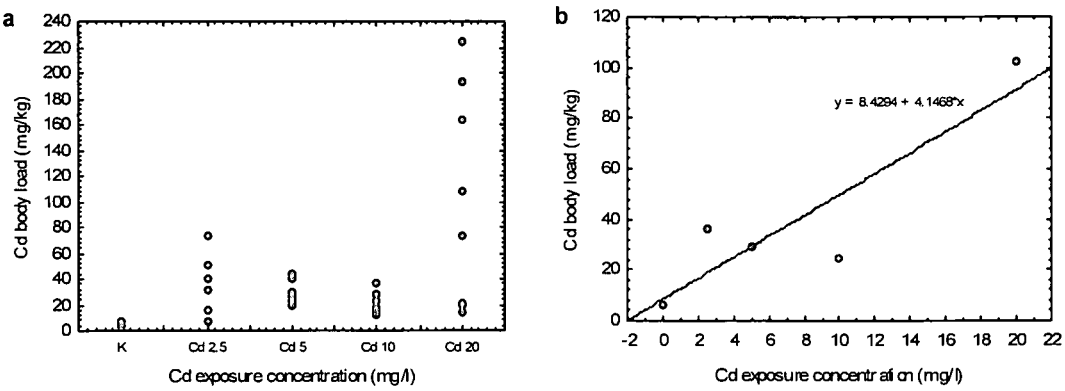

Figure 3.14: Cadmium body loads (mg/kg) for *A. diffringens* exposed to Cd in artificial soil water. (a) Cd body loads for individual earthworms are given per concentration. K = negative control. (b) Mean body load values for each treatment are plotted against exposure concentration for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). For both (a) and (b), the numbers of animals used were: $n = 5$ for K, $n = 6$ for Cd 2.5, $n = 8$ for Cd 5, $n = 7$ for Cd 10 and $n = 8$ for Cd 20.

In *A. caliginosa*, seven (out of 10) worms contained Cd in the negative control, and all worms from the Cd exposure concentrations contained Cd (Fig 3.15a, Appendix B Table 4). The negative control differed significantly from all other treatments ($P < 0.05$, Appendix C Tables 5 & 6). No significant correlation was found between the mean Cd body load per treatment and the Cd exposure concentration (Spearman R = 0.7 $P > 0.05$, Appendix C Table 7, Fig 3.15b), although the correlation coefficient is close to 1. A significant correlation was found between earthworm mass and Cd body load in exposure concentration 20 mg/l Cd ($P < 0.05$, Appendix C Table 8, Fig 3.15c). A significant correlation was found between Cd body load and DNA damage for Tail DNA % in the negative control (Spearman R = 0.8037 $P < 0.05$, Appendix C Table 10), but not in any of the other treatments. No significant correlation was found between Cd body load and DNA damage for Olive tail moment ($P > 0.05$, Appendix C Table 10) in any of the treatments.

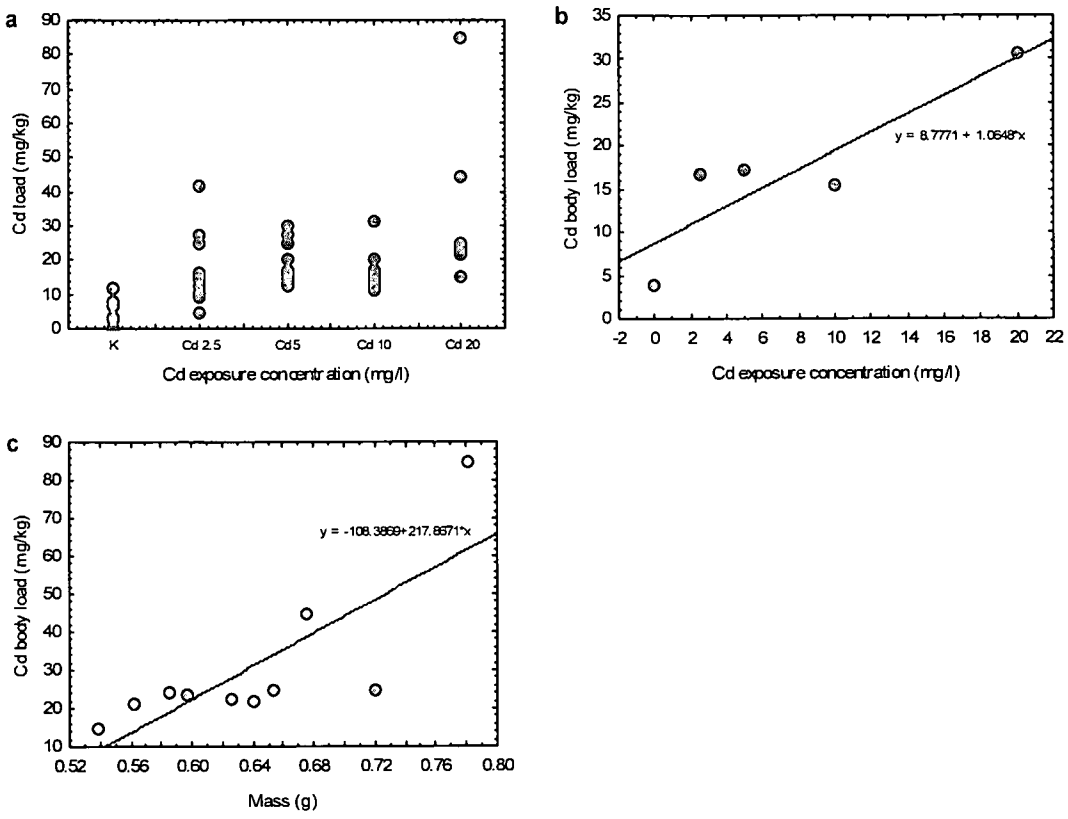

Figure 3.15: Cadmium body loads (mg/kg) for *A. caliginosa* exposed to Cd in artificial soil water. (a) Cd body loads for individual earthworms are given per concentration. K = negative control. (b) Mean body load values for each treatment are plotted against exposure concentration for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). For both (a) and (b), the number of animals used (*n*) was 10 in all treatments. (c) Cd body loads for individual earthworms plotted against their mass for exposure concentration 20 mg/l Cd; $n = 10$.

In *D. rubidus*, not all individuals accumulated Cd in the negative control as well as the Cd exposures (Fig 3.16a, Appendix B Table 5). The negative control differed significantly from exposure concentrations 10 and 20 mg/l Cd ($P < 0.05$, Appendix C Tables 5 & 6). Exposure concentration 20 mg/l Cd differed significantly from exposure concentrations 5 and 10 mg/l Cd ($P < 0.05$, Appendix C Table 6). A significant correlation was found between the mean Cd body load per treatment and the Cd exposure concentration (Spearman R = 0.9 $P < 0.05$, Appendix C Table 7, Fig 3.16b). A significant correlation was found between earthworm mass and Cd body load in exposure concentration 5 mg/l Cd (Spearman R = 0.7511 $P < 0.05$, Appendix C Table 8, Fig 3.16c). A significant correlation was found between Cd body load and DNA damage for Tail DNA % (Spearman R = -0.6869 $P < 0.05$, Appendix C Table 10, Fig 3.16d) in exposure concentration 10 mg/l Cd.

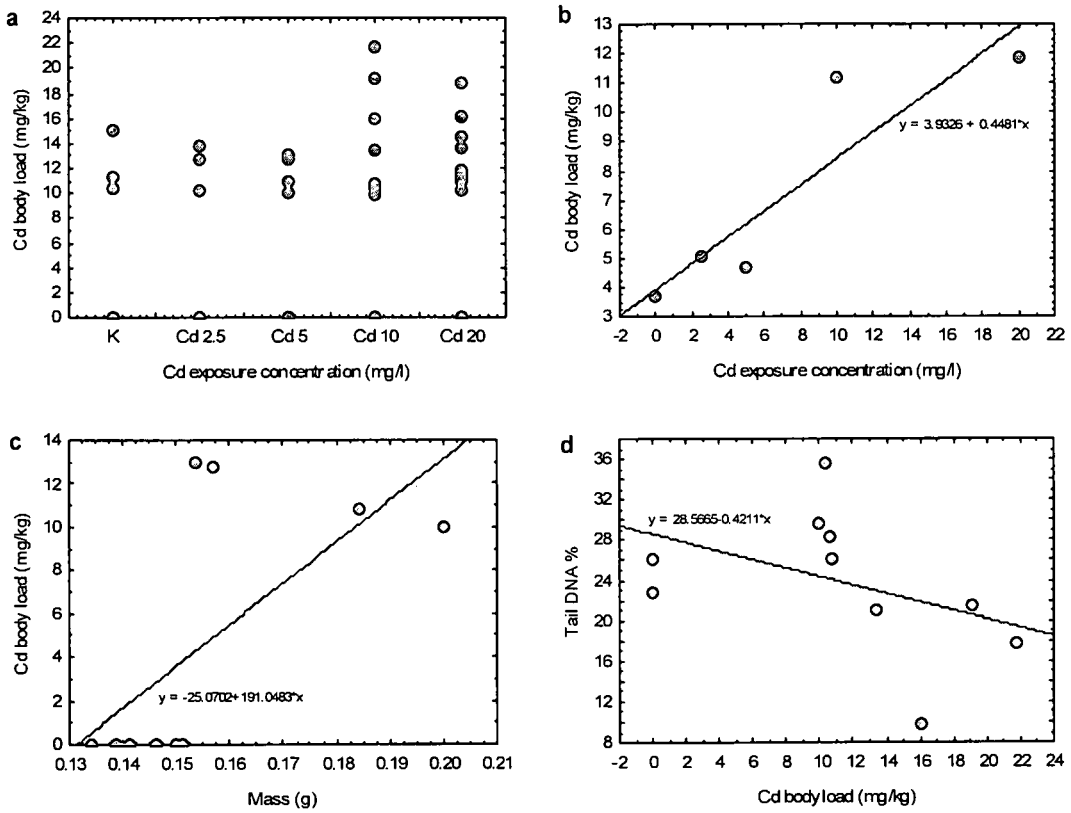

Figure 3.16: Cadmium body loads (mg/kg) for *D. rubidus* exposed to Cd in artificial soil water. (a) Cd body loads for individual earthworms are given per concentration. K = negative control. (b) Mean body load values for each treatment are plotted against exposure concentration for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). (c) Cd body loads for individual earthworms plotted against mass for exposure concentration 5 mg/l Cd. (d) Mean values of DNA damage per animal as measured with comet assay parameter Tail DNA % plotted against their Cd body loads for exposure concentration 10 mg/l Cd. For all treatments, the number of animals used (*n*) was 10.

In *E. fetida*, none of the individuals in the negative control and in exposure concentration 5 mg/l Cd were found to contain Cd (Figure 3.17a, Appendix B Table 6). Three animals exposed to 2.5 mg/l Cd accumulated Cd, and three animals in exposure concentration 10 mg/l Cd. Only five animals were analyzed for metal content in exposure concentration 20 mg/l Cd, and all were found to contain Cd. Exposure concentration 20 mg/l differed significantly from the negative control and exposure concentration 5 mg/l Cd ($P < 0.05$, Appendix C Tables 5 & 6). No significant correlation was found between the mean Cd body load per treatment and the Cd exposure concentration (Spearman R = 0.6156 $P > 0.05$, Appendix C Table 7, Fig 3.17b). A significant correlation was found between earthworm mass and Cd body load in exposure

concentration 10 mg/l Cd (Spearman R = 0.6828 $P < 0.05$, Appendix C Table 8, Fig 3.17c). A significant negative correlation was found in exposure concentration 10 mg/l Cd between Cd body load and DNA damage for Tail DNA % (Spearman R = -0.7442 $P < 0.05$, Appendix C Table 10, Fig 3.17d) and Olive tail moment (Spearman R = -0.7442 $P < 0.05$, Appendix C Table 10, Fig 3.17d).

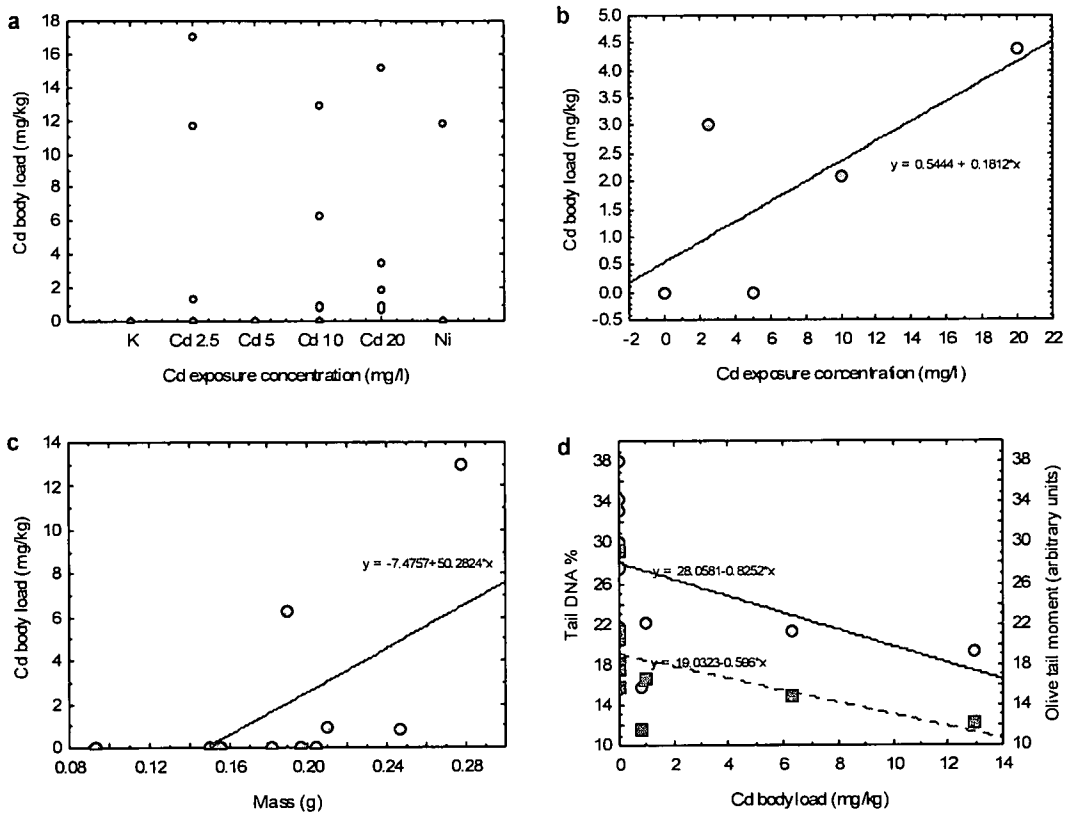

Figure 3.17: Cadmium body loads (mg/kg) for *E. fetida* exposed to Cd in artificial soil water. (a) Cd body loads for individual earthworms are given per concentration. K = negative control. (b) Mean body load values for each treatment are plotted against exposure concentration for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). (c) Cd body loads for individual earthworms plotted against mass for exposure concentration 10 mg/l Cd. (d) Mean values of DNA damage per animal as measured with comet assay parameters Tail DNA % (closed circles ●, solid line) and Olive tail moment (closed squares ■, dashed line) plotted against their Cd body loads for exposure concentration 10 mg/l Cd. For all treatments, the number of animals used (*n*) was 10.

In *M. benhami*, two of 5 animals in the negative control were found to contain Cd (Fig 3.18a, Appendix B Table 7). All animals from all the Cd exposure concentrations were found to contain Cd. The negative control differed significantly from all the Cd exposure concentrations ($P < 0.05$,

Appendix C Tables 5 & 6). A significant correlation was found between the mean Cd body load per treatment and the Cd exposure concentration (Spearman R = 0.9 $P < 0.05$, Appendix C Table 7, Fig 3.18b). No significant correlation was found between earthworm mass and Cd body load in any of the Cd exposures or the negative control ($P > 0.05$, Appendix C Table 8). A significant correlation was found in the negative control between Cd body load and DNA damage for Tail DNA % (Spearman R = 0.8944 $P < 0.05$, Appendix C Table 10, Fig 3.18c) and Olive tail moment (Spearman R = 0.8944 $P < 0.05$, Appendix C Table 10, Fig 3.18c). A significant correlation was found in exposure concentration 2.5 mg/l Cd between Cd body load and DNA damage for Tail DNA % (Spearman R = 1, Appendix C Table 10, Fig 3.18d), but not for Olive tail moment, although the correlation coefficient is close to 1 (Spearman R = 0.8 $P > 0.05$, Appendix C Table 10, Fig 3.18d).

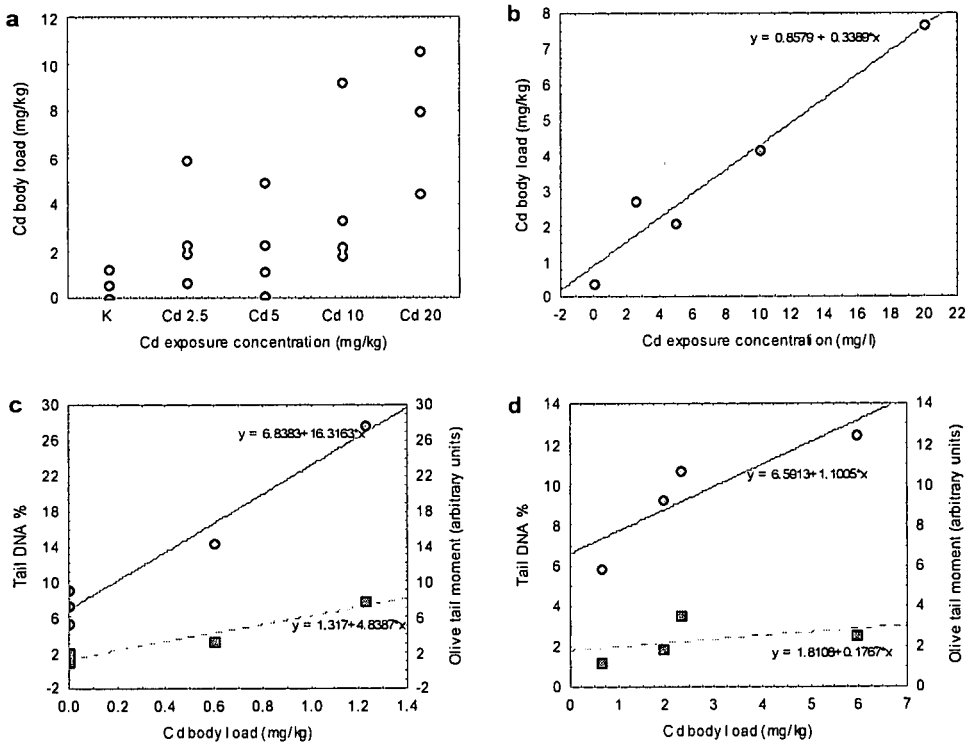

Figure 3.18: Cadmium body loads (mg/kg) for *M. benhami* exposed to Cd in artificial soil water. (a) Cd body loads for individual earthworms are given per concentration. K = negative control. (b) Mean body load values for each treatment are plotted against exposure concentration for the negative control (0 mg/l Cd) and four Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). (c) Mean values of DNA damage for the negative control per animal as measured with comet assay parameters Tail DNA % (closed circles ○, solid line) and Olive tail moment (closed squares □, dashed line) plotted against their Cd body loads. (d) Mean values of DNA damage for exposure concentration 2.5 mg/l Cd per animal as measured with comet assay parameters Tail DNA % (closed circles ○, solid line) and Olive tail moment (closed squares □, dashed line) plotted against their Cd body loads. The numbers of animals used were: $n = 5$ for the negative control, $n = 4$ for 2.5 mg/l Cd, $n = 4$ for 5 mg/l Cd, $n = 4$ for Cd 10 and $n = 3$ for 20 mg/l Cd.

3.2 Patterns between species

3.2.1 Species sensitivity differences

Species differed significantly from each other with the comet parameters Tail DNA % and Olive tail moment when compared to each other in every treatment (Fig 3.19, $P < 0.05$, Appendix C Table 11). For the negative control, damage in all species were similar, except for *D. rubidus* which had significantly lower Tail DNA % values than the other species except *M. benhami* (Table 12, Appendix C). For Olive tail moment, both *D. rubidus* and *M. benhami* showed significantly lower levels of damage than the other species (Table 12, Appendix C). Exposure concentration 20 mg/l Cd was taken as representative to compare species differences in DNA damage, because DNA damage differed significantly from the control in this exposure concentration for most species (Tables 2 and 3, Appendix C), indicating that damage did occur at this exposure concentration and is therefore the most appropriate to use for inter-species comparisons. For Tail DNA %, *E. fetida* differed significantly from all other species ($P < 0.05$, Appendix C Table 12, Fig 3.20) and *M. benhami* differed significantly from all other species ($P < 0.05$, Appendix C Table 12), except from *D. rubidus* ($P > 0.05$, Appendix C Table 12). For Olive tail moment, *E. fetida* and *M. benhami* differed significantly from all other species ($P < 0.05$, Appendix C Table 12).

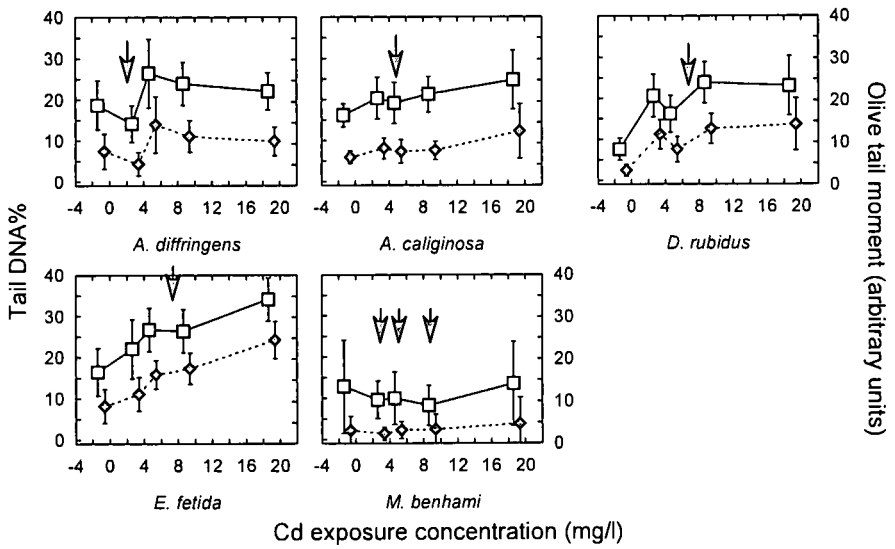

Figure 3.19: Results of comet assay on coelomocytes of five earthworm species exposed to Cd in artificial soil water. Exposures are the negative control (0 mg/l Cd) and a range of Cd concentrations (2.5, 5, 10 and 20 mg/l Cd). DNA damage is Tail DNA % (open squares □, solid line) and Olive tail moment (closed diamonds ♦, dashed line). Open squares and solid diamonds are means and whiskers are 95% confidence intervals. Arrows indicate lower levels of damage than expected in a normal linear or sigmoidal dose-response relationship. Refer to Appendix B Tables 3 to 7 for number of comets from each animal; Table 8 for number of animals analyzed in each species.

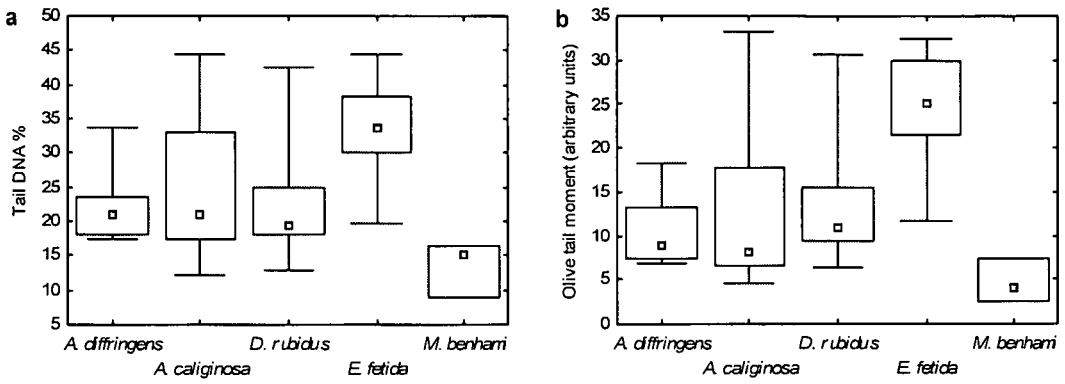

Figure 3.20: Results of the comet assay done on coelomocytes of five earthworm species exposed to 20 mg/l Cd in artificial soil water. DNA damage is depicted by (a) Tail DNA % and (b) Olive tail moment. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. The numbers of animals used were: $n = 8$ for *A. diffringens*, $n = 10$ for *A. caliginosa*, *D. rubidus*, *E. fetida* and $n = 3$ for *M. benhami*.

As previously mentioned, some baseline level of DNA damage is to be expected, because DNA integrity in living cells are constantly changing as a result of natural processes (Shugart 2000). Therefore, the mean Tail DNA % and Olive tail moment values for all the animals in the negative control are considered in the present study as the baseline level of DNA damage in each species. Thus the mean Tail DNA % and Olive tail moment values of the negative control were subtracted from the mean Tail DNA % and Olive tail moment values in all the Cd exposure concentrations (Appendix B Table 8). These "net" levels of DNA damage were compared between species based on the mean values from exposure concentration 20 mg/l and shown in Fig 3.21. *E. fetida* was found to be the most sensitive, followed by *D. rubidus*, *A. caliginosa*, *A. diffringens* and *M. benhami*. The net values for all the Cd exposure concentrations were plotted against Cd treatment concentration and regression equations obtained (Fig 3.22). The gradients were compared, and it was found that *E. fetida* had the steepest gradient (0.6152) for Tail DNA %, followed by *A. caliginosa*, (0.2933) *D. rubidus*, (0.2627) *M. benhami* (0.0415) and *A. diffringens* (0.0287). For Olive tail moment, the same order persisted, with *E. fetida* having the steepest gradient (0.6657), followed by *A. caliginosa*, (0.2569) *D. rubidus*, (0.2318) *M. benhami* (0.094) and *A. diffringens* (-0.0033).

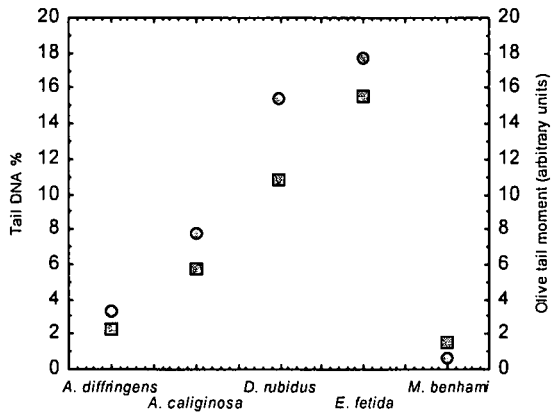

Figure 3.21: Results of the comet assay done on coelomocytes of five earthworm species exposed to 20 mg/l Cd in artificial soil water. Net* DNA damage is depicted by Tail DNA % (closed circles ●) and Olive tail moment (closed squares ■). The numbers of animals used were: $n = 8$ for *A. diffringens*, $n = 10$ for *A. caliginosa*, *D. rubidus*, *E. fetida* and $n = 3$ for *M. benhami*.

* Net DNA damage values are obtained by subtracting the mean Tail DNA % and Olive tail moment values in the negative control from the corresponding values in the exposure treatment.

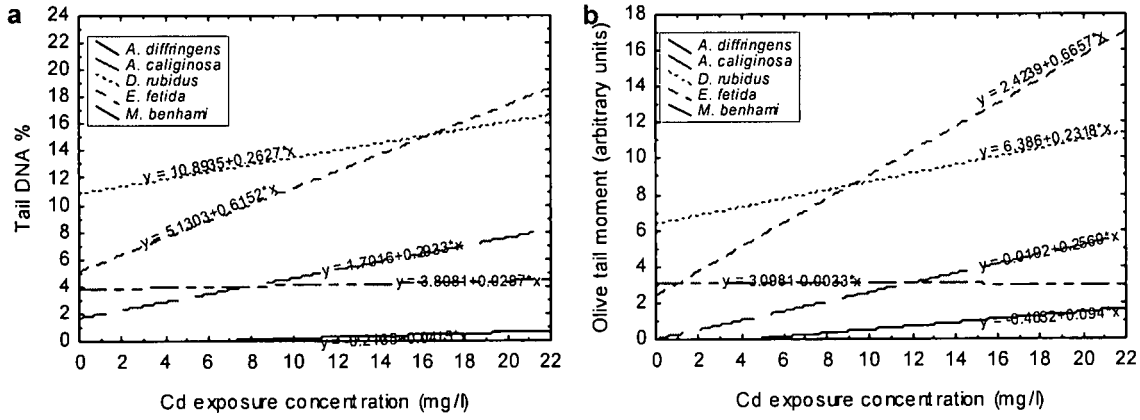

Figure 3.22: Net* DNA damage as measured by the comet assay in five earthworm species exposed to Cd in artificial soil water. Mean values of (a) Tail DNA % and (b) Olive tail moment for each exposure concentration in each species are plotted against the Cd exposure concentration (2.5, 5, 10 and 20 mg/l Cd). The earthworm species are *A. diffringens* (finely dotted line), *A. caliginosa* (large dashed line), *D. rubidus* (dashed line), *E. fetida* (dotted line) and *M. benhami* (solid line). Refer to Appendix B Tables 3 to 7 for the number of comets assayed in each animal, and Table 8 for the number of animals analyzed in each species.

* Net DNA damage values are obtained by subtracting the mean Tail DNA % and Olive tail moment values in the negative control from the corresponding values in the exposure treatment.

3.2.2 Species differences in DNA content of coelomocytes

Head areas from comets without tails were measured in the negative controls for each species. These are considered as the "normal" state where no DNA damage has taken place, and were therefore used as a surrogate of nucleus size and therefore DNA amount in coelomocytes. These nucleus areas differed significantly between all species ($P < 0.05$, Appendix C Tables 13 & 14, Fig 3.23). No significant correlation was found between species nucleus areas and net DNA damage (net values for Tail DNA % and Olive tail moment, obtained as described in the previous paragraph) for any of the Cd exposure concentrations (Appendix C Table 15). As an example, Fig 3.24 illustrates the relationship between species nucleus area and net DNA damage in exposure concentration 20 mg/l Cd.

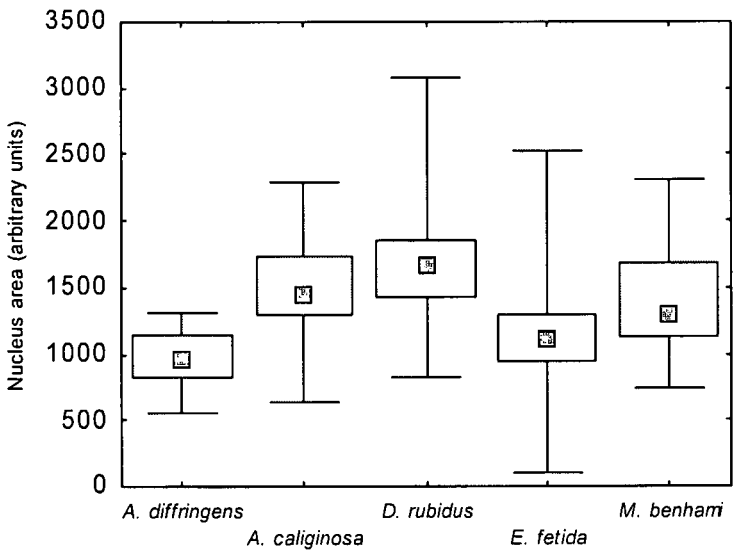

Figure 3.23: Cell nucleus areas* of five earthworm species. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. The numbers of comets analyzed were: $n = 28$ for *A. diffringens*, $n = 109$ for *A. caliginosa*, $n = 581$ for *D. rubidus*, $n = 169$ for *E. fetida* and $n = 118$ for *M. benhami*.

* The head areas of comets without tails represent cell nucleus areas.

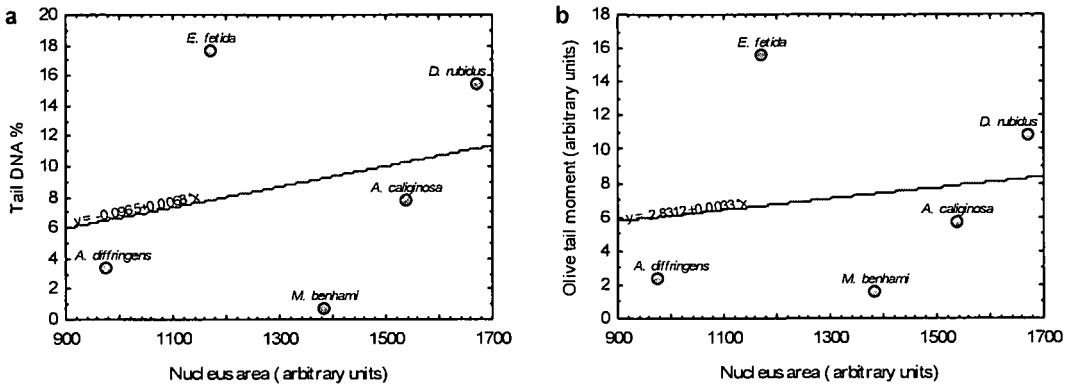

Figure 3.24: Net* DNA damage as measured by the comet assay in five earthworm species exposed to 20 mg/l Cd in artificial soil water plotted against their respective nucleus areas**. Mean values are given for (a) Tail DNA % and (b) Olive tail moment for exposure concentration 20 mg/l Cd. The numbers of animals used were: n = 8 for *A. diffringens*, n = 10 for *A. caliginosa*, *D. rubidus*, *E. fetida* and n = 3 for *M. benhami*.

* Net DNA damage values are obtained by subtracting the mean Tail DNA % and Olive tail moment values in the negative control from the corresponding values in the exposure treatment 20 mg/l Cd.

** The head areas of comets without tails represent cell nucleus areas.

3.2.3 Species differences in body size

All species differed significantly from each other in their body mass (Fig 3.25, $P < 0.05$, Appendix C Tables 16 and 17), except for *A. diffringens* and *A. caliginosa* ($P > 0.05$, Appendix C, Table 17). A significant correlation was found between species mass and net DNA damage (as obtained by the method described above) for Tail DNA % and Olive tail moment in all exposure concentrations ($P < 0.05$, Appendix C Table 18), except for exposure treatment 5 mg/l Cd ($P > 0.05$, Appendix C Table 18). As an example, Fig 3.26 illustrates the relationship between species mass and net DNA damage in exposure concentration 20 mg/l Cd. No significant correlation was found between body size and Cd body load (Appendix C Table 22).

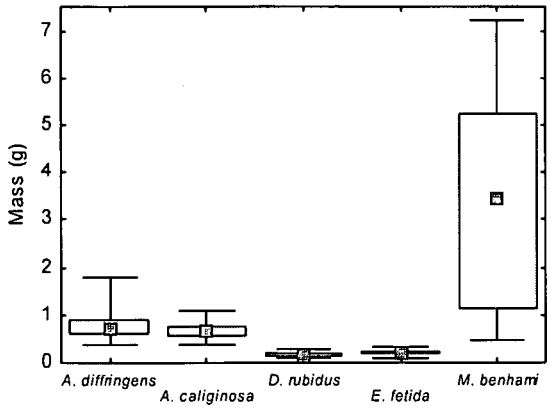

Figure 3.25: Body mass of five earthworm species. To illustrate variation in the data, boxes represent 50% of data, whiskers represent minima and maxima, and solid squares represent medians. The numbers of animals used were: *n* = 40 for *A. diffringens*, *n* = 60 for *A. caliginosa* and *D. rubidus*, *n* = 55 for *E. fetida* and *n* = 27 for *M. benhami*.

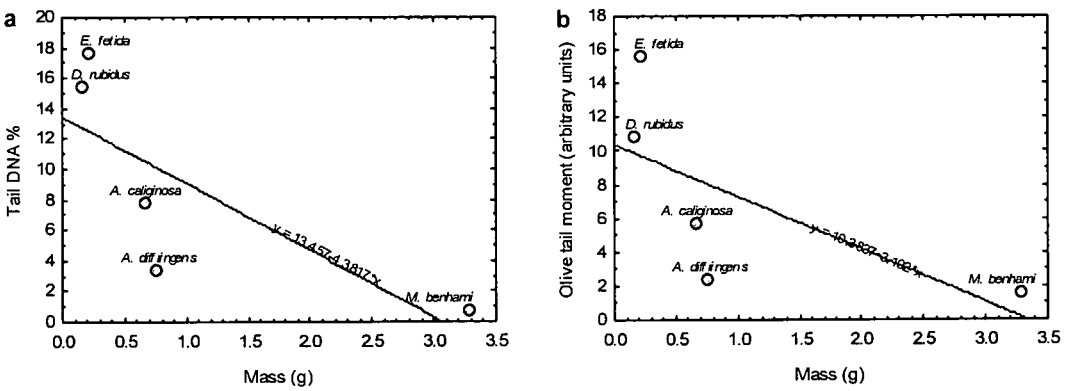

Figure 3.26: Net* DNA damage as measured by the comet assay in five earthworm species exposed to 20 mg/l Cd in artificial soil water plotted against their respective body masses. Mean values are given for (a) Tail DNA % and (b) Olive tail moment for exposure concentration 20 mg/l Cd. The numbers of animals used were: *n* = 8 for *A. diffringens*, *n* = 10 for *A. caliginosa*, *D. rubidus*, *n* = 5 for *E. fetida* and *n* = 3 for *M. benhami*.

* Net DNA damage values are obtained by subtracting the mean Tail DNA % and Olive tail moment values in the negative control from the corresponding values in the exposure treatment 20 mg/l Cd.

3.2.4 Species differences in metal accumulation

Cadmium body loads differed significantly between species (Fig 3.27, $P < 0.05$, Appendix C Table 19). Exposure concentration 20 mg/l Cd was taken as representative to compare species differences in Cd body loads. *A. diffringens* differed significantly from all species ($P < 0.05$, Appendix C Table 20) except for *A. caliginosa* ($P > 0.05$, Appendix C Table 20). *A. caliginosa* also differs significantly from the rest of the species ($P < 0.05$, Appendix C Table 20). No significant correlations were found between Cd body load and net DNA damage (Tail DNA %

and Olive tail moment) in any of the Cd exposure concentrations ($P > 0.05$, Appendix C Table 21). Exposure concentration 20 mg/l Cd was taken as representative to illustrate the relationship between Cd body load and net DNA damage (Fig 3.28).

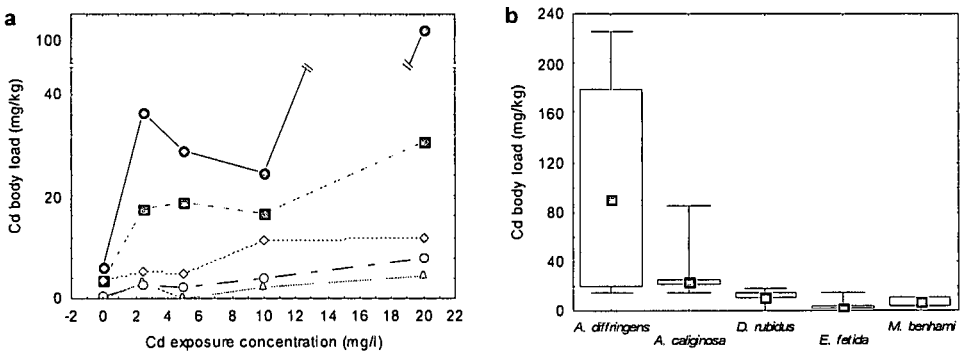

Figure 3.27: Cadmium body loads (mg/kg) for five earthworm species exposed to Cd in artificial soil water. (a) Cd body loads for the negative control and Cd exposure concentrations (2.5, 5, 10 and 20 mg/l Cd). Refer to Appendix B Table 8 for the number of animals analyzed in each species. (b) Body loads for the 20 mg/l Cd exposure concentration. The numbers of animals used were: $n = 8$ for *A. diffringens*, $n = 10$ for *A. caliginosa*, *D. rubidus*, $n = 5$ for *E. fetida* and $n = 3$ for *M. benhami*. For (a), the earthworm species are *A. diffringens* (closed circles ○, finely dotted line), *A. caliginosa* (closed squares ▫, large dashed line), *D. rubidus* (open diamonds ◊, dashed line), *E. fetida* (open triangles Δ, dotted line) and *M. benhami* (open circles ○, solid line).

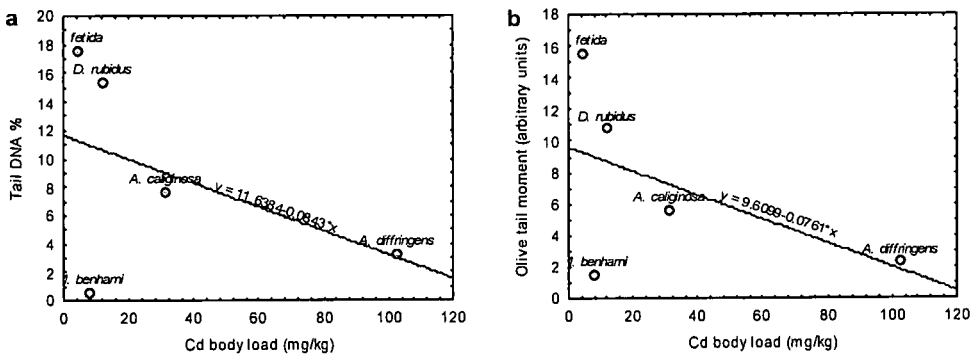

Figure 3.28: Net* DNA damage as measured by the comet assay in five earthworm species exposed to 20 mg/l Cd in artificial soil water plotted against their Cd body loads. Mean values are given for (a) Tail DNA % and (b) Olive tail moment for exposure concentration 20 mg/l Cd. The numbers of animals used were: $n = 8$ for *A. diffringens*, $n = 10$ for *A. caliginosa*, *D. rubidus*, $n = 5$ for *E. fetida* and $n = 3$ for *M. benhami*.

* Net DNA damage values are obtained by subtracting the mean Tail DNA % and Olive tail moment values in the negative control from the corresponding values in the exposure treatment 20 mg/l Cd.

3.2.5 EC50

In order to calculate an EC50, the mean values of Tail DNA % and Olive tail moment in the negative control in each species (Appendix B Table 8) were subtracted from the Tail DNA % and Olive tail moment value in each individual within each species (Appendix B table 11). An EC50 concentration could only be calculated for *E. fetida* (3.706 mg/l Cd) for Tail DNA % and for *A. caliginosa* for Olive tail moment (24.591 mg/l Cd).

3.3 Metal analyses of collecting site soils

Soil samples of collecting sites yielded high concentrations of manganese and zinc (Fig 3.29). Soils from Welgevallen (for *D. rubidus*) and the US botanical garden (for *A. diffringens*) contained higher amounts of copper than Vergenoeg (for *A. caliginosa*) and Middelvlei (for *M. benhami*). All sites contained very low amounts of cadmium.

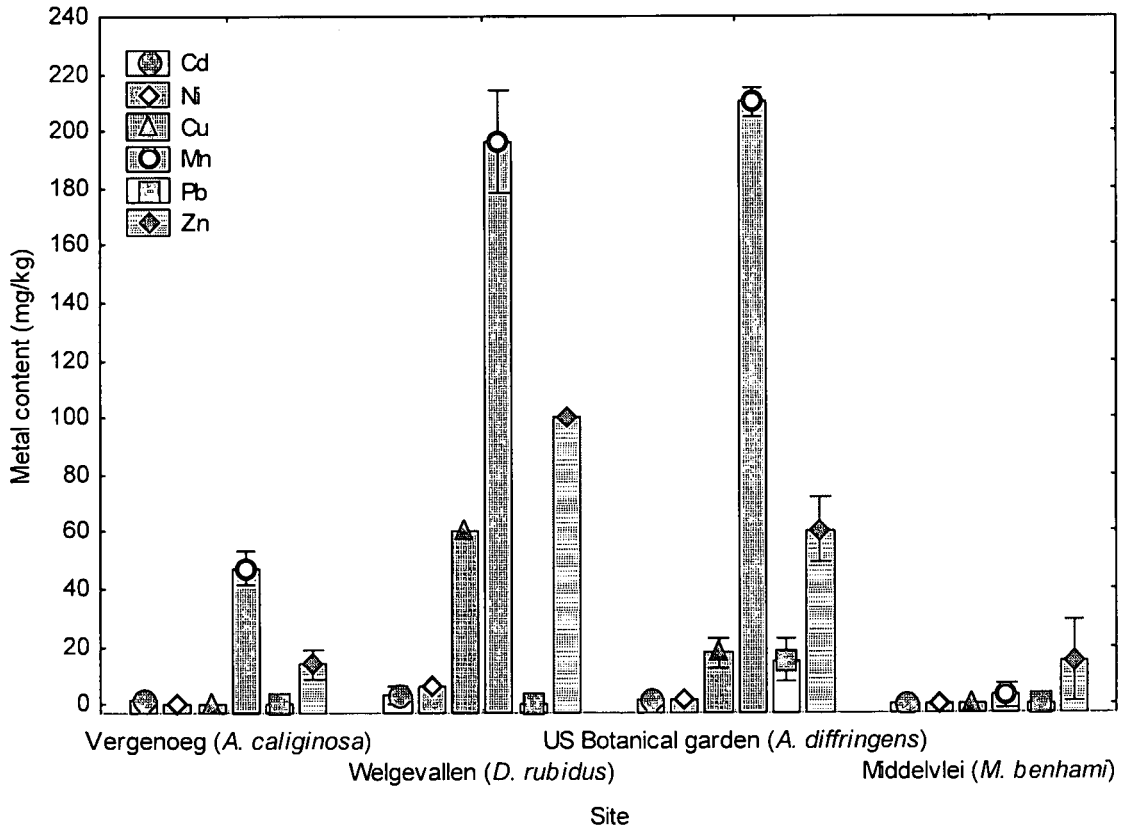

Fig 3.29: Mean metal content of the sampling sites for four earthworm species ($n = 3$ for Welgevallen, Vergenoeg and US botanical garden; $n = 4$ for Middelvlei). Whiskers represent the standard deviation. Where whiskers are not present, standard deviations were zero.

4 Discussion

4.1 DNA damage patterns

4.1.1 Effect of exposure concentration

4.1.1.1 Patterns within species

The results indicated that the five earthworm species showed different DNA damage patterns in relation to Cd exposure concentration. On the whole however, DNA damage, as measured with Tail DNA % and Olive tail moment generally seemed to increase with increasing Cd exposure concentration in all species, except for *Microchaetus benhami* (Fig 3.19).

Significant differences between the negative control and Cd exposure concentrations indicate that Cd induced significant amounts of DNA damage. This was found for *Dendrodrilus rubidus* (Fig 3.5) in all Cd exposure concentrations, and for *Eisenia fetida* (Fig 3.7) in all exposure concentrations except for the lowest concentration (2.5 mg/l Cd). For *Aporrectodea caliginosa* (Fig 3.3) and *M. benhami* (Fig 3.9), the levels of DNA damage induced were not significant in any of the Cd exposure concentrations. For *Amynthas diffringens* (Fig 3.1), DNA damage did not differ between the negative control and the Cd exposure concentrations but the damage in the lowest Cd exposure concentration (2.5 mg/l), were significantly lower than the other Cd exposure concentrations.

Where significant correlations were found between DNA damage and Cd exposure concentration, DNA damage increased consistently with increasing Cd exposure concentration. For the comet parameter Tail DNA %, this was found in *E. fetida* (Fig 3.8) and *A. caliginosa* (Fig 3.4). For Olive tail moment, this was found for *E. fetida* (Fig 3.8), *M. benhami* (Fig 3.10) and *D. rubidus* (Fig 3.6). No such relationships could be found for *A. diffringens* (Fig 3.2).

None of the species showed a clear linear dose-related response between DNA damage and Cd exposure concentration (Fig 3.19). This is in contrast from what is seen in the available literature for other chemicals, where DNA damage in earthworms showed linear increases with increasing doses. Dose-related increases in DNA damage were found in *Aporrectodea longa* by Martin *et al.* (2005) who investigated the genotoxic effects of benzo[*a*]pyrene and lindane in artificial soil on its intestinal and gizzard tissues using the comet assay. Zang *et al.* (2000) conducted the comet assay on coelomocytes of *E. fetida* exposed in solutions of the pesticides Imidacloprid and RH-

5849 in distilled water for 2 hours. Linear dose-effect relationships were found for both pesticides. In a further example, Verschaeve & Gilles (1995) exposed *Lumbricus rubellus* to mitomycin C and x-rays and *E. fetida* to polluted soil from a dumping site (containing e.g. benzene and aniline). For both mitomycin C and x-rays, they found a linear dose-response relationship. They also found higher levels of DNA damage in earthworms exposed to the polluted soils than to the unpolluted control soils. The different results found during the present study can most probably be explained by the fact that a different toxicant having a different mode of action and different pathways within earthworms, was used.

Differences between chemicals with dissimilar modes of toxic action was illustrated by Bierkens *et al.* (1998), who compared 20 bioassays (using 10 species, including earthworms) in both artificial soil as well as aqueous extracts from these soils. They tested for the effects of four toxicants with different modes of toxic action: cadmium, phenol, pentachlorophenol and trifluralin. Tests included acute toxicity, reproduction, weight loss, genotoxicity (comet assay) and immunological tests with coelomocytes in *E. fetida*, as well as various tests on nematodes, springtails, algae and vascular plants. They compared the relative sensitivity of these tests, and found that, for Cd, immunological tests with earthworm coelomocytes (viability, phagocytosis, agglutination, neutral red and rhodamine tests) were the most sensitive for soil assays. Earthworm genotoxicity (*E. fetida* exposed in artificial soil) was the least sensitive for Cd, but the most sensitive test for phenol exposure. It is therefore clear that chemicals with different modes of toxic action will not give similar results in the same organism, and that direct comparisons with other studies should be done cautiously.

An interesting pattern emerging from the results of the present study can be seen in Fig 3.19. In all species, there seems to be a threshold level where DNA damage in a certain exposure concentration is less than that of the previous (lower) exposure concentration (as indicated by the arrows in Fig 3.19). This could possibly be explained by the phenomenon called hormesis (Calabrese & Baldwin 2002). Cadmium-induced hormesis have been reported for growth in the earthworm species *Lumbricus rubellus* (Spurgeon *et al.* 2004b), and metal-induced hormesis for activity of various enzymes involved in xenobiotic metabolism in *A. caliginosa* and *L. terrestris* (Laszczyca *et al.* 2004), therefore it is not an unlikely response in earthworms.

A hormetic dose-response is the result of low dose stimulation (of e.g. the test system, such as growth) and high-dose inhibition (e.g. of growth). This occurs as a compensatory mechanism following a disruption of homeostasis by a toxicant (Calabrese & Baldwin 2002). In other words, when an organism is challenged with a toxicant, homeostasis is disrupted and a compensatory mechanism starts, and damage may be lower than in unexposed organisms. As the toxicant

concentration increases, the compensatory mechanism is overridden, and damage increases concurrently.

From the results of the present study, it can be seen that at a certain low Cd concentration, DNA damage decreases somewhat in relation to the previous (lower) concentrations (Fig 3.19). This is where a compensatory mechanism is initiated. Subsequently, DNA damage does not increase with increasing Cd concentration until the compensatory mechanism is overridden at a higher Cd exposure concentration. Here, a threshold is reached where the Cd concentration exceeds the compensation threshold and DNA damage levels subsequently increase rapidly.

Species differences were found in the range of exposure concentrations where the compensatory mechanism is operative (Fig 3.19). In *A. diffringens* and *M. benhami* it is possible that the concentration at which the compensatory mechanism is initiated, is lower (< 2.5 mg/l Cd) than in the other species. In *A. diffringens*, it seems to be overridden between exposure concentrations of 2.5 and 5 mg/l Cd, but in *M. benhami*, it may be overridden only at very high concentrations, as DNA damage only increased at the highest treatment concentration. Further testing at higher concentrations (> 20 mg/l Cd) for *M. benhami* will be needed to determine whether DNA damage would increase further. For *A. caliginosa* and *D. rubidus*, the compensatory mechanism seems to start between 2.5 and 5 mg/l, and is overridden between 5 and 10 mg/l Cd. For *E. fetida* it seems to start only between 5 and 10 mg/l and is overridden somewhere between 10 and 20 mg/l Cd.

Two possible compensatory mechanisms involved in this hormesis effect are proposed. The first possibility is DNA repair. As previously mentioned, the most likely explanation for Cd toxicity is that natural DNA repair mechanisms are inhibited (Hartwig 1998). Therefore, it is possible that DNA repair is upregulated at low Cd exposure concentrations. At higher exposure concentrations however, the amount of Cd taken up becomes too high and the upregulated repair processes can not compensate for the elevated interference of Cd in DNA repair.

The second, and more likely explanation for the compensatory response is the production of high levels of stress proteins such as metallothionein as well as heat shock proteins 70 and 72, (Homa *et al.* 2005), both playing an important role in the protection of cells against metals (Damelin *et al.* 2000). Metallothioneins are cysteine-rich proteins that bind Cd and other metals such as Zn and Cu (Dabrio *et al.* 2002). According to Spurgeon *et al.* (2004a), earthworms possibly use a detoxification mechanism where Cd is bound to metallothionein (of which production is increased when the earthworm is exposed to Cd) and then compartmentalized within lysosomes and therefore rendered harmless. It has been shown by Homa *et al.* (2005) that

metallothionein and heat shock proteins in earthworms (*E. fetida*) are upregulated within 3 days of dermal exposure to Cd. Fatur *et al.* (2002) suggested that the production of metallothioneins is a protective mechanism to protect cells from Cd-induced DNA damage. This was shown after exposing human cells to $CdCl_2$, and finding an inhibition of DNA damage (as detected with the comet assay) and a concurrent increased production of metallothioneins.

Therefore, in the present study, metallothionein expression was possibly upregulated at low exposure concentrations of Cd, and at higher concentrations, the rate of metallothionein production was not high enough to sequestrate Cd before it could cause damage. The exposure concentration of cadmium inducing the upregulated expression of metallothionein also seem to differ between species (Fig 3.19).

4.1.1.2 Species sensitivity differences

DNA damage was compared between species exposed to a concentration of 20 mg/l Cd. This exposure concentration was used as a representative to compare species, because the highest levels of DNA damage were present in this concentration in most species (Fig 3.19). *E. fetida* had significantly higher and *M. benhami* significantly lower levels of damage than the other species at this exposure concentration (Fig 3.20).

It should however be remembered that some levels of baseline DNA damage occur in all cells (Shugart 2000), therefore this should be accounted for when comparing species. Because the levels of DNA damage differed between species in the negative controls (Appendix C Table 11), these were subtracted from the levels in the exposure concentrations for each species separately to obtain net DNA damage values. Similar to comparing only DNA damage in the 20 mg/l Cd exposure concentration between species, this was also done for the net DNA damage. A clear sensitivity order emerged for both Tail DNA % and Olive tail moment (Fig 3.21) in increasing order of sensitivity: *M. benhami*, *A. diffringens*, *A. caliginosa*, *D. rubidus* and *E. fetida*.

This sensitivity order changed somewhat when the gradients of the regression equations were compared by plotting the net DNA damage against the Cd exposure concentration (Fig 3.22). Sensitivity was in this case measured by the steepness of the gradient. A species with a steeper gradient will have higher levels of DNA damage at lower exposure concentrations relative to a species with a less steep gradient. The order of sensitivity obtained from these regression gradients for both Tail DNA % and Olive tail moment (in increasing order) is: *A. diffringens*, *M. benhami*, *D. rubidus*, *A. caliginosa* and *E. fetida*. This sensitivity order differs from the order when only exposure concentration 20 mg/l is taken into consideration (Fig 3.21).

These discrepancies can be explained by differences between species in their responses to all the exposure concentrations. The sensitivity order of species in the exposure concentrations 2.5, 5, 10 and 20 mg/l Cd differ from each other when net DNA damage levels are compared between species (Appendix B Table 8). Therefore, by using the response to the whole exposure range (by comparing the gradients between species), the increase of DNA damage with exposure concentration is taken into account. A species where this increase is fast (steep gradient) is probably not able to handle the toxicant efficiently and is therefore considered more sensitive than a species with a slow increase in DNA damage. On the other hand, it should be remembered that, although a certain species has a steeper gradient than another species, it may not necessarily have higher net levels of DNA damage in any of the exposure concentrations, as is illustrated for *A. caliginosa* which has a steeper gradient but lower levels of net DNA damage than *D. rubidus* (Fig 3.22). Therefore the amount of DNA damage and the increase of DNA damage with exposure concentration should be considered in combination when comparing species sensitivities.

The results of the present study suggest that the epigeic earthworm *E. fetida* is the most sensitive to Cd in terms of DNA damage of the five species studied. These results do not agree with that of quite a few studies as summarized by Edwards & Coulson (1992), where other chemicals and endpoints were used. As an example, Kula & Larink (1997) compared the sensitivity of *E. fetida*, *A. caliginosa*, *A. rosea* and *Allolobophora chlorotica* to the insecticide dimethoate in terms of acute toxicity (LC50) in two types of test soil (Lufa and OECD soil, (OECD. 1984)). They found that LC50s for these species did not differ much, but did find that *E. fetida* adults were the least sensitive, and *A. caliginosa* juveniles the most sensitive. The order of sensitivity was (with increasing sensitivity): *E. fetida* (adult), *A. chlorotica* (adult), *A. caliginosa* (adult), *E. fetida* (juvenile), *A. rosea* (adult) and *A. caliginosa* (juvenile). Their findings differ from those of the present study, but it must be stressed that, during the present study, a different toxicant (with different physiological effects), as well as a different endpoint was used. It is well known that earthworms do not react in the same way to different toxicants (Edwards & Coulson 1992). It is however interesting that the earthworms (*E. fetida*) used by Kula & Larink (1997) originated from a laboratory culture, and was less sensitive than *A. caliginosa* from the field. In the present study, *E. fetida* (also from laboratory cultures) was found to be more sensitive than *A. caliginosa*. The exposure history of the field populations can be a factor that influenced sensitivity, and might have been different between the two studies, because it is not known. The possible effect of exposure history for the species investigated in the present study is discussed in section 4.4.

Various reasons may exist for the differences in sensitivity observed in the present study, and it may lie either with different ecological groups, or differences as a result of relatedness and phylogeny, such as differences in morphology and physiology. It must be noted that earthworm ecological groups are not delimited on grounds of worm physiology, but rather their morphological characteristics (Bouché 1992). Indeed, these groups are often referred to as "ecological" types e.g. (Langdon *et al.* 2005), and not "ecophysiological" types. Earthworms belonging to the same ecological group do not neccesarily belong to the same taxonomic groups. Through convergent evolution they may assume the same ecological roles and share similar morphological and physiological characters (Bouché 1992).

There seems to be no definite relationship between ecological type and sensitivity in the present study. These results should however be considered cautiously, as all the ecological types are not represented equally (i.e. the anecics and endogeics were represented by only one species each, *M. benhami* and *A. caliginosa* respectively). *D. rubidus* and *E. fetida* are both epigeic earthworms from the family Lumbricidae (originating from the Holarctic and Palearctic respectively), and are the two most sensitive species when net DNA damage in the exposure concentration 20 mg/l Cd is considered (Fig 3. 24). The endogeic *A. caliginosa* also falls within this family (originating from the Palearctic), and is the third most sensitive species. *A. diffringens* is an epigeic species from the family Megascolecidae (originating from Asia), and is the second least sensitive species. The anecic *M. benhami* belongs to the family Microchaetidae (from Africa), and is the least sensitive species. It is possible that the species sensitivity differences observed may be a result of their phylogeny (because the most sensitive three species belong to the same family) or even body sizes, as shown in Fig 3.26, rather than their ecological type. Illustrating that sensitivity may not be influenced by ecological type, Spurgeon & Hopkin (1996a) compared the sensitivity of ecologically different species to metal-contaminated (Cd, Cu, Pb, Zn) soils for mortality, weight change, cocoon production and hatching success of cocoons. They found that the most sensitive species belonged to all three ecological groups (epigeics, endogeics and anecics) and concluded that these groupings are unlikely to predict sensitivity differences of earthworm species. They have proposed that physiology may play a more relevant role, as the more sensitive species e.g. all had a lower calcium gland secretion (involved in the sequestration and detoxification of Pb and Zn) than the more tolerant species. Kiewiet & Ma (1991) have shown that calcium may influence the uptake of Cd negatively by earthworms in artificial soil water. It is therefore possible that differences in Ca gland activity may also affect Cd uptake (and eventually sensitivity) differently in the species used during the present study.

Morgan *et al.* (1989) found that different species of earthworms (*D. rubidus* and *L. rubellus*) possess different isomers of Cd-sequestering metallothioneins. Differences in metallothioneins (which will result in differential sequestration and therefore detoxification between species) could possibly explain differences in sensitivity to Cd between species in the present study. These differences in metallothioneins would most probably coincide with phylogeny, where the genes for these proteins would have evolved to be more similar in closely related species than in less closely related species.

Abdel-Lateif *et al.* (1998) suggested that soil invertebrates are more susceptible to metal toxicity at high and low temperatures, and also that most metal accumulation occurs at high temperatures. Temperature regimes during the exposures in the present study may have had an effect on comet assay results, where higher temperatures may induce higher levels of damage. This effect of temperature was illustrated by Buschini *et al.* (2003) who performed the comet assay on zebra mussels (*Dreissena polymorpha*) and found a positive correlation between DNA damage and temperature. De Andrade *et al.* (2004) found higher DNA damage levels in fish erythrocytes exposed to methylmetane sulfate at 37°C than those exposed at 25°C. For the present study, however, exposures were conducted in a climate-controlled room at a constant temperature of 20°C. It is generally known that different earthworm species have different temperature tolerances, and this could still have influenced the results.

Cell condition could also have an influence on comet assay results, therefore cell viability is checked in many studies prior to performing the comet assay (Collins 2004). It is however suggested by Collins (2004) that an acceptable way of estimating whether the cells are in a good condition for use in the comet assay, is to check the background level of breaks (the level of DNA damage in the negative control). It should be around 10% tail DNA, which was the case in the present study (Appendix B Table 8).

4.1.2 Effect of earthworm body size

The possibility exist that smaller earthworms can take up higher amounts of Cd through the epidermis, because of the greater body surface area in relation to volume (Rozman & Klaassen 2001). This could result in a higher amount of Cd reaching the coelomocytes and potentially causing higher levels of DNA damage.

Clitellate (mature) worms were used in the case of *A. diffringens*, *A. caliginosa*, *D. rubidus* and *E. fetida*, when the ages were not known. Therefore the possibility exists that they could have been of different ages. Older mature earthworms can either be smaller or larger than younger

mature earthworms. Therefore, body mass was taken as surrogate for size (and not age) in these four species. For *A. diffringens* and *A. caliginosa*, body mass did not seem to influence sensitivity (Appendix C Table 9). For *E. fetida*, larger animals had lower levels of DNA damage (only Tail DNA %) in the negative control and the exposure concentration 10 mg/l Cd. For *D. rubidus*, larger animals exhibited higher levels of DNA damage (both Tail DNA % and Olive tail moment) in exposure concentration 10 mg/l Cd. In this species however, at a lower concentration of 2.5 mg/l Cd, smaller animals showed higher levels of DNA damage, although not statistically significantly so (Appendix C Table 9). These discrepancies give rise to the possibility that the sample size was not large enough for adequate comparisons. Further studies with larger sample sizes could be done to clarify this matter for these species.

For *M. benhami*, body mass was taken as a surrogate for age in addition to size. Because sampling proved difficult for *M. benhami*, juveniles were included in the experiments, as sufficient numbers of adults could not be collected. This could possibly have had an effect on the DNA damage results, as age in this sense may be an important factor to determine sensitivity to toxicants. For example, it has been illustrated that juvenile earthworms (*E. fetida*) are more sensitive (survival and growth) than adults to metal contaminated soils (Spurgeon & Hopkin 1996b). In contrast, Rank *et al.* (2005) found a negative correlation between mussel size and DNA strand breaks in mussels, and concluded that juveniles were less sensitive than adults. For *M. benhami*, however, juveniles did not exhibit either higher or lower levels of DNA damage, as could be seen from the lack of a relationship between earthworm mass and DNA damage. Some physiological mechanisms, not influenced by age, possibly play a role in Cd genotoxicity in this species. Age may however be a factor when e.g. dealing with long-term whole-organismal responses such as growth and sexual development (Spurgeon & Hopkin 1996b).

In general, body size within species did not correlate well with DNA damage. Between species however, it seems that larger species exhibited less DNA damage than smaller species (Fig 3.26). If smaller species do take up more Cd because of their larger area to volume ratio, this could be a possible explanation for the higher levels of DNA damage present in the smaller species. The discrepancy between within-species comparisons and between-species comparisons could possibly be due to the differences in body sizes. Within species, the differences in sizes of earthworms were not as large as the differences in sizes between species (Appendix B Tables 3 to 7 and 10).

4.1.3 Effect of nucleus size

No significant correlation between DNA content and body size exist in earthworms, but it appeared from a study by Gregory & Herbert (2002) that some small earthworm species had larger genomes than larger species. For example, they found that *D. rubidus* (body length 20 – 90 mm) has a C-value of 1.24 pg ($2n = 34$) and *E. fetida* (body length 35 – 170 mm) a C-value of 0.7 pg ($2n = 22$). This was reflected in the present study where *D. rubidus* seemed to have larger nuclei than *E. fetida* (Fig 3.23).

Nucleus size did not influence the amount of DNA damage measured in this study (Fig 3.24). This could be attributed to the fact that DNA damage is measured as Tail DNA % (and Olive tail moment, which is dependent on Tail DNA %). Tail DNA % is the percentage of DNA in the tail of the total amount DNA present in the whole comet. This measure of DNA damage is therefore independent of nucleus size.

4.1.4 Tail DNA % versus Olive tail moment

In a review by Collins (2004) the author stated that tail intensity, rather than tail length or tail moment (product of tail intensity and tail length), has been found to exhibit a linear relationship with dose in a number of studies. Collins (2004) therefore recommends the use of relative tail intensity (such as Tail DNA %). In the present study, both these parameters were used in order to compare them. Tail DNA % and Olive tail moment did not yield similar results in some cases, for example where a significant correlation was found between Cd exposure concentration and Tail DNA %, but not Olive tail moment, for *A. caliginosa*, and the opposite for *M. benhami* and *D. rubidus*.

A possible explanation may lie in the experimental method (Collins 2004). It is known that tail length increases at the start of electrophoresis, or with low damage levels. As electrophoresis time or DNA damage increases, the tail intensity (the amount of fluorescence, which indicates the amount of DNA present), but not tail length, increases (Fairbairn *et al.* 1995; Olive & Durand 2005). Therefore, electrophoresis conditions (such as electrophoresis time) could possibly have caused differences between Tail DNA % and Olive tail moment in the present study. Electrophoresis time was however consistent during all the experiments of the present study. An alternative explanation for the difference between the parameters is that the DNA breaks occurred at different sites in different species and that the amount of DNA that formed the tail was not consistent between species.

4.1.5 Nickel as a positive control

Nickel was chosen as the positive control in the present study because it belongs to the same chemical class (Tice *et al.* 2000) as cadmium. Ni induced significant amounts of DNA damage in *E. fetida* and *D. rubidus*, but not in *A. diffringens*, *A. caliginosa* and *M. benhami* (Appendix C Table 2). The findings for *E. fetida* are in agreement with Reinecke & Reinecke (2004b) who exposed *E. fetida* to Ni and found significant DNA damage with the comet assay. Although Ni can be an appropriate positive control for *E. fetida*, where it is known to induce significant levels of DNA damage, it cannot be assumed that it would have the same result in the other species, because the species will most probably also differ in their sensitivity to Ni, as they did to Cd. It is suggested that in future studies where the sensitivities of species are compared, the positive control should be supplemented with a known genotoxicant such as H_2O_2 or UV light, as is used by a number of published studies (e.g. the use of UV in Reinecke & Reinecke (2004b)).

4.1.6 EC50 and SSD

In order to construct a species sensitivity distribution (SSD), an effect concentration for each species is needed (Posthuma *et al.* 2002), such as an EC50 (concentration where 50% of test organisms show an effect). In the present study, EC50s could only be established for *E. fetida* (Tail DNA %, 3.706 mg/l Cd) and *A. caliginosa* (Olive tail moment, 24.591 mg/l Cd), because most of the response data obtained from the other species did not follow a linear dose-response relationship (Fig 3.19). It might be necessary in future to use more treatments, especially in the lower ranges (below 2.5 mg/l) to obtain higher resolution in the dose-response relationships. Other methods than the one used in the present study for obtaining EC50s should also be explored.

As far as could be established, no SSD has yet been constructed for earthworms using sublethal toxicity data. Such a seemingly simple concept is fraught with complications. For example, the choice of biomarker (and organisational level) should be carefully made, as differences between biomarkers within species have been observed when comparing species e.g. (Edwards & Coulson 1992; Bierkens *et al.* 1998). A study aiming to construct an SSD using sublethal data, with a suggested minimum of 4 species, (Aldenberg & Luttik 2002) would be logistically difficult, as a large range of treatment concentrations is required, and a large number of organisms per treatment (Posthuma *et al.* 2002). For some biomarkers, such a vast study design may prove time consuming. Furthermore, the effect concentration also depends on the biomarker used and the chosen endpoint, therefore one species may have several ECs (one for each biomarker) and it may be problematic to choose the most relevant one.

4.2 Metal accumulation patterns

4.2.1 Effect of exposure concentration

A factor that may have influenced the results of the present study is the exposure time. Longer exposure times could alter the dose-responses of the comet assay, or result in higher accumulation of Cd in the earthworms, as accumulation may be time-dependent (Sheppard *et al.* 1998). The exposure time in the present study could however have been long enough, as Homa *et al.* (2005) has shown that when *E. fetida* was exposed for three days to 1.32 µg/cm Cd (and Zn, Cu, Pb) with a filter-paper contact test (dermal exposure), three days were sufficient for Cd to reach coelomocytes and induce responses such as the upregulation of metallothioneins. The earthworms from that study have also accumulated high amounts of Cd (28 mg/kg in treated animals, which was 58 times higher than in untreated animals) in the three days of exposure, which showed that earthworms can be exposed to sufficient concentrations of metals to induce toxic responses in coelomocytes, and to be accumulated, within short exposure periods.

In the present study, all species accumulated Cd (Fig 3.27), with *A. diffringens* accumulating the highest levels, followed by *A. caliginosa*, *D. rubidus*, *M. benhami* and *E. fetida*. Only in *D. rubidus* and *M. benhami* was the correlation between Cd body load and Cd exposure concentration significant (Figs 3.15 & 3.18). *D. rubidus* specimens seemed only to have accumulated either no Cd, or levels of Cd exceeding 10 mg/kg (Fig 3.16). The explanation may lie in the experimental method. As the samples were diluted to the same volume (20 ml) prior to analysis by flame atomic absorption spectophotometry (AA), it is possible that Cd in the *D. rubidus* samples were below the detection limits (0.01 mg/l for Cd) because of the small size (0.159 ± 0.037 g mean mass) of this species relative to the other species (Fig 3.25). In most cases, AA measurements for *D. rubidus* were either 0 mg/l or 0.1 mg/l (Appendix B Table 5), as the instrument only gives reliable results to one decimal (Mr. U. Deutschlander, *Pers. comm.*). Therefore the pattern observed in Fig 3.16, is a function of earthworm mass (the AA measurement is multiplied with the sample volume, which is equal for every sample, and divided by the earthworm mass). It is suggested that small samples, such as from *D. rubidus* individuals should in future not be diluted to such an extent, or that the samples should be evaporated to concentrate the Cd to enable the instrument to detect it. Due to time constraints, this evaporation was not undertaken during the present study. The results obtained for this species should thus be considered cautiously in the light of this dilution factor. Similarly, because *E. fetida* is also fairly small (0.209 ± 0.045 g mean mass), its results should also be regarded with caution.

When comparing the amount of Cd accumulated by the different species, *A. diffringens* and *A. caliginosa* accumulated significantly higher levels of Cd in exposure concentration 20 mg/l Cd than all the other species (Appendix C Table 20). These two species also had consistently higher Cd body loads than the other species for all the other Cd exposure concentrations (Fig 3.27). *D. rubidus* accumulated less Cd than these two species, followed by *M. benhami* and *E. fetida*. It should however be remembered that an experimental problem might have been a factor influencing the results for *D. rubidus* and *E. fetida*, as discussed previously.

Comparing ecological groups, the results from this study suggest that the endogeic *A. caliginosa* accumulates higher levels of Cd than the anecic *M. benhami* and epigeic *D. rubidus* and *E. fetida*. Similar results were obtained by Morgan & Morgan (1992), who found that endogeic species such as *A. caliginosa* and *Allolobophora chlorotica* accumulated higher levels of Cd (up to 344 µg/g) than anecic species (*Aporrectodea longa* and *Lumbricus terrestris*). They also found that endogeic species accumulated more Cd than epigeic species. Soil exposures such as was done by Morgan & Morgan (1992) should however not be compared directly to soil water exposures, as uptake routes (which will influence accumulation ability) may differ between the two exposure systems. For example, in soil, Cd may be taken up by ingestion or exchange through the epidermis, and in water, exchange through the epidermis may occur (Morgan *et al.* 1993; Vijver *et al.* 2003). In soil, there are various physical factors influencing the uptake (and bioavailability) of heavy metals, such as pH, organic content and clay content (Kiewiet & Ma 1991), as well as biological factors such as earthworm feeding strategies and niche separation (Morgan & Morgan 1992; Morgan & Morgan 1999). In water, bioavailability may be determined by the concentration of the metal in the soil water itself (Kiewiet & Ma 1991). Considering these differences between soil and water exposures, one should be careful when extrapolating from water to soil exposures.

Morphological characters could possibly have an influence on uptake and accumulation ability differences between species. For example, nephridia play a role in osmoregulation, and it has been found that Cd accumulates in the nephridia (Prinsloo *et al.* 1999) and that the nephridia possibly excrete the metal (Prinsloo 1999). Most earthworms possess nephridia that open to the exterior, but megascolecid earthworms such as *A. diffringens* have endonephric nephridia. These nephridia open in the gut rather than to the exterior (Oglesby 1978), and could possibly be a mechanism to reduce water loss. If nephridia excrete Cd in *E. fetida*, as is proposed by Prinsloo (1999), and it is also the case in other earthworm species such as *A. diffringens*, the Cd could be released into the intestine by the endonephric nephridia. In this case it would be possible that the Cd can be taken up again from the earthworm intestine, which could explain why *A. diffringens*

accumulated more Cd than the other species. Alternatively, metallothionein expression in this species may differ from that of the other species. These possibilities will have to be investigated further.

4.2.2 Effect of body size

Within species, there was no correlation between body size and Cd accumulation in *A. diffringens* and *M. benhami*. In *A. caliginosa*, larger earthworms accumulated more Cd than smaller worms in the Cd exposure treatment of 20 mg/l (Fig 15). For *E. fetida* at the exposure concentration of 10 mg/l Cd, and for *D. rubidus* at the exposure concentration of 5 mg/l, larger worms seemed to accumulate more Cd than smaller worms. In the light of the previous discussion on the dilution factor, these results should also be considered cautiously.

When looking at the differences between species, it seems that body mass did not have an influence on Cd body load. Although accumulation is dependent on uptake, these two should not be confused. It is possible that some species excrete Cd without sequestrating it; while other species sequestrate most of the Cd they have taken up. Therefore the relationship between accumulation and body size cannot be directly related to the principle of uptake as a function of body size (Rozman & Klaassen 2001).

4.3 Cadmium body load and DNA damage

Considering the possible effect of Cd body load on DNA damage, no such relationship was found in *A. diffringens*. Earthworms containing lower levels of Cd were found to exhibit higher levels of DNA damage for Tail DNA % in the negative control in *A. caliginosa* and in the exposure concentration 10 mg/l Cd in *D. rubidus*. For both comet parameters, earthworms with lower Cd body loads exhibited higher levels of DNA damage in the exposure concentration of 10 mg/l in *E. fetida*, but in *M. benhami*, for both the negative control and the exposure concentration of 2.5 mg/l Cd, animals with higher Cd body loads exhibited higher levels of DNA damage.

In *A. caliginosa*, *D. rubidus* and *E. fetida*, higher levels of DNA damage were found for lower levels of Cd body load. This negative relationship could probably be explained by the following: When metals enter an organism, it may either be partitioned into biologically available fractions that may be toxic, or into biologically unavailable storage fractions that are not toxic (Lanno *et al.* 1998). It is therefore clear that not all the Cd in the earthworm bodies may act as a toxicant, which explains why DNA damage found in the present study did not positively correspond to the

Cd body loads. If bound or sequestered metals would be separated from the bioavailable fraction (Conder *et al.* 2002) prior to metal analyses, it might clarify this problem.

This partitioning of Cd in the earthworms could also explain why no definite relationship could be found between body loads and DNA damage, as compared between species in all the exposure concentrations (Appendix C Table 21). It is also possible that, as more Cd is sequestrated (and therefore accumulated) by earthworms, less is available to induce DNA damage. This would explain the pattern in Fig 3.28 where the species accumulating the least Cd had the highest levels of DNA damage. It should however not be forgotten that uptake and excretion ability may differ between the species, and that it is not known how much Cd is taken up and causes DNA damage but is subsequently excreted without being accumulated.

4.4 Cadmium in the sampling sites and negative control animals

Individuals from the negative controls of all species, except for *E. fetida*, were found to contain levels of Cd, with the highest average of 6.19 ± 1.18 mg/kg for *A. diffringens* (Appendix B Table 8). Low levels of Cd were present in the soils where *A. diffringens*, *A. caliginosa* and *D. rubidus* were collected (Fig 3.29). No Cd could be detected in the soil where *M. benhami* was collected. Therefore the Cd present in the negative control individuals in this species could possibly be explained by accumulation of Cd from either the soil from which they were collected or possibly from water (as these earthworms were collected from waterlogged soil) that subsequently leached out from the substrate, but had persisted in the animals. This presence of Cd in the earthworms, with an absence of detectable levels of Cd in the soil, is most probably due to the depuration rates of Cd that are very slow in earthworms, as has been illustrated by Sheppard *et al.* (1998) who found that the loss half-time for Cd in *L. terrestris* was 150 days.

It is also possible that resistance or tolerance may develop in earthworm species with an exposure history to Cd (Reinecke *et al.* 1999), and that the use of pre-exposed animals may bias the results of ecotoxicological tests. Therefore the results of the present study should be viewed with consideration that the collection sites for three species were found to contain low levels of Cd. Because of the presence of Cd in these sites and animals, the possibility cannot be excluded that this prior exposure to Cd might have caused some change in tolerance or even resistance in the earthworms. This could possibly explain the lack of a definite dose-related response in species such as *A. diffringens* and *A. caliginosa*. In addition, the earthworms originated from different sites with different exposure histories, which may also influence the species sensitivity difference comparisons. Therefore use of a negative control in the present study (by subtracting

the DNA damage in the negative control from the damage in the Cd exposure treatments) could aid in compensating for these different exposure histories.

4.5 Final remarks

Studies comparing the relative sublethal sensitivity of earthworms are scarce (Spurgeon *et al.* 2003). The present study is one of the few, although not the first, to compare sensitivity of ecologically different earthworm species to sublethal levels of heavy metals. Only recently have such studies started to increase in number. For example, sublethal effects of Cu, Pb, Cd and Zn (Spurgeon & Hopkin 1996a; Spurgeon *et al.* 2000; Langdon *et al.* 2005; Lukkari *et al.* 2005; Morgan & Turner 2005), have been compared between different earthworm species. In these studies, the test organisms were exposed in soil, where e.g. differences in feeding strategies and avoidance behaviour (Morgan & Morgan 1992) may influence the results, although such studies are ecologically more relevant than for example artificial soil water exposures or filter paper contact tests. The aim of the present study was to focus on possible differences in physiology, and therefore eliminated the possibility of these ecological factors having and effect by exposing the earthworms to Cd in an artificial soil water medium (Kiewiet & Ma 1991).

The use of the comet assay as a biomarker of genotoxicity to compare species sensitivity is also not a new concept, and has been used to compare e.g. polychaete species (*Capitella* spp.)(Bach *et al.* 2005); mullet (*Mugil* sp.) and catfish (*Netuma* sp.)(De Andrade *et al.* 2004); sea urchins (*Strongylocentrotus droebachiensis*) and mussels (*Mytilus edulis*) (Taban *et al.* 2004). The present study is however the first to utilize the comet assay to compare the sensitivities of terrestrial invertebrates such as earthworms.

It has been illustrated by Bierkens *et al.* (1998) that a single test cannot accurately predict the sensitivity of a certain organism to a single chemical, and that a battery of tests should be used. Also, that a single chemical may not have similar effects in different species, requiring the use of different endpoints to discern its mode of action in different organisms. It is thus desirable that, when a biomarker is used, it should be linked to whole-organism responses such as life-cycle biology (Spurgeon *et al.* 2004a). It is suggested that the comet assay results from the present study should be substantiated in future studies by both LC50s and other biomarkers with endpoints on various levels of biological organisation, and life-cycle parameters such as reproduction tests.

4.6 Conclusions

It can be concluded, with regard to the aims of the study, that

1. DNA damage, as measured with the comet assay did elucidate species differences in five earthworm species exposed to cadmium. It is concluded that the comet assay is useful to determine species sensitivity differences, but that it should be augmented with other biomarkers, as well as whole-organismal responses, such as life-cycle tests.

2. An interesting pattern emerged, where all species exhibited a compensatory response at low levels of Cd. At certain levels of Cd, DNA damage was lower than that in lower Cd exposure concentrations or the negative controls. At higher levels of Cd, DNA damage increased again. This could be explained by the hormetic dose-response, where low levels of a toxicant stimulates a compensatory response, and high levels inhibit this response. Two compensatory mechanisms were proposed. Firstly, DNA repair could have been upregulated at low Cd concentrations and inhibited by high concentrations. Secondly, the production of metal-binding metallothioneins, that bind Cd to be sequestrated, could have been increased at low Cd concentrations. At high Cd concentrations the rate of metallothionein production would not have been high enough to sequestrate all or most of the Cd. Further investigation towards the mechanisms of this compensatory response is needed.

3. The earthworm species studied differed in their sensitivities to cadmium. The order of sensitivity of the species (in order of increasing sensitivity) is: *Microchaetus benhami*, *Amynthas diffringens*, *Aporrectodea caliginosa*, *Dendrodrilus rubidus* and *Eisenia fetida*. Ecological type was not found to predict sensitivity. Sensitivity differences are most probably the result of physiological and morphological differences between species. The relatedness of species possibly also influenced sensitivities (which in turn influences physiology and morphology), as the three most sensitive species belongs to the family Lumbricidae, the second least sensitive species to the family Megascolecidae and the least sensitive species to the family Microchaetidae.

4. The recommended test species *E. fetida* (OECD. 1984) was found to be the most sensitive species, and it is suggested that it is a useful species for genotoxicity testing of the effect of heavy metals such as cadmium.

5. The amount of DNA damage did not correspond with the total amount of Cd

accumulated. It is possible that a certain amount of Cd was sequestrated, therefore rendered unavailable to induce DNA damage. It is suggested that in future, this unavailable fraction should be separated from the available fraction when analysing earthworms for metal contents.

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* Original not seen

APPENDIX A

Chemical solutions

1. Artificial soil water

1 L Distilled water

100 mg $NaHCO_3$

20 mg $KHCO_3$

200 mg $CACl_2 \cdot 2H_2O$

180 mg $MgSO_4$

Mix together and set pH to $\pm$ 8.2.

2. Extrusion solution

0.05 g EDTA

19 ml PBS

0.2 g Guaiacol Gliserol Ether (GGE)

1 ml absolute ethanol

Add EDTA to PBS and mix on stirrer plate until dissolved. Add GGE and stir until dissolved. Store in refrigerator until use. Add 1ml absolute ethanol just before use and keep on ice

3. Agarose gels

3.1. 1% Normal melting point agarose (NMA)

Add 500 mg NMA to 50 ml PBS. Store at 4°C.

3.2. 0.5% Low melting point agarose (LMA)

Add 125 mg LMA to 50 ml PBS. Store at 4°C.

4. Lysing solution

4.1. Lysing solution stock

146.1 g NaCl (2.5 M)

37.2 g EDTA (100mM)

1.2 g Tris (10mM)

dH_20

8 g pellitized NaOH

Add ingredients to ± 700 ml H2O. Then start stirring the mixture. Add 8 g pellitized NaOH and allow the mixture to dissolve for about 20 minutes. Measure the pH (should be ± 10.5). Adjust the pH to 10.0 using concentrated HCl or NaOH. Q.s. to 890 ml with distilled H2O. Store at room temperature.

4.2. Final lysing solution (to be made up just before use)

1% Triton X-100 (10 ml)

10% DMSO (100 ml)

Add 1% Triton X-100 and 10% DMSO, and then refrigerate for 30 – 60 minutes prior to slide addition.

5. Electrophoresis Buffer (for 1000 ml):

5.1. Stock solutions (store them separately at 4°C):

10 M NaOH (made up by adding 200 g to 500 ml dH2O). Store at 4°C.

200 mM EDTA (made up by adding 14.89 g to 200 ml dH2O, pH 10). Store at 4°C.

5.2. Immediately before each electrophoresis run:

30 ml NaOH

5.0 ml EDTA

Mix, q.s. to 1000 ml and mix well.

6. Neutralization buffer

dH_2O

48.5 g Tris (0.4M)

Add Tris to 800 ml dH_2O. Set pH to 7.5. Q.s. to 1000 ml with dH_2O. Set the pH to 7.5 with concentrated HCl (greater than 10 M). Store at 4°C.

7. Staining solution

7.1. Stock

Add 10 mg Ethidium Bromide to 50 ml dH_2O. Store at 4°C.

7.2. Working solution

Obtain 20 µg / ml (add 1 ml stock to 9 ml dH_2O). Store at 4°C.

APPENDIX B

Experimental data

Table 1: Yield of slides made for the comet assay for five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control

Species	Treatment	Animals used	Animals that yielded sufficient cells	% Animals that yielded sufficient cells	Proposed number of animals in experimental setup	% Animals of proposed experimental setup that yielded sufficient cells
A. diffringens	K	18	6	33	10	60
	2.5 mg/l Cd	11	8	73	10	80
	5 mg/l Cd	11	8	73	10	80
	10 mg/l Cd	11	8	73	10	80
	20 mg/l Cd	11	8	73	10	80
	20 mg/l Ni	13	4	31	10	40
A. caliginosa	K	19	16	84	10	160
	2.5 mg/l Cd	19	11	58	10	110
	5 mg/l Cd	18	13	72	10	130
	10 mg/l Cd	16	11	69	10	110
	20 mg/l Cd	17	12	71	10	120
	20 mg/l Ni	18	12	67	10	120
D. rubidus	K	10	10	100	10	100
	2.5 mg/l Cd	11	10	91	10	100
	5 mg/l Cd	12	10	83	10	100
	10 mg/l Cd	12	10	83	10	100
	20 mg/l Cd	12	10	83	10	100
	20 mg/l Ni	11	10	91	10	100
E. fetida	K	11	10	91	10	100
	2.5 mg/l Cd	10	10	100	10	100
	5 mg/l Cd	10	10	100	10	100
	10 mg/l Cd	10	10	100	10	100
	20 mg/l Cd	15	13	87	10	130
	20 mg/l Ni	10	10	100	10	100
M. benhami	K	10	5	50	10	50
	2.5 mg/l Cd	8	4	50	10	40
	5 mg/l Cd	10	4	40	10	40
	10 mg/l Cd	11	4	36	10	40
	20 mg/l Cd	10	3	30	10	30
	20 mg/l Ni	10	7	70	10	70

Table 2: Metal contents per 1 g of soil from collecting sites of four earthworm species. Vergenoeg for *A. caliginosa*, Welgevallen for *D. rubidus*, US Botanical garden for *A. diffringens*, and Middelvlei for *M. benhami*. AA = metal content in mg/l as measured by flame atomic absorption spectrophotometry; Soil = metal concentration (mg/kg) in soil after converting AA measurement. Zero values are indicated by a dash (-).

		Cd		Ni		Cu		Mn		Pb		Zn	
	Sample no	AA (mg/l)	Soil (mg/kg)	AA (mg/l)	Soil (mg/kg)	AA (mg/l)	Soil (mg/kg)	AA (mg/l)	Soil (mg/kg)	AA (mg/l)	Soil (mg/kg)	AA (mg/l)	Soil (mg/kg)
Vergenoeg	**1**	0.1	2.0	-	-	-	-	2.5	50.0	-	-	0.8	16.0
Vergenoeg	**2**	0.1	2.0	-	-	-	-	2.3	46.0	-	-	0.7	14.0
Vergenoeg	**3**	0.1	2.0	-	-	-	-	2.3	46.0	-	-	0.6	12.0
Welgevallen	**1**	0.2	4.0	0.3	6.0	0.3	60.0	9.5	190.0	-	-	5.0	100.0
Welgevallen	**2**	0.2	4.0	0.3	6.0	0.3	60.0	10.2	204.0	-	-	5.0	100.0
Welgevallen	**3**	0.1	2.0	0.3	6.0	0.3	60.0	9.7	194.0	-	-	5.0	100.0
US Botanical garden	**1**	0.1	2.0	0.1	2.0	1.0	20.0	10.4	208.0	0.6	12	2.9	58.0
US Botanical garden	**2**	0.1	2.0	0.1	2.0	0.8	16.0	10.5	210.0	0.8	16	3.3	66.0
US Botanical garden	**3**	0.1	2.0	0.1	2.0	0.9	18.0	10.6	212.0	0.9	18	2.9	58.0
Middelvlei	**1**	-	-	-	-	-	-	-	-	-	-	0.5	10.0
Middelvlei	**2**	-	-	-	-	-	-	0.1	2.0	-	-	0.7	14.0
Middelvlei	**3**	-	-	-	-	-	-	0.3	6.0	-	-	1.4	28.0
Middelvlei	**4**	-	-	-	-	-	-	0.2	4.0	-	-	0.4	8.0

Table 3: Summary (mean ± standard deviation) of comet assay parameters Tail DNA % and Olive tail moment per individual earthworm, with individual weight and metal content for *A. diffringens* exposed to Cd in artificial soil water. K = negative control; Ni = positive control (20 mg/l Ni). AA = Atomic absorption spectrophotometer reading (mg/l); Body = metal body load (mg/kg).

Repli-cate	Treat-ment	Ani-mal name	Mass (g)	AA (mg/l)	Body (mg/kg)	Comet *n*	Tail DNA %	Olive tail moment (arbitrary units)
R1	K	P1	0.970	0.2	4.124	190	13.069 ± 14.064	4.368 ± 8.339
R2	K	P2	0.610	0.2	6.557	107	23.745 ± 16.921	11.760 ± 11.847
R2	K	P3	0.620	0.2	6.452	170	16.194 ± 13.656	6.910 ± 9.918
R2	K	P4	0.600	0.2	6.667	130	16.688 ± 12.903	6.294 ± 8.097
R2	K	P5	0.840	0.3	7.143	98	23.725 ± 18.126	11.753 ± 13.926
R1	2.5 mg/l Cd	P1	1.510	0.8	10.596	170	24.927 ± 17.167	12.500 ± 13.621
R1	2.5 mg/l Cd	P2	0.830	1.0	72.289	100	15.202 ± 14.213	5.949 ± 8.517
R1	2.5 mg/l Cd	P3	1.800	1.8	60.000	213	10.588 ± 13.161	4.457 ± 8.729
R2	2.5 mg/l Cd	P4	0.700	1.4	40.000	109	8.561 ± 6.015	1.789 ± 1.483
R2	2.5 mg/l Cd	P5	0.880	0.7	15.909	121	11.309 ± 10.468	3.301 ± 4.570
R2	2.5 mg/l Cd	P6	1.080	1.7	31.481	102	16.373 ± 14.800	6.139 ± 8.451
R2	2.5 mg/l Cd	P7	0.780	0.3	7.692	129	9.569 ± 9.947	2.782 ± 5.786
R2	2.5 mg/l Cd	P8	0.640	1.6	50.000	127	16.643 ± 10.092	4.467 ± 4.665
R1	5 mg/l Cd	P1	0.900	1.3	28.889	180	36.308 ± 22.109	26.424 ± 23.601
R1	5 mg/l Cd	P2	0.890	1.0	22.472	191	13.729 ± 12.207	4.350 ± 7.974
R1	5 mg/l Cd	P3	0.780	0.9	23.077	238	14.223 ± 10.967	4.588 ± 6.265
R2	5 mg/l Cd	P4	0.560	1.1	39.286	117	21.302 ± 17.001	10.846 ± 13.045
R2	5 mg/l Cd	P5	0.380	0.5	26.316	131	35.679 ± 21.938	19.756 ± 17.208
R2	5 mg/l Cd	P6	0.400	0.4	20.000	142	22.146 ± 14.548	11.818 ± 11.850
R2	5 mg/l Cd	P7	0.460	0.6	26.087	112	29.104 ± 17.910	16.708 ± 14.118
R2	5 mg/l Cd	P8	0.860	1.9	44.186	92	38.901 ± 18.029	22.103 ± 16.210
R1	10 mg/l Cd	P1	0.590	1.1	37.288	188	16.378 ± 15.132	6.064 ± 9.957
R1	10 mg/l Cd	P2	0.970	1.1	22.680	204	19.310 ± 17.963	9.844 ± 15.995
R1	10 mg/l Cd	P3	0.690	0.6	17.391	177	21.021 ± 18.714	10.541 ± 13.899
R2	10 mg/l Cd	P4	0.610	0.4	13.115	138	22.000 ± 13.886	9.506 ± 9.670
R2	10 mg/l Cd	P5	0.750	0.6	16.000	131	28.231 ± 16.645	16.071 ± 15.612
R2	10 mg/l Cd	P6	0.630	0.9	28.571	128	28.504 ± 13.669	12.506 ± 11.447
R2	10 mg/l Cd	P7	1.000	1.8	36.000	166	31.756 ± 14.686	17.895 ± 17.329
R1	20 mg/l Cd	P1	0.440	1.6	72.727	226	21.200 ± 16.312	12.107 ± 14.476
R1	20 mg/l Cd	P2	0.890	2.0	224.719	175	33.742 ± 18.037	18.332 ± 15.575
R1	20 mg/l Cd	P3	0.620	1.5	193.548	169	25.952 ± 17.025	14.426 ± 14.732
R2	20 mg/l Cd	P4	0.720	0.7	19.444	108	21.300 ± 18.665	9.474 ± 12.847
R2	20 mg/l Cd	P5	0.670	1.2	107.463	85	20.554 ± 14.711	7.876 ± 9.039
R2	20 mg/l Cd	P6	0.700	1.9	162.857	135	17.310 ± 13.254	6.989 ± 8.942
R2	20 mg/l Cd	P7	0.560	0.4	14.286	62	18.491 ± 12.096	8.400 ± 10.838
R2	20 mg/l Cd	P8	0.950	1.0	21.053	104	17.849 ± 15.389	6.947 ± 9.330
R2	20 mg/l Ni	P1	0.550	0.9	98.182	150	11.824 ± 10.213	4.099 ± 6.608
R2	20 mg/l Ni	P2	0.460	0.7	30.435	177	12.465 ± 11.831	3.478 ± 5.370
R2	20 mg/l Ni	P3	0.870	2.0	413.793	153	21.087 ± 16.797	10.814 ± 13.876
R2	20 mg/l Ni	P4	0.760	0.8	63.158	155	25.719 ± 13.564	11.276 ± 10.618

Table 4: Summary (mean ± standard deviation) of comet assay parameters Tail DNA % and Olive tail moment per individual earthworm, with individual weight and metal content for *A. caliginosa* exposed to Cd in artificial soil water. K = negative control; Ni = positive control (20 mg/l Ni). AA = Atomic absorption spectrophotometer reading (mg/l); Body = metal body load (mg/kg). * Animals not weighed and analyzed for metal content.

Repli-cate	Treatment	Ani-mal	Mass (g)	AA (mg/l)	Body (mg/kg)	Comet *n*	Tail DNA %	Olive tail moment (arbitrary units)
R1	K	A1	0.736	0.1	2.719	101	13.471 ± 16.630	5.423 ± 8.736
R1	K	A2	0.737	0.0	0.000	54	11.916 ± 15.223	3.352 ± 6.247
R1	K	A3	0.679	0.1	2.947	108	15.296 ± 19.998	7.703 ± 19.404
R1	K	A4	0.735	0.0	0.000	101	13.319 ± 15.045	6.868 ± 12.360
R1	K	A5	0.621	0.0	0.000	100	14.725 ± 15.532	7.050 ± 11.230
R2	K	A6	0.519	0.3	11.561	57	17.170 ± 11.844	6.507 ± 7.794
R2	K	A7	0.639	0.2	6.259	105	24.736 ± 14.749	10.685 ± 13.953
R2	K	A8	0.775	0.3	7.743	116	23.346 ± 14.730	10.367 ± 11.997
R2	K	A9	0.665	0.1	3.007	74	18.500 ± 15.414	7.695 ± 11.451
R2	K	A10	0.686	0.1	2.916	83	17.727 ± 14.770	6.317 ± 8.506
R1	K	A11	*	*	*	109	9.918 ± 14.958	3.501 ± 7.432
R1	K	A12	*	*	*	102	13.495 ± 16.211	4.792 ± 6.410
R1	2.5 mg/l Cd	A1	0.460	0.1	4.352	112	13.025 ± 14.040	5.613 ± 9.495
R1	2.5 mg/l Cd	A2	0.655	0.4	12.219	102	25.794 ± 20.732	13.884 ± 18.202
R1	2.5 mg/l Cd	A3	0.915	1.9	41.512	110	12.726 ± 18.005	6.683 ± 14.208
R1	2.5 mg/l Cd	A4	0.672	0.3	8.930	112	15.984 ± 16.150	6.104 ± 9.392
R1	2.5 mg/l Cd	A5	0.805	1.1	27.329	89	18.384 ± 16.523	7.595 ± 14.154
R2	2.5 mg/l Cd	A6	0.515	0.4	15.540	159	19.857 ± 16.542	8.128 ± 11.267
R2	2.5 mg/l Cd	A7	0.372	0.2	10.744	144	19.630 ± 20.624	6.767 ± 10.228
R2	2.5 mg/l Cd	A8	0.563	0.7	24.880	135	40.398 ± 18.402	17.799 ± 12.721
R2	2.5 mg/l Cd	A9	0.695	0.5	14.397	125	18.571 ± 14.964	7.317 ± 10.556
R2	2.5 mg/l Cd	A10	0.495	0.4	16.168	123	20.480 ± 17.459	8.736 ± 17.671
R1	2.5 mg/l Cd	A11	*	*	*	101	18.177 ± 15.565	8.440 ± 10.295
R1	5 mg/l Cd	A1	0.781	0.5	12.804	111	15.971 ± 15.126	6.205 ± 7.236
R1	5 mg/l Cd	A2	0.701	0.7	19.980	102	12.300 ± 13.570	3.997 ± 5.379
R1	5 mg/l Cd	A3	0.898	0.7	15.583	100	11.290 ± 12.696	3.225 ± 4.753
R1	5 mg/l Cd	A4	0.601	0.9	29.960	127	9.730 ± 11.978	3.529 ± 6.365
R1	5 mg/l Cd	A5	0.746	0.6	16.079	116	15.671 ± 17.961	7.390 ± 13.681
R2	5 mg/l Cd	A6	0.638	0.4	12.539	109	37.902 ± 16.963	16.918 ± 11.205
R2	5 mg/l Cd	A7	0.478	0.6	25.110	159	22.612 ± 17.419	8.931 ± 12.805
R2	5 mg/l Cd	A8	0.738	1.0	27.108	149	14.766 ± 14.164	5.402 ± 11.042
R2	5 mg/l Cd	A9	0.719	0.6	16.685	66	26.930 ± 15.796	15.127 ± 16.766
R2	5 mg/l Cd	A10	0.558	0.4	14.334	113	22.765 ± 17.061	10.332 ± 18.043
R1	5 mg/l Cd	A11	*	*	*	128	16.760 ± 18.927	8.372 ± 15.972
R2	5 mg/l Cd	A12	*	*	*	131	22.807 ± 17.254	7.506 ± 7.665
R1	10 mg/l Cd	A1	0.634	1.0	31.556	117	23.105 ± 16.924	12.593 ± 13.750
R1	10 mg/l Cd	A2	0.792	0.6	15.146	140	19.924 ± 17.460	8.178 ± 12.340
R1	10 mg/l Cd	A3	0.702	0.6	17.092	116	19.602 ± 18.182	9.656 ± 14.971
R1	10 mg/l Cd	A4	1.066	0.6	11.259	134	16.788 ± 16.051	5.892 ± 8.295
R1	10 mg/l Cd	A5	0.636	0.5	15.718	109	13.235 ± 12.927	4.548 ± 6.586
R2	10 mg/l Cd	A6	0.665	0.4	12.030	127	28.620 ± 11.853	9.132 ± 5.886
R2	10 mg/l Cd	A7	0.591	0.6	20.294	120	36.042 ± 15.454	15.226 ± 10.723
R2	10 mg/l Cd	A8	0.637	0.5	15.696	108	15.731 ± 11.400	5.191 ± 6.335

Table 4 (continued)

R2	10 mg/l Cd	A9	0.642	0.4	12.471	106	23.472 ± 15.870	9.080 ± 9.709
R2	10 mg/l Cd	A10	0.597	0.4	13.407	127	19.939 ± 11.669	6.691 ± 6.334
R1	10 mg/l Cd	A11	*	*	*	157	17.112 ± 13.115	5.426 ± 5.977
R1	20 mg/l Cd	A1	0.626	0.7	22.368	78	19.051 ± 14.128	8.310 ± 10.250
R1	20 mg/l Cd	A2	0.721	0.9	24.976	78	29.885 ± 17.479	17.701 ± 17.355
R1	20 mg/l Cd	A3	0.586	0.7	23.907	124	44.574 ± 18.081	33.232 ± 21.685
R1	20 mg/l Cd	A4	0.563	0.6	21.333	108	32.951 ± 13.943	15.173 ± 12.117
R1	20 mg/l Cd	A5	0.597	0.7	23.439	104	33.515 ± 17.679	21.744 ± 16.871
R2	20 mg/l Cd	A6	0.540	0.4	14.829	112	12.133 ± 11.503	4.565 ± 5.933
R2	20 mg/l Cd	A7	0.653	0.8	24.495	101	16.537 ± 12.175	6.657 ± 7.748
R2	20 mg/l Cd	A8	0.675	1.5	44.464	129	21.407 ± 10.300	7.461 ± 5.478
R2	20 mg/l Cd	A9	0.640	0.7	21.882	137	20.239 ± 11.222	7.870 ± 7.989
R2	20 mg/l Cd	A10	0.781	1.1	84.496	111	17.339 ± 12.837	6.381 ± 12.163
R1	20 mg/l Ni	A1	0.924	1.2	181.740	104	14.551 ± 14.553	6.869 ± 10.781
R1	20 mg/l Ni	A2	0.690	0.7	20.278	108	6.967 ± 12.511	2.407 ± 5.696
R1	20 mg/l Ni	A3	0.436	0.3	96.352	79	19.715 ± 15.619	9.695 ± 10.784
R1	20 mg/l Ni	A4	0.840	2.0	238.067	110	17.980 ± 14.636	8.356 ± 12.637
R1	20 mg/l Ni	A5	0.768	1.3	33.841	113	14.848 ± 18.295	5.679 ± 10.312
R2	20 mg/l Ni	A6	0.717	0.8	22.328	127	13.609 ± 10.880	4.905 ± 6.109
R2	20 mg/l Ni	A7	0.855	0.1	2.339	125	21.787 ± 12.195	8.713 ± 8.407
R2	20 mg/l Ni	A8	0.459	0.0	0.000	117	14.952 ± 13.762	4.855 ± 5.921
R2	20 mg/l Ni	A9	0.509	0.7	27.483	152	17.368 ± 10.886	5.323 ± 4.716
R2	20 mg/l Ni	A10	0.536	0.0	0.000	143	24.360 ± 12.817	9.104 ± 7.398
R1	20 mg/l Ni	A11	*	*	*	98	12.747 ± 13.997	4.556 ± 8.273
R1	20 mg/l Ni	A12	*	*	*	112	14.741 ± 16.842	4.710 ± 8.732

Table 5: Summary (mean ± standard deviation) of comet assay parameters Tail DNA % and Olive tail moment per individual earthworm, with individual weight and metal content for *D. rubidus* exposed to Cd in artificial soil water. K = negative control; Ni = positive control (20 mg/l Ni). AA = Atomic absorption spectrophotometer reading (mg/l); Body = metal body load (mg/kg).

Repli-cate	Treat-ment	Ani-mal	Mass (g)	AA (mg/l)	Body (mg/kg)	Comet *n*	Tail DNA %	Olive tail moment (arbitrary units)
R1	K	D1	0.233	0.0	0.000	148	14.463 ± 18.195	7.331 ± 12.522
R1	K	D2	0.196	0.0	0.000	154	10.271 ± 14.753	4.587 ± 11.269
R1	K	D3	0.194	0.1	10.309	155	6.351 ± 10.942	2.180 ± 5.427
R1	K	D4	0.163	0.0	0.000	119	10.842 ± 15.770	5.903 ± 15.213
R1	K	D5	0.132	0.1	15.129	133	7.681 ± 12.869	2.688 ± 5.788
R2	K	D6	0.169	0.0	0.000	112	4.634 ± 8.939	1.397 ± 4.074
R2	K	D7	0.177	0.1	11.331	98	2.422 ± 4.361	1.535 ± 10.228
R2	K	D8	0.262	0.0	0.000	143	9.625 ± 15.368	3.576 ± 7.575
R2	K	D9	0.134	0.0	0.000	110	5.362 ± 9.157	2.575 ± 10.806
R2	K	D10	0.165	0.0	0.000	106	4.085 ± 8.616	2.043 ± 10.244
R1	2.5 mg/l Cd	D1	0.195	0.1	10.256	127	17.059 ± 24.342	10.307 ± 18.438
R1	2.5 mg/l Cd	D2	0.119	0.0	0.000	121	16.712 ± 18.499	8.152 ± 11.281
R1	2.5 mg/l Cd	D3	0.146	0.1	13.717	145	15.124 ± 17.418	7.824 ± 11.432
R1	2.5 mg/l Cd	D4	0.145	0.1	13.793	107	22.858 ± 20.494	12.477 ± 14.535
R1	2.5 mg/l Cd	D5	0.156	0.1	12.796	162	12.843 ± 19.779	6.483 ± 12.346
R2	2.5 mg/l Cd	D6	0.163	0.0	0.000	112	37.005 ± 21.327	21.995 ± 19.333

Table 5 (continued)

R2	2.5 mg/l Cd	D7	0.167	0.0	0.000	107	19.903 ± 23.955	12.334 ± 18.884
R2	2.5 mg/l Cd	D8	0.170	0.0	0.000	108	22.135 ± 24.185	13.191 ± 19.116
R2	2.5 mg/l Cd	D9	0.136	0.0	0.000	120	15.229 ± 19.517	8.047 ± 13.756
R2	2.5 mg/l Cd	D10	0.094	0.0	0.000	130	27.254 ± 23.516	17.967 ± 19.216
R1	5 mg/l Cd	D1	0.139	0.0	0.000	156	14.798 ± 16.358	6.822 ± 9.752
R1	5 mg/l Cd	D2	0.200	0.1	9.990	133	19.045 ± 19.774	8.565 ± 11.048
R1	5 mg/l Cd	D3	0.151	0.0	0.000	158	14.286 ± 19.277	7.043 ± 11.749
R1	5 mg/l Cd	D4	0.134	0.0	0.000	135	6.725 ± 12.713	3.216 ± 10.565
R1	5 mg/l Cd	D5	0.150	0.0	0.000	98	21.252 ± 16.300	11.359 ± 14.363
R2	5 mg/l Cd	D6	0.184	0.1	10.858	145	26.011 ± 21.864	16.986 ± 19.932
R2	5 mg/l Cd	D7	0.146	0.0	0.000	136	22.228 ± 21.152	11.535 ± 14.386
R2	5 mg/l Cd	D8	0.157	0.1	12.747	115	6.845 ± 11.194	2.690 ± 6.317
R2	5 mg/l Cd	D9	0.154	0.1	13.012	115	17.561 ± 20.057	9.064 ± 12.761
R2	5 mg/l Cd	D10	0.141	0.0	0.000	127	13.286 ± 16.824	6.194 ± 11.308
R1	10 mg/l Cd	D1	0.125	0.1	16.026	138	9.820 ± 14.379	4.046 ± 7.926
R1	10 mg/l Cd	D2	0.105	0.1	19.102	123	21.425 ± 18.726	10.121 ± 11.404
R1	10 mg/l Cd	D3	0.194	0.1	10.304	121	35.545 ± 17.704	19.768 ± 14.950
R1	10 mg/l Cd	D4	0.131	0.0	0.000	99	22.696 ± 19.469	11.823 ± 14.374
R1	10 mg/l Cd	D5	0.117	0.0	0.000	102	26.158 ± 21.738	13.329 ± 13.224
R1	10 mg/l Cd	D6	0.202	0.1	9.891	112	29.693 ± 20.791	15.534 ± 14.400
R2	10 mg/l Cd	D7	0.185	0.1	10.793	136	26.151 ± 19.423	13.917 ± 14.019
R2	10 mg/l Cd	D8	0.150	0.1	13.378	110	21.081 ± 23.595	14.933 ± 23.795
R2	10 mg/l Cd	D9	0.092	0.1	21.716	116	17.736 ± 18.754	8.810 ± 12.645
R2	10 mg/l Cd	D10	0.188	0.1	10.655	118	28.251 ± 20.610	20.468 ± 21.163
R1	20 mg/l Cd	D1	0.195	0.1	10.256	149	20.588 ± 17.797	11.315 ± 13.751
R1	20 mg/l Cd	D2	0.248	0.2	16.136	159	18.131 ± 22.840	9.313 ± 13.841
R1	20 mg/l Cd	D3	0.170	0.1	11.772	125	18.663 ± 25.408	11.838 ± 19.537
R1	20 mg/l Cd	D4	0.178	0.1	11.268	109	25.014 ± 17.651	15.396 ± 14.913
R1	20 mg/l Cd	D5	0.138	0.1	14.472	104	39.753 ± 23.640	30.677 ± 23.241
R2	20 mg/l Cd	D6	0.182	0.1	10.965	145	12.862 ± 16.698	7.234 ± 13.401
R2	20 mg/l Cd	D7	0.174	0.1	11.521	155	20.133 ± 19.848	10.495 ± 13.925
R2	20 mg/l Cd	D8	0.125	0.0	0.000	113	18.783 ± 17.930	10.518 ± 12.923
R2	20 mg/l Cd	D9	0.213	0.2	18.824	102	42.478 ± 21.180	29.467 ± 18.917
R2	20 mg/l Cd	D10	0.147	0.1	13.633	120	14.090 ± 13.851	6.368 ± 8.528
R1	20 mg/l Ni	D1	0.219	0.0	0.000	270	16.726 ± 20.311	8.770 ± 14.261
R1	20 mg/l Ni	D2	0.172	0.0	0.000	138	22.954 ± 20.738	13.563 ± 15.523
R1	20 mg/l Ni	D3	0.096	0.0	0.000	118	25.607 ± 23.885	13.704 ± 16.897
R1	20 mg/l Ni	D4	0.140	0.0	0.000	112	20.896 ± 23.566	11.189 ± 16.043
R1	20 mg/l Ni	D5	0.130	0.0	0.000	98	20.425 ± 25.054	10.479 ± 16.948
R2	20 mg/l Ni	D6	0.129	0.0	0.000	130	7.070 ± 15.285	3.630 ± 10.441
R2	20 mg/l Ni	D7	0.129	0.0	0.000	124	16.469 ± 20.886	8.265 ± 14.255
R2	20 mg/l Ni	D8	0.091	0.0	0.000	150	7.788 ± 14.064	3.083 ± 9.411
R2	20 mg/l Ni	D9	0.166	0.0	0.000	134	25.212 ± 24.452	15.041 ± 19.636
R2	20 mg/l Ni	D10	0.134	0.0	0.000	101	15.046 ± 19.499	7.903 ± 13.630

Table 6: Summary (mean ± standard deviation) of comet assay parameters Tail DNA % and Olive tail moment per individual earthworm, with individual weight and metal content for *E. fetida* exposed to Cd in artificial soil water. K = negative control; Ni = positive control (20 mg/l Ni). AA = Atomic absorption spectrophotometer reading (mg/l); Body = metal body load (mg/kg). * Animals not weighed and analyzed for metal content.

Repli-cate	Treat-ment	Ani-mal	Mass (g)	AA (mg/l)	Body (mg/kg)	Comet *n*	Tail DNA %	Olive tail moment (arbitrary units)
R1	K	E1	0.189	0.00	0.000	158	15.645 ± 17.239	8.188 ± 12.231
R1	K	E2	0.172	0.00	0.000	221	21.214 ± 20.674	9.920 ± 12.534
R1	K	E3	0.183	0.00	0.000	243	13.313 ± 13.854	4.811 ± 8.062
R2	K	E4	0.185	0.00	0.000	141	34.074 ± 21.502	21.862 ± 21.362
R2	K	E5	0.257	0.00	0.000	150	10.063 ± 11.966	6.762 ± 13.111
R2	K	E6	0.212	0.00	0.000	145	19.564 ± 16.884	11.712 ± 14.425
R2	K	E7	0.237	0.00	0.000	207	4.236 ± 5.545	0.733 ± 1.091
R2	K	E8	0.231	0.00	0.000	163	10.731 ± 12.318	5.531 ± 11.238
R2	K	E9	0.159	0.00	0.000	141	18.224 ± 19.776	10.639 ± 15.658
R2	K	E10	0.175	0.00	0.000	146	16.703 ± 15.558	9.157 ± 13.378
R1	2.5 mg/l Cd	E1	0.310	0.02	1.290	112	23.943 ± 20.223	14.768 ± 16.205
R1	2.5 mg/l Cd	E2	0.250	0.00	0.000	116	28.496 ± 24.057	16.150 ± 20.201
R1	2.5 mg/l Cd	E3	0.221	0.00	0.000	236	16.207 ± 18.820	7.351 ± 14.455
R1	2.5 mg/l Cd	E4	0.115	0.00	0.000	206	17.314 ± 19.657	7.126 ± 12.757
R1	2.5 mg/l Cd	E5	0.187	0.00	0.000	221	46.346 ± 13.775	21.791 ± 11.093
R2	2.5 mg/l Cd	E6	0.286	0.00	0.000	139	22.880 ± 18.060	14.323 ± 17.360
R2	2.5 mg/l Cd	E7	0.239	0.14	11.715	165	12.908 ± 14.343	6.907 ± 11.122
R2	2.5 mg/l Cd	E8	0.246	0.21	17.073	150	14.664 ± 13.581	7.117 ± 9.123
R2	2.5 mg/l Cd	E9	0.195	0.00	0.000	213	13.364 ± 12.832	5.789 ± 9.079
R2	2.5 mg/l Cd	E10	0.200	0.00	0.000	169	23.869 ± 18.651	15.961 ± 19.802
R1	5 mg/l Cd	E1	0.280	0.00	0.000	105	30.516 ± 22.029	20.738 ± 20.691
R1	5 mg/l Cd	E2	0.190	0.00	0.000	178	27.779 ± 17.528	12.636 ± 12.139
R1	5 mg/l Cd	E3	0.150	0.00	0.000	121	41.038 ± 24.265	21.824 ± 17.394
R1	5 mg/l Cd	E4	0.161	0.00	0.000	133	29.122 ± 22.615	16.831 ± 18.684
R1	5 mg/l Cd	E5	0.215	0.00	0.000	194	14.655 ± 16.562	7.279 ± 13.375
R2	5 mg/l Cd	E6	0.182	0.00	0.000	124	23.384 ± 18.753	16.087 ± 18.958
R2	5 mg/l Cd	E7	0.202	0.00	0.000	156	27.276 ± 20.088	20.708 ± 24.522
R2	5 mg/l Cd	E8	0.196	0.00	0.000	156	27.275 ± 18.378	18.227 ± 19.295
R2	5 mg/l Cd	E9	0.191	0.00	0.000	181	16.954 ± 15.775	11.167 ± 15.885
R2	5 mg/l Cd	E10	0.175	0.00	0.000	142	28.941 ± 18.679	17.965 ± 19.616
R1	10 mg/l Cd	E1	0.204	0.00	0.000	122	33.195 ± 26.135	20.543 ± 21.870
R1	10 mg/l Cd	E2	0.150	0.00	0.000	136	34.365 ± 24.472	21.369 ± 19.650
R1	10 mg/l Cd	E3	0.156	0.00	0.000	106	38.020 ± 22.359	29.214 ± 23.475
R1	10 mg/l Cd	E4	0.093	0.00	0.000	144	27.466 ± 21.792	15.758 ± 17.665
R1	10 mg/l Cd	E5	0.182	0.00	0.000	90	30.107 ± 19.412	17.508 ± 16.380
R2	10 mg/l Cd	E6	0.190	0.06	6.316	179	21.160 ± 14.468	14.778 ± 14.504
R2	10 mg/l Cd	E7	0.210	0.01	0.952	178	22.243 ± 18.098	16.729 ± 18.812
R2	10 mg/l Cd	E8	0.196	0.00	0.000	197	21.595 ± 17.067	18.141 ± 20.805
R2	10 mg/l Cd	E9	0.278	0.18	12.950	185	19.293 ± 15.042	12.115 ± 14.856
R2	10 mg/l Cd	E10	0.246	0.01	0.813	208	15.784 ± 12.115	11.632 ± 15.517
R1	20 mg/l Cd	E1	*	*	*	109	29.212 ± 23.810	19.674 ± 21.666
R1	20 mg/l Cd	E2	*	*	*	106	44.627 ± 21.057	30.331 ± 20.898
R1	20 mg/l Cd	E3	*	*	*	104	44.256 ± 21.926	32.523 ± 24.938
R1	20 mg/l Cd	E4	*	*	*	114	33.029 ± 20.854	21.511 ± 19.091
R1	20 mg/l Cd	E5	*	*	*	147	34.100 ± 21.515	24.742 ± 20.230

Table 6 (continued)

R2	20 mg/l Cd	E6	0.172	0.13	15.116	138	31.004 ± 18.077	25.635 ± 23.513
R2	20 mg/l Cd	E7	0.213	0.01	0.939	125	36.291 ± 16.892	27.125 ± 21.814
R2	20 mg/l Cd	E8	0.214	0.02	1.869	147	38.397 ± 18.763	29.977 ± 23.411
R2	20 mg/l Cd	E9	0.231	0.04	3.463	169	30.149 ± 18.832	22.229 ± 20.152
R2	20 mg/l Cd	E10	0.309	0.01	0.647	159	19.752 ± 16.922	11.567 ± 14.232
R1	20 mg/l Ni	E1	0.193	0.00	0.000	177	19.465 ± 21.412	9.887 ± 14.095
R1	20 mg/l Ni	E2	0.228	0.00	0.000	118	35.704 ± 23.056	23.430 ± 22.716
R1	20 mg/l Ni	E3	0.163	0.00	0.000	250	18.038 ± 18.348	6.521 ± 9.318
R1	20 mg/l Ni	E4	0.232	0.00	0.000	186	25.923 ± 24.392	15.830 ± 23.195
R1	20 mg/l Ni	E5	0.269	0.00	0.000	213	30.196 ± 23.567	17.995 ± 20.126
R2	20 mg/l Ni	E6	0.281	0.00	0.000	164	23.480 ± 19.246	15.604 ± 16.856
R2	20 mg/l Ni	E7	0.260	0.00	0.000	137	21.341 ± 19.913	16.376 ± 22.510
R2	20 mg/l Ni	E8	0.223	0.00	0.000	136	31.625 ± 20.715	24.366 ± 22.042
R2	20 mg/l Ni	E9	0.221	0.13	11.765	176	30.962 ± 17.836	20.319 ± 17.939
R2	20 mg/l Ni	E10	0.214	0.00	0.000	127	29.745 ± 20.217	22.205 ± 19.786

Table 7: Summary (mean ± standard deviation) of comet assay parameters Tail DNA % and Olive tail moment per individual earthworm, with individual weight and metal content for *M. benhami* exposed to Cd in artificial soil water. K = negative control; Ni = positive control (20 mg/l Ni). AA = Atomic absorption spectrophotometer reading (mg/l); Body = metal body load (mg/kg).

Repli-cate	Treat-ment	Ani-mal	Mass (g)	AA (mg/l)	Body (mg/kg)	Comet *n*	Tail DNA %	Olive tail moment (arbitrary units)
R2	K	M1	5.040	0.15	0.595	93	14.490 ± 8.022	3.246 ± 2.851
R1	K	M2	1.290	0.00	0.000	99	7.330 ± 8.046	1.458 ± 3.155
R1	K	M3	1.140	0.00	0.000	102	5.218 ± 5.509	1.061 ± 1.402
R3	K	M4	0.980	0.06	1.224	98	27.819 ± 13.576	7.704 ± 4.102
R2	K	M5	2.240	0.00	0.000	94	9.025 ± 9.652	1.921 ± 2.876
R2	2.5 mg/l Cd	M1	5.350	0.17	0.636	156	5.829 ± 6.188	1.255 ± 1.988
R3	2.5 mg/l Cd	M2	1.080	0.13	2.315	52	10.695 ± 14.961	3.556 ± 8.757
R2	2.5 mg/l Cd	M3	1.950	0.19	1.949	106	9.265 ± 7.577	1.829 ± 1.935
R2	2.5 mg/l Cd	M4	1.750	0.52	5.943	100	12.508 ± 9.702	2.518 ± 2.930
R2	5 mg/l Cd	M1	2.150	0.01	0.093	102	14.182 ± 11.100	3.850 ± 3.085
R2	5 mg/l Cd	M2	0.700	0.04	1.143	110	4.640 ± 6.219	1.224 ± 2.117
R1	5 mg/l Cd	M3	6.170	1.52	4.927	117	9.522 ± 10.987	3.749 ± 7.203
R1	5 mg/l Cd	M4	4.300	0.49	2.279	99	11.342 ± 7.638	3.654 ± 3.798
R2	10 mg/l Cd	M1	7.080	0.78	2.203	114	4.074 ± 4.429	0.930 ± 1.099
R4	10 mg/l Cd	M2	5.660	0.95	3.357	92	10.531 ± 14.992	6.138 ± 13.238
R4	10 mg/l Cd	M3	5.240	0.47	1.794	96	7.694 ± 12.254	3.935 ± 10.646
R2	10 mg/l Cd	M4	1.000	0.46	9.200	105	10.443 ± 10.972	2.432 ± 3.873
R2	20 mg/l Cd	M1	3.900	2.06	10.564	108	16.493 ± 12.427	4.031 ± 4.589
R1	20 mg/l Cd	M2	7.210	2.89	8.017	49	8.814 ± 7.729	2.530 ± 2.984
R1	20 mg/l Cd	M3	6.120	1.36	4.444	98	15.127 ± 15.974	7.443 ± 13.102
R1	20 mg/l Ni	M1	3.890	0.02	0.103	104	13.953 ± 12.839	6.001 ± 9.792
R1	20 mg/l Ni	M2	3.460	0.07	0.405	124	11.856 ± 9.485	3.645 ± 7.037
R3	20 mg/l Ni	M3	0.450	0.00	0.000	45	12.595 ± 8.222	2.990 ± 2.115
R3	20 mg/l Ni	M4	1.380	0.00	0.000	100	16.312 ± 11.371	4.223 ± 3.639
R1	20 mg/l Ni	M5	3.700	0.20	1.081	108	9.384 ± 7.226	2.904 ± 3.000
R1	20 mg/l Ni	M6	0.540	0.00	0.000	104	8.266 ± 5.522	2.440 ± 2.039
R1	20 mg/l Ni	M7	5.120	0.11	0.430	101	18.305 ± 12.863	5.617 ± 5.011

Table 8: Summary (mean ± standard deviation) of earthworm mass, metal body load and comet parameters (Tail DNA % and Olive tail moment) per treatment for five earthworm species exposed in artificial soil water to Cd. K = negative control, 20 mg/l Ni = positive control. * For *E. fetida* exposure 20 mg/l Cd n = 5 for mass and metal body content. ** The mean value for negative control is subtracted from the mean value for each treatment

Species	Treatment	Animal *n*	Mass (g)	Metal body content (mg/kg)	Tail DNA %	Olive tail moment (arbitrary units)	** Net Tail DNA %	** Net Olive tail moment
A. diffringens	K	5	0.73 ± 0.17	6.19 ± 1.18	18.68 ± 4.81	8.22 ± 3.36		
	2.5 mg/l Cd	6	0.82 ± 0.15	36.23 ± 23.47	12.94 ± 3.57	4.07 ± 1.76	-5.74	-4.15
	5 mg/l Cd	8	0.65 ± 0.23	28.79 ± 8.54	26.42 ± 10.01	14.57 ± 8.06	7.74	6.36
	10 mg/l Cd	7	0.75 ± 0.17	24.44 ± 9.73	23.89 ± 5.65	11.78 ± 4.07	5.20	3.56
	20 mg/l Cd	8	0.69 ± 0.17	102.01 ± 83.75	22.05 ± 5.45	10.57 ± 4.08	3.37	2.35
	20 mg/l Ni	4	0.66 ± 0.19	151.39 ± 177.11	17.77 ± 6.77	7.42 ± 4.20	-0.91	-0.80
A. caliginosa	K	10	0.68 ± 0.07	3.72 ± 3.78	17.02 ± 4.25	7.20 ± 2.16		
	2.5 mg/l Cd	10	0.61 ± 0.17	17.61 ± 10.87	20.48 ± 7.96	8.86 ± 3.90	3.46	1.67
	5 mg/l Cd	10	0.69 ± 0.12	19.02 ± 6.25	18.99 ± 8.67	8.11 ± 4.78	1.97	0.91
	10 mg/l Cd	10	0.70 ± 0.14	16.47 ± 5.94	21.65 ± 6.67	8.62 ± 3.34	4.63	1.42
	20 mg/l Cd	10	0.64 ± 0.07	30.62 ± 20.39	24.76 ± 10.05	12.91 ± 9.11	7.74	5.71
	20 mg/l Ni	10	0.67 ± 0.18	62.24 ± 83.71	16.61 ± 4.84	6.59 ± 2.34	-0.41	-0.61
D. rubidus	K	10	0.18 ± 0.04	3.68 ± 6.04	7.57 ± 3.70	3.38 ± 1.98		
	2.5 mg/l Cd	10	0.15 ± 0.03	5.06 ± 6.60	20.61 ± 7.21	11.88 ± 4.92	13.04	8.50
	5 mg/l Cd	10	0.16 ± 0.02	4.66 ± 6.08	16.20 ± 6.32	8.35 ± 4.24	8.63	4.97
	10 mg/l Cd	10	0.15 ± 0.04	11.19 ± 7.11	23.86 ± 7.07	13.27 ± 4.94	16.28	9.89
	20 mg/l Cd	10	0.18 ± 0.04	11.88 ± 4.96	23.05 ± 10.11	14.26 ± 8.70	15.48	10.88
	20 mg/l Ni	10	0.14 ± 0.04	0.00 ± 0.00	17.82 ± 6.54	9.56 ± 4.07	10.25	6.18
E. fetida	K	10	0.20 ± 0.03	0.00 ± 0.00	16.38 ± 8.02	8.93 ± 5.58		
	2.5 mg/l Cd	10	0.22 ± 0.06	3.01 ± 6.15	22.00 ± 10.05	11.73 ± 5.53	5.62	2.80
	5 mg/l Cd	10	0.19 ± 0.04	0.00 ± 0.00	26.69 ± 7.33	16.35 ± 4.69	10.32	7.41
	10 mg/l Cd	10	0.19 ± 0.05	2.10 ± 4.28	26.32 ± 7.38	17.78 ± 5.12	9.95	8.85
	20 mg/l Cd	10*	0.23 ± 0.05	4.41 ± 6.09	34.08 ± 7.40	24.53 ± 6.16	17.70	15.60
	20 mg/l Ni	10	0.23 ± 0.04	1.18 ± 3.72	26.65 ± 5.89	17.25 ± 5.76	10.27	8.32
M. benhami	K	5	2.14 ± 1.69	0.36 ± 0.55	12.78 ± 9.08	3.08 ± 2.71		
	2.5 mg/l Cd	4	2.53 ± 1.91	2.71 ± 2.27	9.57 ± 2.83	2.29 ± 0.99	-3.20	-0.79
	5 mg/l Cd	4	3.33 ± 2.40	2.11 ± 2.08	9.92 ± 4.01	3.12 ± 1.27	-2.86	0.04
	10 mg/l Cd	4	4.75 ± 2.62	4.14 ± 3.44	8.19 ± 3.04	3.36 ± 2.22	-4.59	0.28
	20 mg/l Cd	3	5.74 ± 1.69	7.68 ± 3.07	13.48 ± 4.10	4.67 ± 2.52	0.70	1.59
	20 mg/l Ni	7	2.65 ± 1.84	0.29 ± 0.40	12.95 ± 3.58	3.97 ± 1.38	0.18	0.90

Table 9: Cell nucleus areas (mean ± standard deviation) of five earthworm species.

Species	Nucleus *n*	Nucleus area (arbitrary units)
A. diffringens	28	975.286 ± 191.367
A. caliginosa	109	1535.679 ± 349.969
D. rubidus	581	1670.642 ± 355.396
E. fetida	169	1169.947 ± 367.922
M. benhami	118	1384.424 ± 335.859

Table 10: Mass (mean ± standard deviation) of five earthworm species.

Species	Animal *n*	Mass (g)
A. diffringens	40	0.763 ± 0.273
A. caliginosa	60	0.665 ± 0.130
D. rubidus	60	0.159 ± 0.037
E. fetida	55	0.209 ± 0.045
M. benhami	27	3.292 ± 2.190

Table 11: The number of animals in each treatment that has a mean DNA damage value higher than the mean DNA damage value in the negative control for each species in five earthworm species exposed to Cd in artificial soil water. These values were used to calculate EC50s. K = negative control

Species	Treatment	No animals exposed	Tail DNA % No. of animals higher than K mean	Olive tail moment No. of animals higher than K mean
A. diffringens	K	5	2	2
	2.5 mg/l Cd	8	1	1
	5 mg/l Cd	8	6	6
	10 mg/l Cd	7	6	6
	20 mg/l Cd	8	5	4
A. caliginosa	K	10	5	5
	2.5 mg/l Cd	10	7	5
	5 mg/l Cd	10	4	5
	10 mg/l Cd	10	7	6
	20 mg/l Cd	10	8	7
D. rubidus	K	10	4	3
	2.5 mg/l Cd	10	10	10
	5 mg/l Cd	10	8	8
	10 mg/l Cd	10	10	10
	20 mg/l Cd	10	10	10
E. fetida	K	10	4	5
	2.5 mg/l Cd	10	6	5
	5 mg/l Cd	10	8	9
	10 mg/l Cd	10	9	10
	20 mg/l Cd	10	10	10
M. benhami	K	5	2	1
	2.5 mg/l Cd	4	0	0
	5 mg/l Cd	4	1	0
	10 mg/l Cd	4	0	1
	20 mg/l Cd	3	2	2

APPENDIX C

Statistical results

Table 1: DNA damage as detected with the comet assay (Tail DNA % and Olive tail moment) compared with the Kruskal-Wallis ANOVA by ranks between treatments in five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$).

Species		Tail DNA %	Olive tail moment
A. diffringens	*n*	38	38
	H	13.94518	14.86839
	P	**0.016**	**0.0109**
	df	5	5
A. caliginosa	*n*	68	68
	H	9.918407	6.839886
	P	0.0776	0.2328
	df	5	5
D. rubidus	*n*	60	60
	H	25.0623	25.74623
	P	**0.0001**	**0.0001**
	df	5	5
E. fetida	*n*	60	60
	H	21.19279	26.36525
	P	**0.0007**	**0.0001**
	df	5	5
M. benhami	*n*	27	27
	H	4.96198	4.838624
	P	0.4205	0.4369
	df	5	5

Table 2: Pairwise comparisons (Mann-Whitney U test) between exposure treatments for DNA damage (Tail DNA %) as detected with the comet assay in three earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
A. diffringens	K	vs	2.5 mg/l Cd	5.000	1.826	0.068	5	6
	K	vs	5 mg/l Cd	12.000	-1.171	0.242	5	8
	K	vs	10 mg/l Cd	9.000	-1.380	0.167	5	7
	K	vs	20 mg/l Cd	12.000	-1.171	0.242	5	8
	K	vs	20 mg/l Ni	8.000	0.490	0.624	5	4
	2.5 mg/l Cd	vs	5 mg/l Cd	**6.000**	**-2.324**	**0.020**	**6**	**8**
	2.5 mg/l Cd	vs	10 mg/l Cd	**1.000**	**-2.857**	**0.004**	**6**	**7**

Table 2 (continued)

	2.5 mg/l Cd	vs	20 mg/l Cd	**0.000**	**-3.098**	**0.002**	**6**	**8**
	2.5 mg/l Cd	vs	20 mg/l Ni	6.000	-1.279	0.201	6	4
	5 mg/l Cd	vs	10 mg/l Cd	22.000	0.694	0.487	8	7
	5 mg/l Cd	vs	20 mg/l Cd	21.000	1.155	0.248	8	8
	5 mg/l Cd	vs	20 mg/l Ni	6.000	1.698	0.089	8	4
	10 mg/l Cd	vs	20 mg/l Cd	22.000	0.694	0.487	7	8
	10 mg/l Cd	vs	20 mg/l Ni	7.000	1.323	0.186	7	4
	20 mg/l Cd	vs	20 mg/l Ni	10.000	1.019	0.308	8	4
	K	vs	2.5 mg/l Cd	**1.000**	**-3.704**	**< 0.001**	**10**	**10**
	K	vs	5 mg/l Cd	**12.000**	**-2.873**	**0.004**	**10**	**10**
	K	vs	10 mg/l Cd	**3.000**	**-3.553**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Cd	**2.000**	**-3.628**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Ni	**9.000**	**-3.099**	**0.002**	**10**	**10**
	2.5 mg/l Cd	vs	5 mg/l Cd	34.000	1.209	0.226	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	33.000	-1.285	0.199	10	10
D. rubidus	2.5 mg/l Cd	vs	20 mg/l Cd	43.000	-0.529	0.597	10	10
	2.5 mg/l Cd	vs	20 mg/l Ni	44.000	0.454	0.650	10	10
	5 mg/l Cd	vs	10 mg/l Cd	**18.000**	**-2.419**	**0.016**	**10**	**10**
	5 mg/l Cd	vs	20 mg/l Cd	34.000	-1.209	0.226	10	10
	5 mg/l Cd	vs	20 mg/l Ni	40.000	-0.756	0.450	10	10
	10 mg/l Cd	vs	20 mg/l Cd	37.000	0.983	0.326	10	10
	10 mg/l Cd	vs	20 mg/l Ni	**22.000**	**2.117**	**0.034**	**10**	**10**
	20 mg/l Cd	vs	20 mg/l Ni	42.000	0.605	0.545	10	10
	K	vs	2.5 mg/l Cd	32.000	-1.361	0.174	10	10
	K	vs	5 mg/l Cd	**17.000**	**-2.495**	**0.013**	**10**	**10**
	K	vs	10 mg/l Cd	**15.000**	**-2.646**	**0.008**	**10**	**10**
	K	vs	20 mg/l Cd	**6.000**	**-3.326**	**0.001**	**10**	**10**
	K	vs	20 mg/l Ni	**14.000**	**-2.721**	**0.007**	**10**	**10**
	2.5 mg/l Cd	vs	5 mg/l Cd	28.000	-1.663	0.096	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	33.000	-1.285	0.199	10	10
E. fetida	2.5 mg/l Cd	vs	20 mg/l Cd	**14.000**	**-2.721**	**0.007**	**10**	**10**
	2.5 mg/l Cd	vs	20 mg/l Ni	26.000	-1.814	0.070	10	10
	5 mg/l Cd	vs	10 mg/l Cd	49.000	0.076	0.940	10	10
	5 mg/l Cd	vs	20 mg/l Cd	**17.000**	**-2.495**	**0.013**	**10**	**10**
	5 mg/l Cd	vs	20 mg/l Ni	45.000	-0.378	0.705	10	10
	10 mg/l Cd	vs	20 mg/l Cd	**24.000**	**-1.965**	**0.049**	**10**	**10**
	10 mg/l Cd	vs	20 mg/l Ni	48.000	-0.151	0.880	10	10
	20 mg/l Cd	vs	20 mg/l Ni	**21.000**	**2.192**	**0.028**	**10**	**10**

Table 3: Pairwise comparisons (Mann-Whitney U test) between exposure treatments for DNA damage (Olive tail moment) as detected with the comet assay in three earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
	K	vs	2.5 mg/l Cd	3.000	2.191	0.028	5	6
	K	vs	5 mg/l Cd	11.000	-1.317	0.188	5	8
	K	vs	10 mg/l Cd	10.000	-1.218	0.223	5	7
	K	vs	20 mg/l Cd	10.000	-1.464	0.143	5	8
	K	vs	20 mg/l Ni	6.000	0.980	0.327	5	4
	2.5 mg/l Cd	vs	5 mg/l Cd	**5.000**	**-2.453**	**0.014**	**6**	**8**
	2.5 mg/l Cd	vs	10 mg/l Cd	**1.000**	**-2.857**	**0.004**	**6**	**7**
A. diffringens	2.5 mg/l Cd	vs	20 mg/l Cd	**0.000**	**-3.098**	**0.002**	**6**	**8**
	2.5 mg/l Cd	vs	20 mg/l Ni	6.000	-1.279	0.201	6	4
	5 mg/l Cd	vs	10 mg/l Cd	21.000	0.810	0.418	8	7
	5 mg/l Cd	vs	20 mg/l Cd	23.000	0.945	0.345	8	8
	5 mg/l Cd	vs	20 mg/l Ni	5.000	1.868	0.062	8	4
	10 mg/l Cd	vs	20 mg/l Cd	21.000	0.810	0.418	7	8
	10 mg/l Cd	vs	20 mg/l Ni	8.000	1.134	0.257	7	4
	20 mg/l Cd	vs	20 mg/l Ni	10.000	1.019	0.308	8	4
	K	vs	2.5 mg/l Cd	**1.000**	**-3.704**	**< 0.001**	**10**	**10**
	K	vs	5 mg/l Cd	**11.000**	**-2.948**	**0.003**	**10**	**10**
	K	vs	10 mg/l Cd	**3.000**	**-3.553**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Cd	**2.000**	**-3.628**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Ni	**7.000**	**-3.250**	**0.001**	**10**	**10**
	2.5 mg/l Cd	vs	5 mg/l Cd	28.000	1.663	0.096	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	37.000	-0.983	0.326	10	10
D. rubidus	2.5 mg/l Cd	vs	20 mg/l Cd	47.000	-0.227	0.821	10	10
	2.5 mg/l Cd	vs	20 mg/l Ni	44.000	0.454	0.650	10	10
	5 mg/l Cd	vs	10 mg/l Cd	**20.000**	**-2.268**	**0.023**	**10**	**10**
	5 mg/l Cd	vs	20 mg/l Cd	26.000	-1.814	0.070	10	10
	5 mg/l Cd	vs	20 mg/l Ni	40.000	-0.756	0.450	10	10
	10 mg/l Cd	vs	20 mg/l Cd	44.000	0.454	0.650	10	10
	10 mg/l Cd	vs	20 mg/l Ni	26.000	1.814	0.070	10	10
	20 mg/l Cd	vs	20 mg/l Ni	35.000	1.134	0.257	10	10
	K	vs	2.5 mg/l Cd	36.000	-1.058	0.290	10	10
	K	vs	5 mg/l Cd	**16.000**	**-2.570**	**0.010**	**10**	**10**
	K	vs	10 mg/l Cd	**10.000**	**-3.024**	**0.002**	**10**	**10**
	K	vs	20 mg/l Cd	**4.000**	**-3.477**	**0.001**	**10**	**10**
	K	vs	20 mg/l Ni	**16.000**	**-2.570**	**0.010**	**10**	**10**
	2.5 mg/l Cd	vs	5 mg/l Cd	**23.000**	**-2.041**	**0.041**	**10**	**10**
	2.5 mg/l Cd	vs	10 mg/l Cd	**21.000**	**-2.192**	**0.028**	**10**	**10**
E. fetida	2.5 mg/l Cd	vs	20 mg/l Cd	**7.000**	**-3.250**	**0.001**	**10**	**10**
	2.5 mg/l Cd	vs	20 mg/l Ni	**23.000**	**-2.041**	**0.041**	**10**	**10**
	5 mg/l Cd	vs	10 mg/l Cd	49.000	-0.076	0.940	10	10
	5 mg/l Cd	vs	20 mg/l Cd	**12.000**	**-2.873**	**0.004**	**10**	**10**
	5 mg/l Cd	vs	20 mg/l Ni	46.000	-0.302	0.762	10	10
	10 mg/l Cd	vs	20 mg/l Cd	**18.000**	**-2.419**	**0.016**	**10**	**10**
	10 mg/l Cd	vs	20 mg/l Ni	49.000	-0.076	0.940	10	10
	20 mg/l Cd	vs	20 mg/l Ni	**17.000**	**2.495**	**0.013**	**10**	**10**

Table 4: Spearman Rank order correlation between DNA damage as measured with comet assay parameters Tail DNA % and Olive tail moment and Cd exposure concentration for five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$).

		n	Spearman R	*P*
A. diffringens	Tail DNA %	5	0.5000	0.3910
	Olive tail moment	5	0.5000	0.3910
A. caliginosa	Tail DNA %	**5**	**0.9000**	**0.0374**
	Olive tail moment	5	0.7000	0.1881
D. rubidus	Tail DNA %	5	0.8000	0.1041
	Olive tail moment	**5**	**0.9000**	**0.0374**
E. fetida	Tail DNA %	**5**	**0.9000**	**0.0374**
	Olive tail moment	**5**	**1.0000**	**< 0.05**
M. benhami	Tail DNA %	5	0.1000	0.8729
	Olive tail moment	**5**	**0.9000**	**0.0374**

Table 5: Cd body loads compared with the Kruskal-Wallis ANOVA by ranks between treatments in five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$).

Species		Cd body load (mg/kg)
A. diffringens	*n*	34
	H	15.12875
	P	**0.044**
	df	4
A. caliginosa	*n*	50
	H	27.59589
	P	**< 0.001**
	df	4
D. rubidus	*n*	60
	H	21.1554
	P	**0.008**
	df	5
E. fetida	*n*	45
	H	19.47177
	P	**0.0006**
	df	4
M. benhami	*n*	20
	H	11.89387
	P	**0.0182**
	df	4

Table 6: Pairwise comparisons (Mann-Whitney U test) between exposure treatments for the metal body load in five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
A. diffringens	K	vs	2.5 mg/l Cd	**0.000**	**-2.739**	**0.006**	**5**	**6**
	K	vs	5 mg/l Cd	**0.000**	**-2.928**	**0.003**	**5**	**8**
	K	vs	10 mg/l Cd	**0.000**	**-2.842**	**0.004**	**5**	7
	K	vs	20 mg/l Cd	**0.000**	**-2.928**	**0.003**	**5**	**8**
	K	vs	20 mg/l Ni	**0.000**	**-2.449**	**0.014**	**5**	**4**
	2.5 mg/l Cd	vs	5 mg/l Cd	19.000	0.645	0.519	6	8
	2.5 mg/l Cd	vs	10 mg/l Cd	15.000	0.857	0.391	6	7
	2.5 mg/l Cd	vs	20 mg/l Cd	13.000	-1.420	0.156	6	8
	2.5 mg/l Cd	vs	20 mg/l Ni	5.000	-1.492	0.136	6	4
	5 mg/l Cd	vs	10 mg/l Cd	19.000	1.042	0.298	8	7
	5 mg/l Cd	vs	20 mg/l Cd	23.000	-0.945	0.345	8	8
	5 mg/l Cd	vs	20 mg/l Ni	**2.000**	**-2.378**	**0.017**	**8**	**4**
	10 mg/l Cd	vs	20 mg/l Cd	14.000	-1.620	0.105	7	8
	10 mg/l Cd	vs	20 mg/l Ni	**2.000**	**-2.268**	**0.023**	**7**	**4**
	20 mg/l Cd	vs	20 mg/l Ni	14.000	-0.340	0.734	8	4
A. caliginosa	K	vs	2.5 mg/l Cd	**5.000**	**-3.402**	**0.001**	**10**	**10**
	K	vs	5 mg/l Cd	**0.000**	**-3.780**	**< 0.001**	**10**	**10**
	K	vs	10 mg/l Cd	**1.000**	**-3.704**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Cd	**0.000**	**-3.780**	**< 0.001**	**10**	**10**
	K	vs	20 mg/l Ni	**24.000**	**-1.965**	**0.049**	**10**	**10**
	2.5 mg/l Cd	vs	5 mg/l Cd	37.000	-0.983	0.326	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	46.000	-0.302	0.762	10	10
	2.5 mg/l Cd	vs	20 mg/l Cd	25.000	-1.890	0.059	10	10
	2.5 mg/l Cd	vs	20 mg/l Ni	38.000	-0.907	0.364	10	10
	5 mg/l Cd	vs	10 mg/l Cd	36.000	1.058	0.290	10	10
	5 mg/l Cd	vs	20 mg/l Cd	28.000	-1.663	0.096	10	10
	5 mg/l Cd	vs	20 mg/l Ni	37.000	-0.983	0.326	10	10
	10 mg/l Cd	vs	20 mg/l Cd	**13.000**	**-2.797**	**0.005**	**10**	**10**
	10 mg/l Cd	vs	20 mg/l Ni	34.000	-1.209	0.226	10	10
	20 mg/l Cd	vs	20 mg/l Ni	50.000	0.000	1.000	10	10
D. rubidus	K	vs	2.5 mg/l Cd	45.000	-0.378	0.705	10	10
	K	vs	5 mg/l Cd	46.000	-0.302	0.762	10	10
	K	vs	10 mg/l Cd	**24.000**	**-1.965**	**0.049**	**10**	**10**
	K	vs	20 mg/l Cd	**17.500**	**-2.457**	**0.014**	**10**	**10**
	K	vs	20 mg/l Ni	35.000	1.134	0.257	10	10
	2.5 mg/l Cd	vs	5 mg/l Cd	46.000	0.302	0.762	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	29.000	-1.587	0.112	10	10
	2.5 mg/l Cd	vs	20 mg/l Cd	24.500	-1.928	0.054	10	10
	2.5 mg/l Cd	vs	20 mg/l Ni	30.000	1.512	0.131	10	10
	5 mg/l Cd	vs	10 mg/l Cd	27.000	-1.739	0.082	10	10
	5 mg/l Cd	vs	20 mg/l Cd	**18.000**	**-2.419**	**0.016**	**10**	**10**
	5 mg/l Cd	vs	20 mg/l Ni	30.000	1.512	0.131	10	10
	10 mg/l Cd	vs	20 mg/l Cd	42.000	-0.605	0.545	10	10
	10 mg/l Cd	vs	20 mg/l Ni	**10.000**	**3.024**	**0.002**	**10**	**10**
	20 mg/l Cd	vs	20 mg/l Ni	**5.000**	**3.402**	**0.001**	**10**	**10**

Table 6 (continued)

	K	vs	2.5 mg/l Cd	35.000	-1.134	0.257	10	10
	K	vs	5 mg/l Cd	50.000	0.000	1.000	10	10
	K	vs	10 mg/l Cd	30.000	-1.512	0.131	10	10
	K	vs	20 mg/l Cd	**0.000**	**-3.062**	**0.002**	**10**	**5**
	K	vs	20 mg/l Ni	45.000	-0.378	0.705	10	10
	2.5 mg/l Cd	vs	5 mg/l Cd	35.000	1.134	0.257	10	10
	2.5 mg/l Cd	vs	10 mg/l Cd	48.000	-0.151	0.880	10	10
E. fetida	2.5 mg/l Cd	vs	20 mg/l Cd	11.000	-1.715	0.086	10	5
	2.5 mg/l Cd	vs	20 mg/l Ni	40.500	0.718	0.473	10	10
	5 mg/l Cd	vs	10 mg/l Cd	30.000	-1.512	0.131	10	10
	5 mg/l Cd	vs	20 mg/l Cd	**0.000**	**-3.062**	**0.002**	**10**	**5**
	5 mg/l Cd	vs	20 mg/l Ni	45.000	-0.378	0.705	10	10
	10 mg/l Cd	vs	20 mg/l Cd	11.000	-1.715	0.086	10	5
	10 mg/l Cd	vs	20 mg/l Ni	36.000	1.058	0.290	10	10
	20 mg/l Cd	vs	20 mg/l Ni	**4.000**	**2.572**	**0.010**	**5**	**10**
	K	vs	2.5 mg/l Cd	**1.000**	**-2.205**	**0.027**	**5**	**4**
	K	vs	5 mg/l Cd	3.000	-1.715	0.086	5	4
	K	vs	10 mg/l Cd	**0.000**	**-2.449**	**0.014**	**5**	**4**
	K	vs	20 mg/l Cd	**0.000**	**-2.236**	**0.025**	**5**	**3**
	K	vs	20 mg/l Ni	17.500	0.000	1.000	5	7
	2.5 mg/l Cd	vs	5 mg/l Cd	6.000	0.577	0.564	4	4
	2.5 mg/l Cd	vs	10 mg/l Cd	6.000	-0.577	0.564	4	4
M. benhami	2.5 mg/l Cd	vs	20 mg/l Cd	1.000	-1.768	0.077	4	3
	2.5 mg/l Cd	vs	20 mg/l Ni	**1.000**	**2.457**	**0.014**	**4**	**7**
	5 mg/l Cd	vs	10 mg/l Cd	5.000	-0.866	0.386	4	4
	5 mg/l Cd	vs	20 mg/l Cd	1.000	-1.768	0.077	4	3
	5 mg/l Cd	vs	20 mg/l Ni	4.000	1.890	0.059	4	7
	10 mg/l Cd	vs	20 mg/l Cd	2.000	-1.414	0.157	4	3
	10 mg/l Cd	vs	20 mg/l Ni	**0.000**	**2.646**	**0.008**	**4**	**7**
	20 mg/l Cd	vs	20 mg/l Ni	**0.000**	**2.393**	**0.017**	**3**	**7**

Table 7: Spearman Rank order correlation between mean Cd body load per exposure and Cd exposure concentration for five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$).

	n	Spearman R	*P*
A. diffringens	5	0.6000	0.2848
A. caliginosa	5	0.7000	0.1881
D. rubidus	**5**	**0.9000**	**0.0374**
E. fetida	5	0.6156	0.2690
M. benhami	**5**	**0.9000**	**0.0374**

Table 8: Spearman rank order correlation between earthworm mass and metal body load for five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

		Animal *n*	Spearman R	*P*
A. diffringens	K	5	-0.4000	0.5046
	2.5 mg/l Cd	6	-0.3143	0.5441
	5 mg/l Cd	8	0.2381	0.5702
	10 mg/l Cd	7	0.0000	1.0000
	20 mg/l Cd	8	0.1667	0.6932
	20 mg/l Ni	4	0.8000	0.2000
A. caliginosa	K	10	-0.2761	0.4400
	2.5 mg/l Cd	10	0.5636	0.0897
	5 mg/l Cd	10	-0.2727	0.4458
	10 mg/l Cd	10	-0.5636	0.0897
	20 mg/l Cd	**10**	**0.8667**	**0.0012**
	20 mg/l Ni	10	0.3465	0.3267
D. rubidus	K	10	-0.2759	0.4404
	2.5 mg/l Cd	10	0.0751	0.8366
	5 mg/l Cd	**10**	**0.7511**	**0.0123**
	10 mg/l Cd	10	-0.4924	0.1482
	20 mg/l Cd	10	0.3091	0.3848
	20 mg/l Ni	-	-	-
E. fetida	K	-	-	-
	2.5 mg/l Cd	10	0.4399	0.2033
	5 mg/l Cd	-	-	-
	10 mg/l Cd	**10**	**0.6828**	**0.0296**
	20 mg/l Cd	5	-0.6000	0.2848
	20 mg/l Ni	10	-0.1741	0.6305
M. benhami	K	5	-0.2236	0.7177
	2.5 mg/l Cd	4	-0.8000	0.2000
	5 mg/l Cd	4	0.8000	0.2000
	10 mg/l Cd	4	-0.4000	0.6000
	20 mg/l Cd	3	-0.5000	0.6667
	20 mg/l Ni	**7**	**0.7783**	**0.0393**

Table 9: Spearman Rank order correlation between DNA damage as measured with comet assay parameters Tail DNA % and Olive tail moment and earthworm mass for five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

		Animal *n*	Spearman R	*P*
		Tail DNA %		
	K	5	-0.5000	0.3910
	2.5 mg/l Cd	6	0.0857	0.8717
	5 mg/l Cd	8	0.0000	1.0000
	10 mg/l Cd	7	0.4643	0.2939
	20 mg/l Cd	8	-0.0238	0.9554
A. diffringens	20 mg/l Ni	4	0.6000	0.4000
		Olive tail moment		
	K	5	-0.3000	0.6238
	2.5 mg/l Cd	6	0.5429	0.2657
	5 mg/l Cd	8	0.0476	0.9108
	10 mg/l Cd	7	0.7500	0.0522
	20 mg/l Cd	8	-0.2619	0.5309
	20 mg/l Ni	4	0.8000	0.2000
		Tail DNA %		
	K	10	-0.2727	0.4458
	2.5 mg/l Cd	10	-0.3576	0.3104
	5 mg/l Cd	10	-0.3091	0.3848
	10 mg/l Cd	10	-0.3333	0.3466
	20 mg/l Cd	10	-0.1758	0.6272
A. caliginosa	20 mg/l Ni	10	-0.1758	0.6272
		Olive tail moment		
	K	10	-0.2485	0.4888
	2.5 mg/l Cd	10	-0.0303	0.9338
	5 mg/l Cd	10	-0.4061	0.2443
	10 mg/l Cd	10	-0.2364	0.5109
	20 mg/l Cd	10	-0.2000	0.5796
	20 mg/l Ni	10	0.0667	0.8548
		Tail DNA %		
	K	10	0.3212	0.3655
	2.5 mg/l Cd	10	0.0303	0.9338
	5 mg/l Cd	10	0.4303	0.2145
	10 mg/l Cd	**10**	**0.7455**	**0.0133**
	20 mg/l Cd	10	0.0424	0.9074
D. rubidus	20 mg/l Ni	10	0.3576	0.3104
		Olive tail moment		
	K	10	0.2606	0.4671
	2.5 mg/l Cd	10	0.0909	0.8028
	5 mg/l Cd	10	0.3576	0.3104
	10 mg/l Cd	**10**	**0.8424**	**0.0022**
	20 mg/l Cd	10	-0.0667	0.8548
	20 mg/l Ni	10	0.4545	0.1869

Table 9 (continued)

		Tail DNA %		
E. fetida	K	**10**	**-0.6606**	**0.0376**
	2.5 mg/l Cd	10	0.1152	0.7514
	5 mg/l Cd	10	-0.4182	0.2291
	10 mg/l Cd	**10**	**-0.6848**	**0.0289**
	20 mg/l Cd	5	-0.6000	0.2848
	20 mg/l Ni	10	0.2364	0.5109
		Olive tail moment		
	K	10	-0.4667	0.1739
	2.5 mg/l Cd	10	0.1273	0.7261
	5 mg/l Cd	10	-0.1152	0.7514
	10 mg/l Cd	10	-0.5394	0.1076
	20 mg/l Cd	5	-0.6000	0.2848
	20 mg/l Ni	10	0.1273	0.7261
		Tail DNA %		
M. benhami	K	5	0.0000	1.0000
	2.5 mg/l Cd	4	-0.8000	0.2000
	5 mg/l Cd	4	0.2000	0.8000
	10 mg/l Cd	4	-0.4000	0.6000
	20 mg/l Cd	**3**	**-1.0000**	
	20 mg/l Ni	7	0.4643	0.2939
		Olive tail moment		
	K	5	0.0000	1.0000
	2.5 mg/l Cd	**4**	**-1.0000**	
	5 mg/l Cd	4	0.4000	0.6000
	10 mg/l Cd	4	-0.2000	0.8000
	20 mg/l Cd	3	-0.5000	0.6667
	20 mg/l Ni	7	0.6429	0.1194

Table 10: Spearman Rank order correlation between DNA damage as measured with comet assay parameters Tail DNA % and Olive tail moment and metal body load for five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

		Animal *n*	Spearman R	*P*
		Tail DNA %		
A. diffingens	K	5	0.7000	0.1881
	2.5 mg/l Cd	6	0.4286	0.3965
	5 mg/l Cd	8	0.6429	0.0856
	10 mg/l Cd	7	-0.0714	0.8790
	20 mg/l Cd	8	0.4286	0.2894
	20 mg/l Ni	4	0.0000	1.0000
		Olive tail moment		
	K	5	0.5000	0.3910
	2.5 mg/l Cd	6	0.3714	0.4685
	5 mg/l Cd	8	0.5952	0.1195
	10 mg/l Cd	7	0.0000	1.0000
	20 mg/l Cd	8	0.4524	0.2604
	20 mg/l Ni	4	0.4000	0.6000

Table 10 (continued)

		Tail DNA %		
	K	**10**	**0.8037**	**0.0051**
	2.5 mg/l Cd	10	0.1030	0.7770
	5 mg/l Cd	10	-0.5636	0.0897
	10 mg/l Cd	10	0.0303	0.9338
	20 mg/l Cd	10	0.0545	0.8810
	20 mg/l Ni	10	-0.1702	0.6383
A. caliginosa		Olive tail moment		
	K	10	0.5215	0.1221
	2.5 mg/l Cd	10	0.4545	0.1869
	5 mg/l Cd	10	-0.4545	0.1869
	10 mg/l Cd	10	0.4424	0.2004
	20 mg/l Cd	10	-0.0303	0.9338
	20 mg/l Ni	10	0.2432	0.4984
		Tail DNA %		
	K	10	-0.3206	0.3664
	2.5 mg/l Cd	10	-0.3414	0.3343
	5 mg/l Cd	10	0.1297	0.7209
	10 mg/l Cd	**10**	**-0.6869**	**0.0282**
	20 mg/l Cd	10	0.2242	0.5334
	20 mg/l Ni	-	-	-
D. rubidus		Olive tail moment		
	K	10	-0.3057	0.3903
	2.5 mg/l Cd	10	-0.4302	0.2147
	5 mg/l Cd	10	0.0956	0.7928
	10 mg/l Cd	10	-0.4742	0.1662
	20 mg/l Cd	10	0.2242	0.5334
	20 mg/l Ni	-	-	-
		Tail DNA %		
	K	-	-	-
	2.5 mg/l Cd	10	-0.4101	0.2391
	5 mg/l Cd	-	-	-
	10 mg/l Cd	**10**	**-0.7442**	**0.0136**
	20 mg/l Cd	5	0.2000	0.7471
	20 mg/l Ni	10	0.2901	0.4161
E. fetida		Olive tail moment		
	K	-	-	-
	2.5 mg/l Cd	10	-0.3952	0.2583
	5 mg/l Cd	-	-	-
	10 mg/l Cd	**10**	**-0.7442**	**0.0136**
	20 mg/l Cd	5	0.2000	0.7471
	20 mg/l Ni	10	0.1741	0.6305
M. benhami		Tail DNA %		
	K	**5**	**0.8944**	**0.0405**
	2.5 mg/l Cd	**4**	**1.0000**	
	5 mg/l Cd	4	-0.4000	0.6000
	10 mg/l Cd	4	0.6000	0.4000
	20 mg/l Cd	3	0.5000	0.6667
	20 mg/l Ni	7	0.0371	0.9371

Table 10 (continued 2)

	Olive tail moment		
K	**5**	**0.8944**	**0.0405**
2.5 mg/l Cd	4	0.8000	0.2000
5 mg/l Cd	4	-0.2000	0.8000
10 mg/l Cd	4	0.0000	1.0000
20 mg/l Cd	3	-0.5000	0.6667
20 mg/l Ni	7	0.1482	0.7511

Table 11: DNA damage as detected with the comet assay (Tail DNA % and Olive tail moment) compared with the Kruskal-Wallis ANOVA by ranks between species in each treatment for five earthworm species exposed to Cd in artificial soil water. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

Treatment		Tail DNA %	Olive tail moment
K	*n*	42	42
	H	15.91351	15.28405
	P	**0.0031**	**0.0041**
	df	4	4
2.5 mg/l Cd	*n*	43	43
	H	14.72468	21.30094
	P	**0.0053**	**0.0003**
	df	4	4
5 mg/l Cd	*n*	44	44
	H	17.07121	19.47727
	P	**0.0019**	**0.0006**
	df	4	4
10 mg/l Cd	*n*	42	42
	H	12.43772	22.6633
	P	**0.0144**	**0.0001**
	df	4	4
20 mg/l Cd	*n*	41	41
	H	14.62718	17.47846
	P	**0.0055**	**0.0016**
	df	4	4
20 mg/l Ni	*n*	43	43
	H	17.9087	23.27191
	P	**0.0013**	**0.0001**
	df	4	4

Table 12: Pairwise comparisons (Mann-Whitney U test) between species for DNA damage (Tail DNA % Olive tail moment) as detected with the comet assay in five earthworm species for the negative control and those exposed to 20 mg/l Cd in artificial soil water. Values in bold are significant ($P < 0.05$).

	1st variable	2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
Control Tail DNA %	*A. diffringens*	vs *A. caliginosa*	22	0.843	0.399	5	12
	A. diffringens	vs *D. rubidus*	**1**	**2.939**	**0.003**	**5**	**10**
	A. diffringens	vs *E. fetida*	19	0.735	0.462	5	10
	A. diffringens	vs *M. benhami*	6	1.358	0.175	5	5
	A. caliginosa	vs *D. rubidus*	7	**3.495**	**< 0.001**	**12**	**10**
	A. caliginosa	vs *E. fetida*	59	0.066	0.947	12	10
	A. caliginosa	vs *M. benhami*	17	1.370	0.171	12	5
	D. rubidus	vs *E. fetida*	**14**	**-2.721**	**0.007**	**10**	**10**
	D. rubidus	vs *M. benhami*	16	-1.102	0.270	10	5
	E. fetida	vs *M. benhami*	16	1.102	0.270	10	5
Control Olive tail moment	*A. diffringens*	vs *A. caliginosa*	23	0.738	0.461	5	12
	A. diffringens	vs *D. rubidus*	**5**	**2.449**	**0.014**	**5**	**10**
	A. diffringens	vs *E. fetida*	24	0.122	0.903	5	10
	A. diffringens	vs *M. benhami*	**3**	**1.984**	**0.047**	**5**	**5**
	A. caliginosa	vs *D. rubidus*	**16**	**2.901**	**0.004**	**12**	**10**
	A. caliginosa	vs *E. fetida*	42	-1.187	0.235	12	10
	A. caliginosa	vs *M. benhami*	**10**	**2.108**	**0.035**	**12**	**5**
	D. rubidus	vs *E. fetida*	**15**	**-2.646**	**0.008**	**10**	**10**
	D. rubidus	vs *M. benhami*	19	0.735	0.462	10	5
	E. fetida	vs *M. benhami*	**8**	**2.082**	**0.037**	**10**	**5**
20 mg/l Cd Tail DNA %	*A. diffringens*	vs *A. caliginosa*	38	-0.178	0.859	8	10
	A. diffringens	vs *D. rubidus*	37	0.267	0.79	8	10
	A. diffringens	vs *E. fetida*	**9**	**-2.754**	**0.006**	**8**	**10**
	A. diffringens	vs *M. benhami*	**0**	**2.449**	**0.014**	**8**	**3**
	A. caliginosa	vs *D. rubidus*	44	0.454	0.65	10	10
	A. caliginosa	vs *E. fetida*	**22**	**-2.117**	**0.034**	**10**	**10**
	A. caliginosa	vs *M. benhami*	**2**	**2.197**	**0.028**	**10**	**3**
	D. rubidus	vs *E. fetida*	**19**	**-2.343**	**0.019**	**10**	**10**
	D. rubidus	vs *M. benhami*	4	1.859	0.063	10	3
	E. fetida	vs *M. benhami*	**0**	**2.535**	**0.011**	**10**	**3**
20 mg/l Cd Olive tail moment	*A. diffringens*	vs *A. caliginosa*	37	0.267	0.79	8	10
	A. diffringens	vs *D. rubidus*	31	-0.8	0.424	8	10
	A. diffringens	vs *E. fetida*	**3**	**-3.288**	**0.001**	**8**	**10**
	A. diffringens	vs *M. benhami*	**2**	**2.041**	**0.041**	**8**	**3**
	A. caliginosa	vs *D. rubidus*	41	-0.68	0.496	10	10
	A. caliginosa	vs *E. fetida*	**15**	**-2.646**	**0.008**	**10**	**10**
	A. caliginosa	vs *M. benhami*	**3**	**2.028**	**0.043**	**10**	**3**
	D. rubidus	vs *E. fetida*	**18**	**-2.419**	**0.016**	**10**	**10**
	D. rubidus	vs *M. benhami*	**2**	**2.197**	**0.028**	**10**	**3**
	E. fetida	vs *M. benhami*	**0**	**2.535**	**0.011**	**10**	**3**

Table 13: Cell nucleus areas of five earthworm species compared with the Kruskal-Wallis ANOVA by ranks. Values in bold are significant ($P < 0.05$).

	Nucleus area
n	1005
H	280.94
P	**< 0.001**
df	4

Table 14: Pairwise comparisons (Mann-Whitney U test) between species for cell nucleus areas of five earthworm species. Values in bold are significant ($P < 0.05$).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
Nucleus area	*A. diffringens*	vs	*A. caliginosa*	**223.000**	**-6.955**	**< 0.001**	**28**	**109**
	A. diffringens	vs	*D. rubidus*	**583.000**	**-8.304**	**< 0.001**	**28**	**581**
	A. diffringens	vs	*E. fetida*	**1563.500**	**-2.872**	**0.004**	**28**	**169**
	A. diffringens	vs	*M. benhami*	**484.500**	**-5.803**	**< 0.001**	**28**	**118**
	A. caliginosa	vs	*D. rubidus*	**24854.000**	**-3.566**	**< 0.001**	**109**	**581**
	A. caliginosa	vs	*E. fetida*	**3847.500**	**8.195**	**< 0.001**	**109**	**169**
	A. caliginosa	vs	*M. benhami*	**4717.500**	**3.466**	**0.001**	**109**	**118**
	D. rubidus	vs	*E. fetida*	**14402.500**	**13.995**	**< 0.001**	**581**	**169**
	D. rubidus	vs	*M. benhami*	**18648.000**	**7.816**	**< 0.001**	**581**	**118**
	E. fetida	vs	*M. benhami*	**6185.500**	**-5.472**	**< 0.001**	**169**	**118**

Table 15: Spearman Rank order correlation between net* DNA damage (as measured with comet assay parameters Tail DNA % and Olive tail moment) and nucleus area for five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$). *Net DNA damage was obtained by subtracting the mean DNA damage in the negative control from the mean DNA damage in each Cd exposure treatment.

		n	Spearman R	*P*
Tail DNA %	2.5 mg/l Cd	5	0.616	0.269
	5 mg/l Cd	5	-0.100	0.873
	10 mg/l Cd	5	0.200	0.747
	20 mg/l Cd	5	0.200	0.747
Olive tail moment	2.5 mg/l Cd	5	0.616	0.269
	5 mg/l Cd	5	-0.500	0.391
	10 mg/l Cd	5	0.200	0.747
	20 mg/l Cd	5	0.200	0.747

Table 16: Mass of five earthworm species compared with the Kruskal-Wallis ANOVA by ranks. Values in bold are significant ($P < 0.05$).

	Mass
n	242
H	200.563
P	**< 0.001**
df	4

Table 17: Pairwise comparisons (Mann-Whitney U test) between species for mass of five earthworm species. Values in bold are significant ($P < 0.05$).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
Mass	*A. diffringens*	vs	*A. caliginosa*	953.000	1.738	0.082	40	60
	A. diffringens	vs	*D. rubidus*	**0.000**	**8.443**	**< 0.001**	**40**	**60**
	A. diffringens	vs	*E. fetida*	**0.000**	**8.292**	**< 0.001**	**40**	**55**
	A. diffringens	vs	*M. benhami*	**110.000**	**-5.497**	**< 0.001**	**40**	**27**
	A. caliginosa	vs	*D. rubidus*	**0.000**	**9.448**	**< 0.001**	**60**	**60**
	A. caliginosa	vs	*E. fetida*	**0.000**	**9.238**	**< 0.001**	**60**	**55**
	A. caliginosa	vs	*M. benhami*	**131.000**	**-6.230**	**< 0.001**	**60**	**27**
	D. rubidus	vs	*E. fetida*	**598.000**	**-5.890**	**< 0.001**	**60**	**55**
	D. rubidus	vs	*M. benhami*	**0.000**	**-7.432**	**< 0.001**	**60**	**27**
	E. fetida	vs	*M. benhami*	**0.000**	**-7.326**	**< 0.001**	**55**	**27**

Table 18: Spearman Rank order correlation between net* DNA damage (as measured with comet assay parameters Tail DNA % and Olive tail moment) and mass for five earthworm species exposed to Cd in artificial soil water. Values in bold are significant ($P < 0.05$). *Net DNA damage was obtained by subtracting the mean DNA damage in the negative control from the mean DNA damage in each Cd exposure treatment.

		n	Spearman R	*P*
Tail DNA %	2.5 mg/l Cd	**5**	**-0.975**	**0.005**
	5 mg/l Cd	5	-0.800	0.104
	10 mg/l Cd	**5**	**-0.900**	**0.037**
	20 mg/l Cd	**5**	**-0.900**	**0.037**
Olive tail moment	2.5 mg/l Cd	**5**	**-0.975**	**0.005**
	5 mg/l Cd	5	-0.500	0.391
	10 mg/l Cd	**5**	**-0.900**	**0.037**
	20 mg/l Cd	**5**	**-0.900**	**0.037**

Table 19: Metal body load for five earthworm species exposed to Cd in artificial soil water compared with the Kruskal-Wallis ANOVA by ranks between species in each treatment. K = negative control, 20 mg/l Ni = positive control. Values in bold are significant ($P < 0.05$).

Treatment		Metal body load
K	*n*	40
	H	15.9387
	P	**0.0031**
	df	4
2.5 mg/l Cd	*n*	42
	H	22.44833
	P	**0.0002**
	df	4
5 mg/l Cd	*n*	42
	H	34.58823
	P	**< 0.001**
	df	4
10 mg/l Cd	*n*	41
	H	25.83588
	P	**< 0.001**
	df	4
20 mg/l Cd	*n*	26
	H	25.54917
	P	**< 0.001**
	df	4
20 mg/l Ni	*n*	41
	H	26.70349
	P	**< 0.001**
	df	4

Table 20: Pairwise comparisons (Mann-Whitney U test) between species for Cd body load of five earthworm species exposed to 20 mg/l Cd. Values in bold are significant (P < 0.05).

	1st variable		2nd variable	U	Z	*P*	1st variable *n*	2nd variable *n*
Cd body load	*A. diffringens*	vs	*A. caliginosa*	29.000	0.977	0.328	8	10
	A. diffringens	vs	*D. rubidus*	**3.000**	**3.288**	**0.001**	**8**	**10**
	A. diffringens	vs	*E. fetida*	**1.000**	**2.781**	**0.005**	**8**	**5**
	A. diffringens	vs	*M. benhami*	**0.000**	**2.449**	**0.014**	**8**	**3**
	A. caliginosa	vs	*D. rubidus*	**2.000**	**3.628**	**< 0.001**	**10**	**10**
	A. caliginosa	vs	*E. fetida*	**1.000**	**2.939**	**0.003**	**10**	**5**
	A. caliginosa	vs	*M. benhami*	**0.000**	**2.535**	**0.011**	**10**	**3**
	D. rubidus	vs	*E. fetida*	12.000	1.592	0.111	10	5
	D. rubidus	vs	*M. benhami*	4.000	1.859	0.063	10	3
	E. fetida	vs	*M. benhami*	3.000	-1.342	0.180	5	3

Table 21: Spearman Rank order correlation between net* DNA damage (as measured with comet assay parameters Tail DNA % and Olive tail moment) and Cd body load for five earthworm species exposed to Cd in artificial soil water. Values in bold are significant (P < 0.05). *Net DNA damage was obtained by subtracting the mean DNA damage in the negative control from the mean DNA damage in each Cd exposure treatment.

		n	Spearman R	*P*
Tail DNA %	2.5 mg/l Cd	5	-0.103	0.870
	5 mg/l Cd	5	-0.300	0.624
	10 mg/l Cd	5	-0.100	0.873
	20 mg/l Cd	5	-0.400	0.505
Olive tail moment	2.5 mg/l Cd	5	-0.103	0.870
	5 mg/l Cd	5	-0.100	0.873
	10 mg/l Cd	5	-0.100	0.873
	20 mg/l Cd	5	-0.400	0.505

Table 22: Spearman Rank order correlation between earthworm mass for five earthworm species and Cd body load for these species exposed to 20 mg/l Cd in artificial soil water. Values in bold are significant (P < 0.05).

n	Spearman R	*P*
5	0.200	0.747