Evaluation of the suitability of responses on various organisational levels in terrestrial Oligochaeta to determine species sensitivity relationships

by
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Declaration

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Abstract

Species differ in their sensitivities to toxicants and these differences are exploited in ecological risk assessment methods such as species sensitivity distributions (SSDs). The most commonly used endpoints for ecotoxicity testing and thus to generate data for use in SSDs are on the whole-organismal level, and usually include the evaluation of survival and reproduction. However, suborganismal biomarker responses are in many instances more sensitive than these whole-organismal responses. Therefore, this study investigated and compared responses on various biological organisational levels to determine their suitability for use in SSDs.

Five terrestrial oligochaete species (earthworms) were selected as model test organisms, and were exposed to a range of concentrations of a well-studied pesticide, copper oxychloride. The investigated responses included survival, biomass change and reproduction on the whole-organismal level. In order to investigate responses on the suborganismal level, cells (coelomocytes) were extracted non-invasively. The spectrophotometric neutral red retention (NRR) assay was used to determine cell survival and the MTT assay to determine mitochondrial metabolic activity of the coelomocytes. The alkaline single cell gel electrophoresis (comet) assay was used to assess DNA integrity in these cells. The amount of Cu taken up by earthworms was also determined and compared to their responses.

Species differences were observed in all responses, and EC_{50} and EC_{10} values were calculated for the whole-organismal endpoints and used to generate SSDs. From these SSDs, the hazardous concentrations where 5% of all species would be detrimentally affected (HC_5) were calculated, which indicated that the most sensitive whole-organismal endpoint was mass change, followed by reproduction and survival.

It was found that earthworms avoided feeding on the contaminated substrate in high copper oxychloride concentration exposures. The concentration where this behaviour occurred could be estimated for each species, and an SSD was constructed with these data. The HC_5 value indicated that this response is more sensitive than earthworm survival, but less sensitive than the other responses.

It was shown that the earthworms regulated their body Cu concentrations in a species-specific manner. This regulation of Cu was reflected in the suborganismal responses, and the species that had taken up the highest amount of Cu was the most sensitive species for all three suborganismal assays. Due to this regulation of Cu, the resulting dose-responses for the suborganismal endpoints did not allow for the calculation of EC_{50} values in most of the species and such data could thus not

be used to generate SSDs. Sufficient EC_{10} values were however generated to construct SSDs from the results of the NRR and comet assays.

The HC_5 values obtained from SSDs constructed with EC_{10} values for both suborganismal and whole-organismal endpoints indicated that the NRR assay was the most sensitive endpoint, followed by both the comet assay and earthworm mass change, and subsequently the other whole-organismal endpoints.

In conclusion, the majority of the responses on the various levels of biological organisation investigated during the present study were shown to be suitable to determine species sensitivity relationships in the terrestrial oligochaete species studied.

Opsomming

Spesies verskil van mekaar ten opsigte van hulle sensitiwiteit vir toksikante, en hierdie verskille word in ekologiese risikobepalingsmetodes soos spesie-sensitiwiteitsverspreidings (SSVs) gebruik. Die mees algemene eindpunte vir ekotoksisiteitstoetse, en wat dus gebruik word om data te genereer vir SSVs, is op die heelorganismevlak, en sluit gewoonlik die bepaling van oorlewing en voortplanting van die toetsorganismes in. Hierdie eindpunte is egter in die meeste gevalle minder sensitief as suborganismiese biomerker-response. Hierdie studie het dus die response op verskeie vlakke van biologiese organisasie ondersoek en vergelyk om te bepaal of hulle geskik is vir gebruik in SSVs.

Vyf terrestriële spesies van die klas Oligochaeta is gekies as toetsorganismes en is blootgestel aan 'n reeks konsentrasies van die goed bestudeerde pestisied koperoksichloried. Die response oorlewing, massaverandering en voortplanting is op die heelorganismevlak ondersoek. Vir die suborganismiese response is selle (selomosiete) met behulp van 'n nie-ingrypende proses vanuit die erdwurms geïsoleer. Die suborganismese toetse wat op hierdie selle gedoen is, was die neutraalrooi-retensietoets (NRR toets) om sel-oorlewing te bepaal, die MTT toets om mitochondriese metabolisme te bepaal en die alkaliese komeettoets om DNS-integriteit te bepaal. Die hoeveelheid Cu wat die erdwurms opgeneem het, is ook bepaal en met hulle response vergelyk.

Verskille is tussen die spesies waargeneem vir al die response. Beide EK_{50} en EK_{10} waardes is bereken vir die heelorganismiese eindpunte om SSVs te genereer. Vanaf hierdie SSVs kon die gevaarlike konsenstrasie, waar 5% van alle spesies nadelig beïnvloed kan word (GK_5), bereken word. Hierdie GK_5 waardes het aangedui dat massaverandering die mees sensitiewe heelorganismiese eindpunt was, gevolg deur voortplanting en oorlewing.

Die erdwurms het opgehou vreet aan die gekontamineerde substraat by hoë koperoskichloriedkonsentrasies. Die konsentrasie waar hierdie gedrag plaasgevind het kon vir elke spesie vasgestel word, en 'n SSV is met behulp van hierdie data genereer. Hierdie GK_5 waarde het aangedui dat hierdie respons meer sensitief was as oorlewing, maar minder sensitief as die ander response.

Die erdwurms kon die konsentrasie van Cu in hulle liggame op 'n spesie-spesifieke manier reguleer. Hierdie regulering van interne Cu is weerspieël in die suborganismiese response, waar die spesie wat die meeste Cu opgeneem het, ook die mees sensitiewe was vir al drie suborganismiese toetse. As gevolg van hierdie regulering van Cu en die gevolglike dosis-responsverhoudings, kon EK_{50}-waardes nie vir al die spesies bereken word nie, en dus was daar geen EK_{50}-data beskikbaar

om SSVs mee te genereer nie. Genoegsame EK_{10} waardes kon egter bereken word vir die NRR- en komeettoetse, en gebruik word om SSVs te genereer.

Die GK_5-waardes wat bereken kon word vanuit die SSVs met EK_{10} waardes vir beide suborganismese en heelorganismiese response, het aangedui dat die mees sensitiewe eindpunt die NRR toets was, gevolg deur beide die komeettoets en massaverandering van erdwurms, en daarna die ander heelorganismiese eindpunte.

Die gevolgtrekking is dat daar aangetoon kon word dat die meerderheid van die response wat gedurende hierdie studie ondersoek is, geskik is om sensitwiteitsverhoudings van hierdie groep spesies te bepaal.

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Chapter 1: Introduction

When organisms are subjected to toxic stress, responses occur on different levels of biological organisation (Hyne & Maher 2003; Moore *et al*. 2004; Spurgeon *et al*. 2005), ranging from the community and population levels, through organism and cellular levels to subcellular and molecular levels (McCarthy & Shugart 1990; Spurgeon *et al*. 2005). Responses at each of these levels may be linked to each other in a hierarchical fashion, associated with increasing contaminant exposure (Spurgeon *et al*. 2005). With first exposure to a toxicant, at low concentration levels or short time spans, initial responses will be on suborganismal levels, such as the molecular level, and may include changes in e.g. gene and protein expression. With an increase in time span or exposure level, other responses may follow such as effects on cellular and later tissue level, which could be a direct result of the initial molecular changes. These may, over the long term and also with increasing accumulated body loads of toxicants, result in changes in behaviour and life history parameters such as growth, reproduction and eventually survival, which could eventually affect populations and communities (Spurgeon *et al*. 2005).

Effects at the suborganismal level may be assessed through the measurement of cellular responses or molecular changes with the aid of biomarker assays. A biomarker is defined by Van Gestel & Van Brummelen (1996) as a biological response to an environmental chemical below the individual level, or at the level of biochemical or physiological processes. Biomarkers constitute an array of cellular and sub-cellular endpoints to determine the responses of organisms to toxicants (Schlenk 1999). Because these biomarker or suborganismal endpoints can usually be assessed at lower concentrations or within shorter exposure time intervals than whole-organismal endpoints, they are considered as early warning systems to predict the adverse effects of sublethal concentrations of toxicants on whole-organismal levels (Morgan *et al*. 1999; Spurgeon *et al*. 2005). Nevertheless, biomarker results should be interpreted carefully, and only if definite links between biomarker responses and higher-level effects such as whole-organismal responses and ecological effects are established, should biomarkers be used to predict the effects of toxicants at higher levels of organisation (Moore *et al*. 2004; Forbes *et al*. 2006). Since it is advisable that biomarkers should not be used on their own (Hyne & Maher 2003), they are often used in a supplementary way with other ecologically relevant responses or as part of suites of biomarkers in environmental monitoring and other ecological risk assessment (ERA) procedures (Galay Burgos *et al*. 2005).

For risk assessment purposes, in order to successfully extrapolate from responses on suborganismal levels to the community or ecosystem level, extrapolation between levels of

organisation should be used in conjunction with extrapolation between species. The fact that species differ in their sensitivity to toxicants has always been important in ecotoxicology and ERA, and one of the methods that exploit this variation is the species sensitivity distribution (SSD) approach (Posthuma *et al*. 2002). Species sensitivity distributions have been used for risk assessment worldwide, and extensively in Europe and the USA (Suter II 2002; Van Straalen & Van Leeuwen 2002).

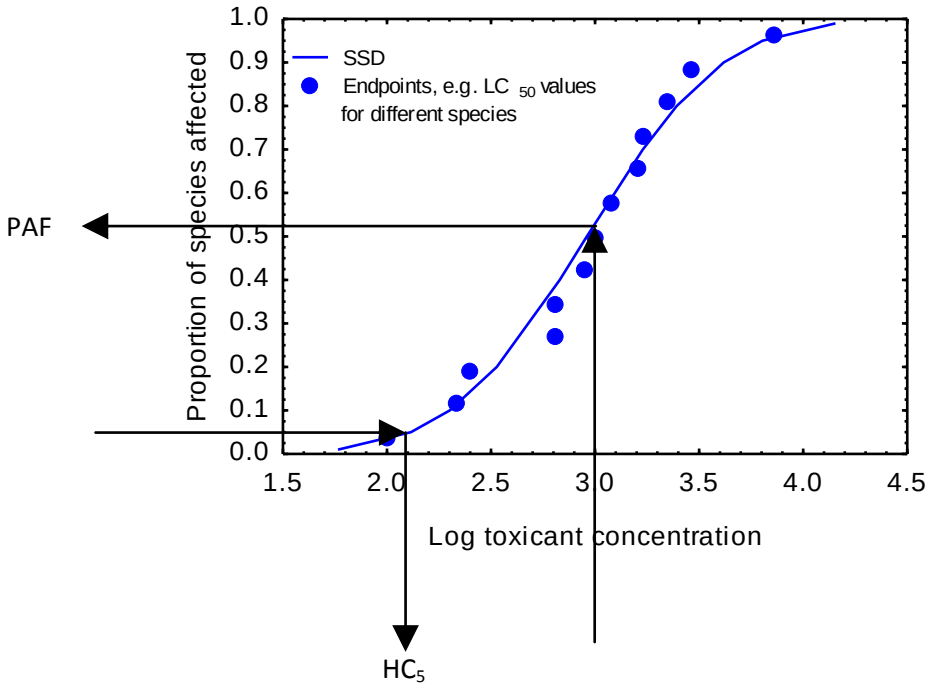


Figure 1.1: An example of a species sensitivity distribution (SSD), showing the forward use to determine the potentially affected fraction (PAF) of species at a measured toxicant concentration, and the inverse use to determine the hazardous concentration where 5% of species will be affected (HC_5). Adapted from Posthuma *et al.* (2002).

A species sensitivity distribution (SSD) is a statistical distribution that describes the variability in measured endpoints for different species exposed to a certain chemical (Calow 1998; Posthuma *et al*. 2002) (Figure 1.1). The endpoints regularly used to construct SSDs, usually gained from published results from various sources of acute and chronic (usually fecundity) tests (Forbes & Calow 2002b), are LC_{50}s for acute tests, and EC_{50}s and NOECs for chronic tests (Suter II *et al*. 2002). Recently, endpoints such as biological traits of organisms (including morphology, life history and physiology), based on the assumption that an organism's sensitivity is a function of its biology (Baird & Van den Brink 2007), as well as the bioavailability of toxicants (Semenzin *et al.* 2007) have been included in SSDs. Usually, these endpoints, as obtained for different species, are distributed according to the normal (Gaussian) or logistic distribution, but sometimes also according to nonparametric types of distributions. Depending on the type of data, analyses can also be done with distribution-free resampling methods (Posthuma *et al*. 2002). The SSD itself is the integral of

the function of such a distribution, usually the Gaussian, and is a sigmoidal graph, with the toxicant concentration (log-converted) on the x-axis and the affected fraction of species on the y-axis.

In ecological risk assessment, SSDs can be used in both forward and inverse approaches to estimate the risk posed by a toxicant (Posthuma *et al*. 2002). Environmental quality criteria (EQC) can be derived using the inverse approach, where the toxicant concentration is estimated where a maximum acceptable fraction of species, chosen by convention to be 5% (thus protecting 95% of species) is adversely affected (Posthuma *et al*. 2002). This is termed the hazardous concentration (HC_5). In the forward approach, the potentially affected fraction (PAF) of species at a measured environmental concentration can be estimated from the SSD.

The construction and use of SSDs in ERA is however not straightforward, since new statistical methods are continuously being developed for SSDs (Chen 2004; Duboudin *et al*. 2004; Fox 2010), and there are many criteria and pitfalls encountered when using this approach (Forbes & Calow 2002a; De Laender *et al*. 2008; Henning-de Jong *et al*. 2009). For example, it has been illustrated that data quality, sample size and different methods for constructing SSDs (Wheeler *et al*. 2002; Henning-de Jong *et al*. 2009), as well as data manipulation (Duboudin *et al*. 2004) have an influence on the outcome and derivation of HC_5 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).

The successful and accurate construction and interpretation of SSDs depend on some important assumptions and criteria. Unique issues and problems exist for each of the three main steps involved in the process of constructing a SSD. These steps include 1) selection of input data, 2) statistical calculation and 3) interpretation of the SSD output (Posthuma *et al*. 2002; Suter II *et al*. 2002). It is not within the scope of the present study to discuss all of these issues, and only the first step, which involves the selection and especially the generation of appropriate input data, is of interest here.

The endpoints regularly used for the construction of SSDs, such as LC_{50} values, may be less sensitive than those obtained from biomarker responses. Although EC_{50} and NOEC values from reproduction tests are more sensitive and ecologically relevant than LC_{50} values, and successfully used to construct SSDs, they can be time-consuming and labour intensive. In addition, reproduction tests are sometimes difficult or even impossible to perform on species where the reproductive biology is not fully known. Since a large dataset is needed to construct SSDs accurately (up to 15 to 20 species, (Suter II *et al*. 2002)), the use of such time-consuming, labour intensive or difficult assays may not facilitate the generation of such a large amount of data in a relatively short time. Therefore, it is proposed here that since the use of biomarker tests can facilitate the generation of a

relatively large amount of data in relatively short time periods, and since data gained from biomarker tests are very sensitive, the use of such data may be promising to construct SSDs.

Until very recently, the concept of using biomarker data in SSDs has not been applied in practice, mainly due to the lack of sufficient data. Only two very recent studies were found in the literature survey that used biomarker and suborganismal data in the construction of SSDs (Smit *et al*. 2009; Fedorenkova *et al*. 2010). In both studies, SSDs constructed from suborganismal response data for marine species were compared to SSDs constructed from whole-organismal response data. In a "pilot study", Smit *et al.* (2009) determined that the HC_5 value obtained from an SSD constructed with biomarker data (LOEC values for DNA damage and oxidative stress) in marine species exposed to oil was lower than the HC_5 value, and corresponded to the HC_{80} value obtained from an SSD constructed with whole-organismal response data (NOEC values for growth, reproduction and survival). The database was however small, and eventually data for only six species were suitable for use in their study. Unfortunately, these data were obtained from different studies utilising different exposure durations (between 3 and 210 days), different oil types and different biomarkers. The biomarkers utilised in these studies included assays for DNA damage such as the comet assay, alkaline unwinding assay, measurement of DNA adducts and micronuclei frequency, and assays for oxidative stress such as glutathione-S-transferase activity, catalase activity and total oxygen radical scavenging capacity. Nonetheless, it could be illustrated by Smit *et al.* (2009) that the biomarker responses were much more sensitive than the whole-organismal responses. Although the authors identified various limitations and uncertainties in their approach, they maintained that it seems to be a promising way to link field monitoring using biomarkers with risk assessment.

In the second study, by Fedorenkova *et al.* (2010), available data (LOEC values) from gene expression studies on marine species exposed to cadmium were used to construct an SSD. This SSD was compared to two SSDs constructed from whole-organismal endpoints: NOEC values from chronic studies and LC_{50} values. It was determined that the HC_5 value obtained from the gene expression LOEC SSD was 3 times higher than the HC_5 value obtained from the whole-organismal NOEC SSD, and 25 times lower than the HC_5 value obtained from the LC_{50} SSD. The authors concluded that the available data for cadmium-exposed marine organisms does not yet confirm that responses on the suborganismal level are more sensitive than those on the whole-organismal level, and that more data need to be generated in order for gene expression changes to be used as early warning indicators of environmental effects of Cd in marine organisms.

When considering the conflicting results from these two studies in terms of the sensitivity of SSDs constructed from biomarker data, it is clear that although the application of biomarker data in

SSDs and environmental monitoring and risk assessment is promising, it should be investigated more thoroughly. It is also clear from these studies that the availability and standardisation of data gained from suborganismal tests should be addressed.

The studies by Smit *et al.* (2009) and Fedorenkova *et al.* (2010) both used data gained from tests on marine species, and the opportunity exist for such a study to be performed on data gained from terrestrial species. It was recently pointed out that comprehensive databases on ecotoxicological data gained from terrestrial organisms, such as earthworms, are still largely lacking (Spurgeon *et al*. 2003b) and that the need exists to increase these databases. The species most often used in ecotoxicological studies belong to Oligochatea (earthworms such as *Eisenia fetida* and *Eisenia andrei*, and enchytraeids such as *Enchytraeus albidus*) and Collembola (springtails such as *Folsomia candida*) (Ratte *et al*. 2003; Solomon 2010).

Terrestrial Oligochaeta, and especially earthworms (suborder Lumbricina) are the preferred model test organisms in a large number of terrestrial ecotoxicological studies (Spurgeon *et al*. 2003b), with a great deal of attention focused on the effects of metals. Various ecological and physiological attributes of earthworms are important factors in rendering them the preferred test species for most ecotoxicological studies (Eijsackers 2004). The ecological roles of earthworms include key functions such as decomposition and soil formation, fertilisation and aeration (Edwards 2004). When exposed to contaminants, earthworms are in many cases not able to perform their valuable ecosystem functions (Edwards & Bohlen 1996). For example, litter consumption (composting) and burrowing activity (soil aeration) may be affected negatively by excess amounts of various toxic substances (Eijsackers *et al*. 2005; Hobbelen *et al*. 2006). Earthworms are an integral part of terrestrial food chains (Edwards 2004), especially since they form a major component of terrestrial faunal biomass. Because earthworms can accumulate toxicants such as heavy metals and some insecticides, their predators may be affected adversely (Reinecke 1992), resulting in negative effects in other trophic levels. In addition, earthworms are vulnerable to physical and chemical changes to soils because they are in close contact with the soil and the pore water (Reinecke & Reinecke 2004a). This, in addition to their relatively limited mobility (Paoletti 1999) renders them ideally suited for ecotoxicological research. In addition, from a practical viewpoint, earthworms are easily available and to handle and use in toxicity tests, and populations of species such as *Eisenia fetida* can be maintained easily under laboratory conditions ensuring a constant and controllable supply of test specimens (Reinecke & Reinecke 2004a).

The species most often used to represent the class Oligochaeta in terrestrial studies (therefore, for which data are readily available for use in SSDs) are the earthworm species *Eisenia andrei* and *E. fetida*, mainly because they are recommended by the OECD and ISO in standardised test

guidelines (OECD 1984; OECD 2004; ISO 2007). Other popular species are *Lumbricus rubellus*, *L. terrestris*, and *Aporrectodea caliginosa* and the enchytraeid (potworm) species *Enchytraeus albidus* (Spurgeon *et al*. 2003b; Nahmani *et al*. 2007). The use of *E. andrei* and *E. fetida* as representatives of the class Oligochaeta in methods such as SSDs could however lead to overestimation of the toxicity of the investigated chemicals, since they have been found on quite a few occasions to be much less sensitive than other earthworm species to toxicants (Spurgeon & Hopkin 1996; Spurgeon *et al*. 2000; Eijsackers 2004; Langdon *et al*. 2005; Lukkari *et al*. 2005). In fact, the ecological relevance and therefore usefulness of *E. andrei* and *E. fetida* in ERA has been a topic of much discussion in the literature (Reinecke & Reinecke 2004a; Nahmani *et al*. 2007; Lowe & Butt 2007).

It is well known that even closely related or ecologically similar earthworm species can differ considerably in their sensitivity towards toxicants (Spurgeon & Hopkin 1996; Spurgeon *et al*. 2000; Christensen & Mather 2004; Rault *et al*. 2007; Fourie *et al*. 2007). It may be possible that the variation within the class Oligochaeta could be greater than the variation between species in this class and other species, although it has been found that earthworms are often much more sensitive than other organisms, depending on the toxicants that they are exposed to (Rundgren & Van Gestel 1998). A number of possible reasons exist for the sensitivity differences between earthworm species. Species can differ in morphology, physiology and behaviour, which would result in differences in uptake, physiological utilisation and sequestration (also immune responses and detoxification), and excretion of toxicants. For example, in smaller species, an increased uptake of toxicants can occur, due to their greater body surface to volume ratios (Rozman & Klaassen 2001). Physiological differences between species may cause differences in sensitivity, such as differences in metal binding proteins (metallothioneins, involved in metal detoxification) (Morgan *et al*. 1989) and calcium gland activity (Spurgeon & Hopkin 1996). Even behavioural differences (Eijsackers 2004), such as avoidance behaviour (Lukkari & Haimi 2005) or differences in feeding strategies between species (Morgan & Morgan 1992) could affect the exposure, uptake and eventual toxicity of chemicals to earthworms. Different feeding strategies in earthworms may be attributed to their different ecological types (Bouché 1992), and earthworm species are often grouped into three basic ecological types (sometimes also referred to as ecophysiological types). This differentiation is based on characteristics such as burrowing activity, body size, shape and pigmentation, and food preferences (Bouché 1992; Paoletti 1999). It is important to note that earthworms in an ecological group do not necessarily belong to the same taxonomic group. Through convergent evolution they can assume similar ecological roles and can share morphological and only rarely certain physiological characters (Bouché 1992). The following ecological types have been characterised by

Bouché (1992): Epigeic species are litter and topsoil inhabiting species and are mostly smaller than earthworms from the other two groups. They are usually fairly darkly pigmented, either red-brown or greenish. They are subject to high predation pressure as a result of their habitat use, but compensate by having an r-selected reproductive strategy (high numbers of small hatchlings). Endogeic species live in horizontal burrows in the upper soil layer, and are usually similar in size or slightly larger than epigeic species. Most of the species in this category lack dark skin pigmentation and may appear greyish or pinkish. This is a very diverse group in terms of feeding ecology, where some species ingest substrates that are relatively rich in organic matter (such as humus) and others ingest substrates poor in organic matter (mineral soil). Anecic species are larger than those of the other two groups (7 cm or more in length), and live in deep vertical burrows (1 to 6 m). They forage nocturnally on the soil surface and drag litter to the lower soil strata. Species from this group can be pigmented dorsally. In many ecotoxicological tests however, it is difficult to establish links between species differences and these ecological groups as defined by Bouché (1992), and it has been suggested that physiological differences, such as those mentioned above, are the main factor determining the different sensitivities of earthworms to metals (Spurgeon & Hopkin 1996; Fourie *et al*. 2007).

Ecotoxicological testing on earthworms involve the measurement of whole-organismal responses such as mortality, growth, reproduction (OECD 1984; OECD 2004) and behavioural responses such as avoidance of contaminated substrates, burrowing activity and feeding activity (Eijsackers *et al*. 2005; Lukkari & Haimi 2005; Hund-Rinke *et al*. 2005; Hobbelen *et al*. 2006). On the cellular level, tests include, amongst others, measures of lysosomal stability (neutral red retention assay), sperm cell responses (quality and quantity), and immunological responses such as phagocytotic activity of coelomocytes (Weeks & Svendsen 1996; Scott-Fordsmand & Weeks 2000). On the molecular level, tests include the assessment of DNA damage with e.g. the alkaline comet assay or the measurement of DNA adducts (Scott-Fordsmand & Weeks 2000; Reinecke & Reinecke 2004b), measurements of gene expression for e.g. metallothionein or heat shock protein regulation (Brulle *et al*. 2006) or reproductive output genes (Ricketts *et al*. 2004).

Many of these suborganismal assays are performed on earthworm coelomocytes. Coelomocytes, occurring in suspension in the coelomic fluid, comprise various types (Dhainaut & Scaps 2001; Olchawa *et al*. 2006; Plytycz *et al*. 2007; Plytycz *et al*. 2010). The classification of coelomocytes is however quite confusing (Dhainaut & Scaps 2001; Adamowicz 2005; Kasschau *et al*. 2007), as the composition of types and number of coelomocytes vary greatly between species (Dhainaut & Scaps 2001; Suavé *et al*. 2002). Two main coelomocyte types may be distinguished (Olchawa *et al*. 2005; Plytycz *et al*. 2007), namely amoebocytes and eleocytes. Amoebocytes, which may be either

hyaline or granular, are believed to originate from the mesothelial lining of the coelom (Hamed *et al*. 2002). Secondly, chloragocytes, which are differentiated into eleocytes, are derived from the chloragogenous tissue that lines the coelom along the digestive tract and blood vessels (Affar *et al*. 1998). Different earthworm species have different ratios of different types of coelomocytes (Kurek & Plytycz 2003), and the ratio of amoebocytes to eleocytes is species specific. For example, eleocytes rarely occur in *Aporrectodea* spp., but are abundant in *Eisenia* spp. (Plytycz *et al*. 2010). Coelomocytes have important immune functions in earthworms, such as phagocytosis (Engelmann *et al*. 2004), encapsulation (Valembois *et al*. 1992) and cytotoxicity (Kauschke *et al*. 2001), as well as nutrition, excretion and detoxification (Dhainaut & Scaps 2001), and are an integral part of the immune system (Cooper & Roch 2003; Cooper *et al*. 2006). Coelomocytes, especially amoebocytes, are also involved in metal trafficking and sequestration (Stürzenbaum *et al*. 2001; Homa *et al*. 2007). The functions of eleocytes include the metabolism and storage of glycogen and lipids, and the transportation of nutrients to diverse cells and tissues (Jamieson 1981; Affar *et al*. 1998). It is thus important to recognise that the condition of the coelomocytes is important in determining the overall health of the organism.

One of the most successful biomarker assays on earthworm coelomocytes is the neutral red retention (NRRT) assay (Svendsen *et al*. 2004). The NRRT assay has been shown to be a sensitive and reliable biomarker in earthworms to assess the effects of a range of toxicants (Svendsen *et al*. 2004), and has also been found to be more sensitive than the assessment of growth and reproduction in many instances (Svendsen *et al*. 2004). Results from the NRRT assay have been closely linked to life-cycle effects (Reinecke *et al*. 2002; Maboeta *et al*. 2003). The NRRT assay works on the premise that when under toxic stress, cellular lysosomes lose their membrane stability or integrity, a process that can be triggered in various ways (Repnik & Turk 2010). The stability of the lysosomal membrane may be measured with the aid of the vital dye neutral red (3-amino-7-dimethyl-amino-2 methyl-phenazine hydrochloride). It is a weak cationic dye which is taken up by cells by non-ionic diffusion through cell membranes and which accumulates in lysosomes of cells (Nemes *et al*. 1979; Babich & Borenfreund 1990). In stressed cells (with damaged lysosomal membranes), the dye may leak, along with the lysosomal contents, through the lysosomal membranes into the cytosol. This process may not occur instantaneously, but may take anything from minutes (in highly damaged cells) up to longer than an hour (in less damaged cells) (Weeks & Svendsen 1996). The measurement of lysosomal membrane stability is seen as a biomarker of general stress, as it may be affected by both chemical and nonchemical factors (Weeks & Svendsen 1996).

The NRRT assay assesses the time taken for the neutral red (NR) dye to leak from the lysosomes into the cytosol in 50% of the cells. It may however be quite time-consuming, as the NRRT in

undamaged cells may be in excess of 90 min (Weeks & Svendsen 1996). The NRRT assay may also be subjective, since the operator assesses a visual colour change (Weeks & Svendsen 1996). It is therefore difficult to perform this assay on many individuals simultaneously, and this relatively small sample size could lead to decreased statistical robustness (Weeks & Svendsen 1996). Another method that utilises NR, is in an automated cytotoxicity assay which is also based on the ability of live, undamaged cells to accumulate NR in their lysosomes and to adhere to surfaces (Borenfreund & Puerner 1985). In order to perform this NR colorimetric assay, cell suspensions are incubated with NR in a 96-well microtiter plate, after which the nonadherent cells and extracellular NR are washed off. The remaining intracellular NR is quantified with the aid of a colorimetric spectrophotometer (Borenfreund & Puerner 1985; Babich & Borenfreund 1990). The amount of remaining NR is subsequently assumed to be proportional to the number of live cells in the sample (Babich & Borenfreund 1990) and is thus an indication of cell viability, which could be used as a biomarker of general stress. The spectrophotometric NRR assay is fast to conduct, and has been adapted for use in ecotoxicological studies. Significant dose response relationships have been found in various invertebrates, such as earthworms, mussels, and crustaceans exposed to various chemicals (Hauton & Smith 2004; Asensio *et al*. 2007; Canty *et al*. 2007). This assay was only very been recently adapted for use in earthworm ecotoxicology, and has been shown to be a promising tool to evaluate cytotoxicity in coelomocytes from earthworms exposed to Cd (Asensio *et al*. 2007; Maleri *et al*. 2008) and to a mixture of Cu, Zn, Cd and Pb (Homa *et al*. 2003). Affar *et al.* (1998) also successfully used this assay to determine the integrity of chloragocytes freshly isolated from the earthworm *Lumbricus terrestris* (without any toxicant exposure).

Another spectrophotometric assay that can be used as a potential biomarker assay on earthworm coelomocytes is the tetrazolium salt (MTT) assay. The MTT assay (Mosmann 1983) is a colorimetric assay in which the water soluble yellow tetrazolium salt (or MTT dye) is converted by means of a redox reaction to a water insoluble purple product (formazan blue) (Mosmann 1983; Carmichael *et al*. 1987). The amount of purple formazan can be assessed colorimetrically with the aid of a spectrophotometer in cell suspensions in a 96-well microtiter plate, and is an indication of the mitochondrial activity of the cells, which is subsequently very often translated into a measure of cell viability (Mosmann 1983).

In earthworms, the MTT assay has mainly been used to check cell viability (Kauschke *et al*. 1997; Affar *et al*. 1998). Recently, it was demonstrated by Maleri *et al*. (2008) that MTT reduction into formazan is significantly inhibited in earthworms (*Eisenia andrei*) exposed to Cd in artificial soil and to ultramafic soils (soils containing naturally occurring elevated amounts of heavy metals such as Cr, Co, Mn and Ni).

Another earthworm biomarker performed on coelomocytes, and which is used increasingly frequently in earthworm ecotoxicology, is the alkaline single cell gel electrophoresis assay, dubbed the comet assay. The comet assay allows for the determination of DNA single strand breaks in single cells (Singh *et al*. 1988; Fairbairn *et al*. 1995). For the alkaline comet assay, cells are embedded in agarose on microscope slides and the cell membranes are subsequently lysed under highly alkaline conditions. The slides are then subjected to electrophoresis in an alkaline buffer, after which they are neutralised and stained with a fluorescing substance such as ethidium bromide and visualised with the aid of fluorescence microscopy (Singh *et al*. 1988). Damaged nuclei will appear as "comets", with "heads" consisting of undamaged, supercoiled DNA retained in the nucleus, and "tails" consisting of damaged or uncoiled DNA loops or strands and fragments that had migrated out of the nucleus as a result of electrophoresis (Duez *et al*. 2003). The extent of DNA damage is assessed by measuring the length of the tail as well as the amount of DNA in the tail as opposed to that in the head (Fairbairn *et al*. 1995; Olive & Durand 2005).

The comet assay has been well established in earthworm ecotoxicology in recent years. It has been successfully illustrated that the comet assay may be used as an indicator of genotoxicity in various earthworm species exposed to various toxicants (Reinecke & Reinecke 2004b; Martin *et al*. 2005; Di Marzio *et al*. 2005; Fourie *et al*. 2007; Piola *et al*. 2009; Hu *et al*. 2010; Voua Otomo & Reinecke 2010; Button *et al*. 2010; Bigorgne *et al*. 2010; Giovanetti *et al*. 2010). The comet assay has also been demonstrated to be effective in determining earthworm species sensitivity differences after exposure to cadmium (Fourie *et al*. 2007). The comet assay, as well as the spectrophotometric NRR and MTT assays, are promising tools to be used to investigate earthworm species sensitivity differences, since they have all been demonstrated to be effective in measuring responses in earthworms exposed to various toxicants.

One of the most thoroughly tested toxicants in earthworm ecotoxicology, but for which suborganismal data obtained trough the use of the NRR, MTT and comet assays are lacking, is the essential metal copper. Various copper salts and other copper formulations, such as fungicides, have been used to expose earthworms in ecotoxicological studies. The effects of the copper-containing fungicide, copper oxychloride, have been well-studied on various organisms (Reinecke *et al*. 2002; Snyman *et al*. 2005; Du Plessis *et al*. 2009). Copper oxychloride is a broad-spectrum fungicide applied to the foliage of a variety of crops to combat a variety of fungal diseases (Vyas 1988). It has been estimated recently that more than 160 t of copper oxychloride is sprayed annually on vineyards in the Western Cape region of South Africa (Du Plessis 2002). Inevitably, some or most of the fungicide end up in the soil after spraying and would eventually result in an increased soil copper concentration (Ayres *et al*. 2002; Komárek *et al*. 2010), even after a single application

(Maboeta *et al*. 2002). Measured concentrations of Cu in vineyards in South Africa range from 11 to 21 mg/kg Cu (Eijsackers *et al*. 2005) and reach up to 47 mg/kg Cu (Maboeta *et al*. 2003). Worldwide, the Cu content of vineyard soils (in the top layers; up to 45 cm deep) range between 4 and 3200 mg/kg Cu (Komárek *et al*. 2010).

The effect of copper oxychloride and other Cu compounds is well-studied in earthworms on the whole-organismal and population levels, and Cu can affect behaviour and induce avoidance responses, reduce feeding activity and affect growth, development, reproduction and survival (Ma 1984; Khalil *et al*. 1996a; Helling *et al*. 2000; Reinecke *et al*. 2002; Spurgeon *et al*. 2004b; Lukkari & Haimi 2005), and decrease earthworm population densities (Paoletti *et al*. 1998; Van Zwieten *et al*. 2004). On the suborganismal level, high concentrations of Cu can decrease earthworm coelomocyte phagocytotic ability and viability in earthworms (Burch *et al*. 1999). Exposure to copper oxychloride and other Cu compounds induce significant dose-response relationships in coelomocytes from various earthworm species as measured with the NRRT assay (Svendsen & Weeks 1997; Maboeta *et al*. 2002; Reinecke *et al*. 2002; Maboeta *et al*. 2003; Maboeta *et al*. 2004).

The effects of exposure to Cu (singly) in earthworms have yet to be assessed with the spectrophotometric NRR and MTT assays and the comet assay. Both the NRR and MTT assays have been demonstrated to successfully detect Cu-induced cytotoxicity in cells isolated from other organisms, such as fish and mammals (Babich *et al*. 1986; Maracine & Segner 1998; Seth *et al*. 2004; Tan *et al*. 2008; Grillo *et al*. 2009; Scheiber *et al*. 2010). The comet assay has been illustrated to be effective in determining DNA damage caused by Cu in freshwater planaria, mice, mussels, fish and polychaetes (Guecheva *et al*. 2001; Banu *et al*. 2004; Villela *et al*. 2006; Bopp *et al*. 2008; Ferreira-Cravo *et al*. 2008).

Aims

The aim of this study was to determine whether sublethal toxicity data obtained from suborganismal responses as well as responses on other levels of biological organisation can be used to determine species sensitivity differences and whether these data can be used in ERA methods such as SSDs. Furthermore, it was sought to determine whether the suborganismal responses were more sensitive than and predictive of whole-organismal responses. It was also sought to determine whether any observed species differences in suborganismal responses could be related to the species-specific ability to take up and regulate the toxicant.

It was hypothesised that suborganismal data can be used in species sensitivity distributions. Thus, it was attempted to reject the null hypothesis that suborganismal data cannot be used in species sensitivity distributions. The hypothesis was tested using three suborganismal assays: the NRR, MTT and alkaline comet assays. The results from these tests were compared with the whole-organismal responses of survival, reproduction, mass change and the avoidance of feeding on contaminated substrates. Experiments were performed on five earthworm species exposed to a range of sublethal concentrations of copper in the form of copper oxychloride.

The specific aims were:

- to study a number of different earthworm species from various ecological niches and to determine EC_{50} values for each species for the following suborganismal responses: coelomocyte cytotoxicity (as measured with the NRR assay), coelomocyte metabolic activity (as measured with the MTT assay) and coelomocyte DNA damage (as measured with the alkaline comet assay),
- to determine EC_{50} values for each of the species for the whole-organismal responses reproduction and mass change, to determine LC_{50} values in species where mortality occurred, and to determine the concentration of toxicant where each species showed a feeding avoidance response,
- to utilise all of the abovementioned EC_{50} and LC_{50} values and feeding avoidance response concentrations to compare species sensitivities and to construct separate SSDs for each endpoint,
- to compare the sensitivity of the suborganismal responses with those of the whole-organismal responses using the LC_{50}, EC_{50} values and the HC_5 values from the constructed SSDs,
- and to determine the amount of toxicant taken up by earthworms and compare it to the various responses, and to discuss the species differences in terms of these accumulation/response relationships.

Chapter 2: Materials and Methods

Adult specimens from five earthworm species were exposed to a range of Cu concentrations in the form of the fungicide copper oxychloride in OECD artificial soil (OECD 2004) for 14 days. After exposure, earthworm body Cu concentrations and soil Cu concentrations were determined. The following endpoints were assessed: On the whole-organismal level, mortalities were recorded, mass changes were monitored and cocoon production was assessed. Responses at the suborganismal level were assessed with the aid of the neutral red retention (NRR) assay, the MTT assay and the alkaline comet assay.

2.1 Earthworms

Five earthworm species were selected: *Amynthas diffringens*, *Aporrectodea trapezoides*, *Eisenia andrei*, *Perionyx excavatus* and a *Chilota* species.

2.1.1 Earthworm taxonomy

The classification of the species is as follows (Reynolds & Cook 1976; Sims & Gerard 1985):

PHYLUM ANNELIDA

Subphylum Clitellata

Class Oligochaeta

Order Haplotaxida

Suborder Lumbricina

Superfamily Lumbricoidea

Family Lumbricidae Rafinesque-Schmaltz 1815

Subfamily Lumbricinae Rafinesque-Schmaltz 1815

Aporrectodea trapezoides Dugès 1828

Eisenia andrei Bouche 1972

Superfamily Megascolecoidea

Family Megascolecidae Rosa 1891

Amynthas diffringens Baird 1869

Perionyx excavatus Perrier 1872

Family Acanthodrilidae Claus 1880

Chilota Michaelsen 1899

2.1.2 Species background information

Amynthas diffringens

This species, previously known as *Pheretima diffringens*, was also considered part of the *Amynthas corticis* complex (Reynolds & Cook 1976; Blakemore 2003) and originates from China, India and Pakistan (Ljungström 1972; Sims & Gerard 1985). Specimens found in South Africa reproduce both parthenogenetically (Ljungström 1972) and sexually (JD Plisko, *Pers. comm.*). No information could be obtained during the literature survey on the life cycle of this species. These reddish brown to dark brown earthworms may reach a length of 49 – 95 mm (Ljungström 1972), and sometimes up to 200 mm (Dlamini & Haynes 2004). They inhabit top-soil and areas with elevated organic content such as compost heaps (Ljungström 1972) and are classified as an epigeic species (Dlamini & Haynes 2004).

Aporrectodea trapezoides

Until recently, it had not been clear whether the phenotypically heterogeneous *Aporrectodea caliginosa* species complex comprised different species or different morphs (Sims & Gerard 1985). Originally, these species were described as *A. caliginosa* Savigny 1826, *A. tuberculata* Eisen 1874, *A. nocturna* Evans 1946 and *A. trapezoides* Dugès 1828 (Sims & Gerard 1985; Pérez-Losada *et al*. 2009). It was also previously classified as belonging to the genus *Allolobophora* (Sims & Gerard 1985), and being a species complex consisting of *Allolobophora caliginosa* Tétry 1937, *Allolobophora trapezoides* Gates 1972, *Allolobophora turgida* Eisen 1873, *Allolobophora tuberculata* Gerard 1964 and *Allolobophora nocturna* Evans 1946. However, many considered these species to be separate morphs within one species, *Aporrectodea caliginosa* (Sims & Gerard 1985). Recently, DNA sequencing, in conjunction with morphological and ecological data, have revealed that these *Aporrectodea caliginosa* "morphs" are in fact separate species (Pérez-Losada *et al*. 2009). The species used during the present study was identified as *A. trapezoides* by Dr. J.D. Plisko (Natal Museum, Pietermaritzburg, South Africa).

This species originates from the western Palearctic and eastern Nearctic, and is now found in the temperate regions of the world where it is fairly abundant in gardens and cultivated land (Sims & Gerard 1985; Baker *et al*. 1997; Blakemore 2003). The life cycle of *A. trapezoides* has been studied to some extent (Reinecke & Visser 1981; McCredie *et al*. 1992; Baker *et al*. 1992).

Specimens of *A. trapezoides* reach lengths of 80 – 140 mm, and the colour is variable and ranges from pale pink to purplish brown (Sims & Gerard 1985). Whilst smaller individuals may inhabit the

topsoil in temporary horizontal burrows, larger individuals will burrow deeper; consequently this species is considered to be endogeic (Sims & Gerard 1985; Bouché 1992).

Chilota sp.

It could not be clearly established to which species of *Chilota* these specimens belong, and the possibility exists that this might even be a new species (J.D. Plisko, *Pers. comm.*). Species in the family Acanthodrilidae are found throughout the southern hemisphere, and representatives of the genus *Chilota* occur throughout the south of South America, on the Southern Ocean Kergeulen and Crozet Islands, in New Zealand (Beddard 1912) and throughout South Africa (Beddard 1912; Pickford 1937; Plisko 2007). Thirteen indigenous South African *Chilota* species have been described (Beddard 1912; Pickford 1937), with a fourteenth species described recently (Plisko 2007).

The lengths of the specimens used for the present study were not measured, but other *Chilota* species reach lengths between 49 – 70 mm (Plisko 2007). The specimens collected during the present study were red-brown dorsally, and unpigmented ventrally. Nothing is known about the life cycle and reproduction of this species. It is unclear as to which ecological category the *Chilota sp.* individuals collected for the present study belong to, since very little is known about their ecology. Pertaining to the soil strata they inhabit, which may give an indication to their ecological category, the specimens collected for the present study were always found in the top 30 cm of the soil. Other species in this genus also inhabit the top soil layers (Plisko 2007). It is therefore assumed this species belongs to the endogeic category.

Eisenia andrei

Eisenia andrei was first described from cultures of *E. fetida* with a low pH (Sims & Gerard 1985) and was until recently regarded as a subspecies of *E. fetida*. It is however clear from recent molecular evidence that *E. andrei* and *E. fetida* are separate species (Domínguez *et al*. 2005; Pérez-Losada *et al*. 2005; Voua Otomo *et al*. 2009). Morphologically, *E. andrei* lacks the intersegmental unpigmented areas or transverse striping that is characteristic to *E. fetida* (OECD 1984; Sims & Gerard 1985). *E. andrei* and *E. fetida* reach 60 – 120 mm in length, and have a reddish colour (Sims & Gerard 1985). They have a Palearctic origin and are presently found in temperate regions all over the world (Sims & Gerard 1985). Both *E. fetida* and *E. andrei* have well-documented life cycles (Venter & Reinecke 1988; Domínguez *et al*. 2003; Domínguez *et al*. 2005; Velando *et al*. 2006), and it has been found that there is reproductive isolation between them (Domínguez *et al*. 2005). *E.*

andrei and *E. fetida* live in areas of high organic content such as compost heaps, manure piles and damp rotting vegetation and both are classified as epigeic species. They are therefore frequently used for vermiculture worldwide and are considered economically important species (Sims & Gerard 1985).

Perionyx excavatus

This epigeic earthworm, originating from tropical Asia (Edwards *et al*. 1998), has a well-studied life cycle and an extremely high reproductive rate (Hallatt *et al*. 1990; Edwards & Bohlen 1996; Edwards *et al*. 1998; Bhattacharjee & Chaudhuri 2002; Suthar 2007), and they have been shown to reproduce parthenogenetically (Hallatt *et al*. 1990). Its preferred habitat is areas of high organic matter content and moisture content such as compost heaps (Edwards *et al*. 1998). It is an economically important species and is commonly used for vermiculture in the Phillipines and Australia (Edwards & Bohlen 1996), Hawaii (Selden *et al*. 2005) and India (Bhattacharjee & Chaudhuri 2002). Individuals from this species may reach 30 – 70 mm in length, and may be dark red or brown (Selden *et al*. 2005).

2.1.3 Earthworm cultures and sampling

Specimens of *A. diffringens* were obtained from a commercial vermiculturist, Wizzard Worms, Greytown, Kwa-Zulu Natal, South Africa. Specimens of *A. trapezoides* were collected by digging and hand-sorting from a historically unpolluted site on the farm Vergenoegd (33°56'28.40"S, 18°53'23.25"E), Stellenbosch, South Africa. *Chilota sp.* specimens were collected by digging and hand sorting on an experimental farm of the South African Agricultural Research Council (ARC), Nietvoorbij (33°54'27.87"S, 18°52'7.52"E) near Stellenbosch. Specimens of *E. andrei* and *P. excavatus* were obtained from mixed laboratory cultures in the Ecotoxicology laboratory, Stellenbosch University. These cultures originated from *E. fetida* specimens obtained from Prof. O. Graff in Braunschweig, Germany. Cultures were augmented periodically with new specimens, thought to be *E. fetida*, from various sources. A recent study by Voua Otomo *et al.* (2009), using DNA barcoding, revealed that specimens from the laboratory cultures by 2008 were from *E. andrei*. It would therefore seem that *E. andrei* specimens were introduced to the laboratory cultures at some time, and possibly outcompeted *E. fetida*. Identification of field-collected species were done with the aid of a key (Sims & Gerard 1985) and in some cases, specimens were sent to an earthworm taxonomist, Dr. J.D. Plisko (Natal Museum, Pietermaritzburg, South Africa) for identification.

In order to obtain some level of standardisation across the five species, fully clitellate earthworms were used for all experiments. For all species, except *Chilota sp.* for which a sufficient number of specimens were not available, biomass was standardised for by selecting specimens within a unique mass category for each species (0.7 – 1.2 g for *A. diffringens*, 0.7 – 1.0 g for *A. trapezoides* and 0.3 – 0.6 g for both *E. andrei* and *P. excavatus*).

In order to acclimatise field-collected earthworms to laboratory conditions, they were maintained in the laboratory (20 °C in constant darkness) in their own substrate for at least 7 days prior to their use in experiments. Two days prior to the commencement of the toxicity tests, all earthworms were acclimatised in uncontaminated test substrate.

2.2 Toxicants

The fungicide copper oxychloride, $Cu_2Cl(OH)_3$, also known as copper (II) chloride oxide hydrate, Bordeaux A or Z and cupric oxide chloride, amongst others, is a bluish-green powder insoluble in water (Richardson 1993). It is however soluble upon decomposition in dilute acids and ammonium hydroxide solutions. It is applied to crops as a wettable powder; it is mixed with water and the substance is dispersed as tiny flakes throughout the water. The source of copper oxychloride used in the present study is a commercially available powder fungicide, Virikop (from Efekto, Registration no. L0527 under Act 36 of 1947), containing 850 g/kg copper oxychloride (Efekto 2010). The Cu content of Virikop is 500 g/kg Cu.

As a positive control (reference substance) for the whole-organismal responses, the broad-spectrum fungicide benomyl ($C_{14}H_{18}N_4O_3$) was selected. This toxicant is recommended by the OECD as a reference substance for toxicity testing (reproduction tests) with earthworms (OECD 2004). Benomyl is known to cause significant decreases in earthworm reproduction at concentrations as low as 3 mg/kg Cu (Van Gestel *et al*. 1992), and is known to affect the ultrastructure of sperm cells (Sorour & Larink 2001).

Benomyl is part of the benzimidazole carbamate chemical group (WHO/FAO 1994) and is a systemic fungicide that is absorbed through the roots and leaves of crop plants after being applied as a wettable powder. Benomyl has a relatively short half-live in soil and can be converted within 19 hours into the fungicide carbendazim (methyl-2-benzimidazole), which in return has a half-life of 25 days at 20 °C. The source of benomyl used in the present study is a commercially available powder fungicide, Demeter (from Volcano Agrosciences, (Pty) Ltd, Registration no. L7291 under Act 36 of 1947), containing 500 mg/kg active ingredient.

For the biomarker tests, hydrogen peroxide (H_2O_2) was selected as an alternative positive control to benomyl to use on coelomocytes *in vitro*. It is known to cause significant DNA damage (as measured with the comet assay) in earthworm coelomocytes at concentrations as low as 37 µM (Di Marzio *et al*. 2005).

2.3 Exposures in artificial soil

An overview of the exposure procedure for all earthworm species, as well as the preceding preparations, is presented in Figure 2.1. All experiments were conducted in a climate-controlled room in darkness at 20 °C and 60% humidity. The exposure period was 14 days for all experiments.

Glass jars with a volume of one litre were used for exposing earthworms, and were covered with fine netting material to allow for aeration and to prevent earthworms from escaping. To minimise water loss, discs of plastic sheeting were placed on top of the soil in each container. The amount of soil placed in each jar was calculated on the basis of the average mass of each earthworm species. One hundred grams (dry weight) of OECD artificial soil (OECD 2004) was used for each 1 g (wet weight) of earthworm. Therefore, for e.g. *A. diffringens*, with an average mass of 1 gram, 400 g of soil was used to expose 4 earthworms.

2.3.1 Preparation of substrate

The OECD artificial soil consisted of (by dry weight) 10% sphagnum peat (Les Tourbes Nirom Peat, Canada), 20% kaolin clay (Serina Kaolin, South Africa) and 69.6% quartz sand (Consol Limited Industrial Minerals, South Africa), of which 50% were of particle size between 50 and 200 µm (OECD 2004). The soil pH was adjusted to 6 ± 0.5 with 0.4% (by dry weight) $CaCO_3$ (Merck, Germany). The pH of 1 g soil in 30 ml distilled water was measured with a Crison micropH 2001 meter (Crison Instruments SA, Spain).

Soils were moistened with distilled water to a moisture content of 60% of the maximum water holding capacity (OECD 2004), which corresponded to a soil moisture content of 32% (w/w). For the negative controls, the required amount of water was thoroughly mixed directly with the soil. For each exposure concentration of copper oxychloride or benomyl, the appropriate amount of toxicant was mixed with the required amount of distilled water prior to mixing with the soil. After moistening and spiking, all soils were allowed to incubate in airtight plastic containers for 48 h prior to introducing the earthworms. The moisture content (% w/w) (measured with a Sartorius MA 45 moisture meter, Sartorius, Germany) and the pH of the soil were measured at the start (Day 0) and end (Day 14) of each exposure period.

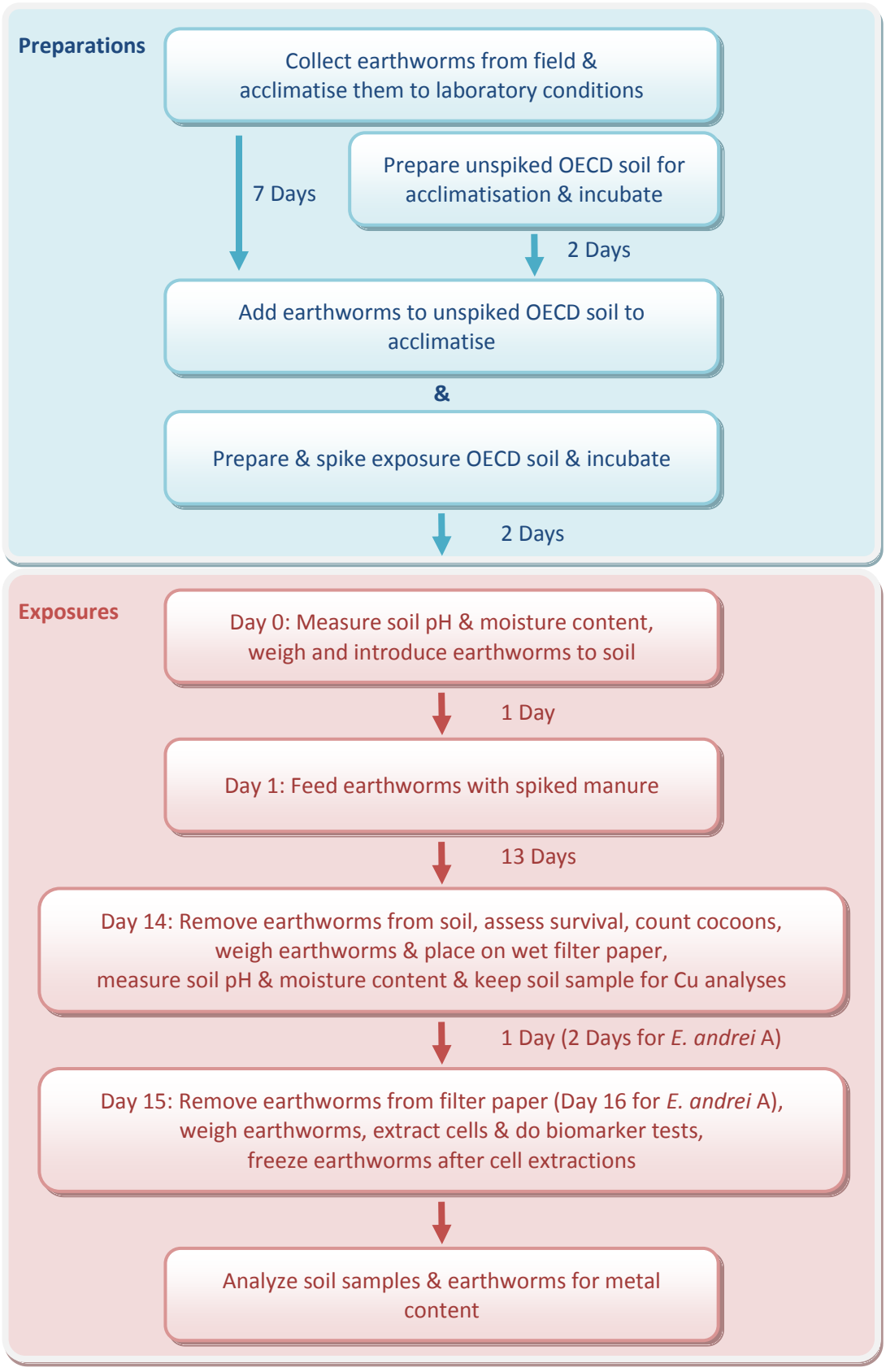


Figure 2.1: The procedure used to expose specimens of five earthworm species to Cu in the form of copper oxychloride in OECD artificial soil for 14 days.

2.3.2 Feeding of earthworms

Earthworms were fed once, at the start of the 14 day exposure period, with 0.5 g (dry weight) dried, ground and sieved urine free cattle manure per 1 g (wet weight) of earthworm. The manure was re-wetted with 4 ml distilled water per 1 g manure, and spiked with the corresponding toxicant concentration. The re-wetted and spiked manure was placed on top of the soil (beneath the plastic sheeting) one day after the earthworms were introduced to the soil (on Day 1). It was decided to spike the earthworm food (cattle manure) for two reasons. Firstly, it was clear from visual inspections in preliminary range finding experiments (where earthworms were supplied with uncontaminated food) that earthworms avoided the spiked soil by crawling into the food layer and lining their tunnels with it. Indeed, it is recommended that food be spiked in order to avoid the possibility that it could serve as a refuge (Spurgeon *et al*. 2003b). Secondly, the situation in the field, where both soil and food sources could be contaminated, would be approximated more closely.

2.3.3 Range finding tests and preliminary exposure experiments

Range-finding tests were conducted with *E. andrei* to determine a suitable range of sublethal concentrations. Although the pesticide copper oxychloride was used, concentrations were determined and are given as Cu concentrations to facilitate comparisons with measured soil and earthworm body Cu concentrations.

The earthworms were exposed to a range of Cu (in the form of copper oxychloride) and benomyl concentrations for 3 weeks. Three replicates were performed with 10, 20, 30, 40, 50, 70, 100, 200, 300 and 500 mg/kg Cu, and 1, 5 and 10 mg/kg (active ingredient) benomyl. Four earthworms were used per concentration per replicate. Mortality for Cu was negligible, with one earthworm (out of a total of 12) dying at each of the following concentrations: 50, 300 and 100 mg/kg Cu. For benomyl, 11% mortality occurred at 1 mg/kg, 22% at 5 mg/kg and 55% at 10 mg/kg benomyl. Subsequently, 10 mg/kg benomyl was chosen for all exposures.

In order to determine whether earthworms would survive at higher Cu concentrations, a second set of range finding tests included 20, 40, 80, 160, 320 and 640 mg/kg Cu. Ten earthworms were used per concentration, and all survived. The Cu concentrations were increased further in a third set of range finding tests (with 10 earthworms per concentration): 20, 40, 80, 160, 320, 640 and 1280 mg/kg Cu. At 1280 mg/kg Cu, mortality was 100%, but no earthworms died at 640 mg/kg Cu and the lower concentrations. Therefore a concentration range of 0, 20, 80, 160, 640 and 960 mg/kg Cu was initially selected for *E. andrei*. In the final exposure experiments, five replicates were initially

performed for *E. andrei* at this concentration range. These replicates will collectively be referred to as *E. andrei* (A).

Range finding tests were subsequently done with both *A. trapezoides* and *P. excavatus* with the abovementioned concentration range, where 50% mortality was found for *A. trapezoides* and 100% mortality for *P. excavatus* at 960 mg/kg. It was therefore decided to exclude the concentration of 960 mg/kg from all experiments with these species, and instead use 0, 20, 80, 160, 320 and 640 mg/kg Cu.

2.3.4 Final exposures

The final concentration ranges and number of replicates for all experiments are summarised in Table 2.1. Both the negative and positive controls and each of the copper (-oxychloride) concentrations will henceforth be referred to as "treatments".

As mentioned above, five replicates were initially done for *E. andrei* (this group is referred to as *E. andrei* (A)) at the concentration range of 0, 20, 80, 160, 640 and 960 mg/kg Cu. The intention was to do only four replicates, but the neutral red retention assay (NRR) and MTT assay data from replicate 3 were lost after a computer failure. Therefore, a fifth replicate was subsequently performed. Also, specimens from this group were depurated on filter paper for 2 days after exposure (Figure 2.1). It was subsequently discovered that 1 day is sufficient to allow for the depuration of guts of earthworms (Arnold & Hodson 2007). It was also thought that Cu detoxification might occur during the second day of depuration, which could possibly influence the NRR, MTT and comet assay results, since the detoxification of Cu can occur in less than a day in some earthworm species (Veltman *et al*. 2007). In addition, the concentration range that was later selected for *A. trapezoides, Chilota sp.* and *P. excavatus*, included the treatment 320 mg/kg Cu which was not in the range for *E. andrei* (A). It was therefore decided to perform another four replicates with *E. andrei* after experimentation with *A. diffringens, A. trapezoides* and *Chilota sp.*. This second group of *E. andrei* replicates will be referred to as *E. andrei* (B).

For *A. diffringens* the first replicate was done before the final concentration range was selected from preliminary results of *A. trapezoides* and *P. excavatus*, and included the same concentrations as for *E. andrei* (A). The other three replicates for *A. diffringens* included the same concentrations as for *A. trapezoides* and the other species (Table 2.1).

The final concentration range was 0, 20, 80, 160, 320 and 640 mg/kg Cu, with a positive control of 10 mg/kg benomyl for *A. trapezoides*, *Chilota sp.* and *P. excavatus*. Five replicates were done with *E. andrei* (A), and four replicates were done for each of *A. diffringens, A. trapezoides, P.*

excavatus and *E. andrei* (B). For *Chilota sp*., three replicates were done because a sufficient number of specimens could not be obtained during the study period. For each species, four earthworms were used per treatment per replicate, except for *E. andrei* (A) where 8 earthworms were used for the first four replicates and 5 earthworms for the fifth replicate.

Table 2.1: The number of replicates used at each treatment for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For all species, the number of specimens used per treatment per replicate was 4, except for *E. andrei* (A), where it was 8 for replicates 1-4, and 5 for replicate 5.

	Negative	Cu (mg/kg)						Benomyl
Species	control	20	80	160	320	640	960	(10 mg/kg)
A. diffringens	4	4	4	4	3	4	1	4
A. trapezoides	4	4	4	4	4	4	--	4
Chilota sp.	3	3	3	3	3	3	--	3
E. andrei (A)	5	5	5	5	--	5	5	5
E. andrei (B)	4	4	4	4	4	4	--	4
P. excavatus	4	4	4	4	4	4	--	4

2.4 Analyses of metal content in soil and earthworms

All soil and earthworm samples were treated according to a protocol modified from Katz & Jenniss (1983).

2.4.1 Preparation of soil for acid digestion

Soil samples from the earthworm collecting sites or their culturing media were prepared and screened for metal (Cd, Ni, Cu, Mn, Pb and Zn) contents. For *A. trapezoides* and *Chilota sp*., three soil samples each from Vergenoegd and Nietvoorbij (respectively) were analyzed. For *E. andrei* and *P. excavatus* (cultured in the same medium) and *A. diffringens*, three samples each from these two culturing media were analyzed. A sample of the dried and ground cattle manure (used for feeding earthworms during exposure, sourced from the Stellenbosch University experimental farm Welgevallen) was also included in the analyses. Copper content was measured in OECD soil samples taken from each container for each species, replicate and treatment (except the benomyl-spiked soil – it is assumed that the Cu content here would be the same as the negative control soil). Soil samples were dried in an oven (Baird & Tatlock Ltd., London) at 60 °C for 48 hours. Subsequently, the soil samples were finely ground with a mortar and pestle and 1 g per sample was used for acid digestion.

2.4.2 Preparation of earthworm specimens for acid digestion

Two earthworms from each treatment, replicate and species, were analyzed for Cu content. These worms were the same earthworms selected for the comet assay, except for *A. trapezoides* (on which the comet assay was not performed) and the first two replicates of *E. andrei* (A), where two specimens from each container were randomly selected. After exposure, all earthworms were weighed and placed on moistened filter paper to void their guts. After the depuration period (24 h for all species, except for *E. andrei* (A), which was 48 h), earthworms were weighed and their coelomocytes were extracted for biomarker tests. Subsequently, they were placed in individually labelled 1.5 ml Eppendorf tubes (1.5 ml micro centrifuge tube, Greiner Bio-One, Germany) and frozen at -18 °C (Defy Multimode freezer, Defy, South Africa) until required for metal analyses. Prior to acid digestions, frozen earthworms were thawed at room temperature and weighed.

2.4.3 Acid digestion procedure

An overview of the acid digestion procedure (modified from Katz & Jenniss 1983) is presented in Figure 2.2. After weighing, all samples were digested with 10 ml (for soil) or 5 ml (for earthworms) nitric acid (55%) (HNO_3, Merck, Germany) for a minimum of 8 hours. Subsequently, the samples were heated to 40-60 °C in a Labcon Dual Digestor heating system (Labdesign Engineering Ltd, South Africa) for 2 hours and then to 110-120 °C until emitting brown fumes. After cooling, 5 ml (for soil) or 2.5 ml (for earthworms) perchloric acid (70%) ($HClO_4$, Associated Chemical Enterprises, South Africa) was added and the samples heated to 110-120 °C until emitting white fumes. After subsequent cooling, 5 ml (for soil) or 2.5 ml (for earthworms) of distilled water was added to each sample, which was reheated to 110-120 °C until emitting white fumes. The samples were then cooled completely and filtered with Whatman no. 6 filter paper (Whatman International Ltd, England) and totalled to 20 ml (for soil) or 10 ml (for earthworms) with distilled water. Subsequently, the samples were filtered through 0.45 µm cellulose nitrate filters (Sartorius, Germany) and stored in polyvinyl containers at 4 °C until metal analyses were performed.

The concentrations of selected heavy metals (Cu, Mn, Zn, Pb, Ni, Cd) in samples of substrates of the sampling sites and culture media from which earthworms were sampled, as well as the cattle manure, were analysed with a Varian Radial ICP-AES (ICP) at the Stellenbosch University Central Analytical Facility. The Cu content in these samples were also analysed with a Varian AA – 1275 flame atomic absorption spectrophotometer (AAS) (Varian Inc., California, USA) at the Department of Physics, Stellenbosch University. The Cu content of the OECD soil and earthworm samples used in experiments were analysed with the AAS. An appropriate range of Cu standards (Merck, Germany), between 1 and 25 mg/l Cu, were made up to calibrate the AAS. The detection

limit for the AAS for Cu is 0.04 mg/l, which corresponds to a body Cu content of 0.8 mg/kg in an average earthworm of 0.5 g (wet weight).

Results from the AAS and ICP readings (in mg/l) were converted to mg/kg (per dry weight soil or wet weight earthworm) with the following formula:

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

where A is the AAS or ICP reading in mg/l; V is the volume of the sample in ml (20 ml for soil and 10 ml for earthworms); and M the mass of the sample in g (1 g for soil). For earthworms, the mass after freezing and subsequent thawing was used.

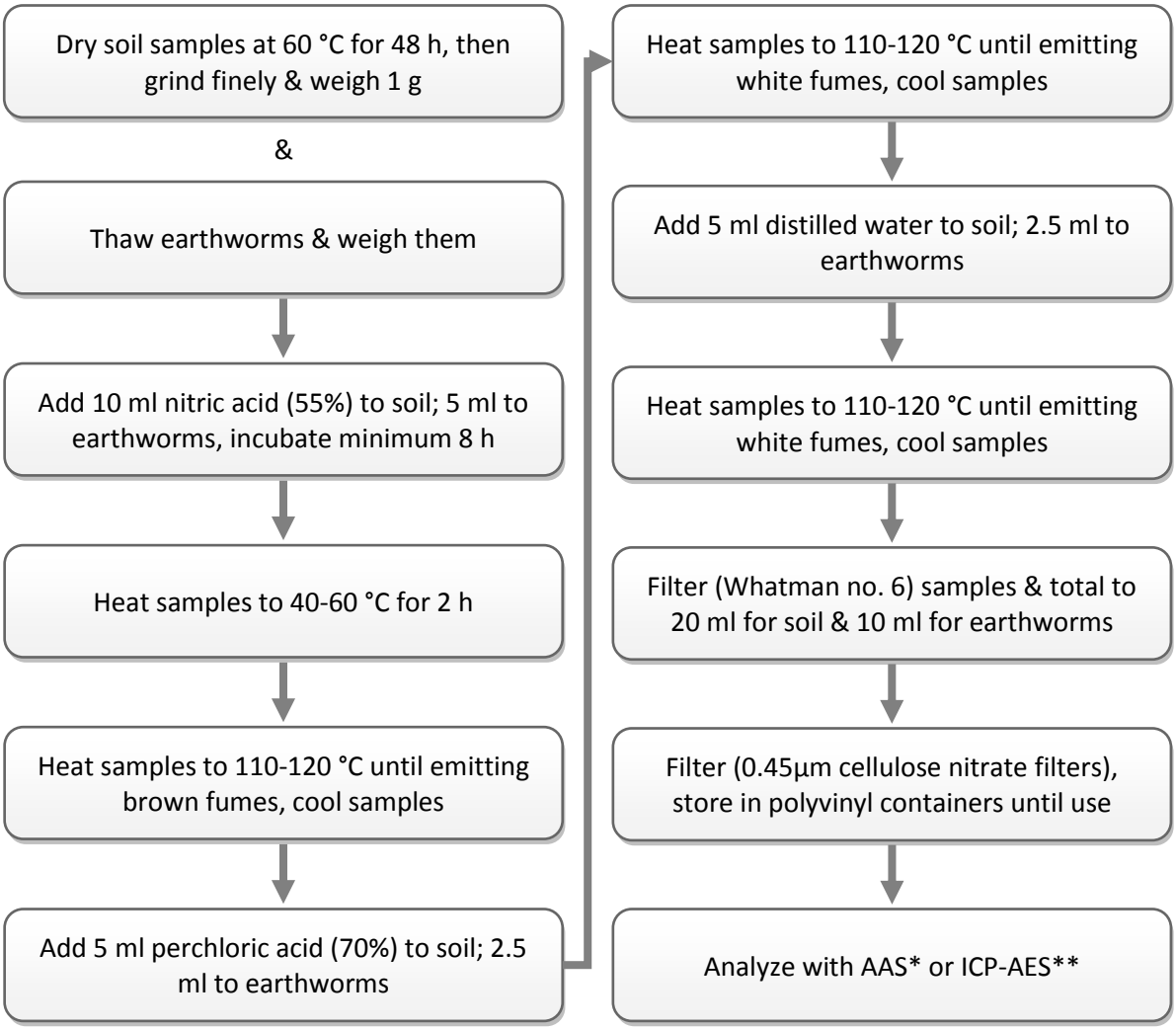


Figure 2.2: Acid digestion procedure (modified from Katz & Jenniss 1983) used for soil samples and specimens of five earthworm species exposed to Cu in the form of copper oxychloride in OECD artificial soil for 14 days. Soil samples include OECD exposure soil, samples from the collecting sites for the earthworms and cattle manure for earthworm feed. * AAS = Atomic absorption spectrophotometer. ** ICP-AES = Inductively coupled plasma atomic emission spectrometer.

For each batch of soil and earthworms that was acid digested, a blank was included to check for possible contamination during the digestion process. In all blanks analysed, the Cu concentrations were below AAS and ICP detection limits (data not shown).

Three spiked reference OECD soil samples (400 mg/kg Cu, spiked with the same Cu standard used for calibrating the AAS) were also acid digested and analysed with both AAS and ICP to determine the recovery efficiency of the method for soil samples. A mean (± std dev) % recovery of 71% (± 8) for Cu concentrations was obtained for AAS results (Appendix B, Table 1), and 85% (± 5) for ICP results. No significant differences were found between the AAS and ICP results (Mann-Whitney U test, $P > 0.05$, Appendix B, Table 2).

To determine the recovery efficiency of biological samples, certified reference material (powdered mussel tissue, ERM-CE278, Institute for Reference Materials and Measurements, Geel, Belgium), containing 9.45 (± 0.13) mg/kg Cu, was digested and analysed with both AAS and ICP in the same manner as the earthworm samples. The mean Cu content of the certified reference material (mussel tissue), as measured with AAS, was 9.77 mg/kg Cu (± 0.22, n = 4) and a mean recovery of 103.4% (± 2.36, n = 4) was found. For ICP measurements, the Cu content was 9.69 mg/kg (± 2.12, n = 4), with a recovery efficiency of 102.56% (± 22.41, n = 4). The measured values were close to the expected values, therefore no correction factor was applied to the results of the AAS measurements of the earthworm samples used in the experiments. No significant differences were found between the AAS and ICP results (Mann-Whitney U test, $P > 0.05$, Appendix B, Table 2).

Three samples of the source of copper oxychloride (Virikop) was analysed for Cu content with both AAS and ICP. In addition, three samples of Virikop from another commercial batch than that used in this study, were included to check for possible differences between batches. The Cu content of the Virikop samples (Appendix B, Table 1) was slightly lower than the expected 500 mg/kg. After applying a correction factor (using the 71% recovery from the AA results of the spiked reference soil), the mean (± std dev) Cu content for Virikop 1 (used to spike substrates in this study) was 473 (± 17) mg Cu /kg Virikop (95 ± 3% recovery after correction) for the AAS measurements and 479 (± 29) mg/kg Cu (96 ± 6% recovery after correction) for the ICP measurements. For Virikop 2 (from the other batch), these values were slightly lower than for Virikop 1, with 435 (± 9) mg/kg Cu (87 ± 2% recovery after correction) for the AAS measurements and 410 (± 8) mg/kg Cu (82 ± 2% recovery after correction) for the ICP measurements. When the Cu content of Virikop 1 and Virikop 2 were compared, no significant differences were found between the two batches (Mann-Whitney U test, $P > 0.05$, Appendix B, Table 3).

A diluted sample of the Cu standard (used for calibrating the AAS and for spiking the reference OECD soil) was analysed with the ICP, and the Cu concentration was measured as 1080 mg/l Cu (108% recovery) (Appendix B, Table 1).

2.5 Whole-organismal parameters

Before exposure, wet weights (g) of individual earthworms were determined (Figure 2.1). Earthworms were weighed individually on a Sartorius balance (Sartorius Handy, Sartorius, Germany) in weighing boats filled with water to avoid desiccation. Subsequently, earthworms were randomly placed into the containers with the OECD soil for the different treatments.

After the 14 day exposure period, the number of mortalities was recorded, as well as the number of cocoons present after wet sieving (1 mm sieve aperture size) of the soil (OECD 2004). The worms were then weighed, after which they were placed on moist filter paper for 24h (or 48 h for *E. andrei* (A)) to void their guts. As previously mentioned, a time period of 24 h was eventually selected, as this is sufficient time for earthworms to void their guts (Arnold & Hodson 2007). The detoxification time of Cu is also less than 24 h in some earthworm species (Veltman *et al*. 2007), therefore the more often used 48 h (e.g. Spurgeon *et al*. 2004a; Vijver *et al*. 2007) was not used.

After depuration of their guts on filter paper, the earthworms were weighed for a third time. Therefore, earthworm biomass was recorded at three time intervals: before exposure ("start mass", Day 0), after exposure ("end mass", Day 14) and after a subsequent period on filter paper ("depurated mass", Day 15; or Day 16 for *E. andrei* (A)). Subsequently, the mass change during exposure (henceforth termed "exposure mass change") was calculated as the end mass minus the start mass and expressed as a percentage of the start mass, to facilitate comparisons between treatments as well as species. In addition, the mass change during depuration ("depuration mass change") was also calculated (depurated mass minus end mass, expressed as a percentage of the end mass). Since the earthworm castings were not weighed, the depuration mass change was assumed to be an indication of the amount of gut contents voided and hence as an indirect indication of the feeding activity of the earthworms.

2.6 Suborganismal parameters

The following procedures were all conducted in a darkened room illuminated with a single yellow 60 Watt light bulb to prevent UV-induced DNA damage of the earthworm coelomocytes.

2.6.1 Cell extraction

Coelomocytes were collected from the coelomic cavity of the earthworms and were used for the neutral red retention (NRR), MTT and alkaline comet assays. After allowing earthworms to depurate their gut contents and subsequently weighing them, coelomocytes were extruded with a non-invasive method (Eyambe *et al*. 1991), adapted by Reinecke & Reinecke (2004b) with some alterations for the present study. An overview of the cell extraction process is presented in Figure 2.3.

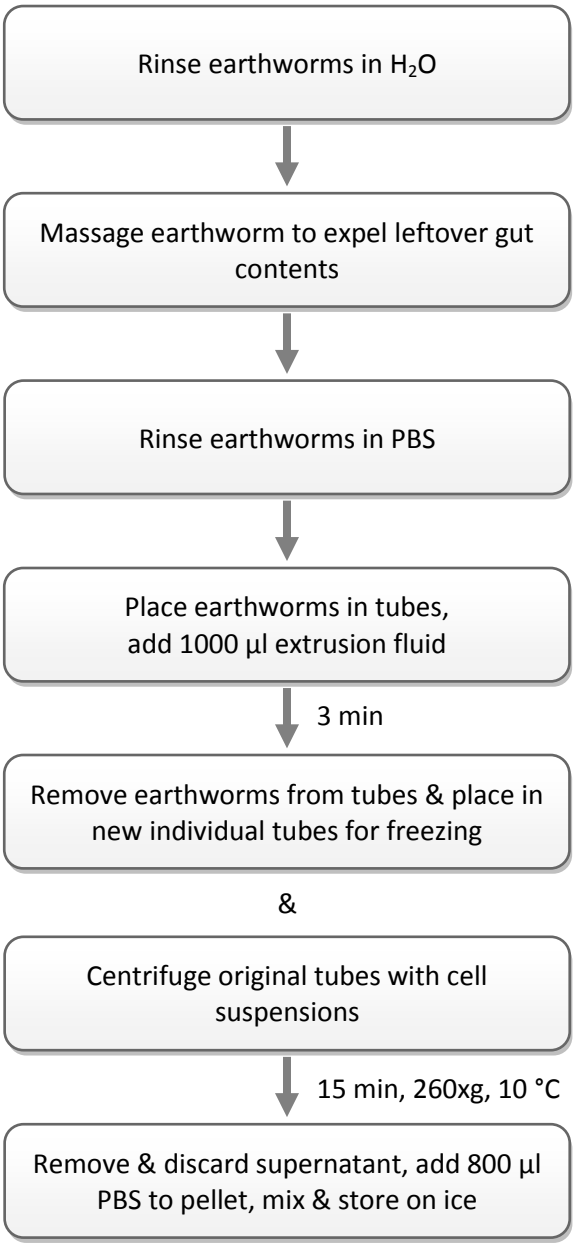


Figure 2.3: Cell extraction protocol, adapted by Reinecke & Reinecke (2004b) after Eyambe *et al.* (1991), used for specimens of five earthworm species after exposure to Cu in the form of copper oxychloride in OECD artificial soil for 14 days.

Each specimen was exposed for 3 minutes in an individually labelled 1.5 ml Eppendorf tube, or a 15 ml polypropylene tube (Greiner Bio-One, Germany) for *A. diffringens*, to 1 ml of an extrusion fluid (Appendix A) which induced it to expel coelomic fluid through its dorsal pores. The specimen was removed from the tube, placed in a clean, individually labelled Eppendorf tube and immediately frozen until used for acid digestions and metal analyses. The cell suspension, left in the original Eppendorf tube (or decanted into a new, labelled 1.5 ml Eppendorf tube for *A. diffringens*), was centrifuged at 260 x g (corresponding to 2000 rpm) in a Biofuge fresco centrifuge (Heraeus Instruments, Germany) for 15 min at 10 °C. Subsequently, the supernatant was removed by drawing it from the tube with a micropipette. The pellet was resuspended in 800 µl PBS (Phosphate Buffered Saline, Sigma-Aldrich) and stored on ice until further use.

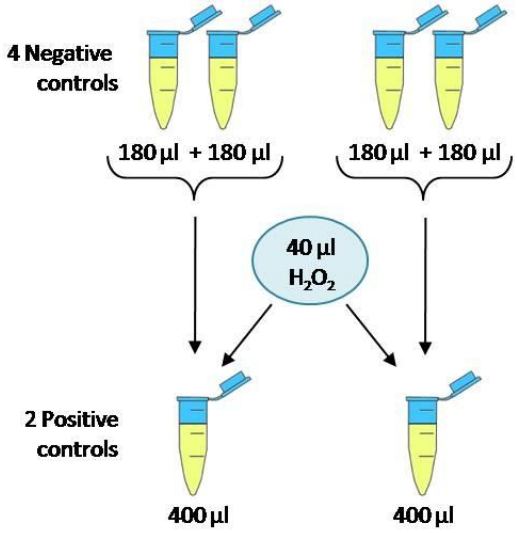


Figure 2.4: Procedure used to spike earthworm cell suspensions with H_2O_2 to obtain two positive controls, obtained from four negative control specimens.

2.6.1.1 Preparation of the in vitro *positive control*

For the NRR, MTT and comet assays, a proportion of coelomocyte suspension taken from the negative control earthworms from each experiment was spiked with 1 mM H_2O_2 (Sigma-Aldrich) to obtain a positive control. This was done because the benomyl-exposed earthworms were not used as a positive control for these biomarker tests. Ideally, a proportion of cell suspension from each of the negative control specimens should be spiked to obtain four separate positive controls. However, this would have resulted in an insufficient volume of cell suspension for all the biomarker assays for the control specimens. Therefore, from each of the four negative control specimens in each replicate, 180 µl of cell suspension was taken (Figure 2.4). The suspensions from specimens 1 and 2 were

mixed to obtain 360 µl of cell suspension, and the same was done for specimens 3 and 4. Subsequently, 40 µl of a 10 mM H_2O_2 stock (in distilled water) was added to each of these two mixtures obtain a final concentration of 1 mM H_2O_2 in 400 µl cell suspension. Therefore, two positive controls were prepared from four negative controls for each replicate.

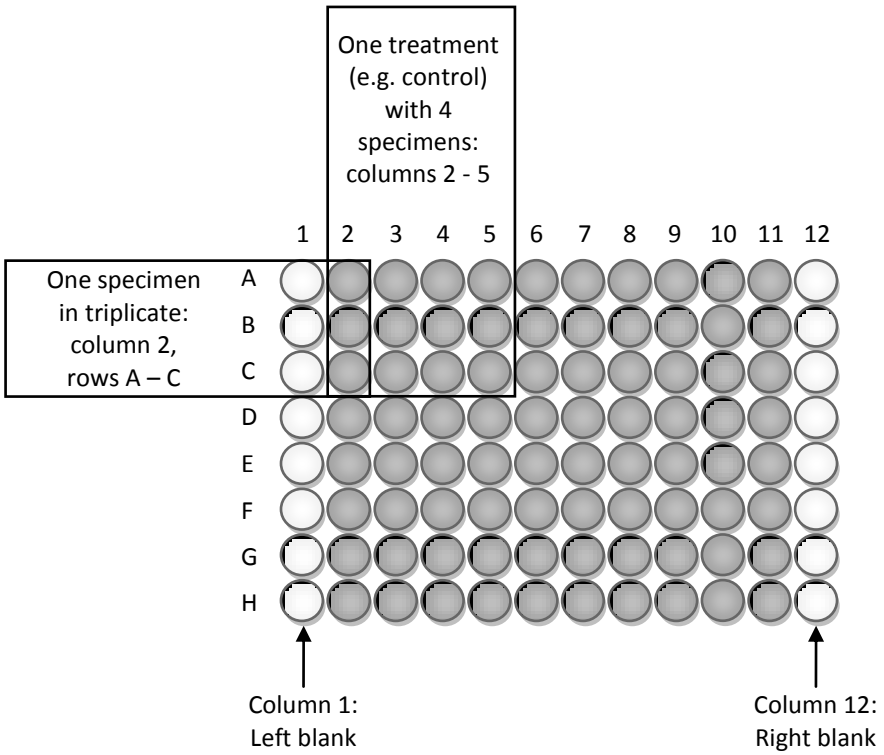


Figure 2.5: Diagram of a 96-well microtiter plate, indicating the position of blanks and cell suspensions for the Bradford, NRR and MTT assays. For each replicate, cell suspensions from all four specimens from each treatment were added in triplicate.

2.6.2 Protein content of coelomic fluid

The protein content of earthworm coelomic fluid was assessed with the Bradford protein assay (Bradford 1976). For the assay, 10 µl of coelomocyte suspension from each specimen was pipetted in triplicate into a 96-well microtiter plate (Greiner Bio-One, Germany). Blanks, consisting of 10 µl distilled water, were added to the first and last columns (1 and 12) of the microtiter plate (Figure 2.5). To both cells and blanks, 180 µl of a 1:4 diluted BioRad solution (containing Coomassie Brilliant Blue G-250, phosphoric acid and methanol, BioRad, Germany) was added, and the plate was incubated for 5 minutes at room temperature (Figure 2.6). Subsequently, the absorption was read at 595 nm on a Multiskan EX spectrophotometer (Thermo Electron Corporation, Finland).

The mean value of the three absorption measurements for each specimen was used in the final analyses, except when one of the three values differed more than 20% from the other two, in which case it was discarded. All readings were corrected for background noise by subtracting the mean of the blanks from the mean value for each specimen.

Since the protein content and the neutral red retention (NRR) and MTT assays were done on the same coelomocyte suspensions from each specimen, the results from the NRR and MTT assays could be divided by the results from the Bradford protein assay to determine the amount of neutral red retention or MTT conversion per cell.

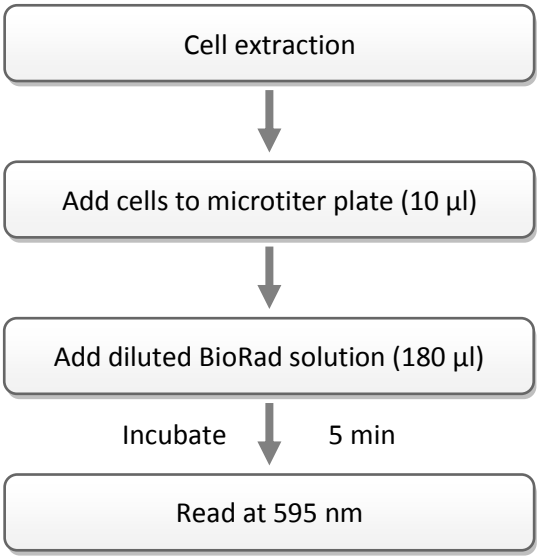


Figure 2.6: Procedure used for the BioRad protein assay (after Bradford 1976) to determine the protein content, which is assumed an indication of cell number, in earthworm coelomocyte cell suspensions. Please see Figure 2.3 for the cell extraction procedure.

2.6.2.1 Standard curves for the protein assay

Before each run of the Bradford assay, a standard curve was created with bovine serum albumin (BSA) (Sigma-Aldrich) to be used for calculating the coelomic fluid protein concentration (µg/µl) for each earthworm specimen from its absorption reading. BSA was added in quadruplicate to a microtiter plate in a concentration range of 0, 2, 4, 6, 8 and 10 µg/µl BSA, and the absorption values were measured with the Bradford assay as described above. The absorption values were plotted against the BSA concentrations, and the resulting functions, correlation coefficients (Pearson correlations) and *P*-values are presented in Appendix B, Table 4. Below 2 µg/µl, no linear relationship could be determined between the protein concentrations and absorption values in any of the standard curves. Therefore, those specimens yielding coelomocyte suspensions with absorption

values below this level were excluded from further analyses with the NRR and MTT assays. Additionally, it was assumed that below 2 µg/µl, coelomocyte cell count would be too low to yield valid results for the biomarker (NRR and MTT) assays.

2.6.2.2 Correlating coelomocyte number and coelomic fluid protein concentration

To determine whether cell numbers in the coelomocyte suspensions correlate with protein concentrations, cells were extracted from two randomly selected *E. andrei* specimens from the laboratory breeding culture. The protein content of serial dilutions from these specimens was determined with the Bradford protein assay, and the cell number concurrently determined with an Improved Neubauer hematocytometer (Boeco, Germany). A significant correlation (Spearman R = 0.96, $P < 0.001$) was found between the protein content (µg/µl) and the number of coelomocytes (cells/µl) (Figure 2.7). Henceforth, the protein content of samples was assumed to be directly related to the cell count.

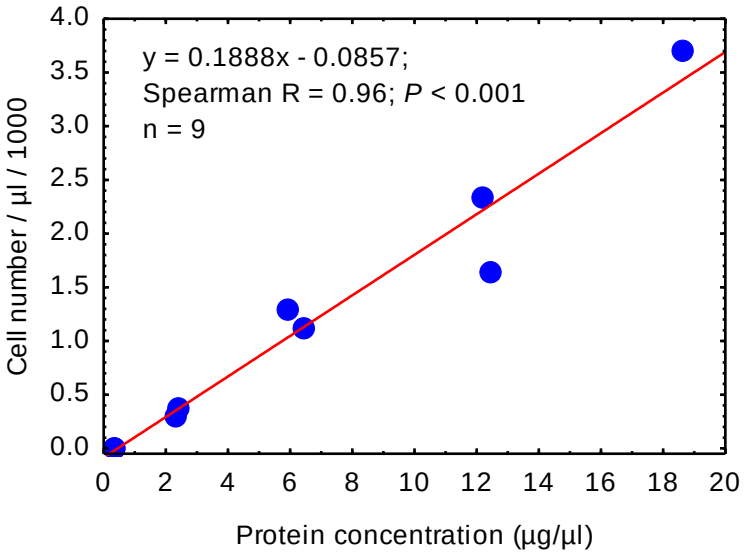


Figure 2.7: Correlation between the number of coelomocytes per µl (presented here as divided by 1000 for illustrative purposes) and the protein content (µg/µl) of coelomic fluid of *E. andrei*.

2.6.3 Spectrophotometric neutral red retention (NRR) and MTT assays

For both the NRR and MTT assays, all live specimens from all treatments, replicates and species, except for *A. trapezoides* and *E. andrei* (A) were used. For *E. andrei* (A), these assays were performed on four specimens from each treatment in all five replicates, but, as previously mentioned, data from replicate 3 were lost due to a computer failure. These assays were not

performed on *A. trapezoides*, because the protein concentrations in the coelomic fluid and thus cell numbers were too low.

The protocols of Maleri *et al*. (2008), adapted from Borenfreund & Puerner (1985) for the NRR assay and from Carmichael *et al*. (1987) for the MTT assay were followed. For both assays, 50 µl of a NRR or MTT colouring solution (Appendix A) was added to 50 µl of coelomocyte suspension, which was added in triplicate for each specimen (as described in Figure 2.5), in two separate 96-well microtiter plates for the two assays. Fifty µl of coelomocyte suspension from each of the two positive controls (as described in section 2.6.1.1 and Figure 2.4) were also added in triplicate to each of the microtiter plates. Blanks (PBS with colouring solution) were added to the first and last lanes of each microtiter plate. For each replicate from each species, one microtiter plate was used for the NRR assay, and one microtiter plate for the MTT assay. Each microtiter plate contained the positive controls and the blanks, as mentioned above.

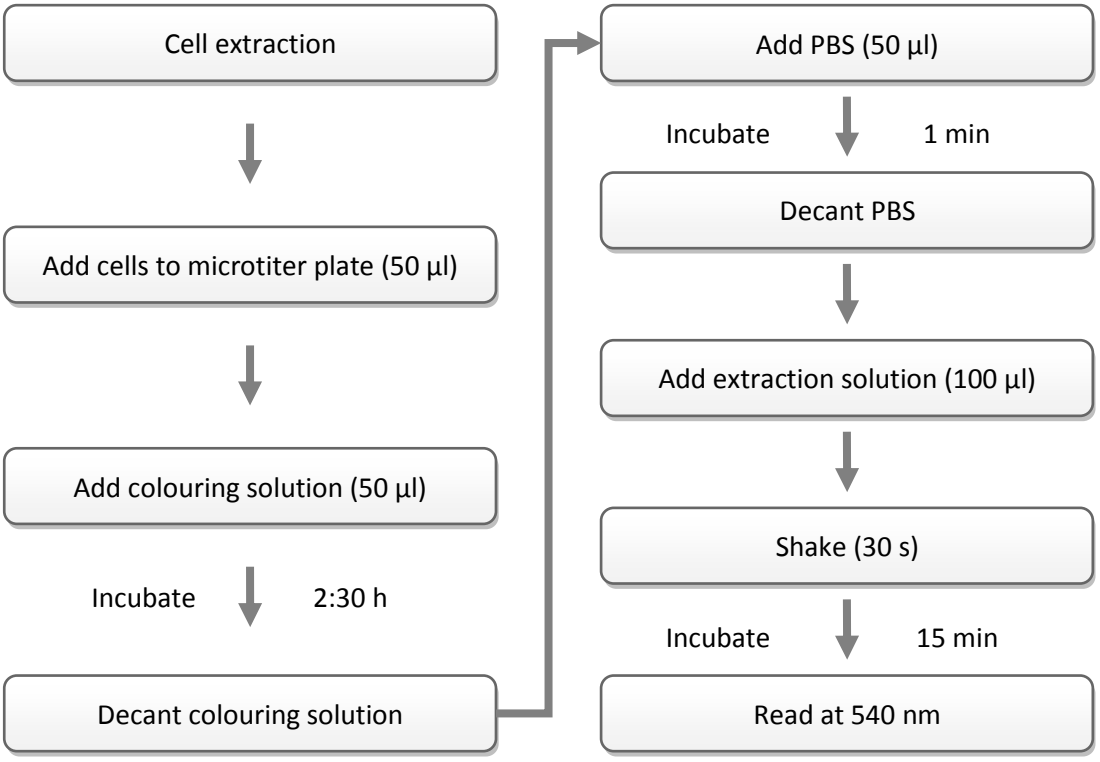


Figure 2.8: Procedure used for the neutral red retention (NRR) assay (after Borenfreund & Puerner 1985; and adapted by Maleri *et al*. 2008) to determine cytotoxicity of Cu to earthworm coelomocytes. Please see Figure 2.3 for the cell extraction procedure.

For the NRR assay, the microtiter plate was subsequently incubated for 2½ hours at 20 °C in darkness, after which the colouring solution was decanted (Figure 2.8). Subsequently, each well

was washed with 50 µl PBS for 1 minute, after which 100 µl of NRR extraction solution (Appendix A) was added to each well. The microtiter plate was then shaken for 30 seconds and left to incubate for 15 min, after which the absorbance was read at 540 nm.

For the MTT assay (Figure 2.9), the microtiter plate was incubated overnight (15 h) at 20 °C in darkness, after which 50 µl of MTT extraction solution (Appendix A) and 50 µl DMSO (dimethyl sulphoxide, Merck) was added to each well. The microtiter plate was allowed to incubate a further 3 hours, after which the absorption was read at 570 nm.

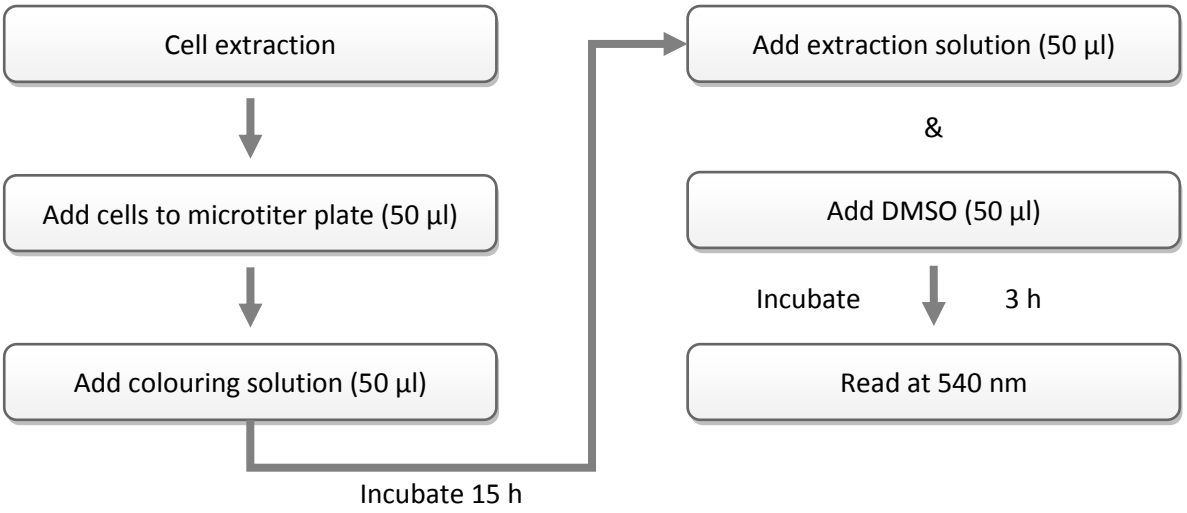


Figure 2.9: Procedure used for the MTT assay (after Carmichael *et al*. 1987; and adapted by Maleri *et al*. 2008) to determine the effect of Cu on the metabolic activity of earthworm coelomocytes. Please see Figure 2.3 for the cell extraction procedure.

The results for each assay were considered valid when the two columns of blanks on opposing sides (column 1 and 12, as shown in Figure 2.5) of the microtiter plate did not differ more than 20% from each other. All readings for NRR and MTT were corrected for background noise by subtracting the mean of the blanks from the mean reading of each specimen. To take the cell number (protein concentration) into account, these mean readings per specimen (with the blanks subtracted) were divided by its protein content to obtain corrected NRR and MTT values. As previously mentioned, only results of specimens where coelomocyte suspension protein concentrations exceeded 2 µg/µl were used in the final analyses. Furthermore, in order to render the NRR and MTT results comparable between replicates and species, the corrected NRR or MTT value for each specimen (termed NRR or MTT (corrected)) was calculated as a percentage of the mean (corrected) NRR or MTT value of all the specimens in the negative control in that replicate.

These NRR and MTT values, calculated as a percentage of the control, termed NRR or MTT (% of control), were used in all further statistical analyses.

2.6.3.1 Validation of in vitro *positive control for NRR assay*

Preliminary experiments were done to test the validity of H_2O_2 as a positive control for the NRR assay. Coelomocyte suspensions, obtained from four randomly selected *E. andrei* specimens from the laboratory breeding culture, were pooled and divided into 8 parts. These were spiked with H_2O_2 concentrations in the following range: 0, 50, 100, 200, 400, 600, 800 and 1000 µM. Coelomocyte suspensions were aliquotted into 8 replicate wells in a 96-well microtiter plate for each H_2O_2 treatment. The NRR assay was performed as described above. Significant differences were found between treatments (ANOVA F = 13.228, N = 63, df = 7, $P < 0.001$, data not shown); these differences were between the controls (without H_2O_2) and all concentrations at or higher than 200 µM (ANOVA post hoc LSD test, $P < 0.05$, data not shown).

2.6.4 Alkaline single cell gel electrophoresis assay

The single cell gel electrophoresis (comet) assay was done according to the protocol of Singh *et al*. (1988), adapted for earthworms by Reinecke & Reinecke (2004b) and modified slightly for the present study. An overview of the procedure is presented in Figure 2.10.

Conventional microscope slides were prepared in advance by covering them with a layer of 1% normal melting point agarose (NMA) (Whitehead Scientific, South Africa). The NMA was dissolved in PBS and heated carefully until melting in a conventional microwave oven. The slides were subsequently allowed to dry at 60 °C in an oven (Memmert, Germany) for 24 h (this is the first gel layer).

After cell extractions from earthworms, two microgel slides were prepared for each specimen, using 0.5% low melting point agarose (LMA) (gel point 24 – 28 °C, LM2) (Whitehead Scientific). The LMA was dissolved in PBS by heating carefully until melting in a conventional microwave oven. Afterwards, it was kept at a constant temperature of 25 °C in a water bath until use. Sixty µl of LMA was mixed with 20 µl coelomocyte suspension and placed on each prepared microscope slide (this is the second gel layer). A coverslip was placed on top, and the gel allowed to solidify on ice for a few minutes. After the coverslip was removed from the solidified gel layer, a third gel layer, 75 µl of 0.5% LMA, was added to each slide. The coverslip was replaced and the slides returned to the ice to solidify for a few minutes. After all gel layers have set, coverslips were

removed and slides were immersed vertically in cold (4 °C) lysing solution (final lysing solution, Appendix A) in Coplin jars and incubated in darkness at 4 °C for 15 ± 1 h.

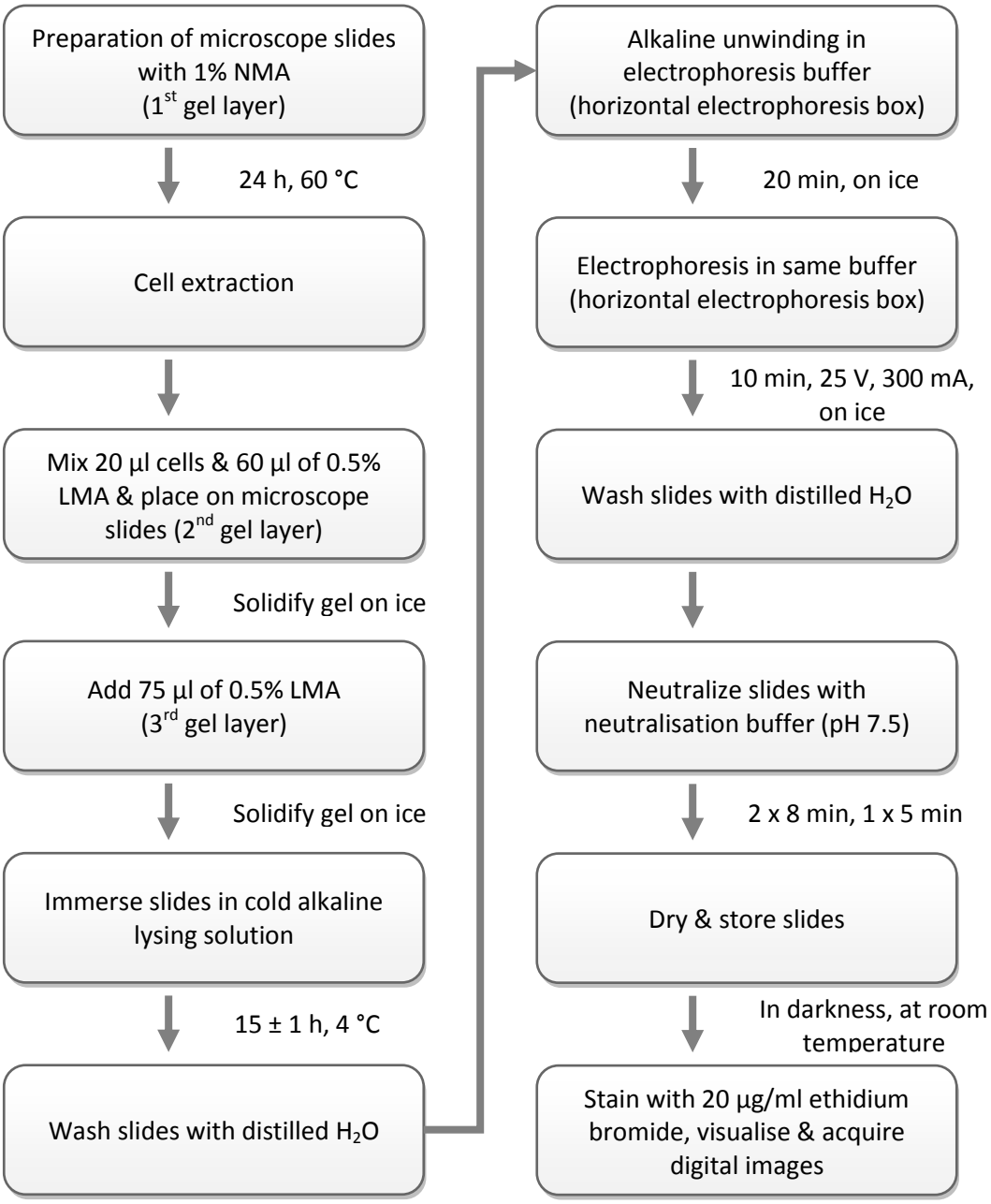


Figure 2.10: Procedure used for the alkaline comet assay (after Singh *et al*. 1988; and adapted by Reinecke & Reinecke 2004b) to determine DNA damage in earthworm coelomocytes. Please see Figure 2.3 for the cell extraction procedure. NMA = normal melting point agarose. LMA = low melting point agarose.

After lysing, the slides were removed and washed with distilled water. After draining the slides, they were placed horizontally in an electrophoresis chamber (Cigen Ltd.) near the anode (+) and were covered with freshly made cold (4 °C) electrophoresis buffer (Appendix A). The

electrophoresis chamber was kept on ice for the duration of its use. The slides were incubated for 20 min to allow for DNA unwinding.

Immediately after unwinding, electrophoresis was done in the same buffer at 25 V and 300 mA for 10 min (Power supply: JD Instruments, South Africa). After electrophoresis, the slides were removed from the buffer and washed with distilled water and drained. Subsequently, the slides were placed on a staining rack and neutralised by flooding (and subsequently draining them) with cold (4 °C) neutralisation buffer (0.4 M Tris, set to pH 7.5 with 70% HCl); twice for 8 min and once for 5 min. The slides were thereafter dried and stored in darkness until analyses.

Immediately before scoring, each slide was stained with 20 µg/ml ethidium bromide and a coverslip was placed on top. Slides were visualised under a Leitz Diaplan (Leitz AG, Wetzlar, Germany) fluorescent microscope (with Ploemopak 2.3: excitation filter 515–650 nm, barrier filter 580 nm). Images were captured with the aid of a black-and-white digital camera (Marlin F046B from Allied Vision Technologies, Germany). Twenty random images, each containing at least 2 nuclei or comet-like structures (comets), were captured per slide (resulting in at least 80 to 100 comets per specimen). When very few comets were found, the whole slide was scanned and images of as many comets as possible were captured.

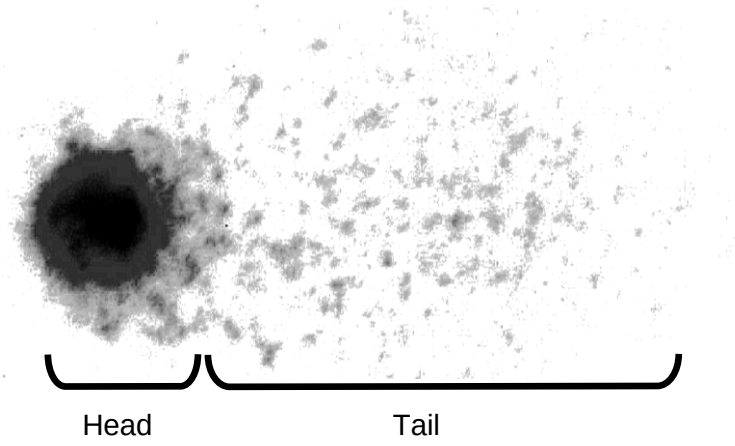


Figure 2.11: A comet of an earthworm coelomocyte (this image was converted to a black and white negative for illustrative purposes; usually the images have a black background with the ethidium bromide-stained DNA fluorescing red). The head is the undamaged DNA contained in the nucleus, and the tail is the damaged DNA migrated from the nucleus during electrophoresis.

Images were analysed with the freely available software program CASP (version 1.2.2) (Konca 2005) and scored for Tail DNA %. This is the percentage of DNA in the comet tail (Figure 2.11), which is measured as the amount of fluorescence (intensity) in the tail and calculated as a percentage of the fluorescence in the entire comet. Tail DNA % was chosen as recommended by Collins (2004). Tail intensity may still increase after a certain point during electrophoresis (i.e. after

a certain electrophoresis time or level of DNA damage) after tail length may have ceased to increase. Therefore, Tail DNA % has been suggested as being much more accurate than tail length (or other indices such as tail moment or Olive tail moment, which are dependent on tail length measurements) (Collins 2004; Kumaravel & Jha 2006).

For the comet assay, the organism (or culture where these are used), rather than the separate cells or nuclei, is considered the unit of analysis (Lee & Steinert 2003; Collins 2004). Therefore, median values (after normality testing) for each specimen (calculated from raw pooled data from both slides) were calculated and then used in further data analyses. These values were termed Tail DNA % (median). For comparative purposes, data analyses were also performed on raw data, where Tail DNA % values from individual comet structures were pooled for all the specimens in a treatment and directly used in data analyses, termed Tail DNA % (raw).

2.7 Data analyses

All data obtained from this study are summarised in Appendix B. Detailed results of statistical analyses (for example the H-values, degrees of freedom, n, and *P*-values for e.g. the Kruskal-Wallis ANOVA by ranks) are also given in Appendix B. The software packages MS Excel, Statistica 8.0 (StatSoft 2008), ToxRAT 2.09 (ToxRat 2006) and an Excel macro, Species Sensitivity Distribution (SSD) Generator (using the linearized log-normal model, obtained from the US Environmental Protection Agency's CADDIS online application (EPA 2009)) were used to analyse data. For all analyses, the level of significance was $P = 0.05$. For all correlations, in addition to $P < 0.05$, only correlation coefficients equal to or higher than 0.8 (or equal to or lower than -0.8), were considered to be significant. In the following results chapter, all statistically significant differences ($P < 0.05$) are referred to as "significant differences".

Normality testing was performed on all data (Shapiro-Wilk test for normality); the results of these tests are however not shown due to the large number of analyses done. Therefore, the use of the appropriate parametric or non-parametric test serves as indication of the normality of the data. Parametric data (Shapiro-Wilk test; $P > 0.05$) were analysed with the applicable parametric tests such as Analysis of Variance (ANOVA), Students T tests and Pearson correlations. Nonparametric data (Shapiro-Wilk test, $P < 0.05$) were analysed with the appropriate nonparametric tests such as Kruskal-Wallis ANOVA (KW ANOVA) by ranks, Mann-Whitney U tests, and Spearman-Rank correlations.

All post-hoc analyses for ANOVA and KW ANOVA are available in Appendix B, and only selected results will be given in the text of the results chapter. For example, where different

treatments within a species are compared to each other, significant differences, if any, between the negative control and other treatments are always given in the text. Any differences between the other treatments are presented in Appendix B.

For graphical representation of summarised parametric data, mean values and standard errors (depicted by whiskers) were used. For non-parametric data, median values were used in box plots, where the boxes represent 25-75% of the data and whiskers the non-outlier ranges.

In order to determine LC_{50}s (concentration that is lethal for 50% of test specimens) and EC_{50}s (concentration where a certain effect is visible in 50% of test specimens), the ecotoxicological statistical package ToxRAT 2.09 (ToxRat 2006) was used. Where possible, SSDs were constructed with the aid of the EPA SSD Generator (EPA 2009).

Chapter 3: Results

3.1 Moisture content and pH of OECD soil

In the majority (97%) of the experiments, the moisture content and pH of the OECD soil did not change more than 10% during the 14 day exposure period (Appendix B, Table 5). When changes were more than 10%, they did not exceed 13% for pH and 17% for moisture content.

3.2 Metal contents in substrates and earthworms

3.2.1 Soil from sampling sites and culturing media

The concentrations (mg/kg dry weight) of selected metals (Cu, Mn, Zn, Pb, Ni, Cd) in substrate samples from the sampling sites or culture media of the earthworms, as well as the cattle manure used for food, are presented in Table 3.1. Concentrations of all the measured metals, except Pb, Ni and Cd, were lower in soils from the farms Vergenoegd (collection site for *Aporrectodea trapezoides*), Nietvoorbij (*Chilota sp.*) and the cattle manure than in the culturing media for *Amynthas diffringens*, *Eisenia andrei* and *Perionyx excavatus*. Levels of Pb and Ni were lower for Vergenoegd soil, Nietvoorbij soil and the laboratory culturing medium, than in the culturing medium of *A. diffringens*. Cadmium was below detection limits in all soil samples except in those from Vergenoegd.

The copper concentrations ranged from 3 mg/kg Cu at Nietvoorbij (*Chilota sp.*) to 25 mg/kg Cu in the culturing media of *A. diffringens*, *E. andrei* and *P. excavatus*. When these Cu concentrations were corrected for a recovery efficiency of 71% for the AAS (atomic absorption spectrophotometer) measurements and the acid digestion method (Materials and Methods, section 2.4.3 and Appendix B, Table 1), they range from 5 to 36 mg/kg Cu (Table 3.1).

3.2.2 OECD artificial soil

The Cu content of the OECD soil at the end of the 14 day exposure period is presented in Figure 3.1 and Appendix B, Table 6. A mean % (± SD) recovery of 61% (± 9) was found for all experiments. There were significant positive correlations between the measured Cu content of the OECD soil and the nominal Cu concentrations for all experiments (Spearman Rank-order correlation R = 0.99, $P < 0.001$ for each experiment) (Figure 3.1). Correcting for a recovery efficiency of 71% for the AAS measurements (Materials and Methods, section 2.4.3 and Appendix

B, Table 1), the Cu concentrations in the OECD soil were slightly higher than before correction (Appendix B, Table 7), and the mean value increased to 87% (± 12) of the nominal concentrations for all experiments.

Table 3.1: Mean (± std dev) metal content (mg/kg dry weight) of soil from the sampling sites or culturing media of five earthworm species, as well as the cattle manure used for food. All values are from ICP measurements, except for Cu (AAS), which were measured by atomic absorption spectrophotometry. For all, n = 3, except for the cattle manure where n = 1. nd = below detection limits. The corrected metal concentrations were calculated according to the 71% recovery efficiency (Material and Methods, section 2.4.3 and Appendix B, Table 1) obtained from AAS measurements of spiked reference soil.

		AAS	ICP					
Species	Sampling site	Cu	Cu	Mn	Zn	Pb	Ni	Cd
A. diffringens	Commercial culture	25 ± 1	27 ± 2	198 ± 16	117 ± 4	49 ± 2	18 ± 1	nd
A. trapezoides	Vergenoegd	6 ± 0	7 ± 1	114 ± 3	21 ± 1	14 ± 0	4 ± 0	1 ± 1
Chilota sp.	Nietvoorbij	3 ± 1	3 ± 1	17 ± 3	7 ± 1	7 ± 1	3 ± 0	nd
E. andrei & *P. excavatus*	Laboratory culture	25 ± 1	22 ± 1	236 ± 17	100 ± 8	2 ± 0	4 ± 0	nd
Cattle manure	Welgevallen	14	12	164	53	2	3	nd
Corrected metal concentrations:								
A. diffringens	Commercial culture	35 ± 2	38 ± 2	280 ± 23	165 ± 5	70 ± 3	26 ± 1	nd
A. trapezoides	Vergenoegd	8 ± 0	10 ± 2	161 ± 4	30 ± 2	20 ± 1	6 ± 0	1 ± 2
Chilota sp.	Nietvoorbij	5 ± 2	5 ± 1	24 ± 4	9 ± 2	10 ± 1	4 ± 1	nd
E. andrei & *P. excavatus*	Laboratory culture	36 ± 2	31 ± 2	334 ± 25	141 ± 11	4 ± 0	5 ± 1	nd
Cattle manure	Welgevallen	20	17	232	75	3	4	nd

3.2.3 Earthworms

The Cu body content of the earthworms after 14 day exposure to copper oxychloride is presented in Figure 3.2 and Appendix B, Table 8. Significant positive correlations (Figure 3.2) were found between soil and body Cu concentrations of all species (Spearman $R > 0.8$, $P < 0.05$), except for *P. excavatus* (Spearman $R = 0.75$, $P < 0.05$). Upon closer inspection of Figure 3.2, it seems that the increase in Cu body content with increasing soil Cu becomes less pronounced at higher Cu concentrations for all species except *E. andrei* (A).

When the Cu body content of the different species are compared (Figure 3.3a), *A. diffringens* had taken up the highest level of Cu, with a maximum mean value of 44 mg/kg Cu (± 17 SD) (Appendix B, Table 8). The second highest level of Cu was taken up by *Chilota sp.* (28 mg/kg Cu ± 5). The maximum level of Cu body content was the lowest for *P. excavatus* (16 mg/kg Cu ± 3). The linear regressions (Figure 3.2) also indicate that the steepest gradient for Cu uptake existed for *A. diffringens*, followed by *Chilota sp.*, then both *A. trapezoides* and *E. andrei* (B), followed by *E. andrei* (A) and *P. excavatus*.

The Cu body contents of *E. andrei* (A) (depurated for 48 h) and (B) (depurated for 24 h) were compared at each treatment to determine whether depuration period had an effect on earthworm body Cu concentration. No significant differences were found (Mann-Whitney U test, $P > 0.05$ at each treatment) between the two *E. andrei* groups at any of the treatments.

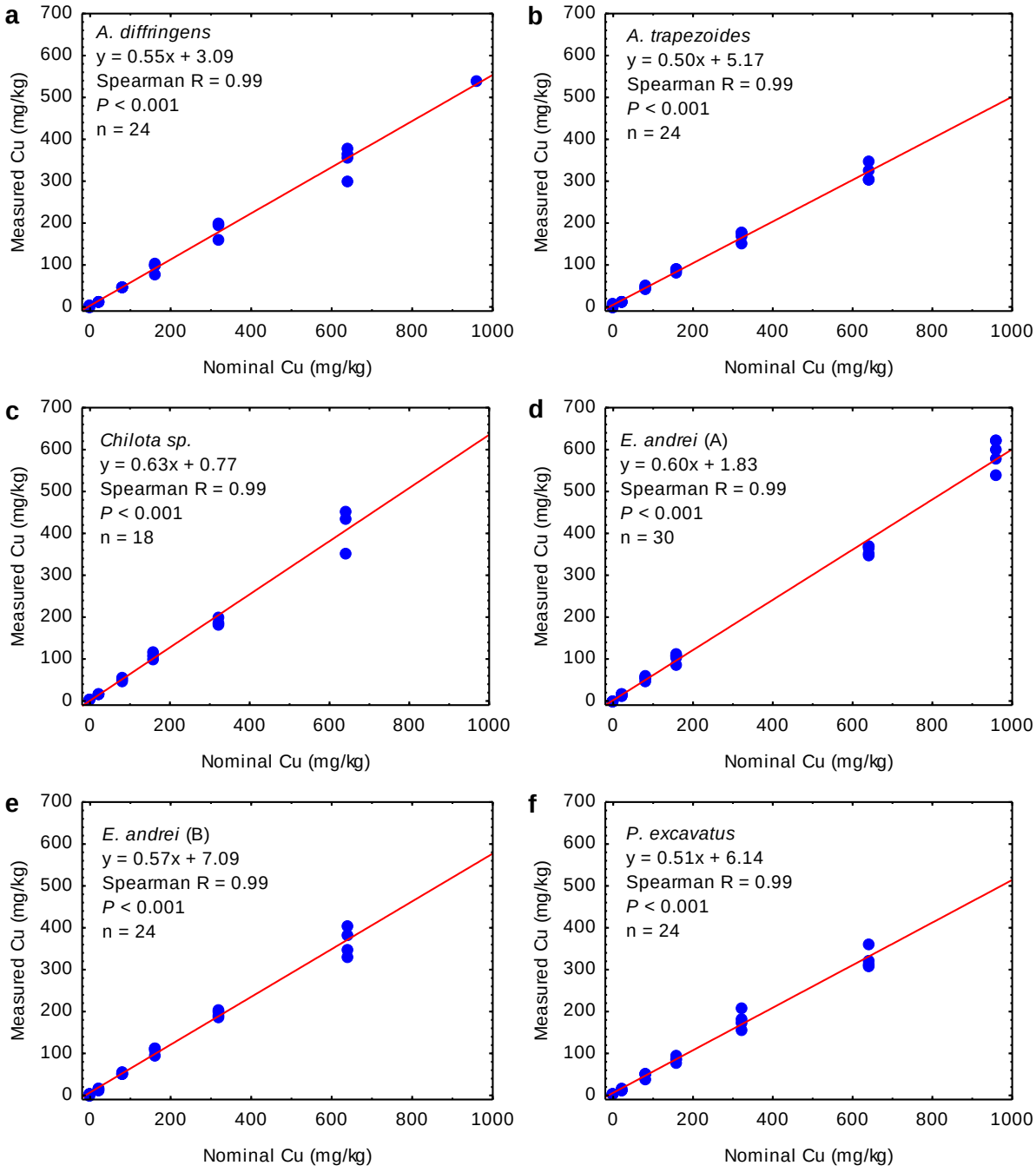


Figure 3.1: Correlations between the nominal and measured soil Cu concentrations (mg/kg) at the end of the 14 day exposure period in OECD soil used to expose specimens of five earthworm species to Cu in the form of copper oxychloride. a) *A. diffringens*, b) *A. trapezoides*, c) *Chilota sp.*, d) *E. andrei* (A), e) *E. andrei* (B) and f) *P. excavatus*.

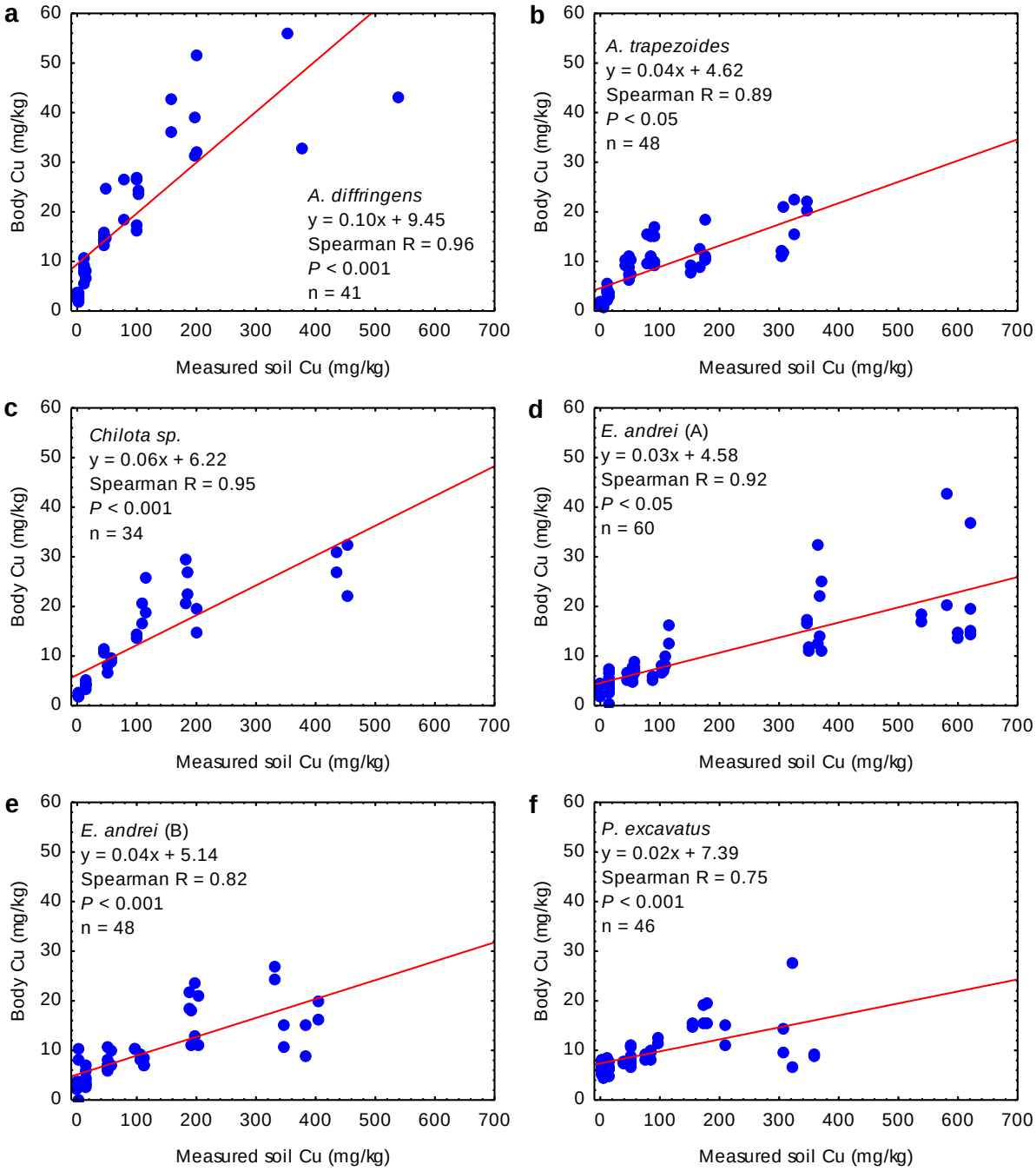


Figure 3.2: Correlations between the measured soil Cu concentrations (mg/kg) and the earthworm body Cu concentrations after exposing specimens of five earthworm species to Cu in the form of copper oxychloride in OECD soil for 14 days. a) *A. diffringens*, b) *A. trapezoides*, c) *Chilota sp.*, d) *E. andrei* (A), e) *E. andrei* (B) and f) *P. excavatus*.

The bioconcentration factor (BCF) for each species at each Cu concentration was calculated by dividing the body Cu concentration by the corrected measured soil Cu concentration (Appendix B, Table 8) and is visually presented in Figure 3.3b. BCF values decreased with increasing Cu concentration for all species. For the Cu exposure treatments, the highest BCF values were in the 20 mg/kg Cu treatments. The species with the highest BCF values at the treatment 20 mg/kg Cu was

A. diffringens (0.5), followed by *P. excavatus* (0.3). The other species all had a BCF of 0.2 at this Cu treatment (Appendix B, Table 8).

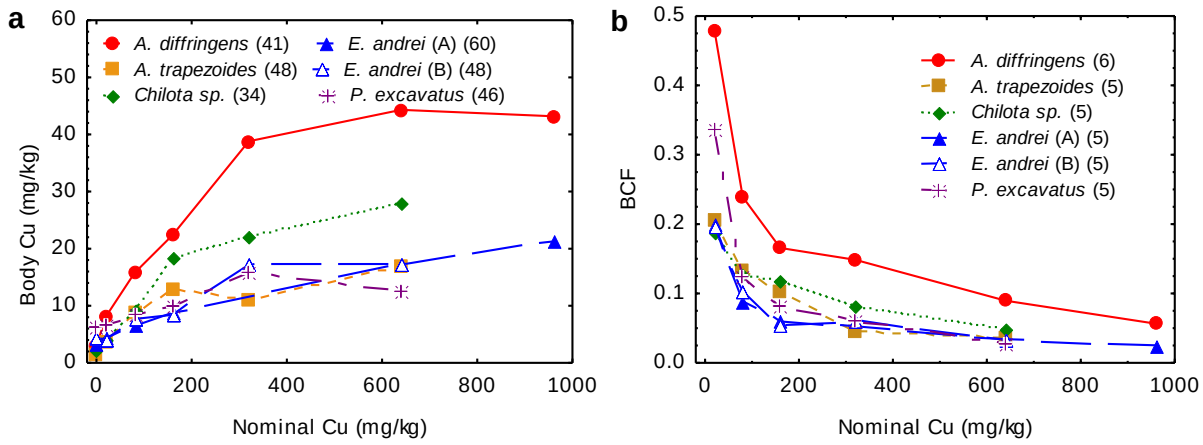


Figure 3.3: The mean copper body concentrations (a), and bioconcentration factors (BCFs) (b), of specimens from five earthworm species, measured after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets in (a) are the amount of specimens used in total for each species; in (b), the numbers in brackets are the number of data points per species. Part (a) of this figure is used in addition to Figure 3.2, and is only for illustrative purposes to enable a visual comparison of Cu body loads between the species. For standard deviations, medians and quartiles, please refer to Appendix B, Table 8. Lines are included in this figure purely to facilitate visual interpretation.

3.3 Earthworm survival

The survival data for earthworms after 14 days exposure in OECD soil to all treatments are presented in Table 3.2. Survival of *A. diffringens* specimens in the copper oxychloride treatments was lower than that of the other species. For *Chilota sp.* and *P. excavatus*, 33% and 44% (respectively) mortality was found at the highest Cu concentration (640 mg/kg). For *E. andrei* (A), mortalities of 3% and 8% were found at 640 and 960 mg/kg Cu respectively. All *A. trapezoides* and *E. andrei* (B) specimens survived the copper oxychloride treatment. Regarding benomyl-exposed specimens, the highest mortality occurred in *A. trapezoides* (75%), followed by *A. diffringens* (13%). All specimens from the other species (*Chilota sp.*, *E. andrei* and *P. excavatus*) survived the benomyl treatment. No significant correlations between % survival and either the measured soil Cu concentrations or body Cu concentrations were found for any of the species where mortality occurred (Spearman R < 0.8 for all).

Table 3.2: The number of earthworms introduced at the start and surviving at the end of 14 days, and the % Survival, of five earthworm species exposed to a range of Cu concentrations, in the form of copper oxychloride, and a positive control, 10 mg/kg benomyl in OECD soil.

Species	Treatment	Introduced (n)	Survived (n)	% Survival
A. diffringens	Control	16	14	87.5
	20 mg/kg Cu	16	15	93.8
	80 mg/kg Cu	16	16	100
	160 mg/kg Cu	16	14	87.5
	320 mg/kg Cu	12	9	75.0
	640 mg/kg Cu	16	2	12.5
	960 mg/kg Cu	4	1	25.0
	Benomyl	16	14	87.5
A. trapezoides	Control	16	16	100
	20 mg/kg Cu	16	16	100
	80 mg/kg Cu	16	16	100
	160 mg/kg Cu	16	16	100
	320 mg/kg Cu	16	16	100
	640 mg/kg Cu	16	16	100
	Benomyl	16	4	25.0
Chilota sp.	Control	12	12	100
	20 mg/kg Cu	12	12	100
	80 mg/kg Cu	12	12	100
	160 mg/kg Cu	13	13	100
	320 mg/kg Cu	11	11	100
	640 mg/kg Cu	12	8	66.7
	Benomyl	12	12	100
E. andrei (A)	Control	37	37	100
	20 mg/kg Cu	37	37	100
	80 mg/kg Cu	37	37	100
	160 mg/kg Cu	37	37	100
	640 mg/kg Cu	37	36	97.3
	960 mg/kg Cu	37	34	91.9
	Benomyl	37	37	100
E. andrei (B)	Control	16	16	100
	20 mg/kg Cu	16	16	100
	80 mg/kg Cu	16	16	100
	160 mg/kg Cu	16	16	100
	320 mg/kg Cu	16	16	100
	640 mg/kg Cu	16	16	100
	Benomyl	16	16	100
P. excavatus	Control	16	16	100
	20 mg/kg Cu	16	16	100
	80 mg/kg Cu	16	16	100
	160 mg/kg Cu	16	16	100
	320 mg/kg Cu	16	16	100
	640 mg/kg Cu	16	9	56.3
	Benomyl	16	16	100

3.3.1 LC, LOEC and NOEC values for survival

LC_{10}, LC_{20} and LC_{50} values could be calculated for *A. diffringens*, *Chilota sp.* and *P. excavatus* (Table 3.3). All specimens of *A. trapezoides* and *E. andrei* (B) survived copper oxychloride exposure, therefore no LC, LOEC and NOEC values could be calculated. Although some mortality occurred in the highest concentrations for *E. andrei* (A), the percentage mortality was too low to accurately calculate LC values. For *E. andrei* (A), the LOEC and NOEC values were both estimated to be higher than the highest exposure concentration (960 mg/kg Cu) (Table 3.3). For *A. diffringens*, the LOEC was 320 mg/kg Cu, and the NOEC 160 mg/kg Cu. For both *Chilota sp.* and *P. excavatus*, both the LOEC and NOEC values were estimated to be higher than the highest exposure concentration (640 mg/kg Cu). The species with the lowest LC_{50} value was *A. diffringens*, followed by *P. excavatus* and *Chilota sp.*.

Table 3.3: LC_{10}, LC_{20} and LC_{50} values, as well as LOEC and NOEC values (in mg/kg Cu), for mortality of five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. nd = Could not be determined. nm - No mortality observed. Confidence limits are not determined for LOEC and NOEC values; in the table these cells are filled with a dash (-).

Species		Cu (mg/kg)	Confidence limit - 95%	Confidence limit + 95%
A. diffringens	LC_{10}	98	nd	nd
	LC_{20}	158	nd	nd
	LC_{50}	393	nd	nd
	LOEC	320	-	-
	NOEC	160	-	-
A. trapezoides	LC_{10}	nm	nm	nm
	LC_{20}	nm	nm	nm
	LC_{50}	nm	nm	nm
	LOEC	nm	-	-
	NOEC	nm	-	-
Chilota sp.	LC_{10}	288	196.3	421.4
	LC_{20}	341	239.2	485.8
	LC_{50}	471	327.5	677.4
	LOEC	>640	-	-
	NOEC	>=640	-	-
E. andrei (A)	LC_{10}	nd	nd	nd
	LC_{20}	nd	nd	nd
	LC_{50}	nd	nd	nd
	LOEC	>960	-	-
	NOEC	>=960	-	-
E. andrei (B)	LC_{10}	nm	nm	nm
	LC_{20}	nm	nm	nm
	LC_{50}	nm	nm	nm
	LOEC	nm	-	-
	NOEC	nm	-	-
P. excavatus	LC_{10}	284	nd	nd
	LC_{20}	332	nd	nd
	LC_{50}	445	nd	nd
	LOEC	>640	-	-
	NOEC	>=640	-	-

3.4 Earthworm reproduction: cocoon production

With the exception of *Chilota sp.*, cocoons were found for all species after the 14 day exposure period (Figure 3.4 and Appendix B, Table 9). For *A. diffringens*, cocoons were only counted in two of the four replicates. Cocoons from this species were extremely small and difficult to identify. It was initially thought that no cocoons were produced, but by the time that the third replicate was analysed, cocoons could be identified.

Although the number of cocoons decreased with increasing soil and earthworm body Cu concentrations for the four species where cocoons were found (Figure 3.4), no significant negative correlations were found between the cocoons produced per earthworm and either the measured soil Cu concentrations or the earthworm body Cu concentrations for any of the species (Spearman rank-order $R > -0.8$ for all species, Appendix B, Table 10).

No cocoons were produced at treatment Cu concentrations equal to or higher than 160 mg/kg Cu for *A. trapezoides*, at 320 mg/kg Cu for both *A. diffringens* and *P. excavatus* and 640 mg/kg Cu for *E. andrei* (B). *E. andrei* (A) produced cocoons at the highest concentrations (Figure 3.4 and Appendix B, Table 9).

Regarding the positive control treatment, benomyl, only *A. trapezoides* and *P. excavatus* failed to produce any cocoons (Figure 3.4). Specimens of *E. andrei* (A) produced fewer cocoons than *E. andrei* (B) and *A. diffringens* at this treatment.

The presence of significant differences in cocoon production between the negative control and the Cu and benomyl treatments for each species was tested for (nonparametric KW ANOVA, Appendix B, Table 11a), using only treatments in which cocoons were produced. These tests were however not performed for *A. diffringens* because data were available for only two replicates, rendering the sample size too small for meaningful statistical analyses. Significant differences between treatments were found only for *E. andrei* (A), where the treatment 80 mg/kg Cu differed from both 960 mg/kg Cu and the positive control, benomyl (Appendix B, Table 11b).

3.4.1 EC, LOEC and NOEC values for cocoon production

EC_{10}, EC_{20} and EC_{50} values for cocoon production were calculated for all four species that produced cocoons (Table 3.4). The species with the lowest EC_{50} value was *A. trapezoides*, followed by *E. andrei* (B), *A. diffringens*, *P. excavatus* and *E. andrei* (A). The lowest NOEC values were calculated for both *E. andrei* experiments (640 mg/kg Cu). The lowest LOEC was calculated for *E. andrei* (A) (160 mg/kg Cu), followed by *E. andrei* (B) (320 mg/kg Cu). For *A. diffringens*, LOEC and NOEC values could not be calculated because data for only 2 replicates were available. For the

other species, both the NOEC and LOEC values were estimated to be above the highest exposure concentration (640 mg/kg Cu).

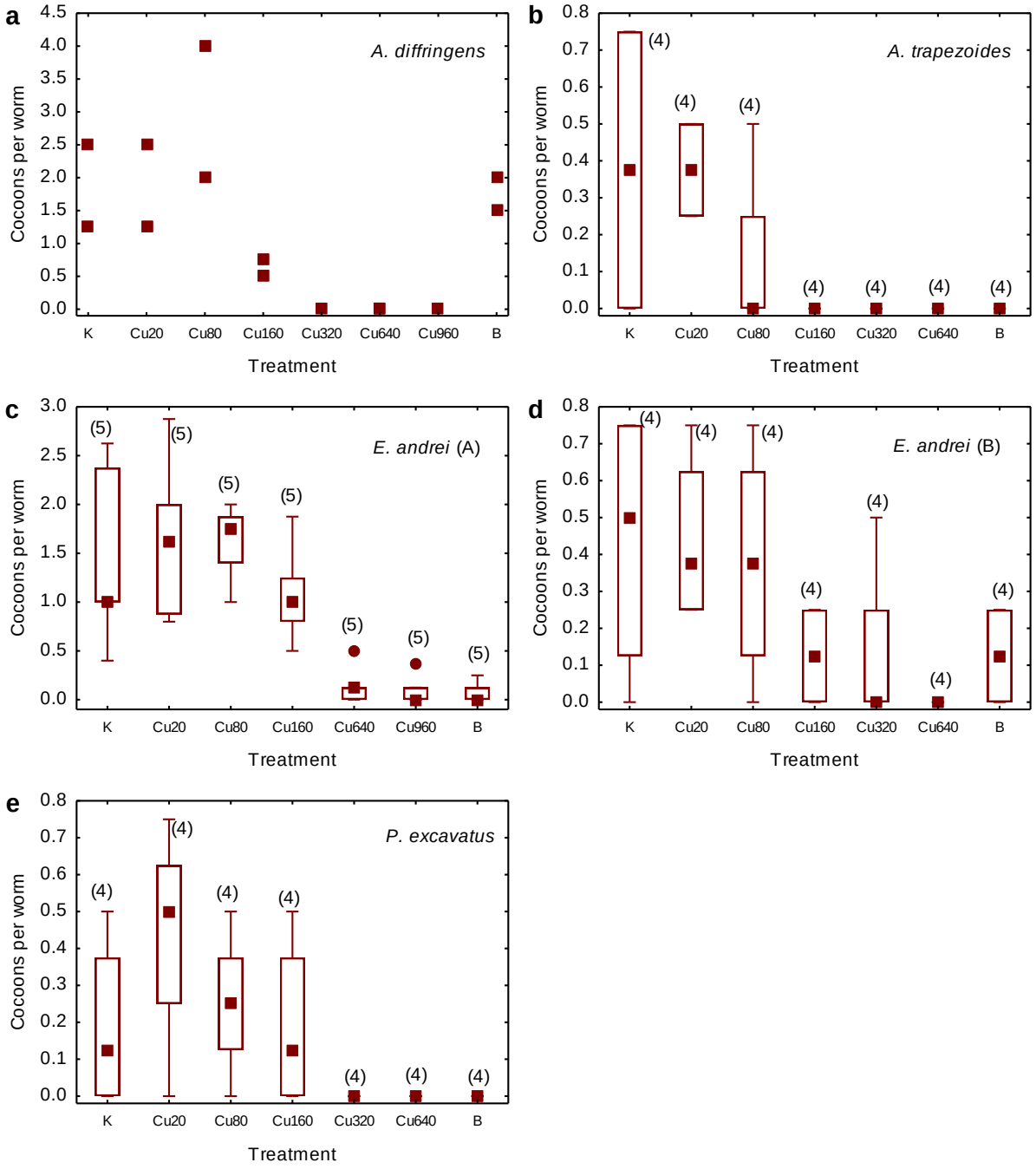


Figure 3.4: The number of cocoons produced per earthworm, determined after a 14 day period for specimens of four earthworm species exposed to Cu in the form of copper oxychloride and 10 mg/kg benomyl (positive control) in OECD soil. The numbers given in brackets are the number of replicates for each treatment (with four specimens per replicate per treatment, except for *E. andrei* (A), where it is 8 for replicates 1 to 4, and 5 for replicate 5), since the number of cocoons per worm is calculated per replicate. a) *A. diffringens*. Please note that for this species cocoons could only be identified in two replicates, therefore the raw data are presented. For this species, the number of replicates is 2 at each treatment, except for treatments 320 mg/kg Cu and 960 mg/kg Cu, where it is 1, b) *A. trapezoides*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. For b) to e), the data are nonparametric and are therefore summarised as median values and represented by squares. Boxes represent 25 – 75% of the data, the whiskers represent the non-outlier range and the circles represent the outliers. K = negative control, B = benomyl (positive control).

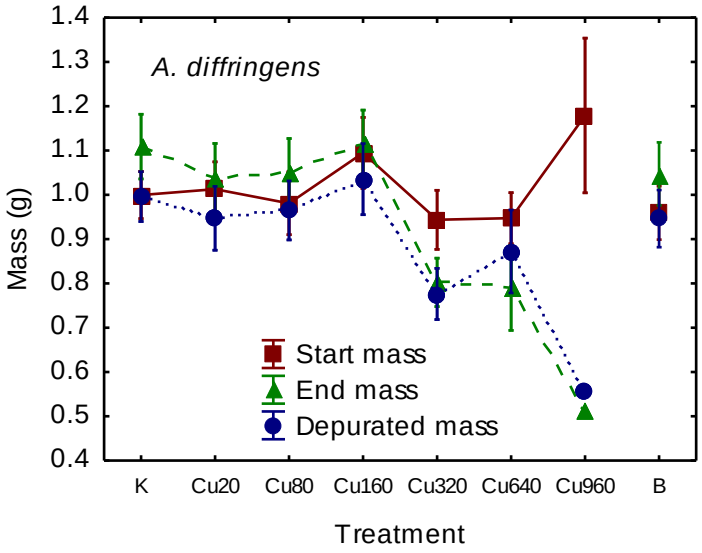
Table 3.4: EC_{10}, EC_{20} and EC_{50} values, as well as LOEC and NOEC values (in mg/kg Cu), for cocoon production for five earthworm species exposed for 14 days to Cu in the form of copper oxychloride in OECD soil. nd = Could not be determined. nc = No cocoons observed. Confidence limits are not determined for LOEC and NOEC values; in the table these cells are filled with a dash (-).

Species		Cu (mg/kg)	Confidence limit - 95%	Confidence limit + 95%
A. diffringens	EC_{10}	111	80.8	124.3
	EC_{20}	122	96.4	132.7
	EC_{50}	146	134.7	150.2
	LOEC	nd	-	-
	NOEC	nd	-	-
A. trapezoides	EC_{10}	49	14.2	59.9
	EC_{20}	55	22.2	64.5
	EC_{50}	71	51.7	74.4
	LOEC	>640	-	-
	NOEC	>=640	-	-
Chilota sp.	EC_{10}	nc	nc	nc
	EC_{20}	nc	nc	nc
	EC_{50}	nc	nc	nc
	LOEC	nc	-	-
	NOEC	nc	-	-
E. andrei (A)	EC_{10}	100	63.9	131.2
	EC_{20}	138	98.6	174.4
	EC_{50}	255	203.6	331.1
	LOEC	640	-	-
	NOEC	160	-	-
E. andrei (B)	EC_{10}	52	nd	nd
	EC_{20}	73	nd	nd
	EC_{50}	141	nd	nd
	LOEC	640	-	-
	NOEC	320	-	-
P. excavatus	EC_{10}	196	195.6	196.4
	EC_{20}	206	205.6	206.3
	EC_{50}	226	225.9	226.7
	LOEC	>640	-	-
	NOEC	>=640	-	-

3.5 Earthworm biomass

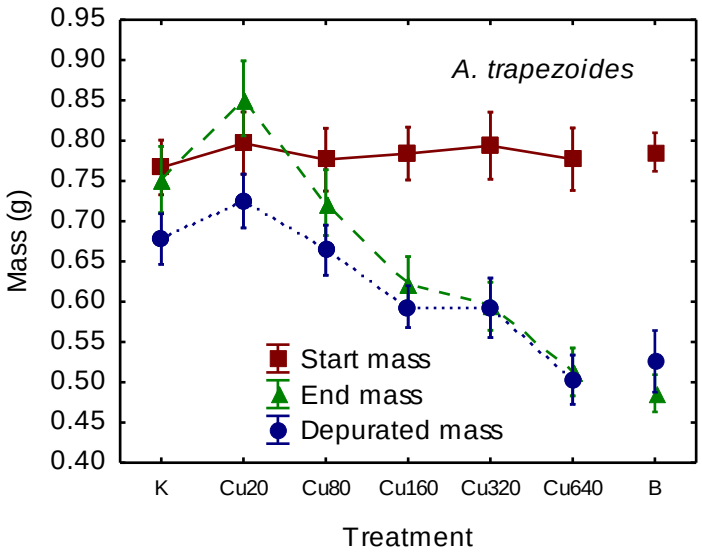
3.5.1 Earthworm mass before exposure

A summary of the earthworm mass before exposure (start mass) is presented in Appendix B, Table 12 and is also depicted (along with the mass after exposure and the mass after subsequent depuration of earthworms) in Figure 3.5 (for *A. diffringens*), Figure 3.6 (*A. trapezoides*), Figure 3.7 (*Chilota sp.*), Figure 3.8 (*E. andrei* (A)), Figure 3.9 (*E. andrei* (B)) and Figure 3.10 (*P. excavatus*).



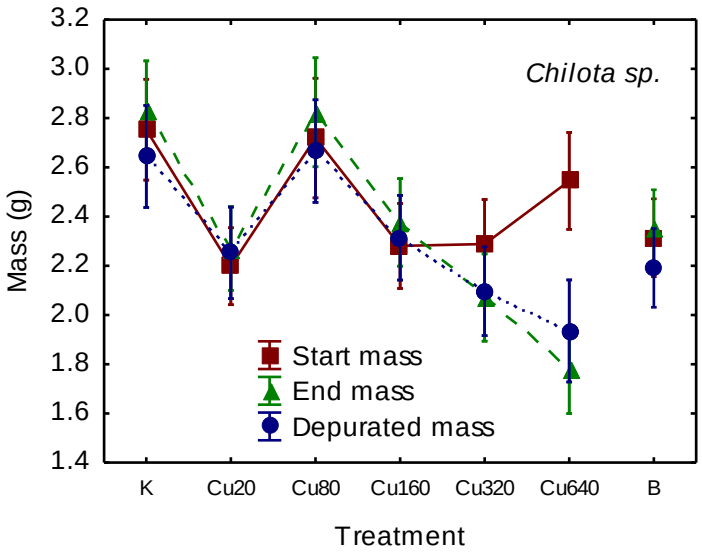
	Start mass n	End mass n	Depurated mass n
K	16	14	14
Cu20	16	15	15
Cu80	16	16	16
Cu160	16	14	14
Cu320	12	9	9
Cu640	16	2	2
Cu960	4	1	1
B	16	14	14

Figure 3.5: Mean (± Std error) earthworm mass of specimens of *A. diffringens* before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 24 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control). Please note that although data are nonparametric for both start, end and depurated mass, means and standard errors are shown to ease visual interpretation.



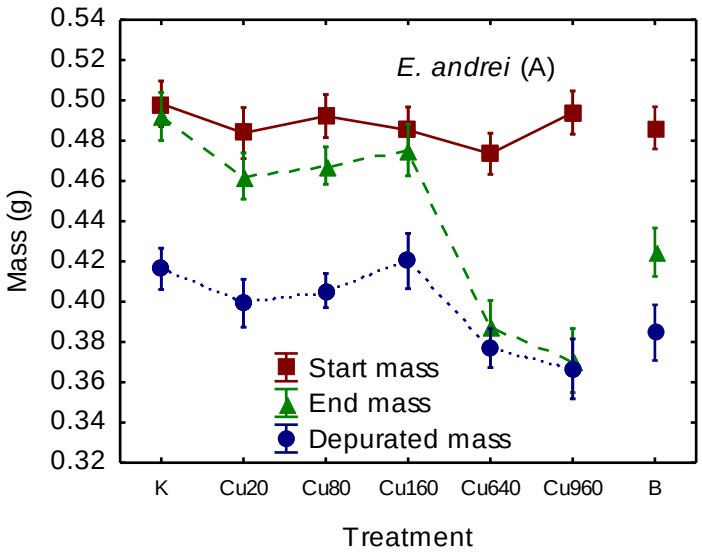
	Start mass n	End mass n	Depurated mass n
K	16	16	16
Cu20	16	16	16
Cu80	16	16	16
Cu160	16	16	16
Cu320	16	16	16
Cu640	16	16	16
B	16	4	4

Figure 3.6: Mean (± Std error) earthworm mass of specimens of *A. trapezoides* before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 24 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control). Please note that although data are nonparametric for both start, end and depurated mass, means and standard errors are shown to ease visual interpretation.



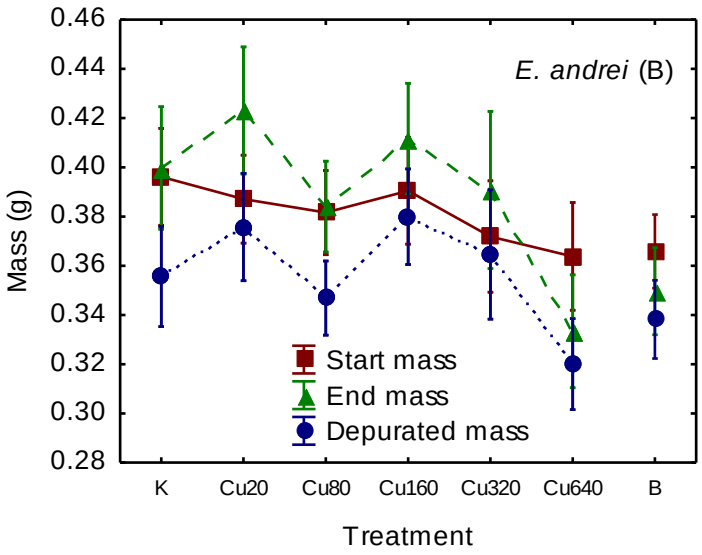
	Start mass n	End mass n	Depurated mass n
K	12	12	12
Cu20	12	12	12
Cu80	12	12	12
Cu160	13	13	13
Cu320	11	11	11
Cu640	12	8	6
B	12	12	12

Figure 3.7: Mean (± Std error) earthworm mass of specimens of *Chilota sp.* before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 24 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control).



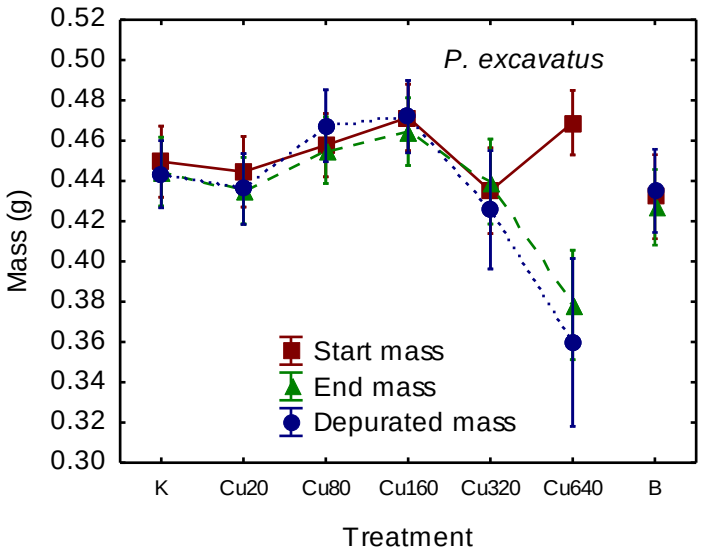
	Start mass n	End mass n	Depurated mass n
K	37	37	36
Cu20	37	37	37
Cu80	37	37	37
Cu160	37	37	37
Cu640	37	36	36
Cu960	37	34	33
B	37	37	21

Figure 3.8: Mean (± Std error) earthworm mass of specimens of *E. andrei* (A) before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 48 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control). Please note that although data are nonparametric for both start and end mass, means and standard errors are shown to ease visual interpretation.



	Start mass n	End mass n	Depurated mass n
K	16	16	16
Cu20	16	16	16
Cu80	16	16	16
Cu160	16	16	16
Cu320	16	16	16
Cu640	16	16	16
B	16	16	16

Figure 3.9: Mean (± Std error) earthworm mass of specimens of *E. andrei* (B) before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 24 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control). Please note that although data are nonparametric for both end and depurated mass, means and standard errors are shown to ease visual interpretation.



	Start mass n	End mass n	Depurated mass n
K	16	16	15
Cu20	16	16	16
Cu80	16	16	16
Cu160	16	16	16
Cu320	16	16	16
Cu640	16	9	8
B	16	16	16

Figure 3.10: Mean (± Std error) earthworm mass of specimens of *P. excavatus* before exposure (start mass, depicted by squares) and after 14 days exposure (end mass, depicted by triangles) to Cu in the form of copper oxychloride, and a positive control 10 mg/kg benomyl, in OECD soil. The mass after subsequent depuration for 24 h on moist filter paper (depurated mass, depicted by circles), is also shown. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. The number of specimens (n) for each treatment is depicted to the right of the graph. K = negative control, B = benomyl (positive control). Please note that although data are nonparametric for both end and depurated mass, means and standard errors are shown to ease visual interpretation.

In order to eliminate the possibility that differences in earthworm mass between treatments after exposure might have been caused by pre-existing differences between treatments in the start mass, the start mass was compared between treatments for each species (nonparametric KW ANOVA,

Appendix B, Table 13). No significant differences were found between treatments for the start mass for any of the species ($P > 0.05$ for all species). Taking the mean start mass for all treatments within a species (Appendix B, last section of Table 12) as an indication of earthworm body size, the largest species was *Chilota sp.* (2.44 g ± 0.67), followed by *A. diffringens* (1.00 g ± 0.26), *A. trapezoides* (0.78 g ± 0.14), *E. andrei* (A) (0.49 g ± 0.07), *E. andrei* (B) (0.38 g ± 0.08) and *P. excavatus* (0.45 g ± 0.07).

3.5.2 Earthworm mass changes during exposure

3.5.2.1 Mass after exposure

A summary of earthworm mass after the 14 day exposure period (end mass) is presented in Appendix B, Table 14 and is also depicted (along with the mass before exposure and the mass after depuration of earthworms) in Figure 3.5 (for *A. diffringens*), Figure 3.6 (*A. trapezoides*), Figure 3.7 (*Chilota sp.*), Figure 3.8 (*E. andrei* (A)), Figure 3.9 (*E. andrei* (B)) and Figure 3.10 (*P. excavatus*). Although the end mass decreased at the highest Cu concentrations for all species, no significant negative correlations were found between the end mass and either soil or earthworm body Cu concentrations (Spearman R > -0.8 for all species, Appendix B, Table 15).

The presence of significant differences between treatments in the mass after exposure for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 16a), and significant differences between treatments were found for *A. trapezoides*, *Chilota sp.* and *E. andrei* (A). Post hoc-testing (Appendix B, Table 16b) indicated that for *A. trapezoides*, the negative control differed significantly from the treatments 640 mg/kg Cu and the positive control (benomyl). For *Chilota sp.*, the negative control differed significantly from the treatments 20, 320 and 640 mg/kg Cu. For *E. andrei* (A), the negative control differed significantly from the treatments 640, 960 mg/kg Cu and the positive control.

3.5.2.2 Mass changes during exposure

For each species, the start mass and end mass was compared at each treatment (nonparametric Mann-Whitney U test or parametric Student t test) to determine the presence of significant mass changes during the 14 day exposure period (Appendix B, Table 17). Significant differences were found between the start and end mass for *A. trapezoides* at the treatments 160, 320 and 640 mg/kg Cu and the positive control. For *E. andrei* (A) significant differences were found at the treatments 80, 640 and 960 mg/kg Cu and the positive control. For both *Chilota sp.* and *P. excavatus*, significant differences were found between the start and end mass at the treatment 640 mg/kg Cu.

In order to facilitate comparisons between treatments and species, the mass change during exposure (the difference between the end mass and the start mass) was calculated as a percentage of the start mass (and termed the exposure mass change). A summary of the exposure mass change for each species and treatment is presented Appendix B, Table 18 and is also depicted in Figure 3.11 (along with the mass change during depuration of earthworms on moist filter paper, as described in section 3.5.3.2).

For all species, the mass change during exposure was more pronounced (more mass was lost during exposure) in the highest Cu concentrations than at the other lower Cu concentrations. A significant negative correlation was found between the exposure mass change and the soil Cu concentration only for *A. trapezoides* (Spearman $R < -0.8$, $P < 0.05$, Appendix B, Table 19).

The presence of significant differences between treatments in the exposure mass change for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 20a), and significant differences between treatments were found only for *A. trapezoides* and *E. andrei* (A). Post hoc-testing (Appendix B, Table 20b) indicated that for *A. trapezoides*, the negative control differed significantly from the treatments 160, 320 and 640 mg/kg Cu and the positive control. For *E. andrei* (A), the negative control differed significantly from the treatments 640 and 960 mg/kg Cu and the positive control.

3.5.2.3 EC, LOEC and NOEC values for mass change during exposure

With the exception of *E. andrei* (A), EC_{10}, EC_{20} and EC_{50} values for the percentage mass change during exposure could be calculated for all species (Table 3.5). The species with the lowest EC_{50} value was *A. diffringens*, followed by *A. trapezoides*, *Chilota sp.*, *E. andrei* (B) and *P. excavatus*. The lowest NOEC and LOEC values were calculated for *A. trapezoides*. For *E. andrei* (B), both NOEC and LOEC values were estimated to be above the highest exposure concentration (640 mg/kg Cu).

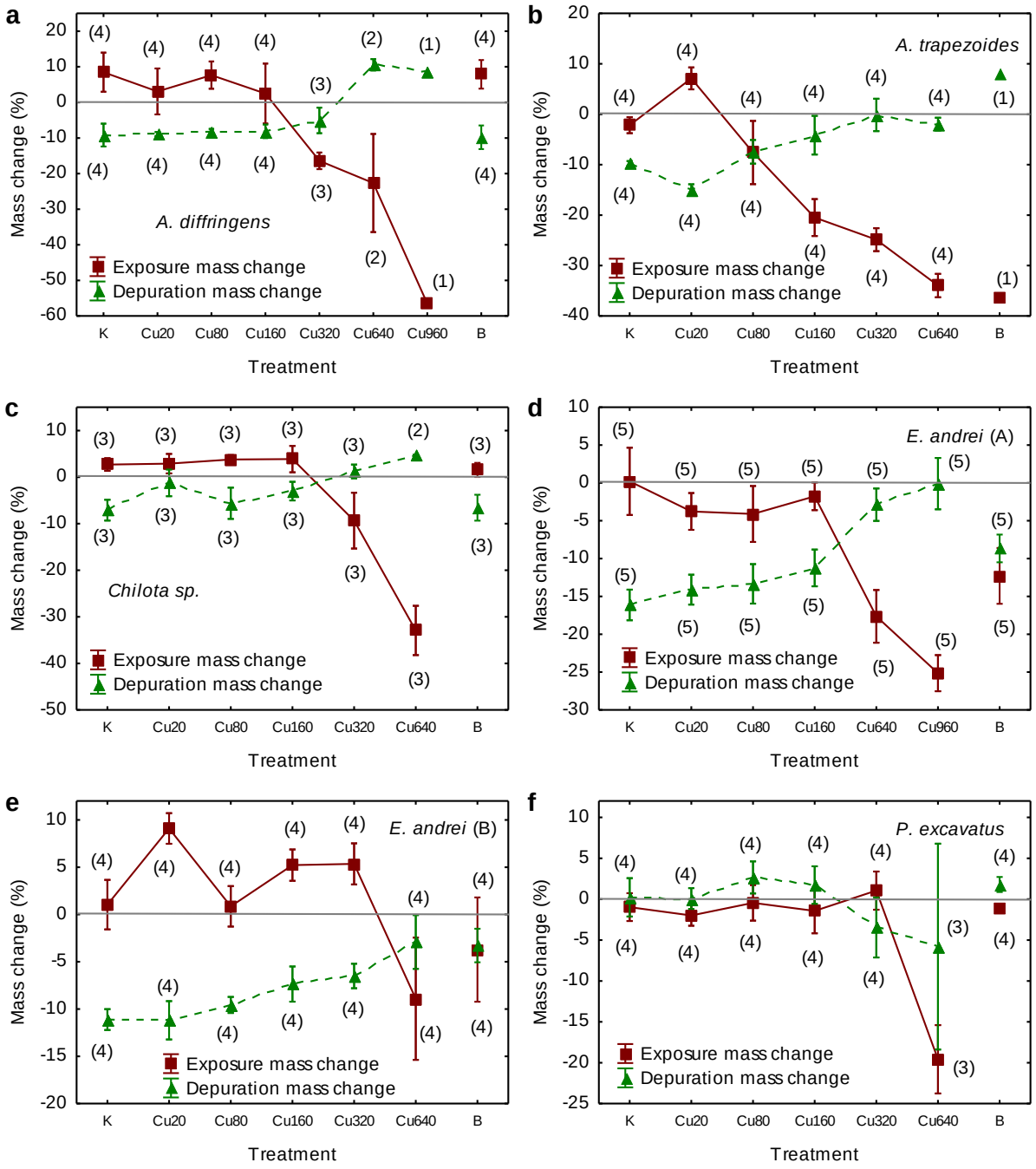


Figure 3.11: Mean (± Std error) earthworm mass change during 14 days exposure (exposure mass change, which is the difference between the mass after exposure and the mass before exposure, calculated as a percentage of the mass before exposure, represented by squares) for specimens of five earthworm species exposed to Cu in the form of copper oxychloride and a positive control, 10 mg/kg benomyl, in OECD soil. The triangles represent the mean (± Std error) mass change during depuration (depuration mass change, which is the difference between the mass after depuration and the mass before depuration (which is the same as the mass after exposure), calculated as a percentage of the mass before depuration) of earthworms kept on moist filter paper for 24 h (or 48 h for *E. andrei* (A)), after the 14 days exposure period, to depurate their guts. The numbers given in brackets are n for each treatment. The connecting lines between the means for each Cu concentration are shown to ease visual interpretation. a) *A. diffringens*, b) *A. trapezoides*, c) *Chilota sp*., d) *E. andrei* (A), e) *E. andrei* (B) and f) P. *excavatus*. K = negative control. B = benomyl.

Table 3.5: EC_{10}, EC_{20} and EC_{50} values, as well as LOEC and NOEC values (in mg/kg Cu), for the endpoint exposure mass change (the difference between mass after exposure and before exposure, calculated as a percentage of the mass before exposure) of five earthworm species exposed for 14 days to Cu in the form of copper oxychloride in OECD soil. nd = Could not be determined. Confidence limits are not determined for LOEC and NOEC values; in the table these cells are filled with a dash (-).

Species		Cu (mg/kg)	Confidence limit - 95%	Confidence limit + 95%
A. diffringens	EC_{10}	12	19.3	3.8
	EC_{20}	16	24.0	6.4
	EC_{50}	27	38.9	16.0
	LOEC	320	-	-
	NOEC	160	-	-
A. trapezoides	EC_{10}	30	32.8	27.2
	EC_{20}	33	36.2	30.2
	EC_{50}	40	43.6	36.7
	LOEC	160	-	-
	NOEC	80	-	-
Chilota sp.	EC_{10}	196	195.6	196.4
	EC_{20}	206	205.6	206.3
	EC_{50}	226	225.9	226.7
	LOEC	320	-	-
	NOEC	160	-	-
E. andrei (A)	EC_{10}	451	nd	nd
	EC_{20}	483	nd	nd
	EC_{50}	592	nd	nd
	LOEC	640	-	-
	NOEC	160	-	-
E. andrei (B)	EC_{10}	392	390.7	393.4
	EC_{20}	412	410.5	413.3
	EC_{50}	453	451.1	454.0
	LOEC	>640	-	-
	NOEC	>=640	-	-
P. excavatus	EC_{10}	45	nd	nd
	EC_{20}	108	nd	nd
	EC_{50}	577	nd	nd
	LOEC	640	-	-
	NOEC	320	-	-

3.5.3 Earthworm mass changes during depuration of guts

3.5.3.1 Mass after depuration

A summary of earthworm mass after depuration on moist filter paper (depurated mass) subsequent to the 14 day exposure period is presented in Appendix B, Table 21 and is also depicted (along with the mass before and after exposure) in Figure 3.5 (for *A. diffringens*), Figure 3.6 (*A. trapezoides*), Figure 3.7 (*Chilota sp.*), Figure 3.8 (*E. andrei* (A)), Figure 3.9 (*E. andrei* (B)) and Figure 3.10 (*P. excavatus*). Although the depurated mass decreased with increasing Cu

concentration for all species (Figures 3.5 to 3.10), no significant negative correlations were found between the depurated mass and either the soil or earthworm body Cu concentrations (Spearman R > -0.8, Appendix B, Table 22).

The presence of significant differences between treatments in the depurated mass for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 23a), and significant differences between treatments were found for *A. trapezoides* and *E. andrei* (A). Post hoc-testing (Appendix B, Table 23b) indicated that for *A. trapezoides*, the negative control differed significantly from the treatment 640 mg/kg Cu. For *E. andrei* (A), the negative control differed significantly from the treatments 640 and 960 mg/kg Cu.

3.5.3.2 Mass changes during depuration

For each species, the end mass and depurated mass was compared at each treatment (nonparametric Mann-Whitney U test or parametric Student t test) to determine the presence of significant mass changes during the depuration period (Appendix B, Table 24). Significant differences were found between the end and depurated mass for *A. trapezoides* at 20 mg/kg Cu. For *E. andrei* (A), significant differences were found at all treatments with exposure concentrations lower than 640 mg/kg Cu and also at the positive control (benomyl).

In order to facilitate comparisons between treatments and species, the mass change during depuration (the difference between the depurated mass and the end mass, which is the mass after exposure and the same as the mass before depuration) was calculated as a percentage of the end mass (mass before depuration), and termed the depuration mass change. A summary of the depuration mass change data is presented in Appendix B, Table 25 and also depicted in Figure 3.11 (along with the exposure mass change). With the exception of *P. excavatus*, the mass change during depuration for all species increased with increasing Cu concentration (Figure 3.11). No significant positive correlations were found between depuration mass change and either the soil or earthworm body Cu concentrations (Spearman R < 0.8, Appendix B, Table 26).

The presence of significant differences in the depuration mass change between treatments for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 27a), and significant differences between treatments were found for *A. trapezoides*, *E. andrei* (A) and *E. andrei* (B). Post hoc-testing (Appendix B, Table 27b) indicated that for *A. trapezoides*, the negative control differed significantly from the treatments 320 and 640 mg/kg Cu and the positive control. For *E. andrei* (A), the negative control differed significantly from the treatments 640 and 960

mg/kg Cu. For *E. andrei* (B), the negative control differed significantly from the treatment 640 mg/kg Cu and the positive control.

3.5.4 Earthworm feeding behaviour (feeding avoidance response)

The earthworm mass loss for all species at high Cu concentrations was more pronounced during exposure than during depuration. These were at concentrations higher than 80 mg/kg Cu for *A. trapezoides*, higher than 160 mg/kg Cu for *A. diffringens*, *Chilota sp.* and *E. andrei* (A), and higher than 320 mg/kg Cu for *E. andrei* (B) and *P. excavatus* (Figure 3.11). At lower Cu concentrations, mass loss was more pronounced during depuration than during exposure. Therefore, earthworm mass loss increased with increasing Cu concentration during exposure and decreased during depuration.

Correlations and linear regressions were performed between the nominal Cu concentrations and the mean values of the exposure and the depuration mass changes for all species in order to determine the x-value (Cu concentration) where the regression lines cross (please see the next paragraph) (Figure 3.12). For *P. excavatus*, linear regressions were done only for the two highest Cu concentrations (320 and 640 mg/kg Cu), where a clear difference between the exposure and depuration mass changes was observed (Figure 3.12f).

Where the regression lines for exposure mass change and depuration mass change cross each other (where y (from the linear function $y = mx + c$) for these two functions are equal to each other), the Cu concentration (x) could be calculated. This is the Cu concentration where the rate of mass loss during depuration became lower than the rate of mass loss during exposure. At this concentration, therefore, it may be assumed that the earthworms changed their feeding behaviour by decreasing feeding activity. This behavioural response will hence be termed "feeding avoidance response". The lowest feeding avoidance response concentration was observed for *A. trapezoides* (104 mg/kg Cu, Figure 3.12b), followed by *Chilota sp.* (162 mg/kg Cu, Figure 3.12c), *A. diffringens* (222 mg/kg Cu, Figure 3.12a), *E. andrei* (A) (332 mg/kg Cu, Figure 3.12d), *P. excavatus* (357 mg/kg Cu, Figure 3.12f) and *E. andrei* (B) (529 mg/kg Cu, Figure 3.12e).

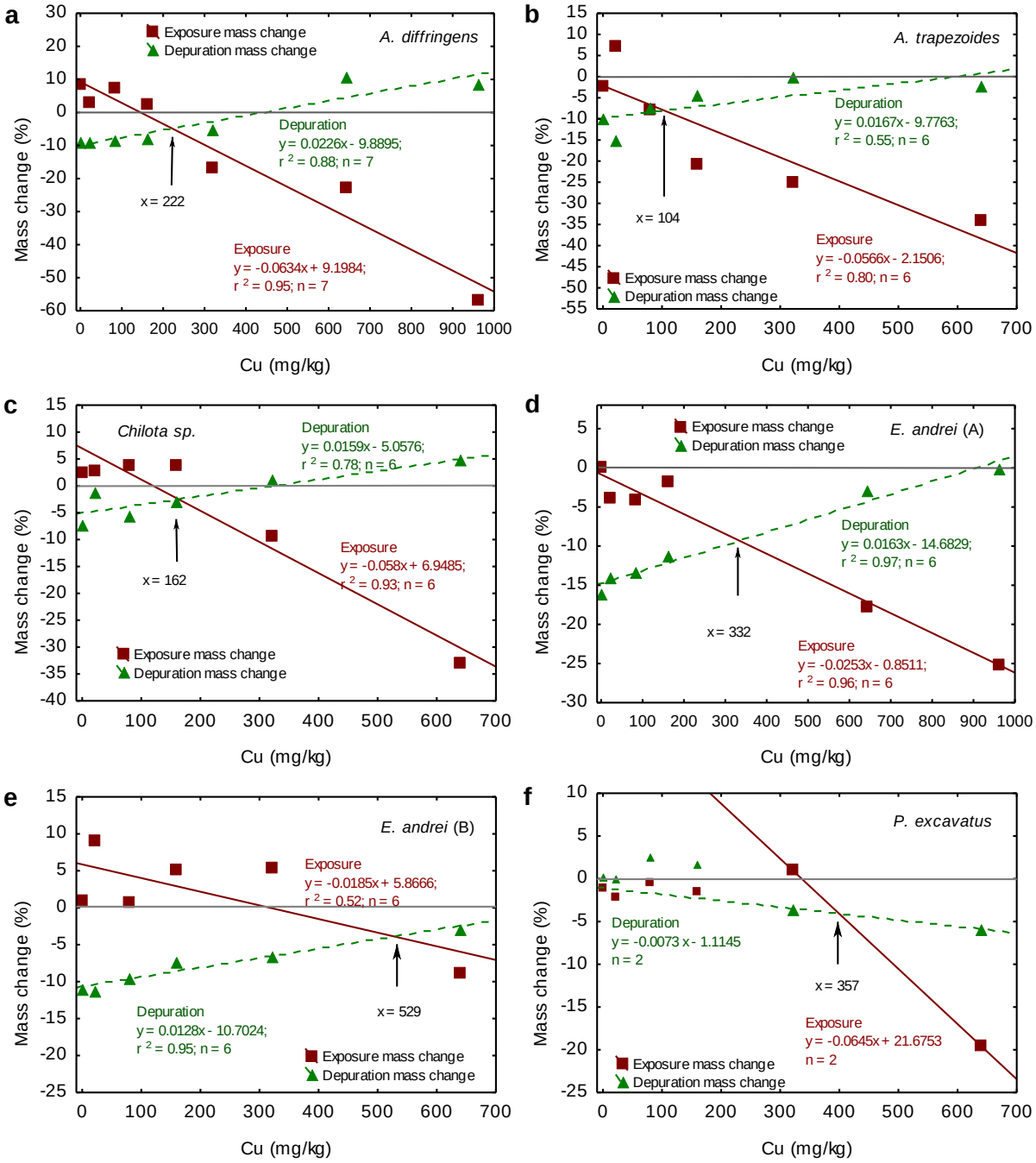


Figure 3.12: Correlation between the nominal soil Cu concentration and the mean earthworm mass change for specimens from five earthworm species during 14 days exposure (exposure mass change, depicted with squares and a solid regression line) to Cu in the form of copper oxychloride in OECD soil. The triangles and the dashed regression line depict the correlation between the nominal soil Cu concentration and the mean mass change during depuration (depuration mass change) of earthworms kept on moist filter paper for 24 h (of 48 h for *E. andrei* (A)) after the 14 days exposure period. The arrows indicate the x-values where the regression lines for exposure mass change and depuration mass change cross. a) *A. diffringens*, b) *A. trapezoides*, c) *Chilota sp.*, d) *E. andrei* (A), e) *E. andrei* (B) and f) *P. excavatus*; please note that for this species, a regression line was only constructed between the two highest Cu concentrations, where a clear difference between the exposure and depuration mass changes was observed. An r^2 value is thus not included for this species.

3.6 Protein content of earthworm coelomic fluid

The protein content of earthworm coelomic fluid extracted from specimens after exposure is presented in Figure 3.13 and Appendix B, Table 28. With the exception of *A. trapezoides*, protein concentrations in coelomic fluid from all species were within the range of 2 to 10 µg/µl. This range of protein concentrations (of bovine serum albumin) was originally used in the standard curves for calculating protein concentrations from absorption values (Materials & Methods, Section 2.6.2). In the standard curves, the protein concentrations below 2 µg/µl did not show a linear relationship with photometric absorption values. Additionally, it was assumed that below 2 µg/µl, the coelomocyte cell count would be too low to yield reliable results for the biomarker (NRR and MTT) assays. Therefore, those specimens yielding coelomocyte suspensions with absorption values below the point corresponding to 2 µg/µl were excluded from further analyses with the NRR and MTT assays.

For *A. trapezoides*, the protein concentrations of 84% of individuals were below 2 µg/µl for the first two replicates. Additionally, visual inspection of comet assay slides made from the same coelomocyte suspensions confirmed that coelomocyte numbers were extremely low. Therefore, protein content analyses of further replicates were abandoned and this species was subsequently excluded entirely from the biomarker (NRR, MTT and comet assay) experiments.

For the remainder of the species, those individuals that yielded coelomocyte suspensions with protein concentrations below 2 µg/µl, were excluded from NRR and MTT analyses. These individuals were however all included in all statistical analyses involving protein concentration.

For *A. diffringens*, *Chilota sp.* and *P. excavatus*, the protein concentration in coelomic fluid was lower in the highest Cu treatment concentrations than in the other treatments (Figure 3.13). No significant correlations were however found between the protein content and either the measured soil Cu concentrations or the body Cu concentrations for any of the species (Spearman $R < 0.8$ and > -0.8, Appendix B, Table 29).

The presence of significant differences in the coelomic fluid protein content between treatments for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 30a), and significant differences between treatments were found only for *E. andrei* (B) (Figure 3.13e). Post hoc-testing (Appendix B, Table 30b) indicated that for *E. andrei* (B), the negative control differed significantly from both 20 and 640 mg/kg Cu.

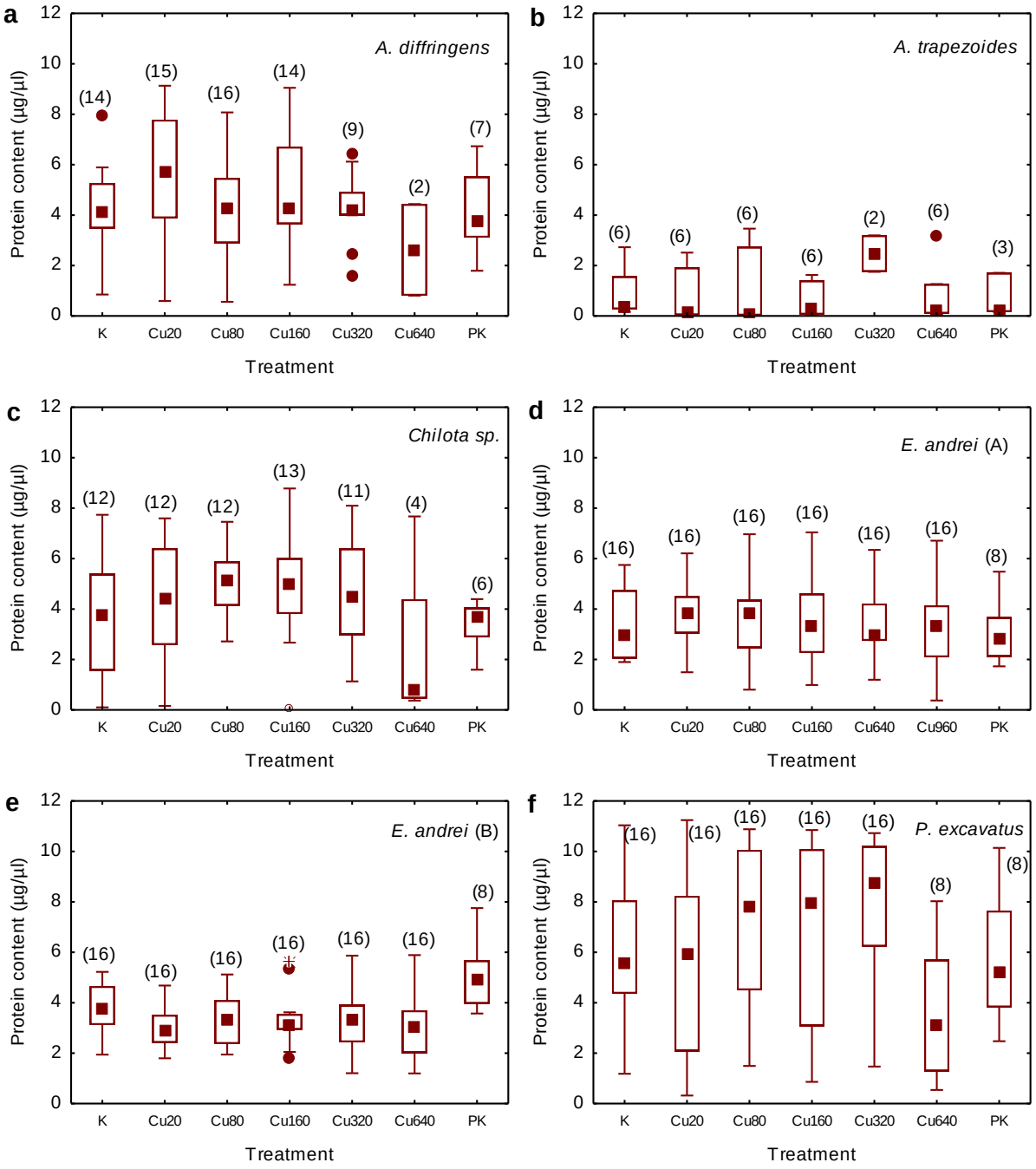


Figure 3.13: Median protein content of coelomic fluid obtained from specimens of five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) *A. diffringens*, b) *A. trapezoides*, c) *Chilota sp*., d) *E. andrei* (A), e) *E. andrei* (B) and f) *P. excavatus*. The numbers given in brackets are n for each treatment. All values (data are nonparametric) are presented as medians (squares), with the boxes representing 25% to 75% of the data, the whiskers the non-outlier range, the circles the outliers and the crosses the extremes. K = negative controls, PK = Positive controls, which are subsamples of the negative controls spiked with 1 mM H_2O_2.

3.7 Neutral red retention (NRR) and MTT assays

3.7.1 NRR assay

The summarised NR retention values of earthworm coelomocytes are presented in Appendix B, Table 31 (for NRR (corrected)) and Table 32 (for NRR (% of control)). The NRR (% of control) results are graphically presented in Figure 3.14. The NRR (% of control) results are also graphically presented in Figure 3.18, along with the MTT (% of control) results, although these assays do not measure the same endpoint. Figure 3.18 was included purely to visualise the results of these two assays.

For *A. diffringens* (Figure 3.14a) and *P. excavatus* (Figure 3.14e), NR retention decreased at 20 mg/kg Cu, then remained at a similar level as Cu concentrations increased, and then increased greatly at 640 mg/kg Cu. For *A. diffringens*, data for only one individual were available at 640 mg/kg. For *Chilota sp.* (Figure 3.14b), NRR was similar to control levels in the treatments 20, 160 and 320 mg/kg Cu, but was increased at 80 mg/kg Cu, and decreased at 640 mg/kg Cu. For *E. andrei* (A) (Figure 3.14c), NRR levels were higher than that of the negative control at all Cu treatments. For *E. andrei* (B) (Figure 3.14d), a similar pattern as for *Chilota sp.* was observed, although the increase in NRR levels was evident at 160 mg/kg Cu and not at 80 mg/kg as for *Chilota sp.*.

Correlations (nonparametric Spearman rank-order correlations) were done between NRR (% of control) and both the soil Cu content and earthworm body Cu content. No significant correlations were found between NRR and the soil Cu content (Appendix B, Table 33) or between NRR and the body Cu content (Figure 3.15 and Appendix B, Table 33).

For all species, the NR retention in the positive control was lower than in the negative control. For *A. diffringens* a mean NR reduction of 93% was observed for the positive control. For *Chilota sp.*, the NR reduction was 35%, for *E. andrei* (A), it was 64%, for *E. andrei* (B) it was 76% and for *P. excavatus* it was 42% (calculated from Appendix B, Table 32).

The presence of significant differences between treatments in NR retention for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 34a), and significant differences were found between treatments for all species, except *Chilota sp.* Post-hoc testing (Appendix B, Table 34b) indicated that for *A. diffringens* (Figure 3.14a), the negative control differed significantly from all other treatments (the treatment 640 mg/kg was not included due to an insufficient number of data points, n = 1). For both *E. andrei* (A) and (B) (Figure 3.14c and d), the negative control differed significantly from the positive control. For *P. excavatus* (Figure 3.14e),

the negative control differed significantly from the positive control and the treatment 640 mg/kg Cu.

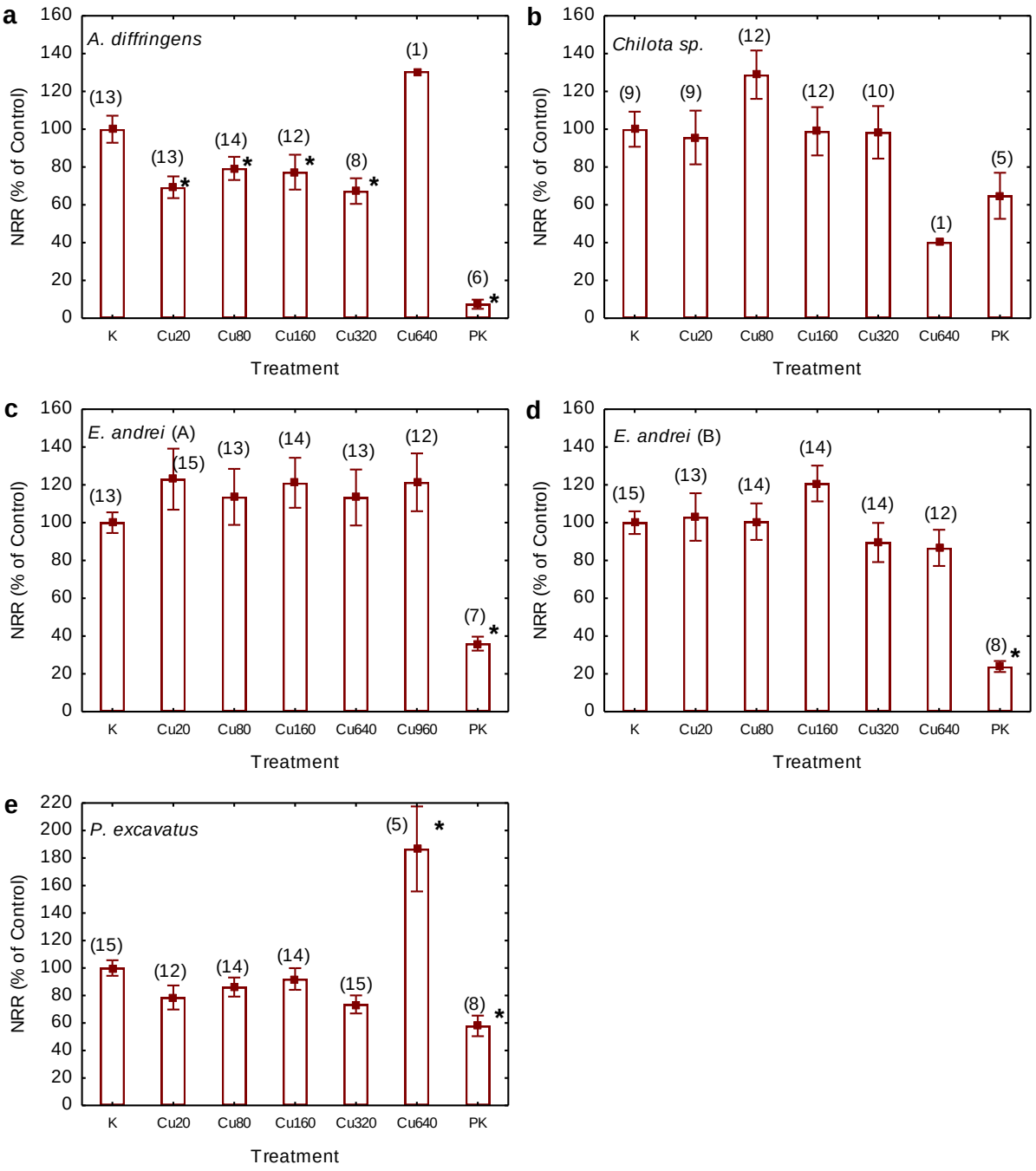


Figure 3.14: Mean (± Std Error) Neutral red retention (NRR), calculated as a % of the negative control response, of coelomocytes from specimens of five earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days. a) *A. diffringens*, b) *Chilota sp*., c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. The asterisks (*) indicate the treatments where NRR is significantly different from the negative control. The numbers given in brackets are n for each treatment. K = negative controls, PK = Positive controls, which are subsamples of the negative controls spiked with 1 mM H_2O_2.

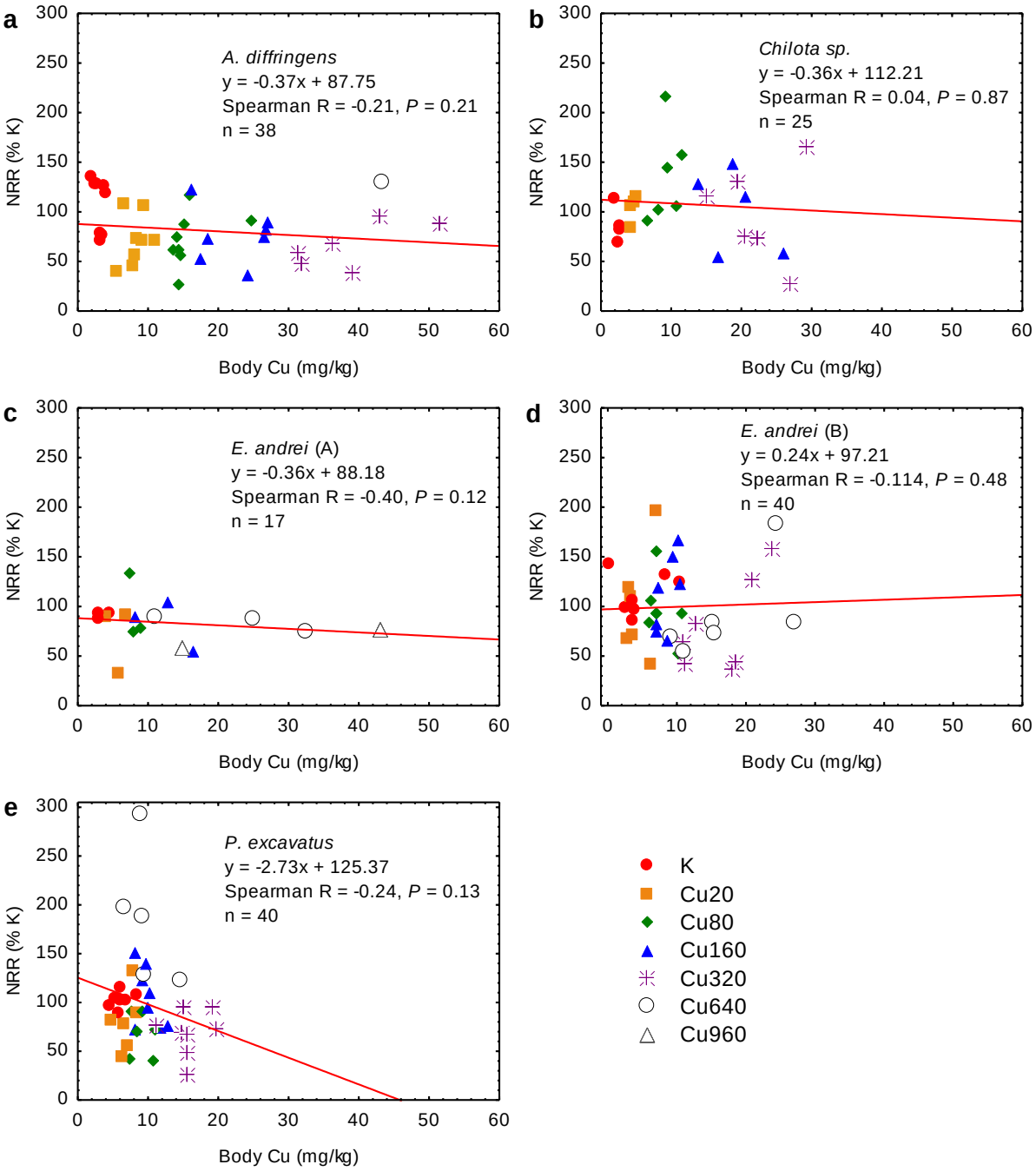


Figure 3.15: Correlations between earthworm body Cu concentrations (mg/kg) and neutral red retention (NRR, calculated as a % of the negative control response) of coelomocytes obtained from specimens of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) *A. diffringens*, b) *Chilota sp.*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. Values for specimens from each treatment were assigned a unique symbol, with the solid circles for specimens from the negative control (K), solid squares for the treatment 20 mg/kg Cu, solid diamonds for 80 mg/kg Cu, solid triangles for 160 mg/kg Cu, crosses for 320 mg/kg Cu, open circles for 640 mg/kg Cu and the open triangles for the treatment 960 mg/kg Cu.

3.7.2 MTT assay

The summarized MTT results are presented in Appendix B, Table 35 (for MTT (corrected)) and Table 36 (for MTT (% of control)). The MTT (% of control) results are graphically presented in Figure 3.16 and also in Figure 3.18 along with the NRR results.

For *A. diffringens* (Figure 3.16a), MTT values decreased at the treatment 20 mg/kg Cu, increased at the treatment 80 mg/kg Cu to a similar level of that of the negative control, decreased again at the treatments 160 and 320 mg/kg Cu. At the treatment 640 mg/kg Cu, the MTT value for the one available specimen was increased up to 159% of the negative control. For *Chilota sp.*, MTT values decreased slightly at the treatment 20 mg/kg Cu, then increased up to the treatment 160 mg/kg Cu, after which the MTT values decreased to levels similar to the negative control at treatments 320 and 640 mg/kg Cu. For both *E. andrei* (A) and (B), MTT values were increased in relation to the negative control at all Cu treatments. For *E. andrei* (A), MTT values increased with increasing Cu concentration, and for *E. andrei* (B), MTT values increased up to the treatment 160 mg/kg Cu, after which it decreased again. For *P. excavatus*, MTT values were similar for the negative control and the treatment 20 mg/kg Cu, and increased at all other Cu concentrations. The highest MTT value was found at treatment 320 mg/kg Cu.

Correlations (nonparametric Spearman rank-order correlations) were done between MTT (% of control) and both the soil Cu content and earthworm body Cu content. No significant correlations were found between MTT and the soil Cu content (Appendix B, Table 37) or between MTT and the body Cu content (Figure 3.17 and Appendix B, Table 37).

For all species, the mean MTT value in the positive control was lower than in the negative control. For *A. diffringens* a mean reduction of 25% in MTT conversion to formazan was observed for the positive control (calculated from Appendix B, Table 36). For *Chilota sp.*, the reduction in formazan production was 29%, for *E. andrei* (A) it was 35%, for *E. andrei* (B) it was 36% and for *P. excavatus* it was 42%.

The presence of significant differences between treatments in MTT conversion for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 38a), and significant differences were found between treatments for all species. Post-hoc testing (Appendix B, Table 38b) indicated that for *A. diffringens* (Figure 3.16a), the negative control differed significantly from the treatments 20 and 320 mg/kg Cu and the positive control (the treatment 640 mg/kg was not included due to an insufficient number of data points, n = 1). For *Chilota sp.* (Figure 3.16b), the negative control differed significantly from the treatment 160 mg/kg Cu. For *E. andrei* (A) (Figure 3.16c), the negative control differed significantly from the treatments 640 and 960 mg/kg Cu and the positive control. For *E. andrei* (B) (Figure 3.16d), no significant differences were found

between the negative control and any of the treatments; however a significant difference was found between the positive control and the treatment 160 mg/kg Cu. For *P. excavatus* (Figure 3.16e), the negative control differed significantly from the treatment 320 mg/kg Cu.

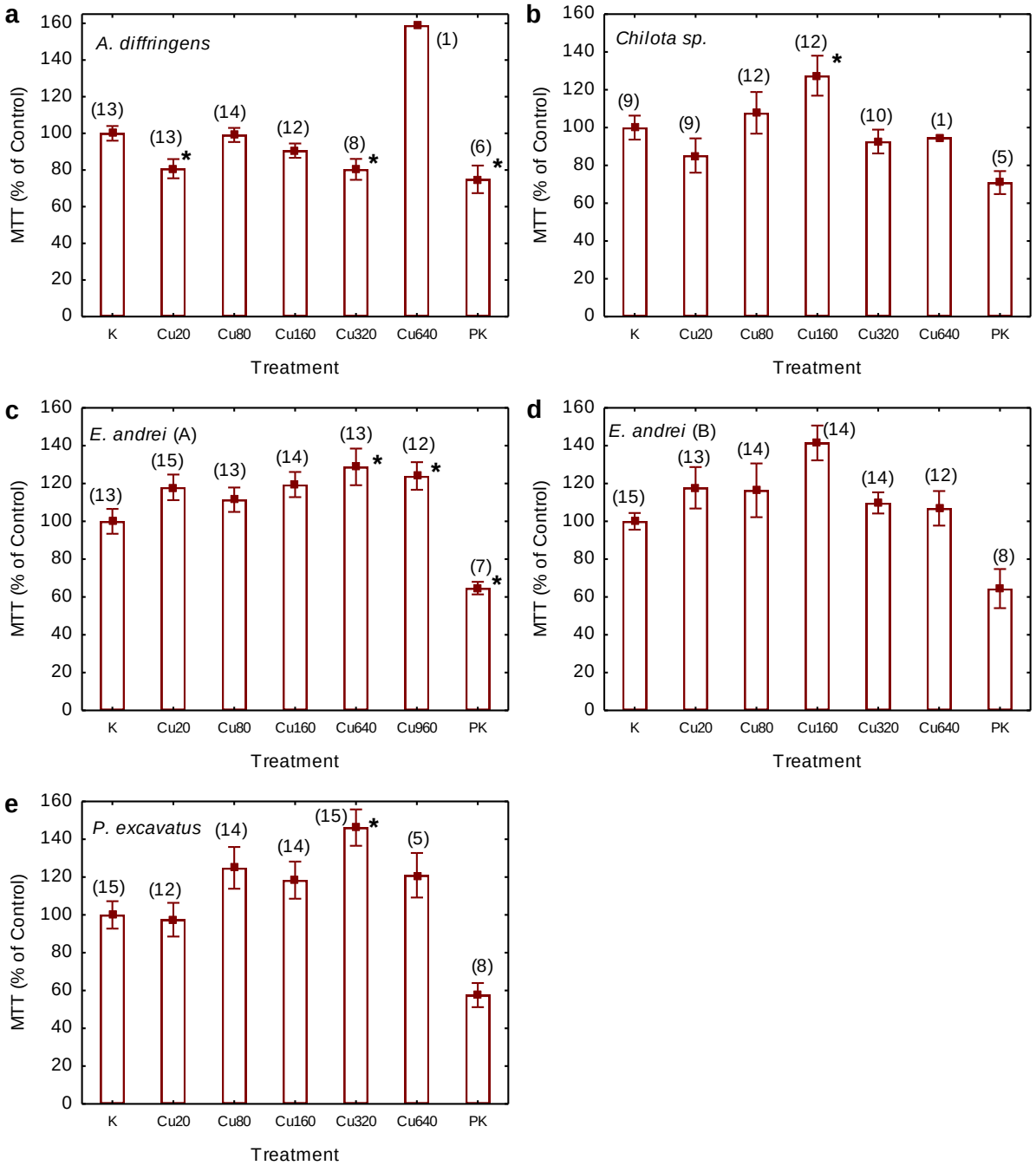


Figure 3.16: Mean (± Std Error) MTT conversion to formazan, calculated as a % of the negative control response, of coelomocytes from specimens of five earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days. a) *A. diffringens*, b) *Chilota sp*., c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. The asterisks (*) indicate the treatments where MTT conversion is significantly different from the negative control. The numbers given in brackets are n for each treatment. K = negative controls, PK = Positive controls, which are subsamples of the negative controls spiked with 1 mM H_2O_2.

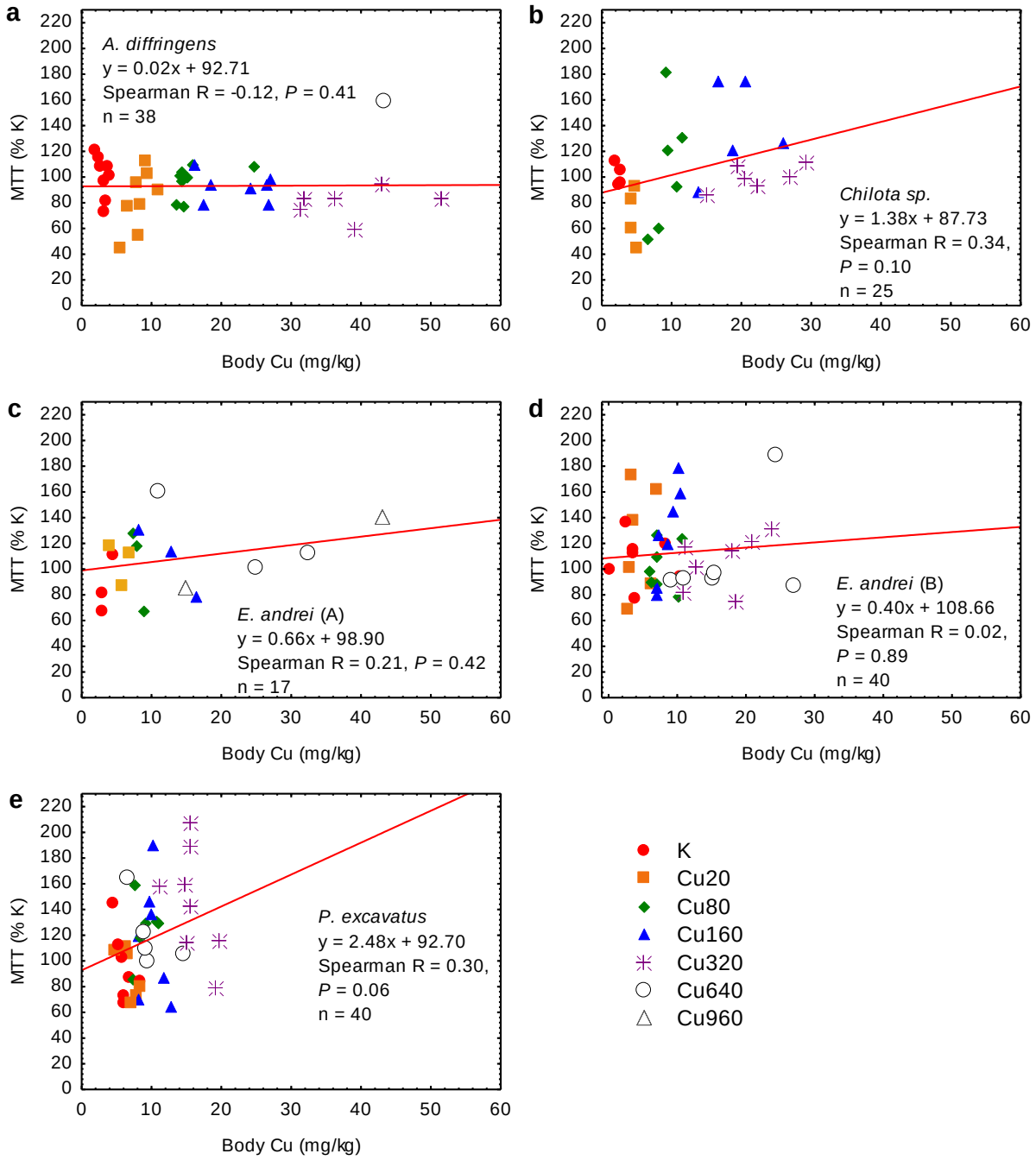


Figure 3.17: Correlations between earthworm body Cu concentrations (mg/kg) and MTT conversion (calculated as a % of the negative control response) of coelomocytes from specimens of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) *A. diffringens*, b) *Chilota sp.*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. Values for specimens from each treatment were assigned a unique symbol, with the solid circles for specimens from the negative control (K), solid squares for the treatment 20 mg/kg Cu, solid diamonds for 80 mg/kg Cu, solid triangles for 160 mg/kg Cu, crosses for 320 mg/kg Cu, open circles for 640 mg/kg Cu and the open triangles for the treatment 960 mg/kg Cu.

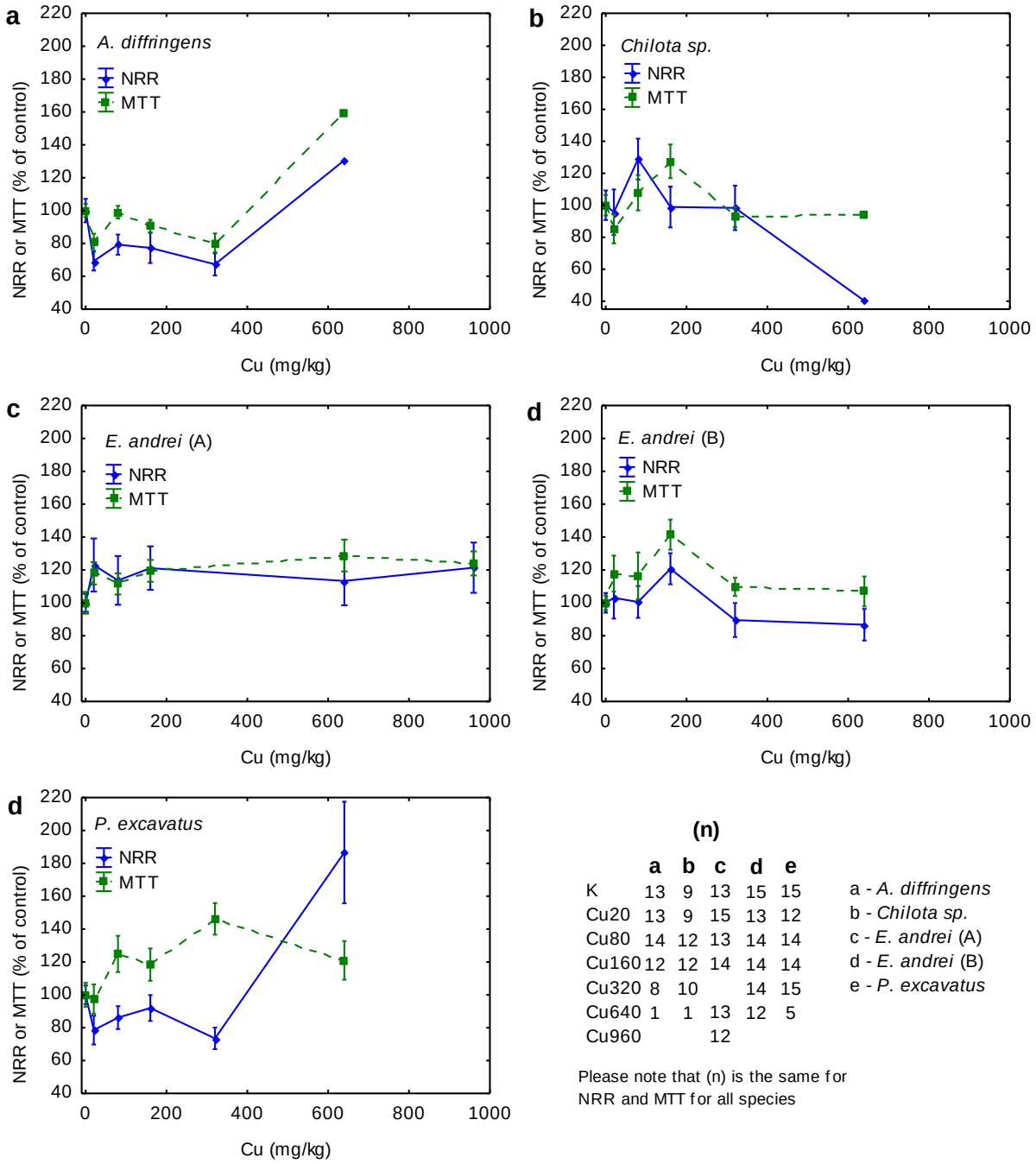


Figure 3.18: Mean (± Std Error) Neutral red retention (NRR) and MTT conversion, calculated as a % of the negative control response, of coelomocytes from specimens of four earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days. The connecting lines between the means for each Cu concentration are shown to aid visual interpretation. a) *A. diffringens*, b) *Chilota sp.*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. K = negative control. Please note that there is no functional link between the NRR and MTT assays, and that this figure is purely for visualisation of the results of these assays.

3.7.3 EC, LOEC and NOEC values for NRR and MTT

Due to the nature of the dose responses obtained in the present study for NRR and MTT, it was not possible to determine EC_{50} values for either NRR or MTT. EC_{10} values were however calculated for NRR (% of control) for *Chilota sp.*, *E. andrei* (B) and *P. excavatus* (Table 3.6). No EC values could be determined for MTT (% of control). LOEC and NOEC values could only be calculated for *A. diffringens* and *P. excavatus* for NRR and *A. diffringens* for MTT (Table 3.6).

Table 3.6: EC_{10}, EC_{20} and EC_{50} values, as well as LOEC and NOEC values (in mg/kg Cu) for neutral red retention (NRR) and MTT conversion (both calculated as a % of the negative control response) in coelomocytes obtained from for four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. nd = Could not be determined. Confidence limits are not determined for LOEC and NOEC values; in the table these cells are filled with a dash (-).

		NRR			MTT		
		Cu	Confidence limit		Cu	Confidence limit	
Species		(mg/kg)	- 95%	+ 95%	(mg/kg)	- 95%	+ 95%
A. diffringens	EC_{10}	nd	nd	nd	nd	nd	nd
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	20	-	-	320	-	-
	NOEC	< 20	-	-	160	-	-
Chilota sp.	EC_{10}	365	92.8	462.9	nd	nd	nd
	EC_{20}	429	165.4	513.2	nd	nd	nd
	EC_{50}	585	458.6	676.4	nd	nd	nd
	LOEC	nd	-	-	nd	-	-
	NOEC	nd	-	-	nd	-	-
E. andrei (A)	EC_{10}	nd	nd	nd	nd	nd	nd
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	nd	-	-	nd	-	-
	NOEC	nd	-	-	nd	-	-
E. andrei (B)	EC_{10}	467	nd	nd	nd	nd	nd
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	nd	-	-	nd	-	-
	NOEC	nd	-	-	nd	-	-
P. excavatus	EC_{10}	4	nd	nd	nd	nd	nd
	EC_{20}	282	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	nd	-	-	nd	-	-
	NOEC	nd	-	-	nd	-	-

3.8 Alkaline comet assay

Summarised values for Tail DNA % (raw) are presented in Appendix B, Table 39. Tail DNA % (raw) values are individual comet Tail DNA % values summarised per treatment per species. Tail DNA % (median) values are presented in Appendix B, Table 40. Tail DNA % (median) values are individual comet Tail DNA % values firstly summarised (median) per specimen and then summarised per treatment per species. Both Tail DNA % (raw) and (median) are graphically presented in Figures 3.19 to 3.23.

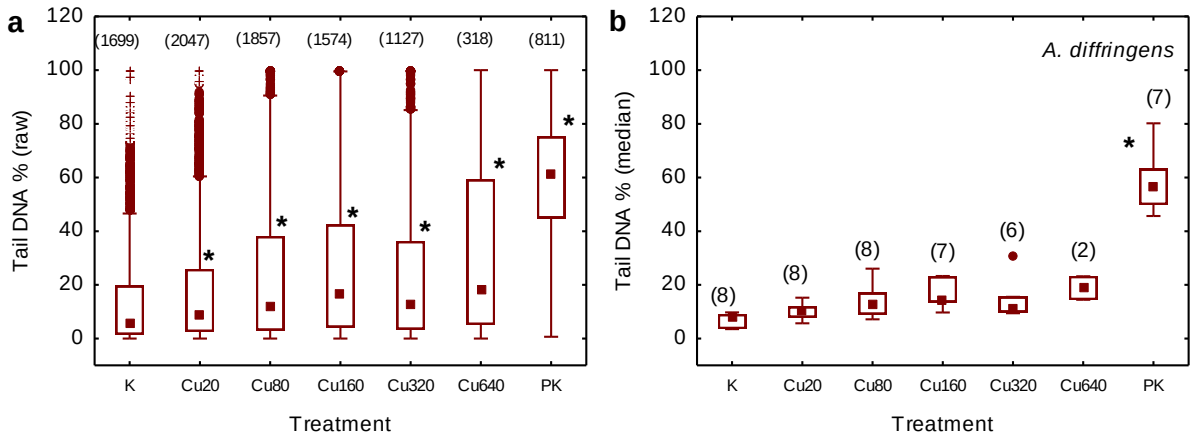


Figure 3.19: DNA damage (Tail DNA %) as measured with the comet assay on coelomocytes from specimens of *A. diffringens* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets are n for each treatment. a) Tail DNA % from raw data from individual comets. b) Tail DNA % values from individual comet data summarised (median) for each specimen. K = negative control. PK = positive control. All values (data are nonparametric) are presented as median (squares), with the boxes representing 25 – 75% of the data, the whiskers the non-outlier range, the circles the outliers and the crosses the extremes. The asterisks (*) indicate the treatments where Tail DNA % values are significantly different from those in the negative control.

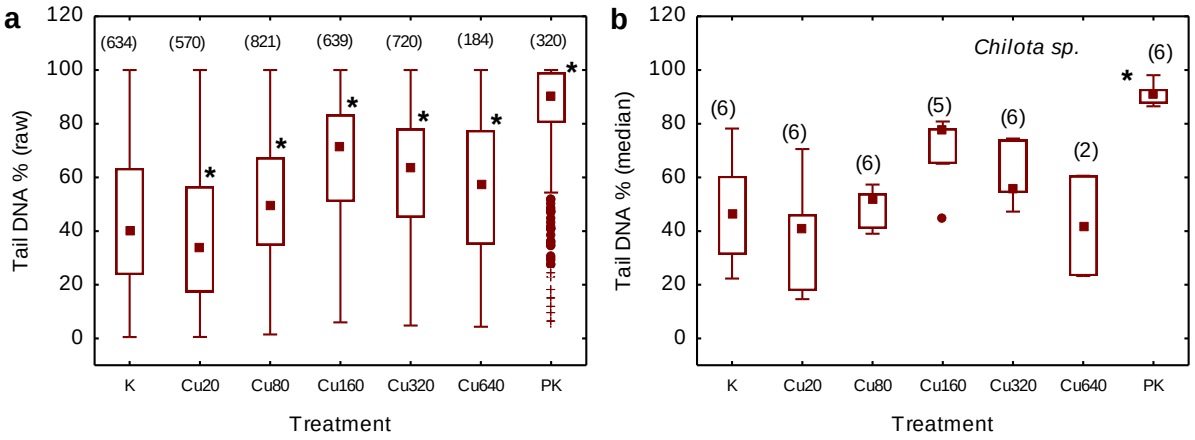


Figure 3.20: DNA damage (Tail DNA %) as measured with the comet assay on coelomocytes from specimens of *Chilota sp.* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets are n for each treatment. a) Tail DNA % from raw data from individual comets. b) Tail DNA % values from individual comet data summarised (median) for each specimen. K = negative control. PK = positive control. All values (data are nonparametric) are presented as median (squares), with the boxes representing 25 – 75% of the data, the whiskers the non-outlier range, the circles the outliers and the crosses the extremes. The asterisks (*) indicate the treatments where Tail DNA % values are significantly different from those in the negative control.

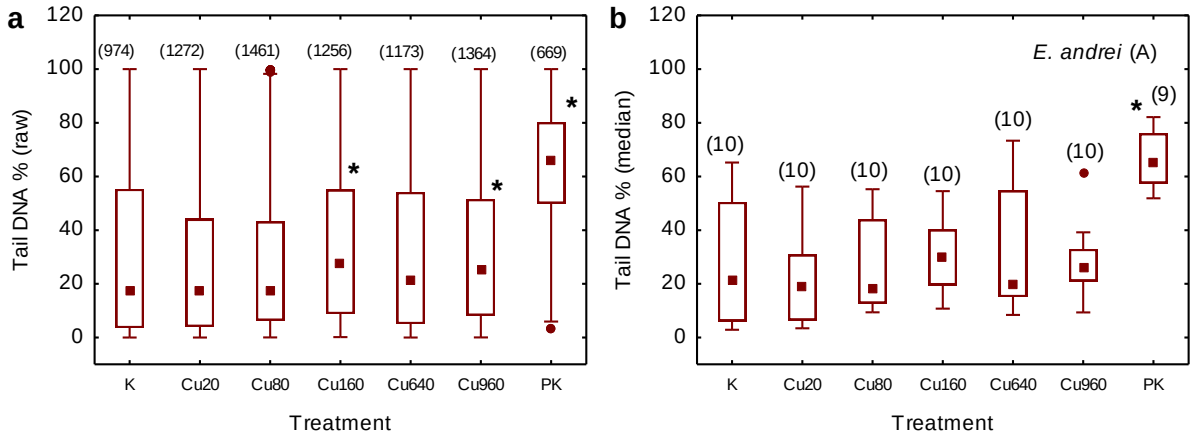


Figure 3.21: DNA damage (Tail DNA %) as measured with the comet assay on coelomocytes from specimens of *E. andrei* (A) after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets are n for each treatment. a) Tail DNA % from raw data from individual comets. b) Tail DNA % values from individual comet data summarised (median) for each specimen. K = negative control. PK = positive control. All values (data are nonparametric) are presented as median (squares), with the boxes representing 25 – 75% of the data, the whiskers the non-outlier range and the circles the outliers. The asterisks (*) indicate the treatments where Tail DNA % values are significantly different from those in the negative control.

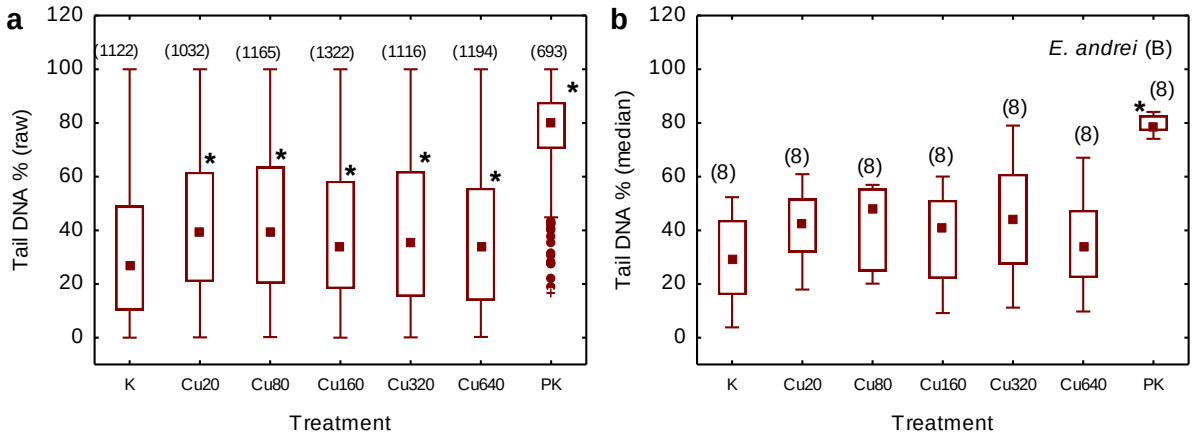


Figure 3.22: DNA damage (Tail DNA %) as measured with the comet assay on coelomocytes from specimens of *E. andrei* (B) after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets are n for each treatment. a) Tail DNA % from raw data from individual comets. b) Tail DNA % values from individual comet data summarised (median) for each specimen. K = negative control. PK = positive control. All values (data are nonparametric) are presented as median (squares), with the boxes representing 25 – 75% of the data, the whiskers the non-outlier range, the circles the outliers and the crosses the extremes. The asterisks (*) indicate the treatments where Tail DNA % values are significantly different from those in the negative control.

For *A. diffringens* (Figure 3.19), there was an increase in DNA damage with increasing Cu concentration, with a less pronounced increase at 320 mg/kg Cu. For *Chilota sp.* (Figure 3.20), DNA damage decreased in the treatment 20 mg/kg Cu in relation to the negative control. At treatments 80 and 160 mg/kg Cu, DNA damage increased, and decreased again at treatments 320 and 640 mg/kg Cu. For *E. andrei* (A) (Figure 3.21), DNA damage in the treatments 20 and 80 mg/kg Cu was similar to that in the negative control. At the treatment 160 mg/kg Cu, DNA damage increased, decreased at 640 mg/kg Cu, and increased again at 960 mg/kg Cu. For *E. andrei* (B) (Figure 3.22), DNA damage in the Cu treatments was higher than in the negative control, although

the damage in treatment 640 mg/kg Cu was lower than at the other Cu treatments. For *P. excavatus* (Figure 3.23), DNA damage in the treatment 20 mg/kg Cu was similar to that of the negative control, and was increased slightly at the higher Cu concentrations.

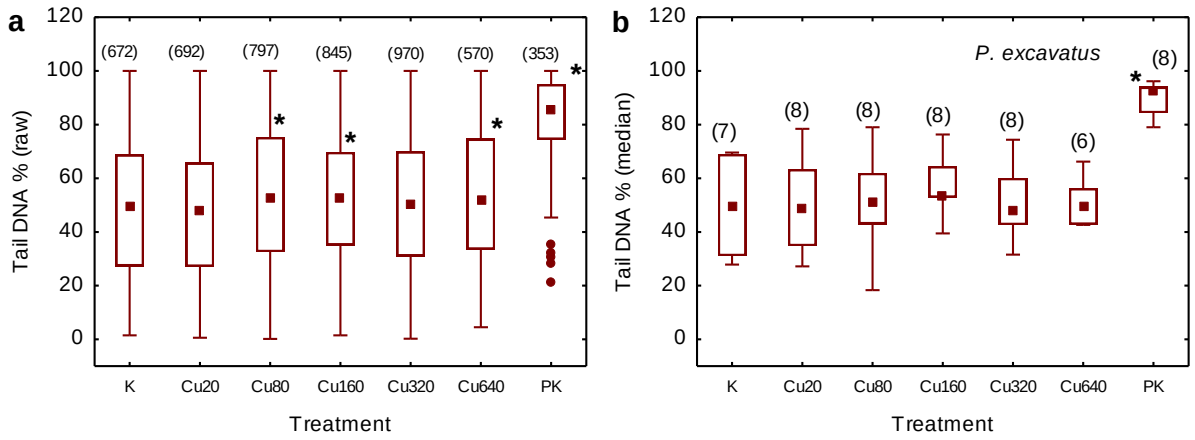


Figure 3.23: DNA damage (Tail DNA %) as measured with the comet assay on coelomocytes from specimens of *P. excavatus* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The numbers given in brackets are n for each treatment. a) Tail DNA % from raw data from individual comets. b) Tail DNA % values from individual comet data summarised (median) for each specimen. K = negative control. PK = positive control. All values (data are nonparametric) are presented as median (squares), with the boxes representing 25 – 75% of the data, the whiskers the non-outlier range and the circles the outliers. The asterisks (*) indicate the treatments where Tail DNA % values are significantly different from those in the negative control.

The presence of significant differences between treatments in Tail DNA % (raw) for each species was tested for (nonparametric KW ANOVA, Appendix B, Table 42a), and significant differences were found between treatments for all species. Post-hoc testing (Appendix B, Table 42b) indicated that for *A. diffringens* (Figure 3.19a), *Chilota sp.* (Figure 3.20a) and *E. andrei* (B) (Figure 3.22a), the negative control differed significantly from all other treatments. For *E. andrei* (A) (Figure 3.21a), the negative control differed significantly from treatments 160 and 960 mg/kg Cu and the positive control. For *P. excavatus* (Figure 3.23a) the negative control differed significantly from all other treatments except 20 and 320 mg/kg Cu.

The presence of significant differences in Tail DNA % (median) between treatments for each species was tested for (ANOVA or nonparametric KW ANOVA, Appendix B, Table 43a), and significant differences were found between treatments for all species. Post-hoc testing (Appendix B, Table 43b) indicated that for all species, the negative control differed significantly only from the positive control.

The relative increase in Tail DNA % (median) values at each treatment was calculated by dividing the Tail DNA % (median) value for each treatment by the Tail DNA % (median) value for the negative control for each species (Appendix B, Table 40), and is presented in Figure 3.24. This was done using the summarised values of Tail DNA % (median) for all replicates for each species.

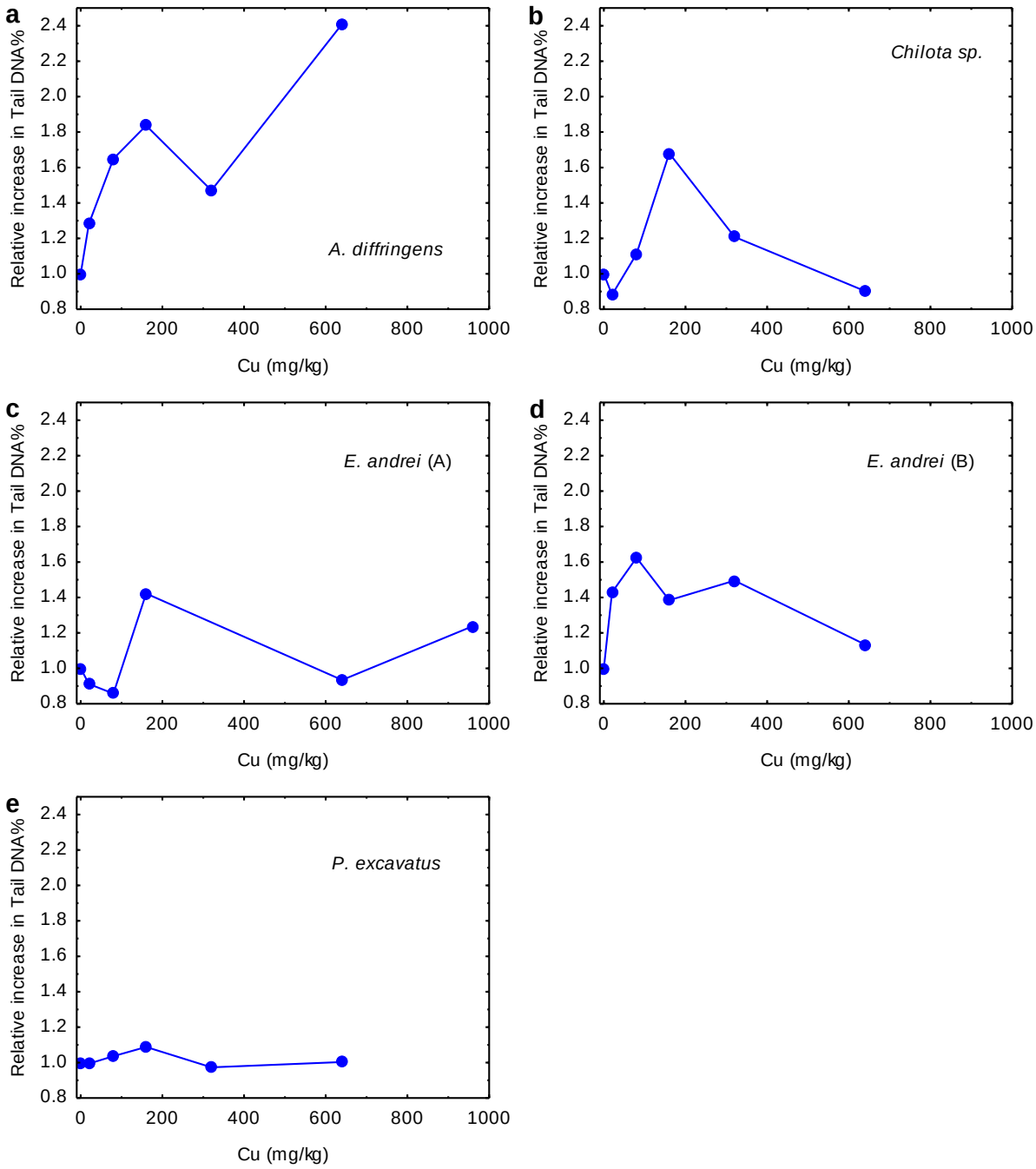


Figure 3.24: The relative increase in Tail DNA % (median) values (Tail DNA % values from individual comet structures summarised (median) for each specimen) as measured with the comet assay on coelomocytes from specimens of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. The relative increase was calculated by dividing the median Tail DNA % value at each Cu treatment by the median Tail DNA % value of the negative control for each species. a) *A. diffringens*, b) *Chilota sp*., c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*.

For *A. diffringens*, the relative increase in Tail DNA % increased up to 1.8-fold at the treatment 160 mg/kg Cu, decreased at the treatment 320 mg/kg Cu, and increased up to 2.4-fold at the treatment 640 mg/kg Cu. For *Chilota sp.*, the relative increase in Tail DNA % decreased at the treatment 20 mg/kg Cu, and increased up to 1.7-fold at the treatment 160 mg/kg Cu, after which it decreased with increasing Cu concentration. For *E. andrei* (A), the relative increase in Tail DNA %

decreased at treatments 20 and 80 mg/kg Cu, and increased up to 1.4-fold at the treatment 160 mg/kg Cu, after which it decreased again. For *E. andrei* B, the relative increase in Tail DNA % increased up to 1.6-fold at the treatment 80 mg/kg Cu, after which it decreased with increasing Cu concentration. For *P. excavatus*, the relative increase in Tail DNA % was increased 1.1-fold at the treatment 160 mg/kg Cu, and decreased at higher Cu concentrations.

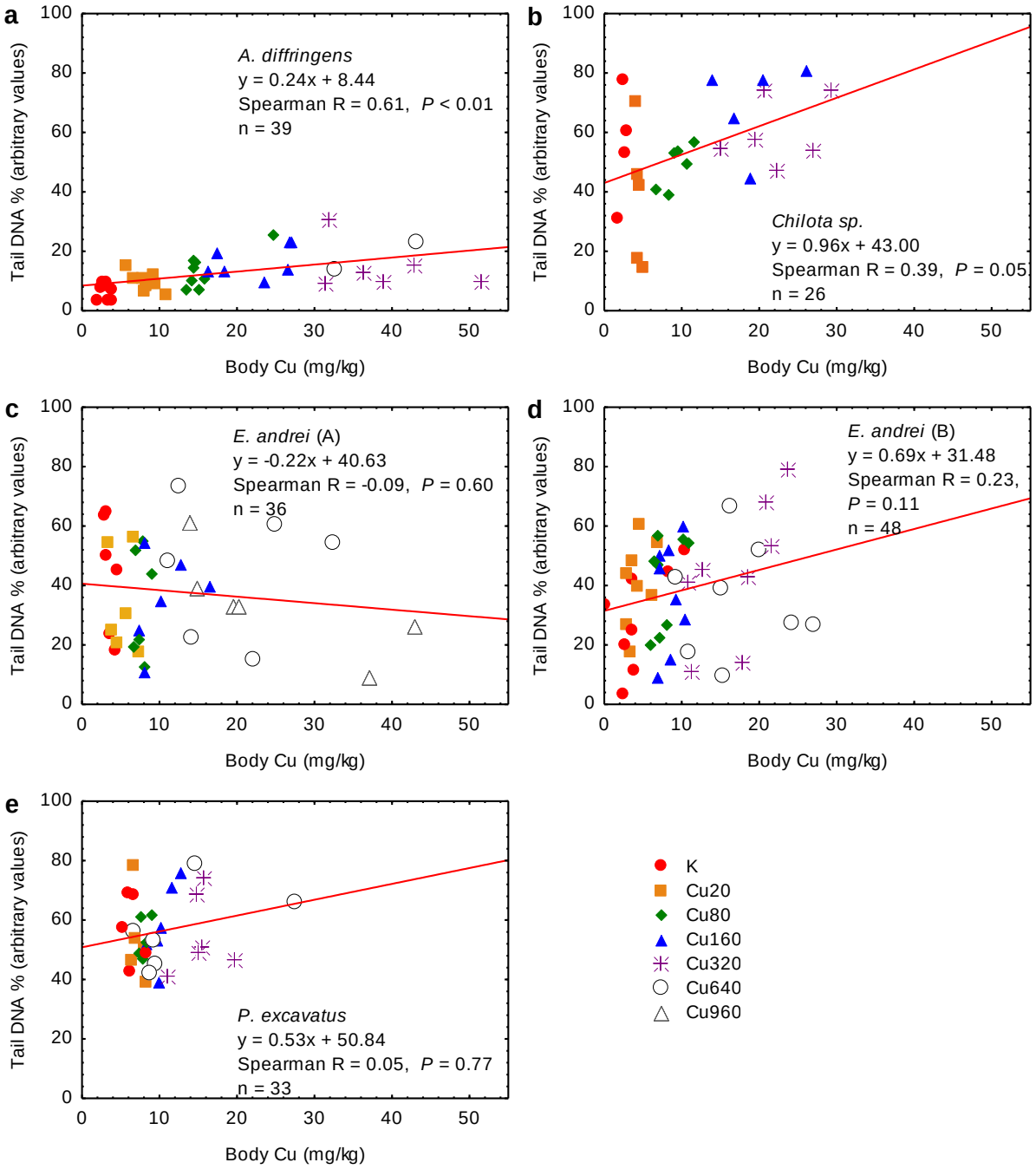


Figure 3.25: Correlations between earthworm body Cu concentrations (mg/kg) and DNA damage (median Tail DNA % value per specimen) in coelomocytes from specimens of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) *A. diffringens*, b) *Chilota sp.*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. Values for specimens from each treatment were assigned a unique symbol, with the solid circles for specimens from the negative control (K), solid squares for the treatment 20 mg/kg Cu, solid diamonds for 80 mg/kg Cu, solid triangles for 160 mg/kg Cu, crosses for 320 mg/kg Cu, open circles for 640 mg/kg Cu and the open triangles for the treatment 960 mg/kg Cu.

Correlations (nonparametric Spearman rank-order correlations) were done between Tail DNA % (median) and both the soil Cu content and earthworm body Cu content, and none of the correlations were significant (Spearman R < 0.8 or > -0.8, Appendix B, Table 41 and Figure 3.25).

In order to determine whether the relatively high Tail DNA % values in the negative control specimens of *E. andrei* (B), *Chilota sp.* and *P. excavatus* could have been due to errors in the experimental procedure, the results of the separate replicates for each species are presented graphically in Figure 3.26. In coelomocytes of *A. diffringens*, the Tail DNA % values in the negative control specimens were similar for all replicates, but there were differences between the replicates for the other species. Because only two specimens were analysed per replicate, statistical analyses were not performed to determine whether these differences were significant. When investigating data for the negative controls of *Chilota sp.*, Tail DNA % in the first replicate was higher than that in the other two replicates. In *E. andrei* (A), replicates 4 and 5 had very high Tail DNA % values, and replicate 3 had intermediate tail DNA % values. In *E. andrei* (B), replicate 4 had intermediate Tail DNA % values, and replicates 1 and 2 had high Tail DNA % values. In *P. excavatus*, replicate 3 had intermediate Tail DNA % values, while the other three replicates all had high Tail DNA % values. When replicates 4 and 5 were removed from *E. andrei* (A), and replicates 1 and 2 are removed from *E. andrei* (B), subsequent statistical analyses (KW ANOVA) revealed that there were still no significant differences between the negative control and any of the Cu treatments for either *E. andrei* (A) or (B) ($P > 0.05$).

3.8.1 EC, LOEC and NOEC values for DNA damage

Due to the nature of the dose responses obtained in the present study for the comet assay, EC_{50} values could not be calculated, except for Tail DNA % (median) for *Chilota sp.*, where the value was 674 mg/kg Cu (Table 3.7). EC_{10} values (Table 3.7) were however obtained for Tail DNA (raw) for *A. diffringens* (355 mg/kg Cu,), *Chilota sp.* (18 mg/kg Cu) and *P. excavatus* (162 mg/kg Cu and for Tail DNA (median) for *A. diffringens* (287 mg/kg Cu) and *Chilota sp.* (106 mg/kg Cu).

LOEC values were calculated for all species for Tail DNA % (raw), and the lowest LOEC value was 20 mg/kg, determined for *A. diffringens*, *Chilota* and *E. andrei* (B). For *P. excavatus*, the LOEC value was 80 mg/kg, and for *E. andrei* (A), the LOEC value was 160 mg/kg Cu. When Tail DNA % (median) data were used, the LOEC values were all above the highest exposure concentration (960 mg/kg for *E. andrei* (A) and 640 mg/kg Cu for the other species).

NOEC values for Tail DNA % (raw) were 20 mg/kg Cu for *P. excavatus* and 80 mg/kg Cu for *E. andrei* (A), and below 20 mg/kg Cu for *A. diffringens*, *Chilota sp.* and *E. andrei* (B). For Tail DNA

% (median), NOEC values were all above or equal to the highest exposure concentration (960 mg/kg for *E. andrei* (A) and 640 mg/kg Cu for the other species).

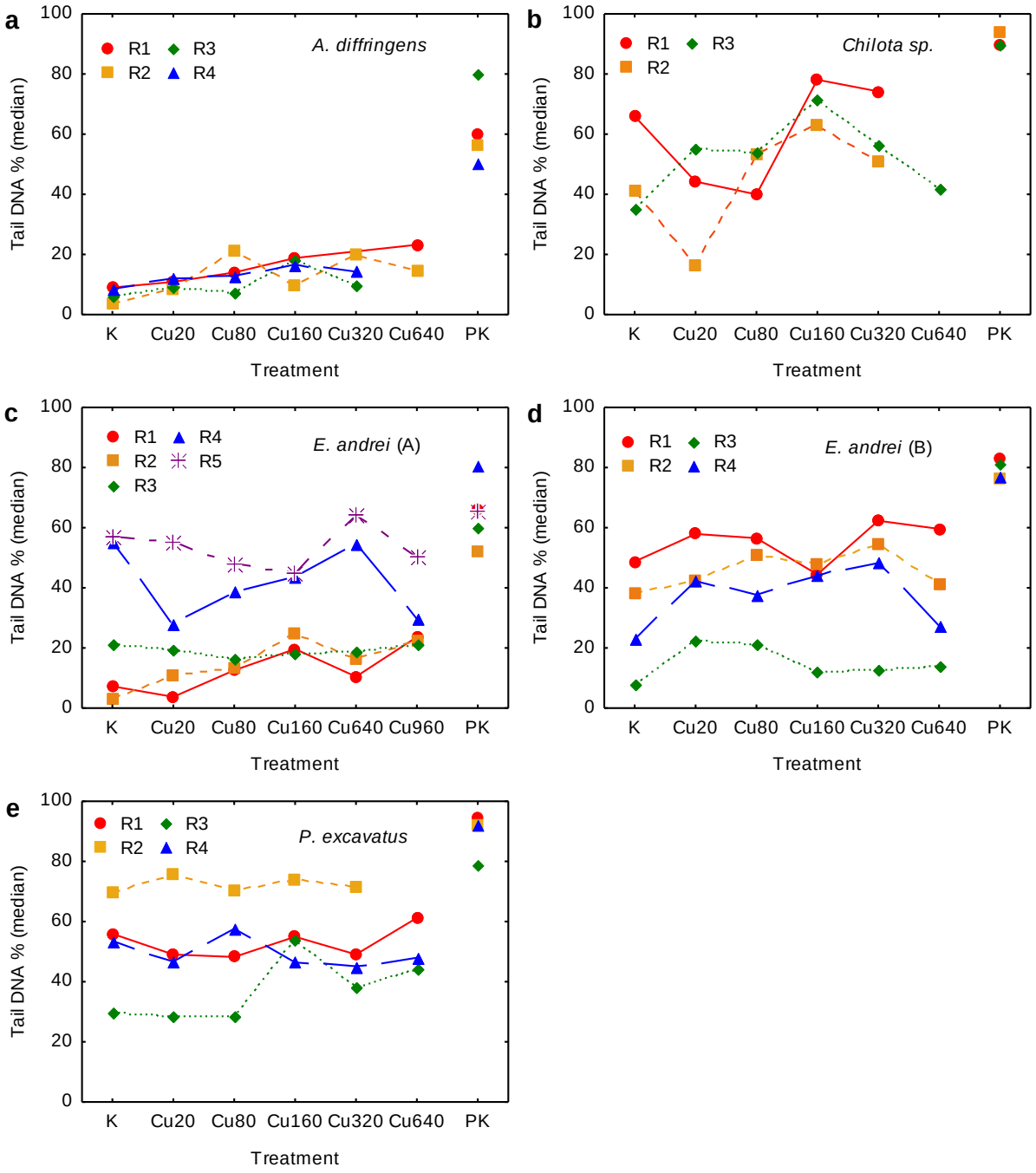


Figure 3.26: Median Tail DNA % values, as an indication of DNA damage as measured with the alkaline comet assay, in coelomocytes obtained from four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. K = negative control. PK = positive control. Median values of each replicate are given, and the connecting lines between treatments serve purely for visual purposes. This figure is presented to visually compare the replicates with each other, therefore please see Figures 3.19 to 3.23 for the box plots, with median values and 25 – 75% percentiles and the number of specimens for each species.

Table 3.7: EC_{10}, EC_{20} and EC_{50} values, as well as LOEC and NOEC values (in mg/kg Cu) for DNA damage (Tail DNA %) as measured with the comet assay in coelomocytes of four earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days. nd = Could not be determined. Tail DNA % (raw) = Tail DNA % values from individual comet structures used per treatment per species. Tail DNA % (median) = Tail DNA % values from individual comet structures summarised (median) per specimen and then used per treatment per species. Confidence limits are not determined for LOEC and NOEC values; in the table these cells are filled with a dash (-).

		Tail DNA % (raw)			Tail DNA % (median)		
		Cu	Confidence limit		Cu	Confidence limit	
Species		(mg/kg)	- 95%	+ 95%	(mg/kg)	- 95%	+ 95%
A. diffringens	EC_{10}	355	nd	nd	287	92.1	1898.8
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	20	-	-	> 640	-	-
	NOEC	< 20	-	-	>=640	-	-
Chilota sp.	EC_{10}	18	nd	nd	106	nd	nd
	EC_{20}	99	nd	nd	200	nd	nd
	EC_{50}	nd	nd	nd	674	nd	nd
	LOEC	20	-	-	> 640	-	-
	NOEC	< 20	-	-	>=640	-	-
E. andrei (A)	EC_{10}	nd	nd	nd	nd	nd	nd
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	160	-	-	> 640	-	-
	NOEC	80	-	-	>=640	-	-
E. andrei (B)	EC_{10}	nd	nd	nd	nd	nd	nd
	EC_{20}	nd	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	20	-	-	> 640	-	-
	NOEC	< 20	-	-	>=640	-	-
P. excavatus	EC_{10}	162	nd	nd	nd	nd	nd
	EC_{20}	334	nd	nd	nd	nd	nd
	EC_{50}	nd	nd	nd	nd	nd	nd
	LOEC	80	-	-	> 640	-	-
	NOEC	20	-	-	>=640	-	-

3.9 Species sensitivity distributions

Species sensitivity distributions (SSDs) were constructed from the available EC_{10}, EC_{50} and LOEC values for earthworm survival (Figure 3.27), reproduction (Figure 3.28), mass change during exposure (Figure 3.29), feeding avoidance response (Figure 3.30) and the NRR (Figure 3.31) and comet (Figure 3.32) assays. The HC_5 values (Hazardous concentration where 5% of species are affected) and 95% prediction intervals (PI) for all the SSDs that were constructed are presented in Table 3.8.

3.9.1 Earthworm survival

A species sensitivity distribution (SSD) was constructed with the available three LC_{50} values (Figure 3.27a). The HC_5 was 371 mg/kg Cu (95% prediction interval: 274.35 – 500.03) (Table 3.8).

Another SSD was constructed with the LC_{10} values (Figure 3.27b), and the HC_5 was 60 mg/kg Cu (95% prediction interval: 0.225 – 15968.85). A SSD could not be constructed with LOEC values, because only one LOEC value was available (for *A. diffringens*).

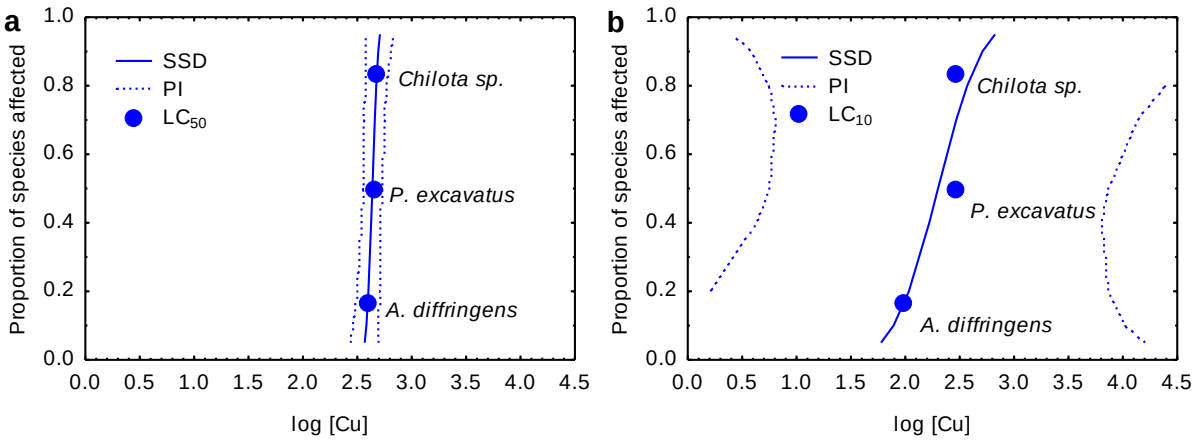


Figure 3.27: Species sensitivity distribution, from survival data, for three earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) LC_{50} values were used, and b) LC_{10} values were used. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

3.9.2 Earthworm reproduction: cocoon production

From the SSD constructed with EC_{50} values for cocoon production (Figure 3.28a), an HC_5 of 63 mg/kg Cu (95% prediction interval: 35.61 – 112.84) was calculated (Table 3.8). Another SSD was constructed with the EC_{10} values (Figure 3.28b), with a HC_5 of 33 mg/kg (95% prediction interval: 17.5 – 60.3). A SSD could not be constructed with LOEC values, because only two LOEC values were available (for *E. andrei* (A) and (B)).

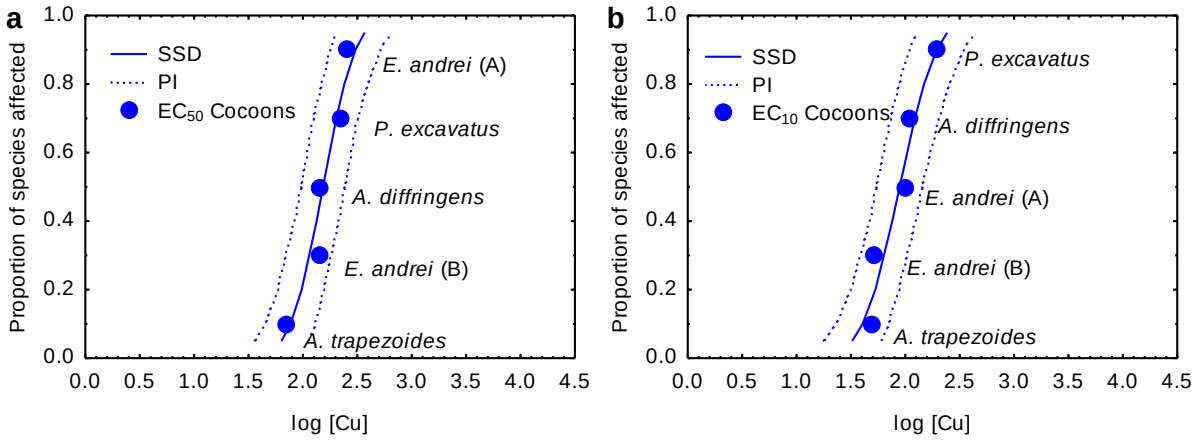


Figure 3.28: Species sensitivity distribution for cocoon production for five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) EC_{50} values were used, and b) EC_{10} values were used. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

3.9.3 Earthworm mass change during exposure

A SSD was constructed with the EC_{50} for the % mass change during exposure (Figure 3.29a) and a HC_5 of 15 mg/kg Cu (95% prediction interval: 2.10 – 102.76) was calculated (Table 3.8). From the SSD constructed with the EC_{10} values (Figure 3.29b), a HC_5 of 7 mg/kg (95% prediction interval: 1.85 – 25.11) was calculated. A SSD was also constructed with the LOEC values (Figure 3.29c), and a HC_5 of 130 mg/kg Cu (95% prediction interval: 103.54 – 162.43) was calculated.

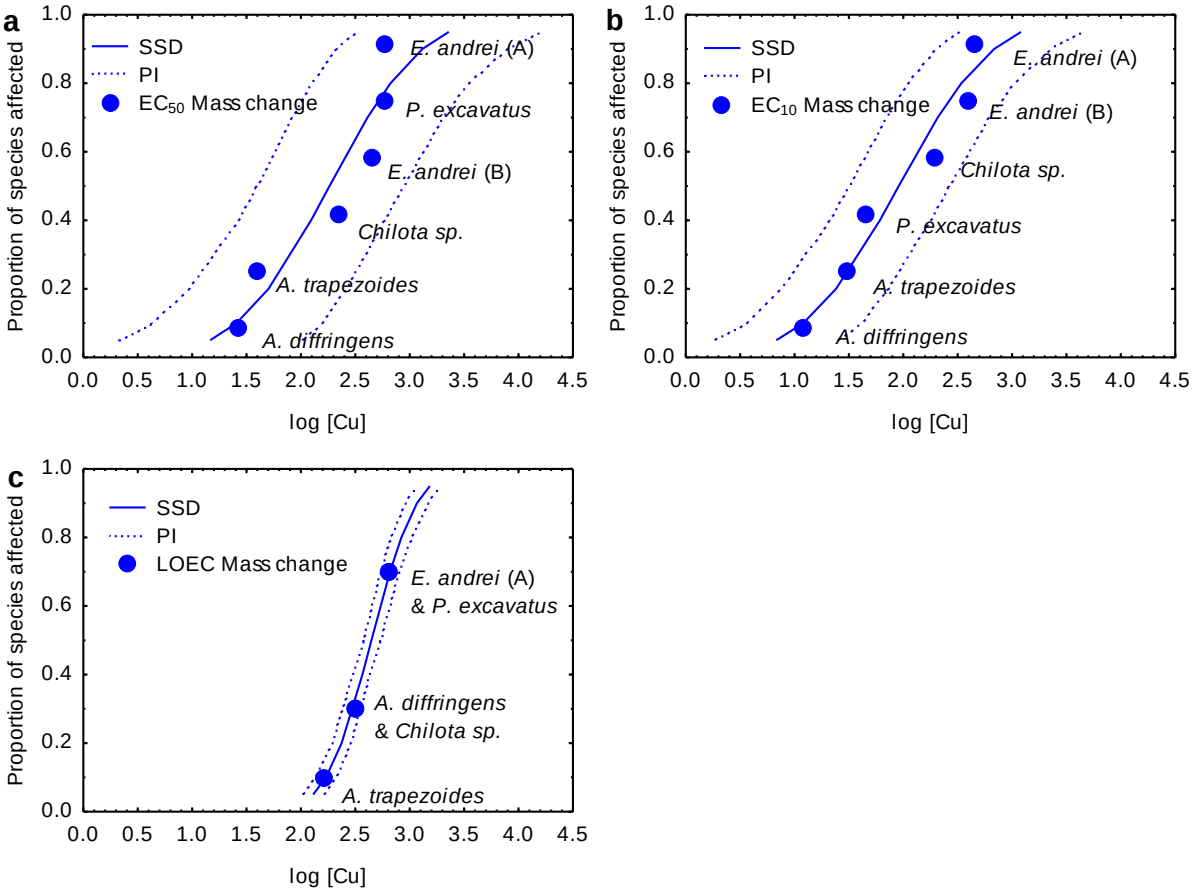


Figure 3.29: Species sensitivity distribution for mass change, for five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) EC_{50} values were used, b) EC_{10} values were used, and c) LOECs were used. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

3.9.4 Earthworm feeding behaviour (feeding avoidance response)

A SSD was constructed with the feeding avoidance response concentrations (Figure 3.30), and a HC_5 of 91.5 mg/kg Cu (95% prediction interval: 69.8 – 119.93) was determined (Table 3.8).

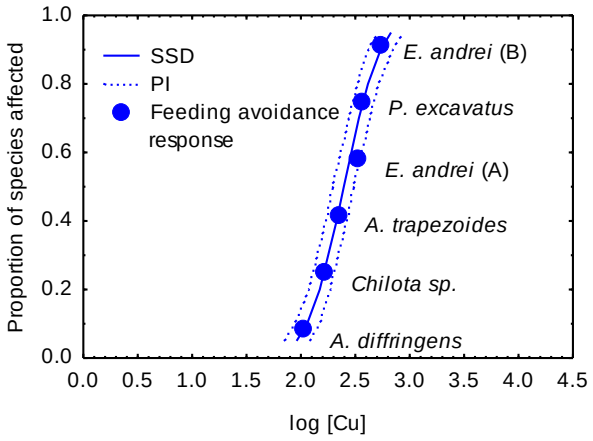


Figure 3.30: Species sensitivity distribution, for the feeding avoidance response for five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

3.9.5 NRR and MTT assays

A SSD was constructed with the available EC_{10} values for NRR (Figure 3.31) and the HC_5 was calculated as 0.52 mg/kg Cu (95% prediction interval: 1.4×10^{-10} – 1.94×10^{9}) (Table 3.8). Sufficient data were not available to construct a SSD for the MTT assay results.

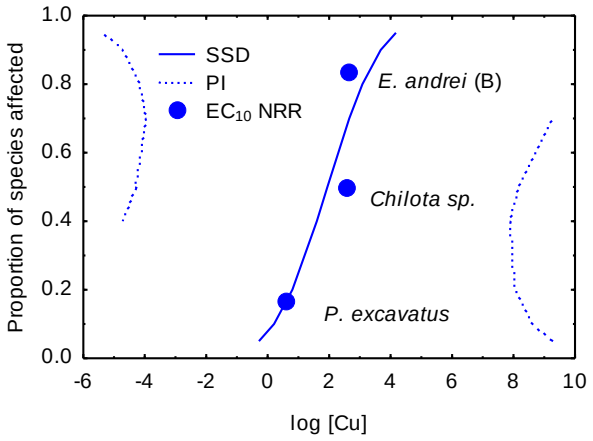


Figure 3.31: Species sensitivity distribution, based on EC_{10} values for neutral red retention (NRR, calculated as a % of the control response), in coelomocytes of three earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

3.9.6 Comet assay

A SSD was constructed from the EC_{10} values for Tail DNA % (raw) (Figure 3.32a), with a HC_5 of 6.6 (95% prediction interval: 0.01 – 4049.91) (Table 3.8). A SSD could also be constructed for Tail DNA % (raw) from the LOEC values (Figure 3.32b), and a HC_5 of 14.44 mg/kg Cu (95%

prediction interval: 8.01 – 29.80) could be calculated. No SSDs could be constructed for Tail DNA % (median).

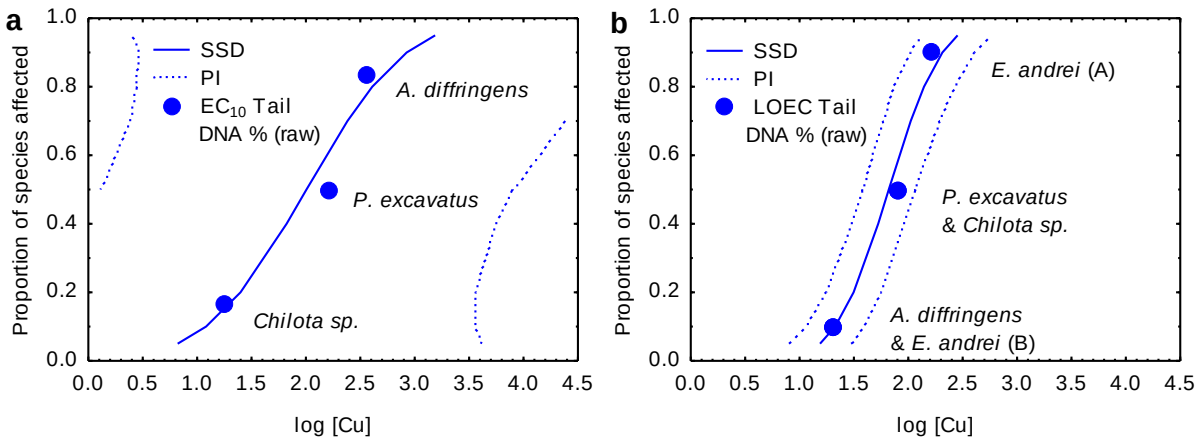


Figure 3.32: Species sensitivity distribution for DNA damage (Tail DNA % (raw), which are the Tail DNA % values obtained from individual comet structures used per treatment per species), performed on four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) EC_{10} values were used, and b) LOEC values were used. SSD = Species Sensitivity Distribution. PI = 95% Prediction Interval.

Table 3.8: HC_5 values (hazardous concentration where 5% of species are affected) from SSDs (species sensitivity distributions) constructed with LC_{50}, LC_{10}, EC_{50}, EC_{10} and LOEC values for seven endpoints from five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil for 14 days. PI = 95% prediction interval. nd = Could not be determined. The feeding avoidance response concentrations are not EC or LOEC values, but the concentrations where earthworms are assumed to decrease their feeding activities; therefore a dash (-) is used to fill the cell in the second column of the table.

Endpoint	LC, EC or LOEC	HC_5	Lower PI	Upper PI
Survival	LC_{10}	60	0.2	15968.8
	LC_{50}	371	274.4	500.0
	LOEC	nd	nd	nd
Cocoon production	EC_{10}	32	17.5	60.3
	EC_{50}	63	35.6	112.8
	LOEC	nd	nd	nd
Mass change	EC_{10}	7	1.8	25.1
	EC_{50}	15	2.1	102.8
	LOEC	130	103.5	162.4
Feeding avoidance response	-	91	69.8	119.9
NRR	EC_{10}	0.5	$1.4x10^{-10}$	$1.9x10^{+9}$
	EC_{50}	nd	nd	nd
	LOEC	nd	nd	nd
MTT	EC_{10}	nd	nd	nd
	EC_{50}	nd	nd	nd
	LOEC	nd	nd	nd
Comet assay	EC_{10}	7	0.01	4049.9
	EC_{50}	nd	nd	nd
	LOEC	15	8.01	29.80

3.10 Comparing different endpoints within each species

Within each species, the endpoints cocoon production, exposure mass change, NR retention, MTT conversion and Tail DNA % were compared with each other in order to determine whether significant correlations or notable patterns, indicating possible relationships, emerged. A notable pattern was only observed for cocoon production and exposure mass change (Figure 3.33).

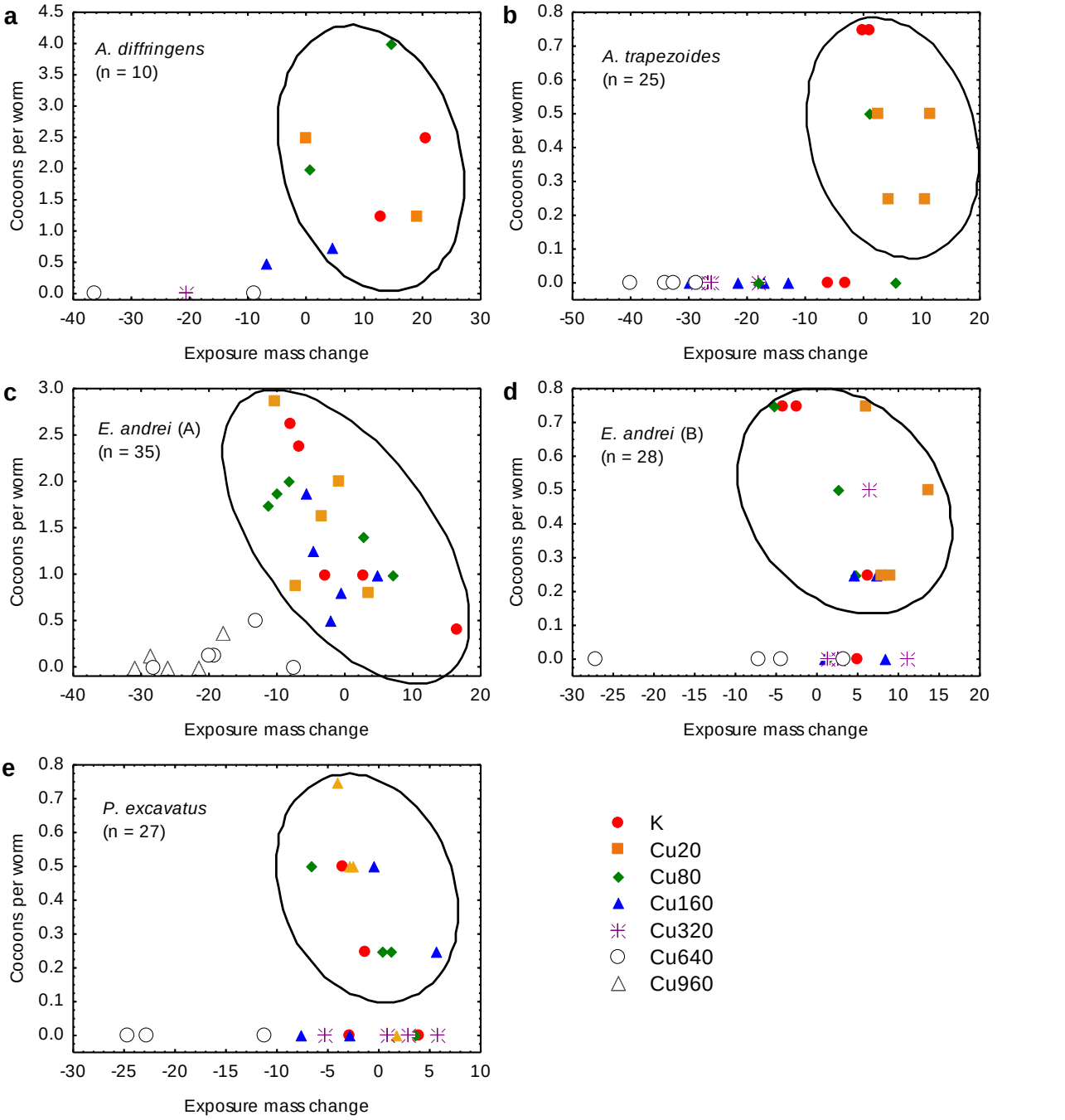


Figure 3.33: The number of cocoons produced per earthworm plotted against the mass change during exposure (exposure mass change, which is the difference between the mass after exposure and the mass before exposure, calculated as a percentage of the mass before exposure) for specimens of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. a) *A. diffringens*, b) *A. trapezoides*, c) *E. andrei* (A), d) *E. andrei* (B) and e) *P. excavatus*. The data points that are encircled indicate to an inverse relationship between the exposure mass change and the cocoons per worm.

For the comparison exposure mass change *versus* cocoons produced per earthworm, no significant positive correlations were found (Spearman R < 0.8, Appendix B, Table 44), although decreased cocoon production was noted to be coinciding with increased mass loss in *A. diffringens* (Figure 3.33a). Upon closer inspection of Figure 3.33, it can be seen that in *E. andrei* (A) (Figure 3.33c) and (B) (Figure 3.33d), *P. excavatus* (Figure 3.33e) and to a lesser extent *A. trapezoides* (Figure 3.33b), this relationship is inverted at the lower Cu concentrations, where cocoon production seems to be decreased when mass loss is decreased. When separate correlations were done between cocoons produced per earthworm and exposure mass change for each treatment in each species, a significant negative correlation emerged only for *E. andrei* (A) in the negative control (Spearman R = -0.97, $P < 0.01$, n = 5).

3.11 Visual comparison of biological parameters

A summary of the LC_{50}, feeding avoidance response concentrations and EC_{50} values determined for the whole-organismal and suborganismal responses are presented in Table 3.9.

The summarised results of the biological parameters (including the earthworm body Cu concentrations) are presented in Figures 3.34 (*A. diffringens*), 3.35 (*A. trapezoides*), 3.36 (*Chilota sp.*), 3.37 (*E. andrei* (A)), 3.38 (*E. andrei* (B)) and 3.39 (*P. excavatus*) to enable visual comparison between endpoints within a species as well as between species. In these figures, all endpoints, including earthworm body Cu, % survival, cocoons per earthworm, exposure mass change, depuration mass change, NR retention, MTT conversion and Tail DNA %, are plotted against the measured soil Cu concentration for each species. The measured background Cu concentrations in the sampling sites or culturing substrates are represented in these figures as a vertical dashed line. A second vertical line (dotted) represents the feeding avoidance response concentration (Section 3.5.6), calculated from the measured Cu concentrations, of each species.

Table 3.9: LC_{50} values, feeding avoidance response values and EC_{50} values for cocoon production, mass change, NRR-, MTT- and comet assays (in mg/kg Cu) for five earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days, summarised from Tables 3.3 to 3.7. nm - no mortality observed. nc - no cocoons observed. na - not analysed for NRR, MTT and comet assays. nd - not determined.

		Feeding avoidance response	EC_{50} (mg/kg)				
Species	LC_{50} (mg/kg)	(mg/kg)	Cocoon production	Mass change	NRR	MTT	Comet assay (Tail DNA % (median))
A. diffringens	393	222	146	27	nd	nd	nd
A. trapezoides	nm	104	71	40	na	na	na
Chilota sp.	471	162	nc	226	585	nd	674
E. andrei (A)	nd	332	255	592	nd	nd	nd
E. andrei (B)	nm	529	141	453	nd	nd	nd
P. excavatus	445	357	226	577	nd	nd	nd

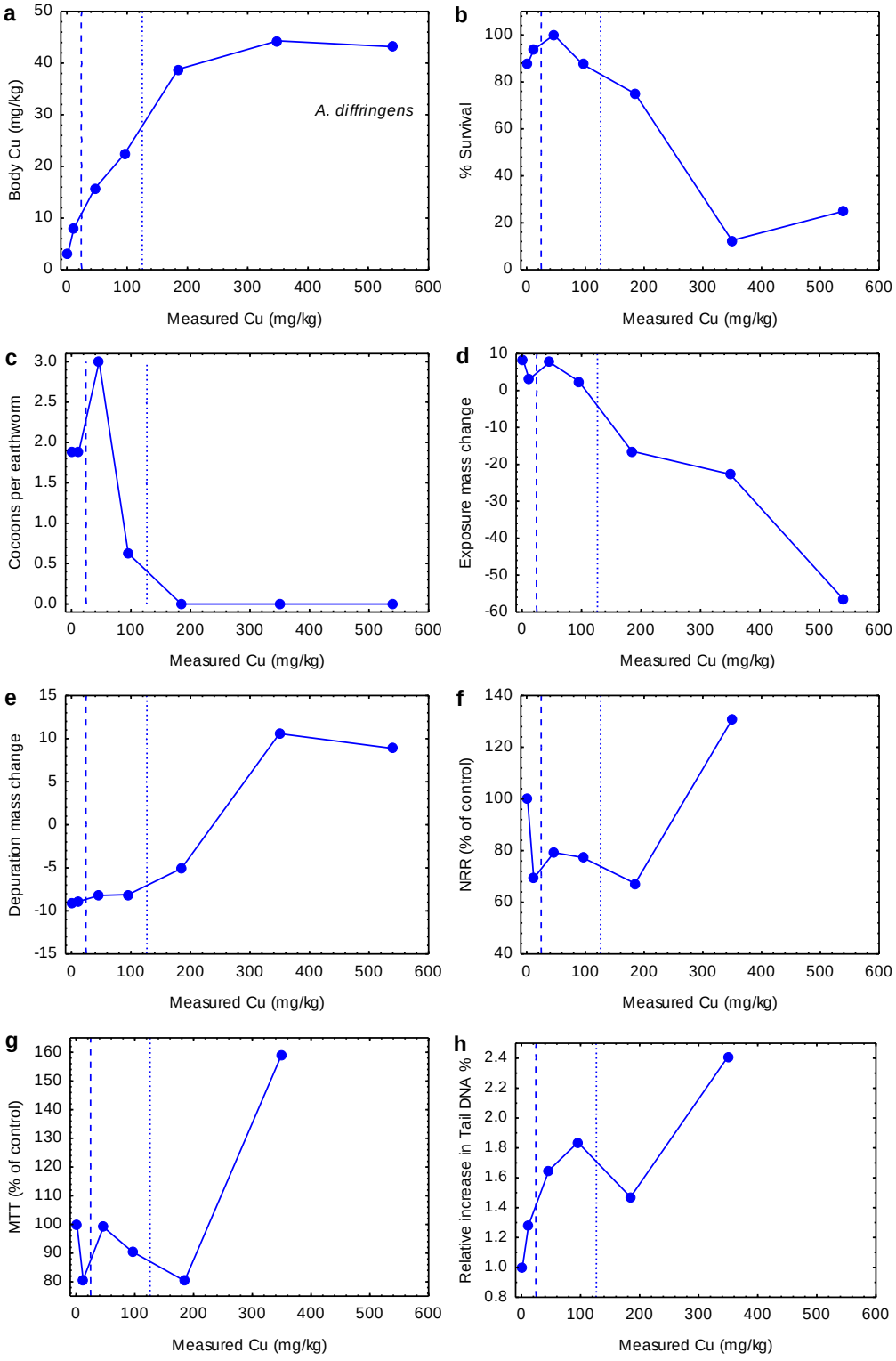


Figure 3.34: The mean values of results from eight endpoints measured in specimens of *A. diffringens* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (25 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (126 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival. c) The number of cocoons produced per earthworm. d) Mass change during the 14 day exposure period. e) Mass change during 24 h depuration on moist filter paper after exposure. f) Neutral red retention (NRR) of earthworm coelomocytes, calculated as a % of the control response. g) MTT conversion in earthworm coelomocytes, calculated as a % of the control response. h) The relative increase in Tail DNA % in earthworm coelomocytes, calculated as the Tail DNA % value at each concentration divided by the Tail DNA % value at the negative control.

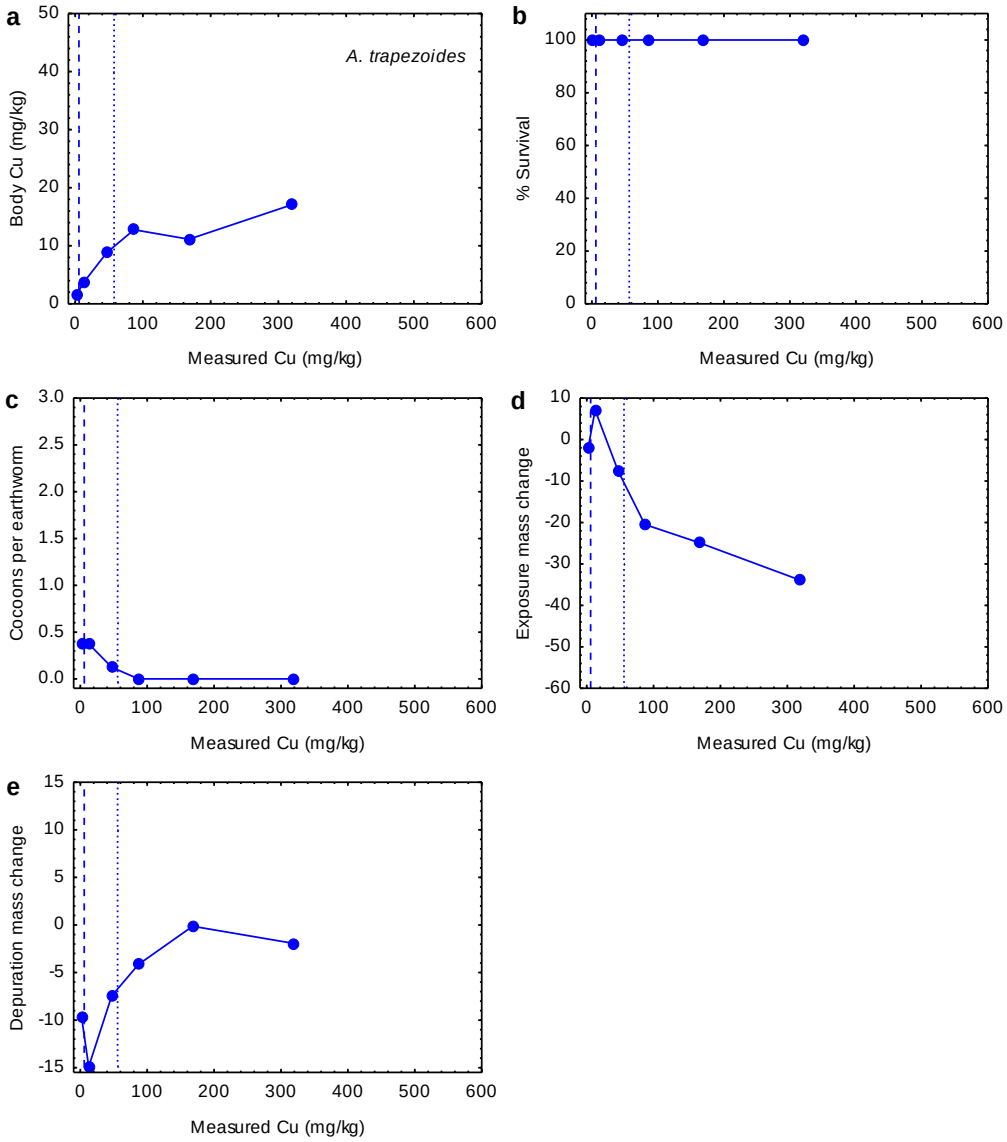


Figure 3.35: The mean values of results from five endpoints measured in specimens of *A. trapezoides* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (6 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (57 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival (all earthworms survived). c) The number of cocoons produced per earthworm during the 14 day exposure period. d) Mass change during the 14 day exposure period. e) Mass change during 24 h depuration on moist filter paper after exposure.

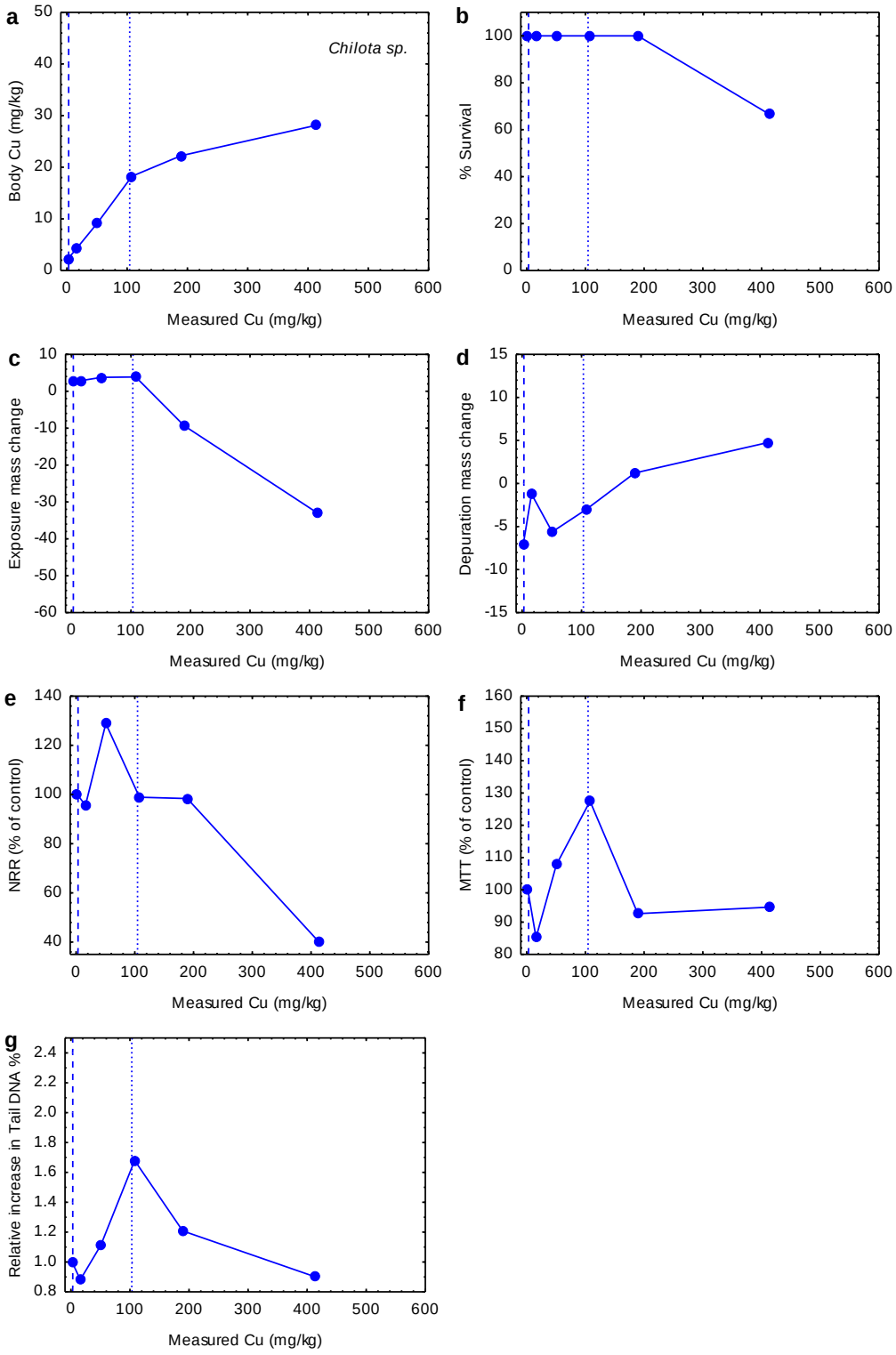


Figure 3.36: The mean values of results from seven endpoints measured in specimens of *Chilota sp.* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (3 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (104 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival. c) Mass change during the 14 day exposure period. d) Mass change during 24 h depuration on moist filter paper after exposure. e) Neutral red retention (NRR) of earthworm coelomocytes, calculated as a % of the control response. f) MTT conversion in earthworm coelomocytes, calculated as a % of the control response. g) The relative increase in Tail DNA % in earthworm coelomocytes, calculated as the Tail DNA % value at each concentration divided by the Tail DNA % value at the negative control.

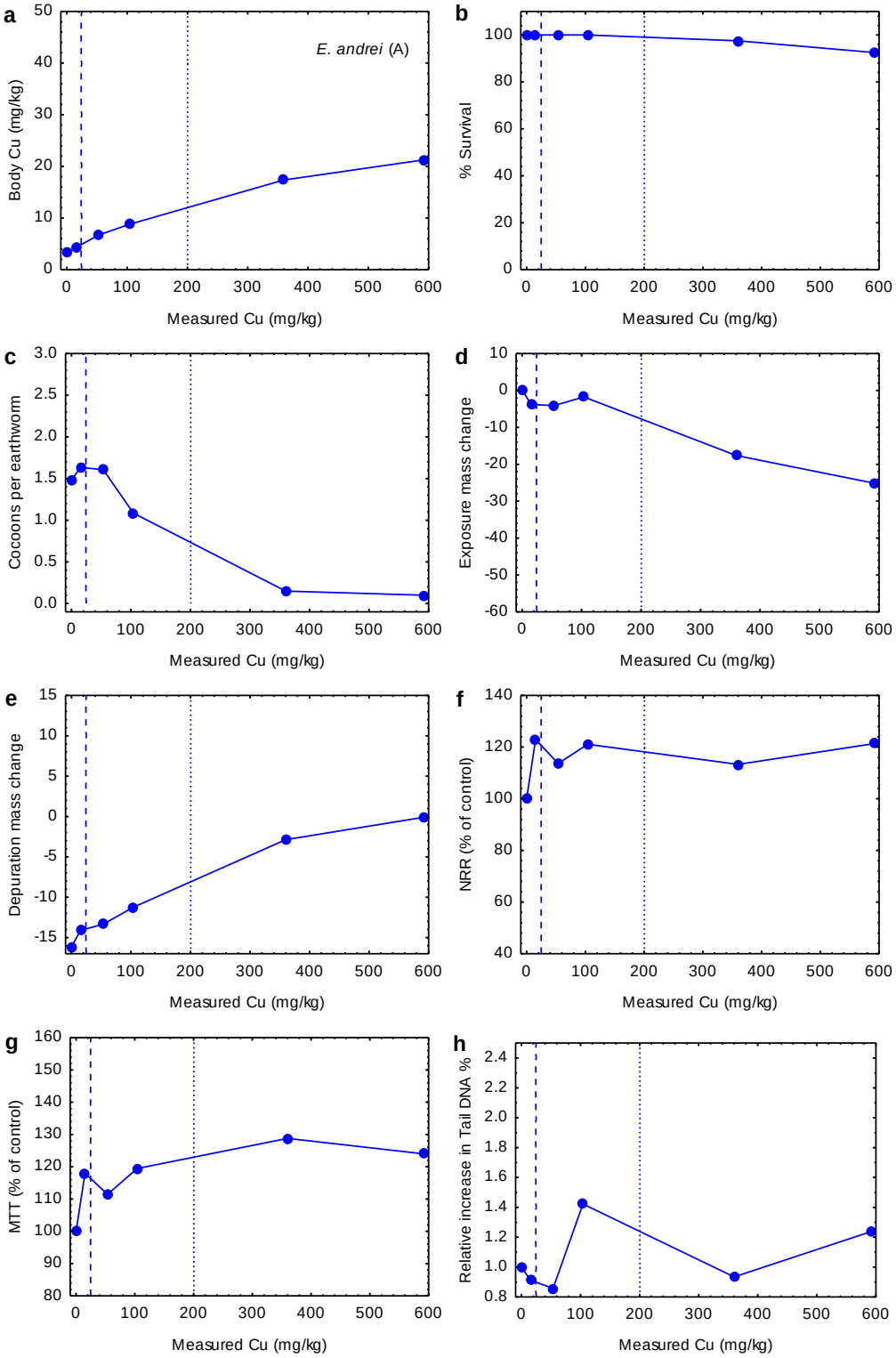


Figure 3.37: The mean values of results from eight endpoints measured in specimens of *E. andrei* (A) after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (25 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (201 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival. c) The number of cocoons produced per earthworm during the 14 day exposure period. d) Mass change during the 14 day exposure period. e) Mass change during 48 h depuration on moist filter paper after exposure. f) Neutral red retention (NRR) of earthworm coelomocytes, calculated as a % of the control response. g) MTT conversion in earthworm coelomocytes, calculated as a % of the control response. h) The relative increase in Tail DNA % in earthworm coelomocytes, calculated as the Tail DNA % value at each concentration divided by the Tail DNA % value at the negative control.

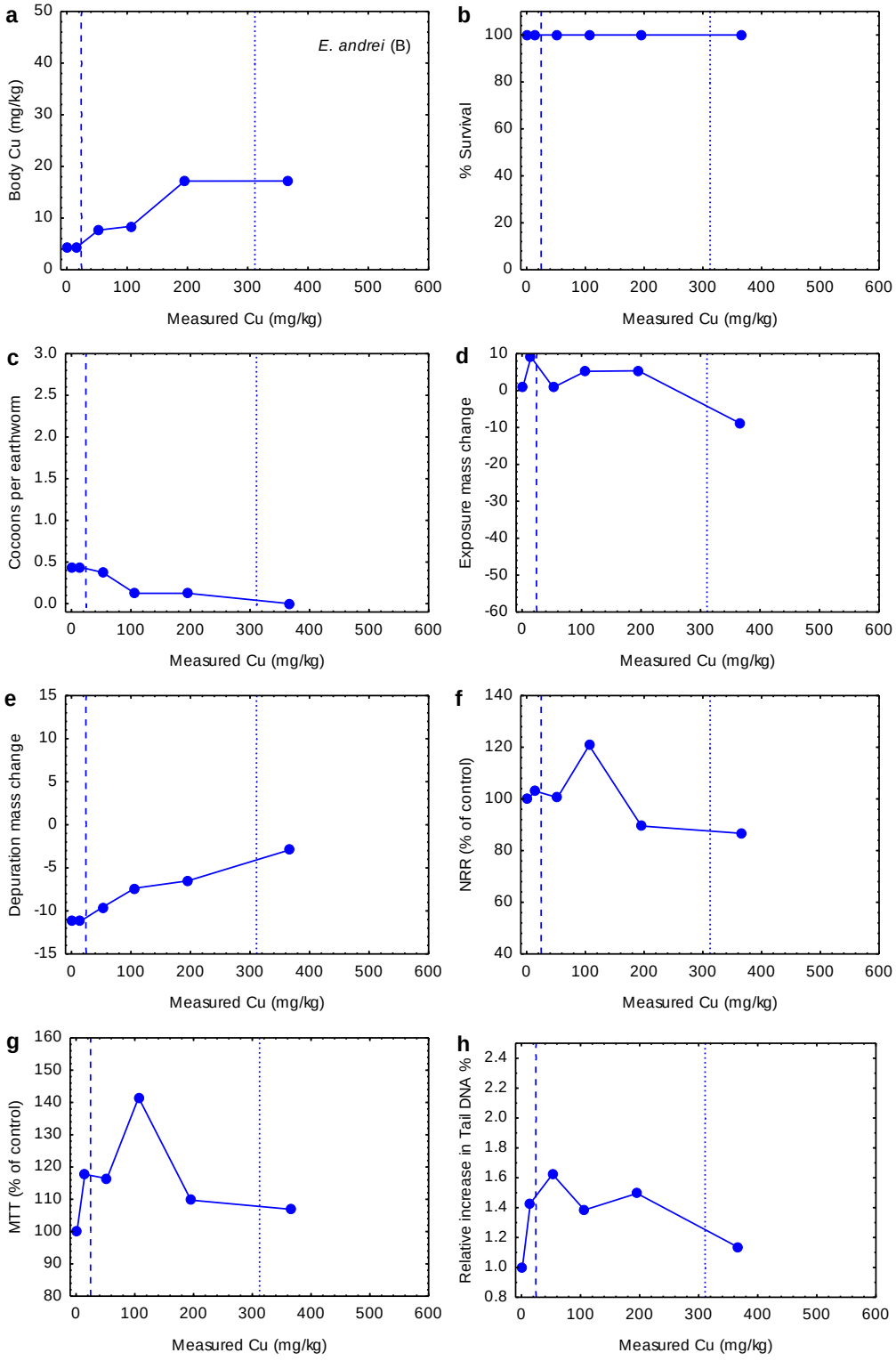


Figure 3.38: The mean values of results from eight endpoints measured in specimens of *E. andrei* (B) after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (25 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (312 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival (all earthworms survived). c) The number of cocoons produced per earthworm during the 14 day exposure period. d) Mass change during the 14 day exposure period. e) Mass change during 24 h depuration on moist filter paper after exposure. f) Neutral red retention (NRR) of earthworm coelomocytes, calculated as a % of the control response. g) MTT conversion in earthworm coelomocytes, calculated as a % of the control response. h) The relative increase in Tail DNA % in earthworm coelomocytes, calculated as the Tail DNA % value at each concentration divided by the Tail DNA % value at the negative control.

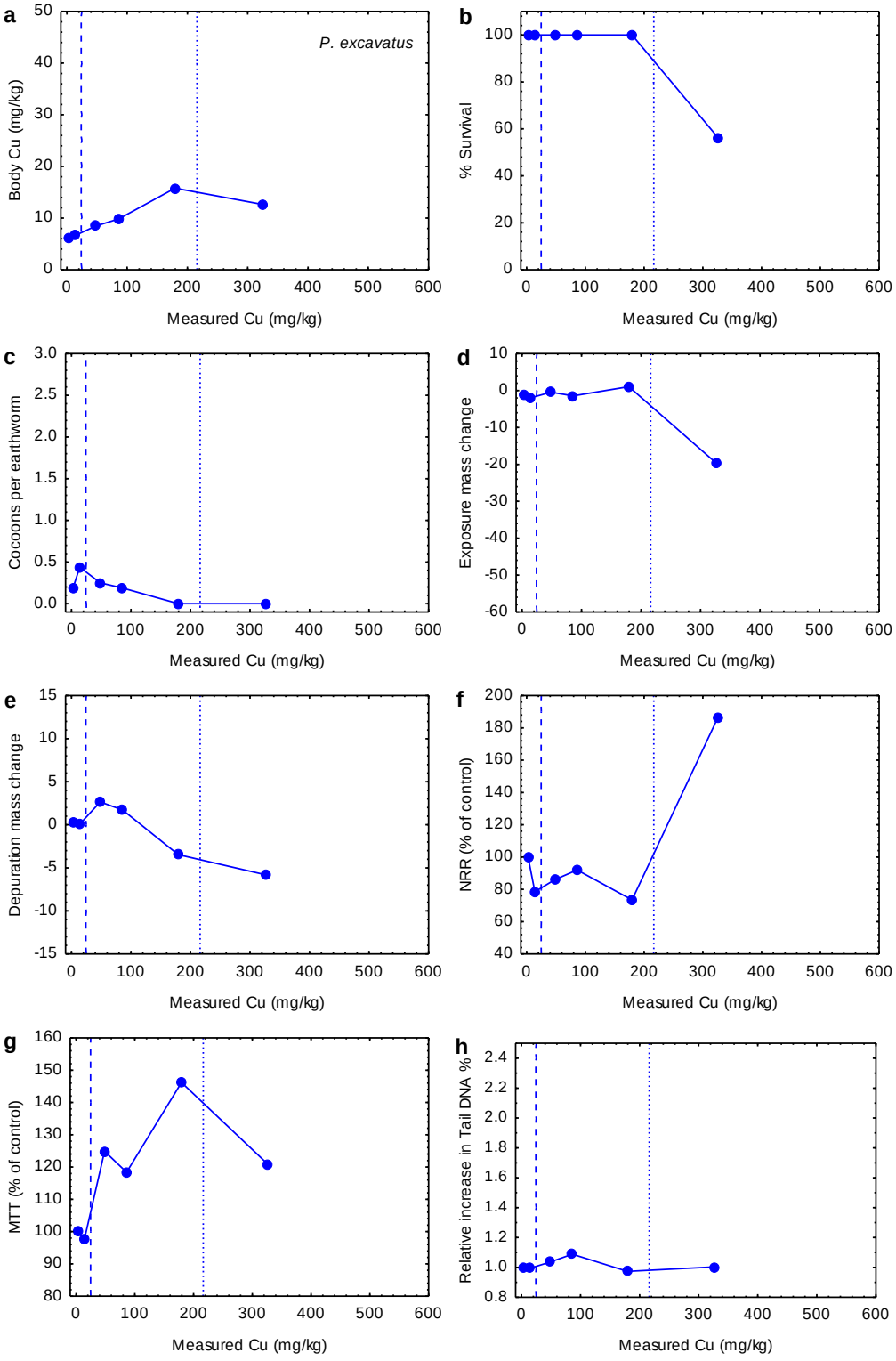


Figure 3.39: The mean values of results from eight endpoints measured in specimens of *P. excavatus* after 14 days exposure to Cu in the form of copper oxychloride in OECD soil, plotted against the measured Cu concentrations in the soil. The first vertical line (dashed), depicts the Cu concentration (25 mg/kg Cu) corresponding to the background levels in the earthworm culturing substrate. The second vertical line (dotted), depicts the Cu concentration (216 mg/kg Cu) corresponding to the feeding avoidance response. This figure serves an illustrative purpose; therefore only means are given without error bars; data with error bars and corresponding n are represented fully in figures and tables previously presented in this text. a) Cu in earthworm bodies. b) % Survival. c) The number of cocoons produced per earthworm during the 14 day exposure period. d) Mass change during the 14 day exposure period. e) Mass change during 24 h depuration on moist filter paper after exposure. f) Neutral red retention (NRR) of earthworm coelomocytes, calculated as a % of the control response. g) MTT conversion in earthworm coelomocytes, calculated as a % of the control response. h) The relative increase in Tail DNA % in earthworm coelomocytes, calculated as the Tail DNA % value at each concentration divided by the Tail DNA % value at the negative control.

Chapter 4: Discussion

4.1 Dose-response relationships for whole-organismal responses

During the present study, LC_{50} values were calculated for three earthworm species subsequent to 14 days exposure to copper oxychloride. Cocoon production EC_{50} values were calculated for four species, and mass change EC_{50} values and feeding response avoidance concentrations for all five species (Table 3.9). Comparisons of species sensitivities could therefore be made for the four whole-organismal endpoints studied using these LC_{50} and EC_{50} values and feeding avoidance response concentrations. No single species was the most sensitive for all four whole-organismal endpoints, and the order of species sensitivity was different for each of the whole-organismal endpoints. *Aporrectodea trapezoides* was the most sensitive species for both the feeding avoidance response and cocoon production, and *Amynthas diffringens* was the most sensitive species for both survival and mass change. Comparisons of species sensitivity were therefore done for each endpoint separately, and will be discussed in the following sections.

4.1.1 Survival

The most sensitive species in terms of LC_{50} values was *A. diffringens*, followed by *Perionyx excavatus* and *Chilota sp.* (Table 3.9). The least sensitive species was *A. trapezoides*, where no mortality occurred at any of the Cu concentrations, followed by *Eisenia andrei* (Table 3.2). The lack of earthworm mortality in *E. andrei* (B), and the low levels or mortality in *E. andrei* (A) in the treatments 640 and 960 mg/kg Cu are to be expected, since the majority of LC_{50} values reported for the closely related *E. fetida* (LC_{50} values for *E. andrei* could not be found during the literature survey) (Table 4.1), are above the exposure range used during the present study.

Table 4.1: LC_{50} values for various earthworm species exposed to various copper formulations on various types of substrates, as gained from the literature

Species	LC_{50} (mg/kg Cu)	Exposure time	Cu formulation	Substrate	Reference
Various species	218 to 1000	various	various	various	Wightwick *et al.* (2008)
A. caliginosa	640	8 weeks	$CuSO_4$	field soil	Khalil *et al.* (1996b)
P. excavatus	103	4 days	$CuSO_4$	unspecified soil	Vaidya & Kulkarni (2006)
E. fetida	643	2 weeks	$Cu(NO_3)_2$	artificial soil[a]	Neuhauser *et al.* (1985)
E. fetida	683	2 weeks	$Cu(NO_3)_2$	OECD soil	Spurgeon *et al.* (1994)
E. fetida	> 1400	3 weeks	$CuCl_2$	field soil	Scott-Fordsmand *et al.* (2000)
E. fetida	1002, 453[b]	4 weeks	$CuCl_2$	OECD, LUFA[b]	Kula & Larink (1998)
E. fetida	519	4 weeks	copper oxychloride	artificial soil[a]	Maboeta *et al.* (2004)

a – has the same composition as OECD artificial soil
b – for OECD and LUFA soil, respectively

This lower sensitivity of *E. andrei* to Cu, compared to those of the other species used during the present study, in terms of survival as well as for most of the other investigated endpoints (Table 3.9), is in agreement with a number of studies that found *E. andrei* and *E. fetida* less sensitive than other earthworm species to toxicant exposure (Spurgeon & Hopkin 1996; Spurgeon *et al*. 2000; Eijsackers 2004; Langdon *et al*. 2005; Lukkari *et al*. 2005). Information on the lethality of Cu towards *A. trapezoides* could not be found in the literature and it is therefore assumed that the present study is the first to show that this species can survive Cu concentrations of up to 640 mg/kg in OECD soil during 14 days of exposure. It is also assumed that the present study is the first to report 14-day LC_{50} values for Cu for *A. diffringens* and *Chilota sp.*, as no LC_{50} values for Cu for these species were found in the literature during the present study. The LC_{50} values for these species that were calculated during the present study, as well as that of *P. excavatus* (Table 3.3) are within the range reported in a study by Wightwick *et al.* (2008) for various earthworm species exposed to Cu (Table 4.1). The LC_{50} value calculated for *P. excavatus* during the present study is higher than that reported by Vaidya & Kulkarni (2006) (Table 4.1), the only other study presenting an LC_{50} value for Cu for this species. This difference is most probably due to the use of different exposure substrates and different copper formulations.

4.1.2 Cocoon production

Cocoons were produced by all species (Figure 3.4) except by *Chilota sp.*. The general biology and life cycle of this species is unknown, and no information could be found in the literature on the life cycle of any species from the genus *Chilota*. It can therefore only be speculated that either the 14 day period was too short for cocoon production, or that laboratory conditions were not ideal for the earthworms. Cocoon production was thus not considered to be a valid endpoint for determining sensitivity in this species. The present study is the first to use an indigenous South African earthworm species from the genus *Chilota* to investigate the effects of toxicant exposure, and the results obtained here for the other endpoints investigated (discussed in the next sections) indicate that this is a sensitive endemic South African organism and a promising species to use in ecotoxicological research. It is recommended that its life-cycle and suitability for laboratory culture and testing is investigated in future.

Clear dose responses were observed for cocoon production in the other species used during the present study (Figure 3.4). The most sensitive species in terms of EC_{50} values was *A. trapezoides*, followed by *E. andrei* (B), *A. diffringens*, *P. excavatus* and *E. andrei* (A) (Table 3.4).

Cocoon production decreased with increasing Cu concentration, but no statistically significant correlations were found between cocoon production and either the soil Cu concentrations or the body Cu concentrations in any of the species. This lack of significant correlation for any of the species is due to the shape of the dose-response curves, where cocoon numbers were higher than in the negative controls at the treatments with 20 and 80 mg/kg Cu in *E. andrei* (A) and *P. excavatus*, and at 80 mg/kg Cu in *A. diffringens* (Figure 3.4). This type of dose-response is not uncommon for earthworms exposed to Cu, and have been observed in other studies (Van Gestel *et al*. 1989; Spurgeon *et al*. 2003a; Bindesbøl *et al*. 2007). It is possible that, since Cu is an essential metal, the Cu exposure concentrations of 20 and 80 mg/kg used during the present study may be optimal for cocoon production (Van Gestel *et al*. 1989; Spurgeon *et al*. 2003a), but that the lower levels of Cu in the negative control are sufficient to sustain cocoon production in the earthworms. Alternatively, this increased cocoon production at the lower Cu exposure concentrations, as was observed during the present study, may be a result of some kind of compensatory mechanism, perhaps to ensure survival of the species in elevated stress conditions. However, for an essential metal such as Cu, it is difficult to distinguish between hormesis, which is linked to such a compensatory mechanism (Stebbing 1982) and essentiality (Svendsen & Weeks 1997). This type of dose-response curve could also be due to the inhibition of parasites (Holmstrup *et al*. 2010a), such as nematodes (Bindesbøl *et al*. 2007) and bacteria (Olchawa *et al*. 2006) by Cu concentrations lower than those which inhibit earthworm cocoon production.

The differences between species sensitivity observed during the present study in terms of cocoon production could have been due to the combined stress of exposure to Cu and sub-optimal exposure conditions for some species, since exposure conditions were standardised for all species in order to facilitate comparisons of species sensitivity. The type of exposure substrate could cause differences in cocoon production between species. For example, Kula & Larink (1998), using *Aporrectodea caliginosa* which is closely related to *A. trapezoides* used during the present study, found an unsatisfactory low cocoon production rate in OECD soil as opposed to LUFA soil. It is possible that the rate of cocoon production in *A. trapezoides* could similarly have been inhibited to some extent due to the use of OECD soil during the present study. Some earthworm species may prefer a higher organic matter content or a different type of food than that supplied during the experiments, and the available palatable food can be less than optimal for these species, which could lead to decreased cocoon production rates. It is known that a lack of food leads to decreased cocoon production in some earthworm species, as has been demonstrated by Reinecke and Viljoen (1990) for *E. fetida*. Other exposure conditions, such as temperature and soil moisture requirements for optimal cocoon production vary between species (Reinecke *et al*. 1992; Lowe & Butt 2005) and could have caused

the observed differences in species sensitivity in cocoon production during the present study. For example, both the optimum temperature and soil moisture content for cocoon production in *P. excavatus* are higher (25 °C and 80% moisture content) (Hallatt *et al*. 1992) than those used during the present study. It is therefore possible that *P. excavatus* specimens did not produce the maximum number of cocoons that it potentially could produce during the present study.

From the literature it is deduced that the present study is the first to report on the effects of copper oxychloride on cocoon production in *A. diffringens*, *P. excavatus* and *A. trapezoides*, since no such literature could be found. The EC_{50} value of 71 mg/kg Cu calculated during the present study for *A. trapezoides* is close to that found for the closely related *A. caliginosa* (Table 4.2) by Ma (1988), despite the fact that different copper formulations and substrate types were used. It is possible that these two species are very similar in terms of their sensitivity towards copper.

Table 4.2: EC_{50} values for cocoon production in various earthworm species exposed for various times to various copper compounds on various types of substrates, as gained from the literature

Species	EC_{50} (mg/kg Cu)	Exposure time	Cu formulation	Substrate	Reference
A. caliginosa	68	4 weeks	$CuCl_2$	field soil	Ma (1988)
E. andrei	331[a]	4 weeks	$CuCl_2$	OECD soil	Kula & Larink (1997)
E. andrei	62 & 191	1 & 3 weeks[b]	$CuCl_2$	artificial soil[c]	Van Gestel *et al.* (1989)
E. fetida	53	8 weeks	$Cu(NO_3)_2$	OECD soil	Spurgeon *et al.* (1994)
E. fetida	309	4 weeks	copper oxychloride	OECD soil	Owojori *et al.* (2009)
E. fetida	210 & 517[d]	3 weeks	$CuCl_2$	field soil	Scott-Fordsmand *et al.* (2000)
E. fetida	138	4 weeks	$CuCl_2$	artificial soil[c]	Kula & Larink (1998)

a – calculated from their data during the present study
b – the 3-week period was subsequent to the 1-week period
c – has the same composition as OECD artificial soil
d – in freshly contaminated and soils contaminated for 70 years, respectively

The EC_{50} values for cocoon production calculated during the present study for *E. andrei* (A) and (B) (255 and 141 mg/kg Cu, respectively) falls within a range of values gained from the literature for *E. andrei* and *E. fetida*, as presented in Table 4.2. The difference in EC_{50} values between *E. andrei* (A) and *E. andrei* (B), calculated during the present study, could be due to higher variation in *E. andrei* (B) (Figure 3.4) and thus a higher margin of statistical error. The confidence intervals could thus not be determined for the EC_{50} value calculated for *E. andrei* (B) (Table 3.4), and this value should be interpreted cautiously.

Selective allocation of energy to either reproduction or maintenance of body mass could have played a role in determining the observed differences between the two *E. andrei* groups, since specimens of *E. andrei* (B) gained more mass during exposure than *E. andrei* (A) (Figure 3.11). This trade-off between cocoon production and body mass maintenance was also evident to some

degree for the other species used in the present study (Figure 3.33). This type of selective allocation of energy during toxic stress is well-studied in earthworms (Kokta 1992; Nisbet *et al*. 2000; Van Gestel & Hoogerwerf 2001; Widarto *et al*. 2004; Galay Burgos *et al*. 2005; Leduc *et al*. 2008). Species-specific mechanisms of selective energy allocation would thus have resulted in differences in sensitivity between species.

Benomyl was selected as positive control for cocoon production as it is recommended by the OECD (2004) for *E. fetida* and *E. andrei*. Exposure to benomyl did not cause a reduction in cocoon production in *A. diffringens* (Figure 3.4), and the mass of these specimens increased during exposure (Figure 3.5). This apparent of sensitivity raises concern that benomyl might not be an appropriate reference substance for species such as *A. diffringens*. It is therefore recommended that the effects of benomyl and other possible reference substances on the life-cycle parameters of this species should be investigated thoroughly in further experiments.

4.1.3 Biomass change and feeding avoidance behaviour

Although copper oxychloride treatment caused mass loss at high concentrations during exposure in all species during the present study (Figure 3.11), no significant negative correlations were found between any of these endpoints and either the soil or earthworm body Cu concentrations for any of the species, except for *A. trapezoides*. In this species, a significant negative correlation was found between the exposure mass change and the soil Cu concentration. The lack of significant correlations in the other species is due to the shape of the dose-response curves (Figures 3.5 to 3.11), where earthworm mass changes during exposure only decreased drastically at concentrations higher than 160 mg/kg Cu in *A. diffringens*, *Chilota sp.* and *E. andrei* (A), and at concentrations higher than 320 mg/kg Cu in *E. andrei* (B) and *P. excavatus*. This type of dose-response may be due to the essential nature of Cu, as was discussed in the previous section for cocoon production. Since Cu is an essential metal, the low Cu exposure concentrations used during the present study may be optimal for the maintenance of earthworm body mass.

The EC_{50} values for mass loss indicated that the most sensitive species was *A. diffringens* (Table 3.9), followed by *A. trapezoides*, *Chilota sp.*, *E. andrei* (B), *P. excavatus* and *E. andrei* (A). These species differences in loss of biomass could have been caused by species-specific feeding behaviour. Not only could the suitability and palatability of the food supplied during the present study have differed between species, but the presence of Cu could have led to reduced feeding activity in the earthworms. It is well known that the presence of Cu in soil can reduce the feeding activity of earthworms (Ma 1984; Svendsen & Weeks 1997; Kula & Larink 1998; Depta *et al*.

1999; Spurgeon *et al*. 2004a). A decrease in feeding activity at high metal concentrations could result in less energy available for growth and survival (Holmstrup *et al*. 2010a) and detoxification processes, resulting in increased susceptibility to the toxicant (Nisbet *et al*. 2000). Therefore, species differences in feeding activity of earthworms can determine species differences in their eventual sensitivities to a toxicant. The results of the present study indicated that the different species started to avoid feeding on the Cu-contaminated food at different concentrations (Figure 3.12), which could have resulted in the observed differences between species in their mass changes. These feeding avoidance response concentrations were between the NOEC and LOEC values for mass change (Table 3.5) for all species where these values were calculated. The feeding avoidance response concentrations can therefore be seen as an approximate indication of the concentration where earthworm feeding activity could have started to decrease and thus cause mass loss during exposure.

The most sensitive species in terms of the feeding avoidance responses was *A. trapezoides*, followed by *Chilota sp.*, *A. diffringens*, *E. andrei* (A), *P. excavatus* and *E. andrei* (B). Both *A. trapezoides* and *Chilota sp.* can be classified as endogeic species, and their increased sensitivity in terms of their feeding behaviour could be due to their feeding preferences, since endogeic species mainly ingest mineral soil (Curry & Schmidt 2007). It is possible that, although *A. trapezoides* and *Chilota sp.* did incorporate the contaminated cattle manure into the OECD soil as witnessed during experimentation, they could have ceased feeding on the contaminated manure at an earlier stage than the other species due to the combined stress of toxicant exposure and sub-optimal food quality. It is also possible that an exposure history of elevated Cu concentrations could have resulted in increased tolerance in *A. diffringens*, *E. andrei* and *P. excavatus* in terms of their feeding behaviour, since they were collected from substrates containing higher Cu concentrations than those where *A. trapezoides* and *Chilota sp.* were collected (Table 3.1).

4.2 SSDs for whole-organismal responses

It was possible to construct SSDs using the feeding avoidance response concentrations and the LC_{50} or EC_{50} values for the other whole-organismal responses (Figures 3.26 to 3.29) calculated during the present study. The 95% prediction intervals of the resulting whole-organismal HC_5 values were fairly large (Table 3.8), and the HC_5 values should therefore be interpreted carefully. These large prediction intervals were most probably due to the small number of data points used for SSD construction (from three values for the endpoint survival to six for mass change and feeding avoidance response), which would have decreased statistical reliability. Although minima of 3, 5 or

8 data points (species) are acceptable for the construction of SSDs (Suter II *et al*. 2002), the recommended minimum number of data points is generally agreed to be at least between 10 and 15 (Wheeler *et al*. 2002) to increase statistical robustness.

The HC_5 value obtained from the SSD constructed with LC_{50} values during the present study (371 mg/kg Cu), is higher than an HC_5 value (353 mg/kg Cu) calculated by Frampton *et al.* (2006) for Cu. These authors used data obtained from the literature to construct a SSD from LC_{50} values (in mg/kg Cu) for a range of terrestrial invertebrates exposed to a variety of copper-containing pesticides in standard test conditions similar to those utilised during the present study. Only three of the 12 invertebrate species used by Frampton *et al.* (2006) were earthworm species: *E. andrei* / *E. fetida* (grouped together as one species), *Lumbricus terrestris* and *Aporrectodea caliginosa*. It is possible that the difference between the HC_5 value calculated during the present study and the value calculated by Frampton *et al.* (2006) is due to their inclusion of other invertebrate species with lower LC_{50} values, as well as their use of data obtained for a variety of copper formulations, which could have affected LC_{50} values.

The HC_5 value for cocoon production calculated during the present study (63 mg/kg) is higher than the HC_5 value (55 mg/kg Cu) calculated by Jänsch *et al.* (2007) based on EC_{50} values from chronic tests in soil invertebrates exposed to various copper compounds. This difference is most probably also due to the inclusion of other invertebrate species with lower EC_{50} values in their dataset, as was discussed above for the HC_5 values calculated from SSDs constructed with LC_{50} values.

4.3 Dose-response relationships for suborganismal responses

On the suborganismal level, EC_{50} values could only be calculated for *Chilota sp.* for the NRR and comet assays during the present study (Table 3.9). EC_{10} values could however be calculated for three species from the results of the NRR and comet assays (Tables 3.6 and 3.7, respectively). LOEC values were calculated from the results of the comet assay for all species. Species sensitivities could therefore be compared using these EC_{10} and LOEC values, and as with the whole-organismal responses, no single species was the most sensitive for all endpoints. Comparisons of species sensitivity were therefore done for each endpoint separately, and will be discussed in the following sections.

4.3.1 Neutral red retention (NRR) assay

During the present study, species sensitivity differences in neutral red (NR) retention responses were observed in earthworms exposed to copper oxychloride (Figure 3.14). Such species differences on the suborganismal level could be due to different amounts of toxicant reaching the target due to differences between species in the uptake, regulation and accumulation of the toxicant. The regulation of a toxicant such as a metal in an organism can be facilitated by compensatory mechanisms involving stress proteins such as metallothioneins, metallothionein-like metalloproteins, non-metallothionein-like metalloproteins, heat shock proteins and histidine (Dhainaut & Scaps 2001). Species differences in regulation of Cu in earthworms, for example, could be due to the presence of different metallothionein isoforms (Stürzenbaum *et al*. 1998b) that possess different abilities to bind to Cu (Stürzenbaum *et al*. 2001). The metallothionein isoforms may differ between species, which would result in species differences in regulation and detoxification ability of metals such as Cu. It was shown during the present study that body Cu was regulated in all species to some extent, evidenced by the decreasing bioconcentration factors (BCFs) with increasing Cu exposure concentration (Figure 3.3b), and that this regulation varied between species (Figures 3.2 and 3.3). Specimens of *A. diffringens* had taken up the highest body Cu when exposed to 640 mg/kg Cu, indicating that regulation of Cu probably had occurred to a lesser extent in this species than in the other species (Figure 3.3). *A. diffringens* was the only species where statistically significant reductions in NRR were observed in specimens exposed to any of the Cu treatments. Statistically significant differences were found for this species between the negative control and all Cu treatments except 640 mg/kg Cu (Figure 3.14). It is therefore possible that body Cu levels were sufficiently high to cause significant decreases in NR retention in coelomocytes of this species.

Body Cu levels were lower in *Chilota sp., E. andrei* and *P. excavatus* than in *A. diffringens*, possibly due to regulation of this essential metal, as shown by the decreasing BCFs with increasing Cu exposure concentration (Figure 3.3b). Body Cu concentrations were probably not high enough to cause statistically significant decreases in NR retention in coelomocytes obtained from these species, although some decreases in NRR values could be seen to correspond to increases in body Cu (Figure 3.15) in some specimens. Although no significant correlations were found between body Cu content and NR retention in any of the species (Figure 3.15), NRR values did decrease with increasing body Cu concentration in *E. andrei* (A) and *P. excavatus*, and also in *A. diffringens* at body Cu concentrations lower than 40 mg/kg Cu, and in *Chilota sp.* specimens exposed to 160 and 320 mg/kg Cu, except for one specimen. NRR values also decreased with increasing Cu body concentration in *E. andrei* (B) specimens exposed to 80 to 640 mg/kg Cu, except for four specimens

exposed to 320 and 640 mg/kg Cu. In those specimens of *A. diffringens*, *Chilota sp.* and *E. andrei* (B) exposed to 320 and 640 mg/kg Cu where NRR values did not decrease with increasing body Cu concentration (Figure 3.15), it is possible that a compensatory mechanism, such as those mentioned above, was activated that would have aided in the detoxification of Cu. This would have resulted in decreased amounts of available Cu and thus decreased levels of cytotoxicity in coelomocytes. It is also possible that apoptotic cell death, as discussed below, resulted in a smaller healthier remaining coelomocyte population in those specimens, which would have resulted in elevated NRR values.

Neutral red retention was increased drastically after exposure to 640 mg/kg Cu in *A. diffringens* and *P. excavatus* (Figure 3.14). Upon investigation of Figure 3.15, it can be seen that *P. excavatus* specimens exposed to the treatment with 640 mg/kg Cu had similar body Cu concentrations than those exposed to the lower Cu concentration treatments. It is thus possible that at the treatment with 640 mg/kg Cu, a compensatory mechanism, linked to Cu detoxification, could have been activated in these specimens, as mentioned above. It is also possible that the decreased levels of cytotoxicity in *A. diffringens* and *P. excavatus* specimens exposed to 640 mg/kg Cu, as reflected by the increased NRR values in these specimens (Figure 3.14), could have been due to a less damaged coelomocyte population remaining after apoptotic cell death of the majority of the most damaged coelomocytes. It is known that exposure to metals such as Cu, Pb and Cd leads to decreases in coelomocyte numbers through apoptosis (Homa *et al*. 2007). It was also observed during the present study that the protein content and thus coelomocyte number in coelomic fluid obtained from *A. diffringens* and *P. excavatus* specimens from the 640 mg/kg Cu treatment were lower than that of specimens exposed to lower Cu concentrations (Figure 3.13), suggesting that apoptotic cell death could have occurred. It is therefore possible that the NRR values were increased due to the removal of the most damaged coelomocytes through apoptosis. These increased NRR values could also be due to the following: When the final NRR values were calculated, the spectrophotometric NRR value obtained after correction for background noise for each specimen was divided by the protein concentration of the coelomic fluid of that specimen to determine the relative amount of NRR retained per cell (as described in section 2.6.3). Since the NRR assay measures the amount of NR dye retained in adherent, live coelomocytes after a washing step, this amount of NR dye in the sample would be the same whether or not the damaged and nonadherent cells were still intact (and could therefore be detected with the Bradford protein assay) or disintegrated prior to coelomocyte extraction due to apoptosis. Since the NRR value is divided by the protein concentration value to obtain a final NRR value, division by a low protein concentration value (such as in the case of a small coelomocyte population due to apoptosis) would result in a high final NRR value, and

division by a high protein concentration value (where no or little apoptosis occurred) would result in a lower final NRR value.

Other increases in NRR in relation to the negative control were observed for *Chilota sp.* at the treatment 80 mg/kg Cu (Figure 3.14), and in *E. andrei* (B) at the treatment 160 mg/kg Cu. In *E. andrei* (A) coelomocytes, NRR values in all Cu treatments were higher than those in the negative control. In *Chilota sp.* and *E. andrei* (B), NRR values decreased again at higher exposure concentrations. It is possible that exposure to Cu had induced compensatory mechanisms to regulate and detoxify body Cu in these species, such as those mentioned above, which would have resulted in elevated NR retention at the intermediate concentrations. At higher exposure concentrations, it is possible that such a compensatory mechanism might be inhibited or downregulated, similar to what was shown by Galay Burgos *et al.* (2005). They showed that the expression of four genes (metallothionein isoforms 1 and 2, amine oxidase and the lysosomal associated glycoprotein) involved in metal sequestration and detoxification in *Lumbricus rubellus*, are upregulated at low Cu exposure concentrations (5 and 25 mg/kg Cu) and subsequently returned to background levels at higher Cu concentrations (125 and 200 mg/kg Cu). It is therefore possible that such detoxification mechanisms, which could have been induced at the lower Cu concentrations during the present study, such as at 80 mg/kg Cu in *Chilota sp.* and 160 mg/kg Cu in *E. andrei* (B), would not have been activated or would have been downregulated at the higher concentrations, which could explain the increasing and subsequent decreasing NRR values.

The data generated from the NRR assay during the present study were not conducive to the calculation of EC_{50} values for *A. diffringens*, *E. andrei* (A), *E. andrei* (B) and *P. excavatus*. An EC_{50} value could only be calculated for *Chilota sp.* (Table 3.6). It was however possible to calculate EC_{10} values for *Chilota sp.*, *E. andrei* (B) and *P. excavatus*. Based on EC_{10} values, the most sensitive species, where these values could be calculated, was *P. excavatus*, followed by *Chilota sp.* and *E. andrei* (B). These values should however be interpreted cautiously, since confidence intervals for the EC_{10} values for *P. excavatus* and *E. andrei* (B) could not be calculated, and the confidence interval for *Chilota sp.* was extremely large.

The species differences in NRR results observed during the present study could not only depend on the amount of Cu reaching the coelomocytes within the earthworm bodies, but also the inherent sensitivity of coelomocytes to Cu, as well as the inherent ability of different coelomocytes to retain the NR dye. Kurek & Plytycz (2003) demonstrated, with the use of a spectrophotometric NRR assay, that NR retention differ inherently between coelomocytes obtained from various earthworm species. In addition, it is known that different earthworm species possess different ratios of different types of coelomocytes (Kurek & Plytycz 2003) and that not all coelomocyte types are equally

effective in retaining the NR dye. This has been demonstrated by Plytycz *et al.* (2007) who used flow cytometry to determine the proportion of NR accumulating cells in different earthworm coelomocyte types. Plytycz *et al.* (2007) also incubated coelomocytes extruded from two earthworm species (*Dendrobaena veneta* and *Lumbricus castaneus*) *in vitro* with $CuCl_2$ in order to determine whether coelomocytes from different species differ inherently in their sensitivity to Cu. The dose-response relationships differed between the species, and *D. veneta* coelomocytes displayed a sharper decrease in NR retention with increasing Cu concentration than those of *L. castaneus*. Therefore, inherent differences in coelomocyte ability to retain NR, and coelomocyte sensitivity to Cu could have caused the differences between the species observed during the present study.

When the changes in NR retention of earthworm coelomocytes after Cu exposure during the present study were compared with responses on the whole-organismal level, different patterns emerged for the different species. In coelomocytes of *A. diffringens* and *P. excavatus*, reductions in NR retention were observed at the treatment 20 mg/kg Cu, which were at concentrations lower than the concentrations where cocoon production and mass started to decrease, and lower than the feeding avoidance response concentrations (Figures 3.34 and 3.39 respectively). In these species, the NRR assay therefore has the potential to be used as a biomarker to predict changes in reproduction and earthworm body mass at a later stage or at higher concentrations.

In coelomocytes of *Chilota sp.*, NR retention decreased at the treatment with 640 mg/kg Cu, a concentration higher than those at which earthworms started to lose mass, as well as the feeding avoidance response concentration (Figure 3.36). The decrease in NR retention was also observed at the same concentration where mortality started to occur, and it can be concluded that the NRR assay may not be a good biomarker for predicting toxic effects at higher levels of organisation in this *Chilota* species.

In *E. andrei*, the depuration time after exposure seemed to have an influence on the NRR assay results, and thus whether decreases in NRR could be detected at lower concentrations than those where changes in whole-organismal responses were observed. In coelomocytes of *E. andrei* (A), where specimens were depurated for 48 hours after exposure to Cu, the NRR assay failed to detect cytotoxic effects of Cu (Figure 3.37), and can thus not be used to predict effects of Cu exposure on the whole-organismal level. In *E. andrei* (B), depurated for 24 hours like the other species used during the present study, NR retention decreased at a concentration lower than the feeding avoidance response concentration and the concentration where earthworm mass decreased (Figure 3.38). However, cocoon production started to decrease at a lower concentration than the concentration where NRR values started to decrease, and it would therefore seem that should specimens of *E. andrei* be depurated for shorter times instead of longer times, the NRR assay could

perhaps be used to predict changes in earthworm mass, but not in cocoon production. The NRR assay is however less sensitive than cocoon production in this species and may thus not be a good biomarker for predicting toxic effects at higher levels of organisation.

The NRR assay could potentially be predictive of endpoints such as cocoon production and mass change in earthworm species such as *A. diffringens* and *P. excavatus*, but it should not be considered a representative assay for all earthworm species. Therefore, the NRR assay could be useful as one endpoint in an array of assays in testing sublethal effects in selected species where it is known to successfully detect cytotoxicity at low exposure concentrations.

It is known that hydrogen peroxide can cause significant reductions in NR retention in fish cells incubated *in vitro* (Wright *et al*. 2000; Reeves *et al*. 2008), but no literature could be found that reported on responses of earthworm coelomocytes to this substance as measured with the spectrophotometric NRR assay. It is therefore deduced that the present study is the first to use this NRR assay to determine responses of earthworm coelomocytes after *in vitro* exposure to H_2O_2. Exposure to H_2O_2 caused significant decreases in NRR values in all species, except *Chilota sp.* Hydrogen peroxide therefore has the potential to be used as a positive control for *A. diffringens*, *E. andrei* and *P. excavatus*, since it is known to cause cytotoxicity in cells, which can be detected with the NRR assay (Reeves *et al*. 2008).

The extent of decreases in NRR after *in vitro* exposure to H_2O_2 differed between species, and NRR values were decreased by 35% in *Chilota sp.* coelomocytes, 42% in *P. excavatus*, 64% in *E. andrei* (A), 76% in *E. andrei* (B) and 93% in *A. diffringens* (values were calculated from Table 32 in Appendix B). These data show that species differences exist in earthworm coelomocyte sensitivity when exposed *in vitro* to a toxicant. As discussed above, these inherent differences in coelomocyte sensitivity would play a role in determining species differences to a toxicant as measured with a suborganismal assay.

Chilota sp. was the only earthworm species used during the present study where H_2O_2 did not induce significant cytotoxicity in coelomocytes as measured with the NRR assay. This apparent lack of sensitivity to H_2O_2 could possibly be linked to the fact that the coelomic fluid of this species displays bioluminescence. During experimentation, the coelomic fluid emitted a faint green glow immediately after extrusion, which faded within a few minutes. This bioluminescence was however briefly stimulated again when the H_2O_2 was added to the coelomocyte suspension to obtain the positive controls. It is known that the addition of H_2O_2 to earthworm coelomic fluid *in vitro* can stimulate bioluminescence in chloragogen cells of bioluminescent earthworm species such as *Diplocardia longa* (Rudie & Wampler 1978). In coelomic fluid obtained from bioluminescent species exposed *in vitro*, the reaction of H_2O_2 and luciferin, through the action of luciferase, result

in light and other products (Bellisario & Cormier 1971; Wampler 1980). In earthworms *in vivo*, however, oxygen is required for light emission, and it has been postulated that an oxidase is present to generate the required H_2O_2 *in vivo* to supply the bioluminescent reaction (Bellisario & Cormier 1971; Rudie & Wampler 1978; Wampler 1980). This dependence upon H_2O_2 for the bioluminescent reaction in species such as *D. longa* could be the reason why coelomocytes obtained from *Chilota sp.* during the present study did not seem as sensitive to H_2O_2 as those of the other species as measured with the NRR assay. It is recommended that the reaction of the coelomic fluid of *Chilota sp.* to H_2O_2 should be investigated in future in order to determine the possible dependence of this species on this substance for the bioluminescent reaction.

The present study was the first, according to the literature surveyed, that used the spectrophotometric NRR assay to determine the effects of Cu on earthworms and to compare species sensitivity differences in earthworms exposed to Cu. This NRR assay has however been performed on other invertebrates, such as mussels exposed to Cu (Pipe *et al*. 1999; Gómez-Mendikute & Cajaraville 2003), but significant reductions in NRR values after Cu exposure were not found in any of these studies. It would seem that internal regulation of this essential metal reduces the cytotoxic effects in coelomocytes and haemocytes of invertebrates such as earthworms and mussels.

The NRR assay, as performed during the present study, is however not the only method of using the neutral red (NR) dye to determine effects of toxicants on cells. Other methods that utilise the NR dye to determine earthworm sensitivity include the use of flow cytometry to determine the proportion of NR accumulating cells in different earthworm coelomocyte types (Plytycz *et al*. 2007), and the assessment of the integrity of lysosomal membranes in earthworm coelomocytes (Weeks & Svendsen 1996). The latter method is termed the neutral red retention time assay (NRRT assay) and is a sensitive sublethal subcellular assay that is widely used to assess the effects of toxicants on earthworms (Svendsen *et al*. 2004). The NRRT assay has been shown to accurately predict responses on higher levels of biological organisation in various earthworm species exposed to various copper formulations (Weeks & Svendsen 1996; Maboeta *et al*. 2002; Reinecke *et al*. 2002; Maboeta *et al*. 2003; Maboeta *et al*. 2004; Svendsen *et al*. 2004). For example, significant reductions in neutral red retention times can occur at soil concentrations as low as 18 mg/kg Cu in *Aporrectodea caliginosa* (Maboeta *et al*. 2003), at 21 mg/kg Cu in an indigenous South African *Microchaetus* species (Maboeta *et al*. 2002) and at 73 mg/kg Cu in *E. fetida* (Maboeta *et al*. 2004). In *E. andrei*, Svendsen & Weeks (1997) found significant decreases in NRRT in specimens exposed at 80 mg/kg Cu. In contrast, during the present study, NRR values were not statistically significantly decreased at any of the Cu exposure concentrations in *E. andrei*. This difference is

most probably due to the different endpoints measured by these two assays. Since the NRRT assay is a subcellular assay that measures the integrity of lysosomal membranes in live cells (Weeks & Svendsen 1996), it should be able to detect the adverse effects of toxicant exposure at lower concentrations than the spectrophotometric NRR assay, used during the present study, which is used to determine the extent of cell viability in a sample (Borenfreund & Puerner 1985).

4.3.2 MTT assay

The amount of tetrazolium converted into formazan in earthworm coelomocytes varied between the species (Figure 3.16). The only species where statistically significant reductions in MTT conversion were observed in any of the Cu treatments was *A. diffringens*. Statistically significant differences were found for this species between the negative control and the treatments 20 and 320 mg/kg Cu (Figure 3.16). This species had had taken up the highest levels of body Cu (Figure 3.3), and it is possible that body Cu levels were sufficiently high to cause significant decreases in MTT conversion in coelomocytes of this species. The MTT value for the single available specimen of *A. diffringens* exposed to 640 mg/kg Cu was higher than that of those in the negative control, possibly due to a detoxification mechanism or a less damaged coelomocyte population remaining after possible apoptotic death of the highly damaged coelomocytes, as was discussed for the NRR assay.

MTT values were higher than those of the negative control in specimens of *Chilota sp.* exposed to 80 and 160 mg/kg Cu and in specimens of *E. andrei* (A), *E. andrei* (B) and *P. excavatus* exposed to all Cu concentrations (Figure 3.16). MTT values were the highest at the intermediate concentrations (at 160 mg/kg Cu for *Chilota sp.* and *E. andrei* (B) and 320 mg/kg Cu for *P. excavatus*), and decreased again at the highest Cu exposure concentrations. Two possible interpretations of these results are suggested. Firstly, it is possible that at the intermediate concentrations, some kind of compensatory mechanism, as discussed previously for the NRR assay, could have been activated to aid in the protection of coelomocytes. This would have been reflected in the elevated formazan production and thus mitochondrial activity as measured by the MTT assay. At the highest concentrations, such a compensatory mechanism could have been inhibited or downregulated, resulting in increased damage to coelomocytes and thus lower mitochondrial activity. Secondly, it can alternatively be reasoned that if an increase in Cu detoxification would have occurred in the specimens exposed to the intermediate concentrations, the increased energy demand of the detoxification process would have resulted in an increased metabolic activity in the coelomocytes, which would be detected by the MTT assay, since the MTT assay measures metabolic the activity of cells (Mosmann 1983). Heavy metal regulation and detoxification in earthworms can be energy intensive (Holmstrup *et al*. 2010b), and the increase in energy demand

due to increased heavy metal detoxification in earthworms has been shown to be reflected in the expression of mitochondrial genes (Stürzenbaum *et al*. 1998a). Therefore, the increases in mitochondrial activity at the intermediate Cu exposure concentrations, as measured with the MTT assay during the present study, could have been due to an energy-intensive detoxification process. It is therefore suggested that such an energy-intensive Cu regulation or detoxification process in *P. excavatus* specimens exposed during the present study could have resulted in low body Cu concentrations (Figure 3.3) and high MTT values (Figure 3.16). In contrast to *P. excavatus*, specimens of *A. diffringens* had the highest Cu body loads and MTT values were lower than those of the other species. This could indicate a possible lack of Cu detoxification in *A. diffringens* and thus the increased energy production that such a process would have demanded.

Because food consumption is a way of energy acquisition, it can have an effect on metabolic rate and thus mitochondrial function. When earthworms decreased their feeding activity during the present study, this lack of energy could have resulted in decreases in metabolic activity and thus MTT values. It was observed that MTT values and the exposure mass change decreased drastically at the feeding response avoidance concentration in *Chilota sp.* (Figure 3.36). Similar results were observed in *P. excavatus*, where the exposure mass change and MTT values decreased at concentrations higher than the feeding avoidance response concentration (Figure 3.39). In *A. diffringens*, both the MTT values and the exposure mass change decreased at concentrations higher than 80 mg/kg Cu (Figure 3.34). This was however not evident in either *E. andrei* (A) or (B), where the feeding avoidance response concentration was high, indicating that earthworms were probably still feeding sufficiently to maintain energy levels.

Since significant decreases in MTT values were not observed in *Chilota sp.*, *E. andrei* or *P. excavatus* during the present study (Figure 3.16), and since statistically significant decreases were not observed at the intermediate exposure concentrations in *A. diffringens*, it is concluded that this assay may not be a good biomarker to determine decreases in mitochondrial metabolism of earthworm coelomocytes. Due to the increases found in MTT values in all species (Figure 3.16), and the possibility that these increases could be due to an increased energy demand as a result of regulation of the toxicant, it is suggested that the potential of the MTT assay as a biomarker to measure compensatory responses, along with the energy demands of toxicant regulation in earthworms, is investigated in future.

Due to the dose-responses found for the MTT assay during the present study, the calculation of EC_{50} values could not be done. EC_{50} values could therefore not be used to compare species sensitivities, but differences between the dose-response curves of the species could be observed

(Figure 3.16). These differences between species most probably reflected the different abilities of earthworm species to detoxify and regulate body Cu.

The literature survey conducted during the present study indicated that this study is the first to perform the MTT assay on earthworm coelomocytes exposed *in vitro* to H_2O_2. Difference in the inherent sensitivity of coelomocytes to H_2O_2 were found, and the highest decrease in MTT values after *in vitro* exposure to this substance was observed for *P. excavatus* (42%), followed by *E. andrei* (B) (36%), *E. andrei* (A) (35%), *Chilota sp.* (29%) and *A. diffringens* (25%) (values calculated from Table 36 in Appendix B). These data show that species differences exist in earthworm coelomocyte sensitivity when exposed *in vitro* to a toxicant, and as discussed above, these inherent differences in coelomocyte sensitivity would play a role in determining species differences to a toxicant.

4.3.3 Alkaline comet assay

The two ways of comet assay data analyses used during the present study yielded different statistical results. When the median Tail DNA % value for each specimen was calculated and statistical analyses performed on these values (called Tail DNA % (median), Figures 3.19b to 3.23b), none of the Cu treatments differed statistically significantly from the negative control in any of the species. On the other hand, when the raw Tail DNA % values for the separate comets were pooled per treatment (called Tail DNA % (raw), Figures 3.19a to 3.23a) and subsequently analysed, significant increases in Tail DNA % were observed in specimens exposed to all or most of the Cu treatments, depending on the species (Figures 3.19b to 3.23b). The type of data treatment can therefore influence the eventual conclusions of the study. Therefore, should the animal be regarded as the experimental unit, the conclusion would be that earthworm exposure to copper oxychloride does not induce significant amounts of DNA damage in earthworm coelomocytes. On the other hand, if the individual cell nucleus is regarded as the experimental unit, the conclusion would be that exposure to copper does induce significant DNA damage in earthworm coelomocytes. However, it has been stated clearly by Lovell *et al.* (1999) that failure to use the animal as the experimental unit, as is recommended, would lead to overestimation of significance and therefore misinterpretation of results. Further discussion on the comet assay results will hence pertain only to the Tail DNA % (median) results, and it can thus be concluded that although copper oxychloride exposure did induce some DNA damage in coelomocytes obtained from the selected earthworm species, it is not significant at the applied exposure concentrations.

Species differences in the dose-responses could be observed. In Figure 3.24, it can be seen that the highest relative increase in Tail DNA % (which was calculated by dividing the median Tail

DNA % value at each Cu treatment by the median Tail DNA % value of the negative control for each species) was observed in coelomocyte nuclei of *A. diffringens*, and the lowest in *P. excavatus*. These species differences could possibly due to differences in regulation of Cu, as was discussed in previous sections.

In coelomocytes of *A. diffringens*, DNA damage increased with increasing Cu exposure concentration (Figure 3.19). The relative increase in Tail DNA % (Figure 3.24) also increased with increasing exposure concentration, except between the treatments with 320 and 160 mg/kg Cu. In coelomocytes of *Chilota sp.*, *E. andrei* (A) and *P. excavatus*, the relative increase in Tail DNA % reached a maximum at the treatment with 160 mg/kg Cu, after which it decreased again. In coelomocytes of *E. andrei* (B), this maximum was reached at 80 mg/kg Cu. It is possible that at Cu concentrations higher than 80 or 160 mg/kg Cu, a threshold was reached where either DNA repair mechanisms were induced or not hindered anymore.

These results can be interpreted by considering the mode of toxic action of Cu on DNA, and also the regulation of body Cu by earthworms. There will always be natural levels of DNA damage in cells (Lindahl 1993; Shugart 2000), and certain toxicants interfere with DNA repair processes rather than, or in addition to, causing direct damage to the DNA molecule (Shugart 2000). Although the complete mechanism of exactly how Cu influences DNA repair mechanisms in earthworms is not fully understood, it is known that Cu may compete with Zn in zinc finger structures of mammalian DNA repair proteins (Asmuß *et al*. 2000). Copper may therefore cause DNA damage both indirectly by interfering with DNA repair and directly, at higher concentrations, where Cu is involved in the formation of ROS through Fenton-type reactions (Gaetke & Chow 2003) that may cause DNA strand breaks.

Physiological processes such as Cu regulation with the aid of e.g. metallothioneins, combined with DNA repair, could have caused the increases and subsequent decreases in DNA damage as observed during the present study. For example, Spurgeon *et al.* (2004a) and Bundy *et al.* (2008) found that that earthworm *mt2* (metallothionein isoform 2) transcription in *Lumbricus rubellus* was upregulated at an exposure concentration of 160 mg/kg Cu. This increase in metallothionein at 160 mg/kg Cu could result in a decreased amount of free Cu ions available to induce e.g. DNA damage or compete with Zn in DNA repair proteins. This could explain the results found during the present study, where the relative increase in DNA damage (Figure 3.24) decreased at exposure concentrations higher than 160 mg/kg Cu for most of the species.

Taking these processes into consideration, and regarding the DNA damage results found during the present study, it can be speculated that in the specimens exposed to the lower concentrations of copper oxychloride, Cu concentrations could have been high enough to outcompete Zn in the DNA

repair proteins. The Cu concentration would however not be high enough to stimulate the production of sufficient amounts of DNA repair proteins, and this would lead to higher levels of DNA damage. At the higher Cu exposure concentrations, the production of higher amounts of DNA repair proteins would be stimulated. At the same time, body Cu concentrations would have been regulated at the higher Cu exposure concentrations, as is evidenced by the decreased BCF values (Figure 3.3), possibly by a detoxification mechanism involving metallothioneins as mentioned above. Therefore, at the high Cu exposure concentrations, the concentration of affected DNA repair proteins (where Cu outcompeted Zn) would be lower than unaffected DNA repair proteins, and DNA repair can be resumed.

The relatively low levels of DNA damage at exposure concentrations higher than 160 mg/kg Cu observed during the present study could also be due to apoptosis in coelomocytes at the higher Cu concentrations. As was mentioned in the sections for the NRR and MTT assays, exposure to Cu is known to cause apoptosis in coelomocytes of earthworms exposed to Cu (Homa *et al*. 2007). It has also been shown by Bundy *et al.* (2008) that apoptotic regulators are down-regulated with increasing exposure Cu concentration in *Lumbricus rubellus*. It is therefore possible that apoptosis could have occurred in coelomocytes of earthworms exposed to the highest Cu concentrations during the present study, leaving only intact cells and nuclei to be scored and a resulting decrease in Tail DNA %.

Due to the dose-responses found for the comet assay results during the present study, an EC_{50} value could only be calculated for *Chilota sp.* using Tail DNA % (median) values (Table 3.7). EC_{10} values were however calculated using Tail DNA % (median) values for *A. diffringens* and *Chilota sp.* The most sensitive species, based on these values, was *Chilota sp.*. Tail DNA % (raw) values were also used to calculate EC_{10} values for *A. diffringens*, *Chilota sp.*, and *P. excavatus*, for comparative purposes. Again, *Chilota sp.* was the most sensitive species. Although it is not recommended to use Tail DNA % (raw) data, as mentioned above, these data could be useful to determine species sensitivity differences.

As previously mentioned, increases in Tail DNA % with increasing exposure concentration were only observed for *A. diffringens* exposed to the full range of Cu concentrations used during the present study. Although these increases were not statistically significant (when the animal is considered as the experimental unit, as discussed above), they were observed at concentrations lower than those where reductions in cocoon production and mass change were observed, and at concentrations lower than the feeding avoidance response concentration (Figure 3.34). The comet assay therefore has potential to be used as a biomarker to predict toxic effects of Cu on the whole-organismal level in *A. diffringens*. Since levels of DNA damage, as detected with the comet assay,

decreased at the highest Cu exposure concentrations in the other species, this assay may not be suitable as a biomarker to predict responses at higher levels of biological organisation in those earthworm species.

Although the comet assay has been performed on earthworms in numerous studies, thus far, no published work could be found on earthworms exposed singly to Cu. Similar results to those in the present study were found in comet assay results for freshwater planarians (*Dugesia shubarti*) exposed to $CuSO_4$ for 7 days by Guecheva *et al.* (2001). These authors found that DNA damage increased with increasing $CuSO_4$ concentrations up to 0.25×10^{-5} M Cu, levelled off at intermediate Cu concentrations and decreased at the highest exposure concentration (1×10^{-5} M Cu). It would therefore seem that the regulation of Cu by organisms can reduce the availability of Cu to induce DNA damage, which could influence the results of the comet assay.

During the present study, it was found that background levels of DNA damage in the negative control specimens differed between species, and were 8% Tail DNA for *A. diffringens*, 21% for *E. andrei* (A), 30% for *E. andrei* (B), 47% for *Chilota sp.* and 49% for *P. excavatus*. The Tail DNA % values in the negative controls for *E. andrei*, *Chilota sp.* and *P. excavatus* were higher than the 10 % recommended by Collins (2004), although it has been noted by Button *et al.* (2010) that this recommendation is for the use of human cells for which the assay has been developed. The Tail DNA % values in the negative control specimens of *E. andrei* (B), *Chilota sp.* and *P. excavatus* found during the present study are however also higher than those reported in other earthworm species by Fourie *et al.* (2007) (8 and 19%) and Button *et al.* (2010) (16 to 24%). It is therefore possible that some experimental errors could have occurred during the present study, such as accidental exposure to bright light during the comet assay procedure. To investigate whether sample preparation could have had an effect on the comet assay results, the replicates for each species were investigated separately (Figure 3.26). Differences in Tail DNA % values between replicates of the negative controls were observed for *Chilota sp.*, *E. andrei* (A), *E. andrei* (B) and *P. excavatus*, indicating that errors could have occurred during the experimental procedure for the replicates with high Tail DNA % values in the negative controls. After removal of the replicates with high Tail DNA % values in the negative controls for both *E. andrei* groups, statistical analyses revealed that there were still no significant differences between the negative control and any of the Cu treatments for both *E. andrei* groups, and that Cu still does not seem to have a significant influence on the DNA integrity of coelomocytes of these species.

In coelomocytes of *Chilota sp.* and *P. excavatus* specimens from the negative controls, DNA damage in all replicates are higher than that in negative control specimens of *A. diffringens* and *E. andrei* (A) and (B). It is possible that inherent levels of DNA damage in *Chilota sp.* and *P.*

excavatus could be higher than those of the other species, since inherent levels of DNA damage in coelomocytes may differ between species. Different coelomocyte types display different inherent levels of DNA damage, as Di Marzio *et al.* (2005) demonstrated in coelomocytes obtained from *E. fetida*. They found that the median Tail DNA % value was 4.45% for the total coelomocyte population, 3.9% for eleocytes, 8.28% for amoebocytes and 20.5% for granulocytes. Although it was advised by Di Marzio *et al.* (2005) that only eleocytes should thus be used in the comet assay, since this coelomocyte type comprised more than 70% of the coelomocytes that were found in *E. fetida*, it is known that the composition of coelomocyte populations differ very much between species (Cholewa *et al*. 2006). Indeed, eleocytes, for example, are numerous in species such as *Eisenia fetida, Allolobophora chlorotica* and *Dendrobaena veneta*, but are rarely found in species such as *Lumbricus terrestris* and *Aporrectodea caliginosa* (Kurek & Plytycz 2003; Adamowicz 2005; Cholewa *et al*. 2006; Kasschau *et al*. 2007). The exact composition of coelomocyte types are not known in all the species used in the present study, therefore whole coelomocyte populations were used. This could however explain the differences observed during the present study in negative control Tail DNA % values between species, as well as the relatively high Tail DNA % values in negative control specimens of *Chilota sp.* and *P. excavatus*.

4.3.4 Protein content of earthworm coelomic fluid

It was observed during the present study that the protein content in earthworm coelomic fluid decreased at the treatment 640 mg/kg Cu in *A. diffringens*, *Chilota sp.* and *P. excavatus* (Figure 3.13), although not statistically significantly so. Since it was found during the present study that a significant correlation exists between protein content and cell number (Figure 2.7), a decrease in protein content of earthworm coelomic fluid could be a useful indicator of a decrease in coelomocyte cell number. Decreases in coelomocyte numbers in earthworm species exposed to toxicants have been successfully used as suborganismal indicators of toxic stress in various studies. For example, it has been shown that exposure to Cu leads to a decrease in coelomocyte numbers in *E. fetida* (Homa *et al*. 2005; Homa *et al*. 2007), *Allolobophora chlorotica* (Homa *et al*. 2007) and *Amynthas hawayanus* (Nusetti *et al*. 1999). Since the protein content can be linked to the cell number, and since the measurement of protein content can be conducted faster than manual counting of cells, the measurement of coelomic fluid protein content may be a promising alternative indicator of coelomocyte number in ecotoxicological studies with earthworms.

4.4 General comments on suborganismal results

4.4.1 The effect of depuration period length on suborganismal responses

Despite the fact that *E. andrei* (A) was depurated for 24 hours longer than *E. andrei* (B), the body Cu concentrations at each treatment did not differ statistically significantly between the two groups. This indicates that more Cu was not excreted during the extra 24 h in *E. andrei* (A).

Differences between the two groups, although not statistically significant, were found in the results from the suborganismal assays, where NRR and MTT values decreased in *E. andrei* (B) at concentrations of 320 mg/kg Cu and higher, but not in *E. andrei* (A) (Figures 3.18 and 3.24). The results of the comet assay indicated that levels of DNA damage were higher in *E. andrei* (B) (Appendix B, Table 40), and that statistically significant differences between the two groups were found for specimens exposed to 80 mg/kg Cu (Mann-Whitney U test, $P < 0.05$).

These results indicate that lower levels of damage were observed in coelomocytes of *E. andrei* (A) than in *E. andrei* (B), and that some degree of detoxification by sequestration, but not excretion, of Cu could have occurred in earthworms during the second day of depuration. Additionally, regeneration or replacement of coelomocytes could have occurred during the extra day of depuration in *E. andrei* (A), but not in *E. andrei* (B), where time for coelomocyte generation could have been too short. It is known that earthworm coelomocytes can regenerate within 2 days after depletion through extrusion (Homa *et al*. 2008), or after 3 days on uncontaminated substrate subsequent to dermal exposure to Cu (Olchawa *et al*. 2006). The suborganismal assays used during the present study would therefore have been performed on a mixture of original and newly generated coelomocytes, which would have led to increased NRR and MTT values, or decreased DNA damage levels. The depuration time after exposure therefore seems to be an important factor when performing these suborganismal assays in earthworms.

4.4.2 Regulation of body Cu and its energetic costs

Regulation of the body Cu load, in order to prevent direct toxic effects of Cu on target organs or tissues, may have energetic costs, which can cause a reduction in e.g. biomass or a reduction in reproductive capabilities. Species differences in this allocation of energy may therefore cause differences in their sensitivity in terms of reproduction and the maintenance of biomass. The results of the present study indicate that this may have occurred in the species used. For example, when the results of *P. excavatus* and *A. diffringens* are compared, it can be seen that although the body Cu concentrations in *P. excavatus* were lower than those of *A. diffringens*, specimens of *P. excavatus* produced lower numbers of cocoons than *A. diffringens*. This could be a result of an energy-

intensive detoxification process. On the suborganismal level, Cu did not seem to affect the results of the NRR, MTT and comet assays in specimens of *P. excavatus*, but in *A. diffringens*, exposure to Cu caused significant decreases in NRR and MTT values and high increases in Tail DNA % values. Therefore, it seems that in specimens of *A. diffringens*, more energy was allocated to reproduction, and in *P. excavatus*, more energy was allocated to detoxification. It can be concluded that the species differences in sensitivity observed during the present study could have been due to species-specific allocation of energy to handle toxic stress.

4.5 SSDs for suborganismal responses

SSDs were constructed using EC_{10} values for the NRR and comet assays, as well as LOEC values for the comet assay (Figures 3.30 and 3.31). The HC_5 values that were calculated from the SSDs constructed with EC_{10} values (Table 3.8) indicated that the most sensitive endpoint was NR retention. The 95% prediction intervals for these HC_5 values were however fairly large, and should therefore be interpreted carefully. These large prediction intervals were most probably due to the small number of data points used for SSD construction (three values for both endpoints), which would have reduced statistical robustness, as was discussed in section 4.2.

4.6 Comparison of whole-organismal responses with suborganismal responses using ECs and HC_5s

As was discussed for the whole-organismal and suborganismal endpoints above, comparisons between the endpoints using EC, LC or feeding response concentration values are difficult, since no single endpoint was the most sensitive for all species, and no species was the most sensitive for all endpoints. It is however possible to compare the relative sensitivities of the endpoints using the HC_5 values obtained from the SSDs. Comparisons between whole-organismal and suborganismal endpoints, using EC_{50} and LC_{50} values could however not be done, since sufficient suborganismal EC_{50} values could not be calculated for use in SSDs. HC_5 values obtained from SSDs constructed with EC_{10} values could however be compared between the whole-organismal and suborganismal endpoints. The suborganismal endpoints had lower or similar HC_5 values than the whole-organismal endpoints (Table 3.8). The most sensitive endpoint was NRR, followed by both Comet assay and mass change, and then cocoon production and survival. It can be concluded that selected suborganismal responses, such as NR retention in earthworm coelomocytes can be sensitive tools to

be used in determining species sensitivity relationships and for use in risk assessment procedures such as SSD models.

4.7 Possible factors determining similarities in species sensitivities

It was attempted to explain the observed species differences in Cu uptake and sensitivity on the various levels of organisation in terms of species body size, ecological grouping, possible tolerance due to exposure history, and morphological and physiological differences between species. Relationships between species in the amount of Cu taken up, and body Cu regulation ability, were not evident when investigating some of the factors that could cause similarities in species, as listed in Table 4.3. It can therefore not be concluded that e.g. epigeic species have taken up higher levels of Cu than endogeic species, or that larger species have taken up higher levels of body Cu than smaller species. There is however a possible reason that could explain the higher body Cu levels in specimens of both *A. diffringens* and *Chilota sp.* (Figure 3.3) than in the other species. The high levels of Cu in *A. diffringens* could be due to the presence of enteronephric nephridia, a characteristic of many megascolecid species (Oglesby 1978). It is known that coelomocytes traffic metals such as Cd and Cu to the nephridia (Stürzenbaum *et al*. 2001; Homa *et al*. 2005; Homa *et al*. 2007), and that the nephridia are directly involved in excretion of metals such as Cd (Prinsloo *et al*. 1999; Stürzenbaum *et al*. 2001). In the enteronephric condition, where nephridiopores discharge into the intestine rather than to the exterior of the earthworm body, it is thus possible that these metals are available for uptake through the intestinal wall before leaving the body with the faecal matter. It is possible that this type of excretion system could also have resulted in the elevated body Cu of specimens of *Chilota sp.* observed during the present study. Although the type of nephridia is not known in the *Chilota* species used in the present study, the presence of external nephridiopores have not been observed in a number of indigenous South African *Chilota* and closely related *Parachilota* species (Plisko 2007; Plisko 2008), which indicate the possibility that nephridia are enteronephric in these *Chilota* species.

Since no single species was the most sensitive to Cu for all of the whole-organismal or suborganismal responses (Table 3.9), the endpoints were investigated separately to determine whether the most sensitive or least sensitive species for any endpoint shared any common characteristics. It was found that the most sensitive species in terms of survival to Cu exposure were *A. diffringens* and *P. excavatus*, both belonging to the family Megascolecidae, and the least sensitive species were *A. trapezoides* and *E. andrei*, belonging to the family Lumbricidae (Table 4.3). These similarities between species in sensitivity to Cu were not observed for the other

endpoints, and it is possible that other factors than species relatedness determined the observed differences in species sensitivity.

Table 4.3: Summary of selected results from the present study, including earthworm body Cu concentrations for five earthworm species after 14 days exposure to 640 mg/kg Cu, the bioconcentration factors (BCF), the Cu concentrations in the soil from which the earthworm specimens originated (Habitat Cu) and the earthworm body mass (the mean start mass for each species) and the feeding avoidance response concentration. The family, ecological type and the preferred type of substrate that the earthworms ingest are also included.

Species	Mean body Cu (mg/kg)	BCF	Habitat Cu (mg/kg)	Body mass (g)	Feeding avoidance response (mg/kg)	Family	Ecological type	Preferred ingesta
A. diffringens	44	0.090	35	1.00	222	Megascolecidae	Epigeic	Organic matter
Chilota sp.	28	0.048	5	2.44	162	Acanthodrilidae	Endogeic[a]	Soil[a]
E. andrei	17.3	0.034	36	0.46	332 (A) 529 (B)	Lumbricidae	Epigeic	Organic matter
A. trapezoides	17.1	0.038	8	0.78	104	Lumbricidae	Endogeic	Soil
P. excavatus	13	0.027	36	0.45	357	Megascolecidae	Epigeic	Organic matter

a – for *Chilota sp.*, information on the ecological type and food type preferences are not available in the literature; these are presumed to be endogeic and soil, respectively, as deduced from the habitat preferences of the specimens observed during sampling and from the literature on closely related *Chilota* species (Plisko 2007).

A possible factor that could have contributed to the species sensitivity differences observed during the present study is the exposure history of each species. The exposure history of a test organism plays a significant role in the outcome of toxicity tests, where previous exposure to the toxicant could lead to the development of resistance or acclimation to the toxicant (Reinecke *et al*. 1999). Cross-resistance, where previous exposure to one toxicant, or a mixture, may lead to heightened resistance to another toxicant, is also possible (Voua Otomo & Reinecke 2010). Copper concentrations were the highest in the substrates in which specimens of *E. andrei*, *P. excavatus* and *A. diffringens* had been cultured (Table 4.3). Should an exposure history of elevated Cu concentrations influence the sensitivity of the earthworms, it could be expected that the results from the present study would indicate increased tolerance to Cu in these three species, but not in *A. trapezoides* and *Chilota sp.* When investigating the earthworm responses to copper oxychloride exposure in terms of the EC_{50}, LC_{50} and feeding avoidance response values (Table 3.9), no single species seemed to be the most tolerant to Cu, since the order of species sensitivity differed between endpoints. *A. trapezoides* and *Chilota sp.* were therefore not consistently the most sensitive species, but on the other hand, *E. andrei* and *P. excavatus* did seem to be more tolerant than the other species regarding most of the whole-organismal endpoints. However, *A. diffringens* was the most sensitive species in terms of survival and mass change, and was the only species for which significant decreases in neutral red retention and MTT conversion was found. The results of the

present study therefore could not conclusively give evidence that previous exposure to Cu could have resulted in increased tolerance to Cu in any of the species investigated.

4.8 Conclusions

The main aim of the present study, which was to determine whether sublethal toxicity data obtained from various levels of biological organisation could be used to determine species sensitivity differences and whether these data could be used in SSDs, was achieved. Species sensitivity differences could be determined using whole-organismal and suborganismal responses, and data from most of these endpoints could be used to construct SSDs. The null hypothesis, that stated that suborganismal data cannot be used in species sensitivity distributions, is therefore rejected.

It was also sought to determine whether the suborganismal responses were more sensitive than, and predictive of whole-organismal responses. Not all of the suborganismal responses were shown to be more sensitive than the whole-organismal responses, but the NRR assay was found to detect adverse effects on earthworm coelomocytes of some of the species at exposure concentrations below those where adverse effects on reproduction, maintenance of body mass and feeding avoidance behaviour were evident.

Species differences in the suborganismal responses could be related to the species-specific ability to take up and regulate the toxicant, and it was shown that the species that had taken up the highest amount of toxicant also had the most drastic decreases in coelomocyte viability and metabolic activity, as well as the most drastic increases in DNA damage. Conversely, the species that had the lowest body loads of toxicant, also had the lowest levels of damage, as revealed by the suborganismal assays.

The specific aims were met to some degree, and EC_{50}, LC_{50} values, as well as the toxicant concentration where each species showed a feeding avoidance response could be calculated for all or most of the species for the whole-organismal responses, depending on the endpoint. EC_{50} values could however not be calculated for all species for all of the suborganismal endpoints, and EC_{10} values were used for species comparisons.

These LC_{50}, LC_{10}, EC_{50}, EC_{10} and feeding avoidance response concentrations could be used to compare species sensitivities, and also to construct SSDs. The relative sensitivities of the suborganismal and whole-organismal responses could be compared by using the HC_5 values obtained from the SSDs constructed with LC_{10}, EC_{10} and feeding avoidance responses concentrations. Using these HC_5 values, it was demonstrated that those suborganismal responses for which SSDs could be constructed, detected adverse effects of toxicant exposure at lower concentrations than the whole-organismal responses.

In conclusion, the majority of the responses on the various levels of biological organisation investigated during the present study were suitable to determine species sensitivity relationships in the terrestrial oligochaete species studied. In terms of the contribution of this study towards policy

and guidelines in ecological risk assessment, it could be demonstrated that, with further research, it would be possible to include biomarkers into SSDs as a fast, effective and sensitive indicator of species sensitivity.

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Appendix A: Chemical solutions

1. Coelomocyte extrusion solution

0.05 g EDTA

19 ml PBS (Phosphate Buffered Saline, Ca and Mg free)

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

2. NRR colouring solution

2.1 Stem solution

0.4% neutral red powder (4 mg)

1000 µl distilled water

- Mix well.

2.2 Colouring solution

2 ml Stem solution in 158 ml PBS (1:80 stem solution / PBS)

- Mix well and use immediately.

3. NRR extraction solution

50% Absolute ethanol (5 ml)

1% Acetic acid (100 µl)

distilled water (4.9 ml)

- Mix well and use immediately.

4. MTT colouring solution

10 mg MTT (3-(4,5dimethyldiazol-2-yl)-2,5-dipenyl)

20 ml PBS

- Mix well and use immediately.

5. MTT extraction solution

0.1 N of 70% HCl (20 µl) in Isopropanol (18 ml)

10% Triton X (2 ml)

- Add the HCl to the Isopropanol, then add the Triton X, mix well and use immediately.

6. Comet assay lysing solution

6.1. Lysing solution stock

2.5 M NaCl (146.1 g)

100mM EDTA (37.2 g)

10mM Tris (1.2 g)

distilled water

8 g pellitized NaOH

- Add ingredients to ± 700 ml distilled water.
- 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) and adjust the to 10.0 using concentrated HCl or NaOH.
- Q.s. to 890 ml with distilled H_2O. Store at room temperature.

6.2. 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 to the stock solution, and then refrigerate for 30 – 60 minutes prior to slide addition.

7. Comet assay electrophoresis buffer

for 1000 ml

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

10 M NaOH (made up by adding 200 g to 500 ml dH_2O). Store at 4 °C.

200 mM EDTA (made up by adding 14.89 g to 200 ml dH_2O, pH 10). Store at 4 °C.

7.2. Immediately before each electrophoresis run:

30 ml NaOH

5.0 ml EDTA

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

Appendix B: Summary of experimental data and statistical results

Table 1 (data): Mean (± std dev) Cu concentrations (mg/kg) in OECD soil spiked with a Cu standard and of two different batches of Virikop (the source of Cu). Virikop 1 is the batch used in this study, and Virikop 2 was included from another batch for comparative purposes. An additional sample of the standard (used for spiking the abovementioned OECD soil and calibrating the AAS) was diluted and included in the ICP analyses (Cu Standard); value in mg/l. The % Recovery is the measured Cu concentration calculated as a percentage of the nominal concentration. The Corrected Cu is the measured Cu concentration corrected for the recovery efficiency of 71% (the % Recovery obtained for the AAS measurements of the OECD soil spiked with the Cu standard, as given in the first row of this table). The Corrected % Recovery is the Corrected Cu calculated as a percentage of the nominal concentration. AAS: measured with atomic absorption spectrophotometry. ICP: measured with inductively coupled plasma spectrometry. For all n = 3, except for the Cu Standard where n = 1.

Measurement method	Sample	NominalCu (mg/kg)	Measured Cu (mg/kg)	% Recovery	Corrected Cu (mg/kg)	Corrected % Recovery
AAS	Soil + standard	400	283 ± 31	71 ± 8		
	Virikop 1	500	334 ± 12	67 ± 2	473 ± 17	95 ± 3
	Virikop 2	500	308 ± 7	62 ± 1	435 ± 9	87 ± 2
ICP	Soil + standard	400	341 ± 18	85 ± 5		
	Virikop 1	500	339 ± 21	68 ± 4	479 ± 29	96 ± 6
	Virikop 2	500	290 ± 6	58 ± 1	410 ± 8	82 ± 2
ICP	Cu Standard	1000	1080	108		

Table 2 (statistical results): Pairwise comparisons (Mann-Whitney U Test) between Cu values measured with AAS (atomic absorption spectrophotometry) and those measured with ICP (inductively coupled plasma spectrometry) of OECD soil spiked with a Cu standard (Soil + Standard), two different batches of Virikop (source of Cu) and certified biological reference material (ERM-CE278, Mussel tissue), for both the measured Cu values and the % Recovery (the measured Cu concentration calculated as a percentage of the nominal concentration). Virikop 1 is the batch used in this study, and Virikop 2 was included from another batch for comparative purposes. None of the *P*-values were significant ($P < 0.05$).

				Cu values			% Recovery			1st variable	2nd variable
Sample	1st variable		2nd variable	U	Z	*P*	U	Z	*P*	n	n
All samples	AAS	vs	ICP	28.00	-1.06	0.29	37.00	-0.26	0.79	9	9
Soil + Standard	AAS	vs	ICP	0.00	-1.75	0.08	0.00	-1.75	0.08	3	3
Virikop 1	AAS	vs	ICP	4.00	0.00	1.00	4.00	0.00	1.00	3	3
Virikop 2	AAS	vs	ICP	0.00	1.75	0.08	0.00	1.75	0.08	3	3
Mussel tissue	AAS	vs	ICP	4.00	1.01	0.31	4.00	1.01	0.31	4	4

Table 3 (statistical results): Pairwise comparisons (Mann-Whitney U Test) between Cu values measured in samples of two different batches of Virikop (source of Cu), as measured by AAS (atomic absorption spectrophotometry) and ICP (inductively coupled plasma spectrometry). Virikop 1 is the batch used in this study, and Virikop 2 was included from another batch for comparative purposes. None of the *P*-values were significant ($P < 0.05$).

Measurement method	1st variable		2nd variable	U	Z	*P*	1st variable n	2nd variable n
AAS	Virikop 1	vs	Virikop 2	0.00	1.75	0.08	3	3
ICP	Virikop 1	vs	Virikop 2	0.00	1.75	0.08	3	3

Table 4 (statistical results): Functions and correlation coefficients (parametric Pearson correlation) for standard curves obtained with the Bradford protein assay using bovine serum albumin (BSA). This was done each time before protein content determination of earthworm coelomic fluid extracted from five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. For the function, x = BSA (µg/µl) and y = Absorption (nm). Significant correlations (both $P < 0.05$ and $r^2 > 0.8$ or < -0.8) are in bold. For each correlation, n = 6.

Experiment with				
Species	Replicate	Function	r^2	*P*
A. diffringens	1	y = 0.075x + 0.239	**0.94**	**0.006**
	2	y = 0.067x + 0.163	**0.89**	**0.016**
	3	y = 0.079x + 0.219	**0.94**	**0.006**
	4	y = 0.091x + 0.120	**0.97**	**0.003**
A. trapezoides	1	y = 0.078x + 0.285	**0.96**	**0.003**
	2	y = 0.076x + 0.240	**0.94**	**0.007**
Chilota sp.	1	y = 0.073x + 0.270	**0.95**	**0.004**
	2	y = 0.078x + 0.257	**0.96**	**0.003**
	3	y = 0.085x + 0.253	**0.92**	**0.011**
E. andrei (A)	1	y = 0.075x + 0.281	**0.95**	**0.004**
	2	y = 0.080x + 0.290	**0.95**	**0.006**
	4	y = 0.074x + 0.247	**0.95**	**0.005**
	5	y = 0.073x + 0.292	**0.98**	**0.005**
E. andrei (B)	1	y = 0.083x + 0.318	**0.92**	**0.010**
	2	y = 0.083x + 0.318	**0.92**	**0.010**
	3	y = 0.082x + 0.271	**0.93**	**0.008**
	4	y = 0.079x + 0.320	**0.96**	**0.004**
P. excavatus	1	y = 0.089x + 0.245	**0.96**	**0.003**
	2	y = 0.079x + 0.231	**0.98**	**0.001**
	3	y = 0.077x + 0.266	**0.94**	**0.006**
	4	y = 0.080x + 0.274	**0.91**	**0.011**

Table 5 (data): pH and moisture content (% w/w) of OECD soil used for exposing five earthworm species to Cu in the form of copper oxychloride and benomyl (positive control) for 14 d. Measurements were made before exposure (on day 0 = Start) and after exposure (on day 14 = End). The percentage change (% Change) in pH and moisture content is the difference between Start and End measurements calculated as a percentage of the Start measurement. Values for % Change that are higher than 10% or lower than -10% are in bold. For all species, n = 28, except *Chilota sp.* where n = 21 and *E. andrei* (A) where n = 34.

			pH			Moisture content		
Species	Replicate	Treatment	Start	End	% Change	Start	End	% Change
A. diffringens	1	Control	6.50	6.92	6.46	32.17	30.38	-5.56
		20 mg/kg Cu	6.29	6.80	8.03	32.80	31.48	-4.02
		80 mg/kg Cu	6.06	6.75	**11.39**	32.77	30.71	-6.29
		160 mg/kg Cu	5.95	6.54	9.83	33.92	29.19	**-13.94**
		640 mg/kg Cu	6.00	6.57	9.59	30.90	30.69	-0.68
		960 mg/kg Cu	6.16	6.74	9.42	32.83	32.41	-1.28
		Benomyl	6.19	6.61	6.87	31.36	31.00	-1.15
	2	Control	6.49	6.49	0.00	32.94	31.41	-4.64
		20 mg/kg Cu	6.37	6.46	1.41	33.92	33.07	-2.51
		80 mg/kg Cu	6.34	6.39	0.87	32.59	31.61	-3.01
		160 mg/kg Cu	6.18	6.29	1.78	32.99	31.89	-3.33
		320 mg/kg Cu	6.23	6.46	3.69	31.17	33.18	6.45
		640 mg/kg Cu	6.26	6.51	3.91	32.80	34.19	4.24
		Benomyl	6.16	6.20	0.73	33.37	34.52	3.45
	3	Control	6.30	6.05	-3.89	32.14	30.51	-5.07
		20 mg/kg Cu	6.12	6.25	2.04	31.76	31.33	-1.35

Table 5 continued (pH and Moisture content of OECD soil)

			pH			Moisture content		
Species	Replicate	Treatment	Start	End	% Change	Start	End	% Change
A. diffringens	3	80 mg/kg Cu	6.10	6.22	1.97	32.09	31.06	-3.21
		160 mg/kg Cu	6.04	6.24	3.31	31.88	31.64	-0.75
		320 mg/kg Cu	6.03	6.31	4.73	32.80	31.69	-3.38
		640 mg/kg Cu	5.94	6.41	7.83	31.88	31.59	-0.91
		Benomyl	5.98	6.53	9.20	31.06	33.72	8.56
	4	Control	6.22	6.29	1.13	32.75	30.72	-6.20
		20 mg/kg Cu	6.31	6.51	3.09	32.27	32.77	1.55
		80 mg/kg Cu	6.20	6.40	3.15	32.95	32.17	-2.37
		160 mg/kg Cu	6.04	6.28	3.97	31.74	34.14	7.56
		320 mg/kg Cu	6.08	6.38	4.94	31.94	29.42	-7.89
		640 mg/kg Cu	5.99	6.50	8.43	34.19	33.29	-2.63
		Benomyl	6.31	6.78	7.53	31.60	34.21	8.26
A. trapezoides	1	Control	6.15	6.39	3.90	32.77	31.17	-4.88
		20 mg/kg Cu	6.24	6.75	8.09	33.40	33.61	0.63
		80 mg/kg Cu	6.25	6.58	5.28	32.06	32.48	1.31
		160 mg/kg Cu	6.41	6.65	3.74	32.85	31.15	-5.18
		320 mg/kg Cu	6.35	6.68	5.20	32.31	31.19	-3.47
		640 mg/kg Cu	6.35	6.50	2.28	33.59	30.59	-8.93
		Benomyl	6.37			32.09	31.63	-1.43
	2	Control	6.07	6.18	1.90	31.99	34.19	6.88
		20 mg/kg Cu	6.12	6.56	7.28	34.02	32.47	-4.56
		80 mg/kg Cu	5.79	6.18	6.74	32.74	30.64	-6.41
		160 mg/kg Cu	6.04	6.21	2.73	34.41	32.97	-4.18
		320 mg/kg Cu	6.20	6.29	1.45	32.38	31.95	-1.33
		640 mg/kg Cu	6.14	6.33	3.01	33.49	33.61	0.36
		Benomyl	6.29	6.76	7.39	32.89	33.37	1.46
	3	Control	6.02	6.04	0.33	33.46	32.60	-2.57
		20 mg/kg Cu	6.11	6.27	2.70	32.25	30.35	-5.89
		80 mg/kg Cu	6.02	6.33	5.15	33.06	32.26	-2.42
		160 mg/kg Cu	6.13	6.17	0.65	33.50	31.84	-4.96
		320 mg/kg Cu	6.16	6.15	-0.08	33.14	33.60	1.39
		640 mg/kg Cu	6.09	6.12	0.49	32.67	32.34	-1.01
		Benomyl	6.02	6.13	1.91	33.89	34.05	0.47
	4	Control	5.76	6.17	7.03	31.57	33.12	4.91
		20 mg/kg Cu	5.94	6.25	5.13	33.29	30.01	-9.85
		80 mg/kg Cu	6.02	6.22	3.24	32.43	30.72	-5.27
		160 mg/kg Cu	6.04	6.25	3.48	32.05	32.81	2.37
		320 mg/kg Cu	5.93	6.23	5.06	31.64	31.58	-0.19
		640 mg/kg Cu	5.85	6.10	4.28	31.41	33.19	5.67
		Benomyl	6.03	6.41	6.39	34.10	29.81	**-12.58**
Chilota sp.	1	Control	6.08	6.04	-0.66	32.17	28.85	**-10.32**
		20 mg/kg Cu	6.13	6.33	3.27	32.42	29.94	-7.65
		80 mg/kg Cu	6.07	6.30	3.79	31.95	31.06	-2.79
		160 mg/kg Cu	5.99	6.25	4.34	32.44	27.70	**-14.61**
		320 mg/kg Cu	6.02	6.05	0.50	31.92	31.17	-2.35
		640 mg/kg Cu	6.04	6.07	0.41	33.59	31.15	-7.26
		Benomyl	6.15	6.28	2.03	32.55	29.00	**-10.91**
	2	Control	5.97	5.93	-0.67	33.33	34.67	4.02
		20 mg/kg Cu	6.06	6.06	0.00	32.95	34.18	3.73
		80 mg/kg Cu	6.04	6.13	1.49	33.25	30.73	-7.58
		160 mg/kg Cu	6.13	6.12	-0.08	33.53	33.18	-1.04
		320 mg/kg Cu	6.39	6.14	-3.99	33.36	31.07	-6.86

Table 5 continued (pH and Moisture content of OECD soil)

			pH			Moisture content		
Species	Replicate	Treatment	Start	End	% Change	Start	End	% Change
Chilota sp.	2	640 mg/kg Cu	6.17	6.35	2.84	34.68	30.93	**-10.81**
		Benomyl	6.18	6.27	1.46	32.82	32.89	0.21
	3	Control	6.38	6.19	-2.98	32.47	34.07	4.93
		20 mg/kg Cu	6.06	6.03	-0.41	32.36	30.33	-6.27
		80 mg/kg Cu	6.12	6.27	2.53	32.06	33.92	5.80
		160 mg/kg Cu	6.20	6.46	4.11	34.20	28.56	**-16.49**
		320 mg/kg Cu	6.32	6.30	-0.40	32.79	31.86	-2.84
		640 mg/kg Cu	6.25	6.43	2.88	33.73	35.33	4.74
		Benomyl	6.22	6.36	2.33	33.12	31.49	-4.92
E. andrei (A)	1	Control	6.70	6.34	-5.37	28.57	30.04	5.15
		20 mg/kg Cu	6.37	6.51	2.20	28.80	30.13	4.62
		80 mg/kg Cu	6.66	6.62	-0.60	29.25	30.13	3.01
		160 mg/kg Cu	6.80	6.45	-5.15	29.85	29.69	-0.54
		640 mg/kg Cu	6.92	6.56	-5.20	28.97	30.23	4.35
		960 mg/kg Cu	6.51	6.42	-1.38	29.40	29.18	-0.75
		Benomyl	6.55	6.46	-1.37		29.91	
	2	Control	7.45	7.37	-1.01	30.46	31.16	2.30
		20 mg/kg Cu	7.31	7.49	2.46	30.04	30.68	2.13
		80 mg/kg Cu	7.48	7.45	-0.40	31.26	30.09	-3.74
		160 mg/kg Cu	7.36	7.16	-2.72	31.60	31.08	-1.65
		640 mg/kg Cu	7.20	7.02	-2.50	30.26	29.66	-1.98
		960 mg/kg Cu	7.08	6.86	-3.11	31.22	29.44	-5.70
		Benomyl	7.28	7.12	-2.20	30.89	31.56	2.17
	3	Control	6.28	6.48	3.27	31.60	34.56	9.37
		20 mg/kg Cu	6.04	6.67	**10.35**	31.42	32.63	3.85
		80 mg/kg Cu	6.03	6.71	**11.28**	31.23	31.67	1.41
		160 mg/kg Cu	6.10	6.50	6.64	30.68	31.37	2.25
		640 mg/kg Cu	6.05	6.61	9.26	31.19	33.31	6.80
		960 mg/kg Cu	5.82	6.54	**12.47**	30.12	32.13	6.67
		Benomyl	6.02	6.18	2.74	31.47	32.09	1.97
	4	Control	5.68	6.42	**12.94**	29.29	33.65	**14.89**
		20 mg/kg Cu	5.68	6.38	**12.32**	32.34	33.15	2.50
		80 mg/kg Cu	5.61	5.90	5.26	30.94	31.23	0.94
		160 mg/kg Cu	5.57	5.66	1.62	31.49	32.22	2.32
		640 mg/kg Cu	5.60	6.11	9.02	31.29	31.17	-0.38
		960 mg/kg Cu	5.65	6.01	6.37	30.93	30.95	0.06
		Benomyl	5.60	5.83	4.02	31.75	31.71	-0.13
	5	Control	6.31	6.47	2.62	31.98	32.14	0.50
		20 mg/kg Cu	6.32	6.85	8.47	31.99	32.58	1.84
		80 mg/kg Cu	5.98	6.54	9.37	31.77	33.15	4.34
		160 mg/kg Cu	6.45	6.81	5.59	32.15	30.80	-4.20
		640 mg/kg Cu	6.00	6.56	9.25	32.54	33.67	3.47
		960 mg/kg Cu	6.30	6.71	6.43	32.96	33.10	0.42
		Benomyl	6.19	6.46	4.37	32.19	31.03	-3.60
E. andrei (B)	1	Control	6.32	6.15	-2.77	32.37	31.97	-1.24
		20 mg/kg Cu	6.41	6.37	-0.70	33.07	34.22	3.48
		80 mg/kg Cu	6.33	6.42	1.42	30.61	29.08	-5.00
		160 mg/kg Cu	6.28	6.37	1.51	31.10	31.97	2.80
		320 mg/kg Cu	6.27	6.40	1.99	32.42	31.98	-1.36
		640 mg/kg Cu	6.20	6.29	1.45	33.17	30.92	-6.78
		Benomyl	6.41	6.38	-0.47	32.41	31.83	-1.79
	2	Control	6.45	6.60	2.25	32.01	32.64	1.97

Table 5 continued (pH and Moisture content of OECD soil)

			pH			Moisture content		
Species	Replicate	Treatment	Start	End	% Change	Start	End	% Change
E. andrei (B)	2	20 mg/kg Cu	6.35	6.45	1.65	32.00	31.71	-0.91
		80 mg/kg Cu	6.15	6.35	3.25	31.99	31.25	-2.31
		160 mg/kg Cu	6.14	6.42	4.56	31.49	33.39	6.03
		320 mg/kg Cu	6.07	6.21	2.31	31.95	30.71	-3.88
		640 mg/kg Cu	6.09	6.21	1.89	30.97	32.64	5.39
		Benomyl	6.19	6.23	0.73	32.82	32.62	-0.61
	3	Control	5.71	5.60	-2.01	31.87	32.97	3.45
		20 mg/kg Cu	5.73	5.87	2.36	32.22	30.04	-6.77
		80 mg/kg Cu	5.70	5.73	0.44	32.89	33.91	3.10
		160 mg/kg Cu	5.85	5.77	-1.37	31.86	31.84	-0.06
		320 mg/kg Cu	5.89	5.80	-1.44	31.23	32.85	5.19
		640 mg/kg Cu	5.74	5.68	-1.13	34.52	32.05	-7.16
		Benomyl	5.88	5.86	-0.34	32.71	31.52	-3.64
	4	Control	5.81	6.01	3.44	32.47	32.31	-0.49
		20 mg/kg Cu	5.97	5.99	0.34	33.39	31.53	-5.57
		80 mg/kg Cu	5.99	6.12	2.26	33.87	33.98	0.32
		160 mg/kg Cu	5.96	6.08	1.93	31.90	31.08	-2.57
		320 mg/kg Cu	5.90	6.15	4.15	32.21	32.17	-0.12
		640 mg/kg Cu	5.85	5.98	2.14	32.48	31.76	-2.22
		Benomyl	5.90	6.08	2.97	32.19	30.72	-4.57
P. excavatus	1	Control	6.08	6.26	2.88	34.32	33.91	-1.19
		20 mg/kg Cu	6.35	6.35	0.08	32.39	34.04	5.09
		80 mg/kg Cu	6.26	6.43	2.64	33.33	32.69	-1.92
		160 mg/kg Cu	6.28	6.41	2.07	32.61	32.55	-0.18
		320 mg/kg Cu	6.29	6.36	1.03	32.91	32.88	-0.09
		640 mg/kg Cu	6.31	6.38	1.11	32.27	30.67	-4.96
		Benomyl	6.30	6.49	3.02	32.35	31.10	-3.86
	2	Control	6.16	6.03	-2.11	32.21	32.93	2.24
		20 mg/kg Cu	6.32	6.36	0.55	32.69	33.10	1.25
		80 mg/kg Cu	6.38	6.32	-0.94	33.24	33.15	-0.27
		160 mg/kg Cu	6.29	6.42	2.15	32.35	32.64	0.90
		320 mg/kg Cu	6.30	6.40	1.51	32.53	31.64	-2.74
		640 mg/kg Cu	6.29	6.64	5.56	34.42	33.23	-3.46
		Benomyl	6.45	6.56	1.78	31.76	33.70	6.11
	3	Control	6.22	6.17	-0.80	32.39	33.11	2.22
		20 mg/kg Cu	6.37	6.33	-0.63	32.23	33.16	2.89
		80 mg/kg Cu	6.35	6.32	-0.47	33.19	35.07	5.66
		160 mg/kg Cu	6.33	6.37	0.55	33.86	32.84	-3.01
		320 mg/kg Cu	6.26	6.30	0.64	31.22	34.08	9.16
		640 mg/kg Cu	6.25	6.44	3.04	32.97	30.80	-6.58
		Benomyl	6.33	6.47	2.21	33.59	34.33	2.20
	4	Control	6.15	6.25	1.63	32.70	33.24	1.65
		20 mg/kg Cu	6.36	6.38	0.31	33.20	32.31	-2.68
		80 mg/kg Cu	6.37	6.40	0.39	33.27	32.74	-1.59
		160 mg/kg Cu	6.29	6.43	2.23	33.47	31.78	-5.05
		320 mg/kg Cu	6.24	6.44	3.12	31.97	32.69	2.25
		640 mg/kg Cu	6.23	6.48	4.01	33.00	32.13	-2.64
		Benomyl	6.39	6.63	3.84	33.74	34.70	2.85

Table 6 (data): Summarised Cu concentrations measured after the exposure period in OECD soil used for exposing five earthworm species to Cu in the form of copper oxychloride for 14 days. The % Recovery is the measured Cu concentration calculated as a percentage of the nominal concentration. The nominal Cu concentrations are given in the Treatment column (Control = 0 mg/kg Cu).

			Measured Cu (mg/kg)					% Recovery				
Experiment with	Treatment	n	Mean	Std Dev	Median	Q25	Q75	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	4	1.5	1.0	2.0	1.0	2.0					
	20 mg/kg Cu	4	12.0	1.6	12.0	11.0	13.0	60.0	8.2	60.0	55.0	65.0
	80 mg/kg Cu	4	46.5	1.0	46.0	46.0	47.0	58.1	1.3	57.5	57.5	58.8
	160 mg/kg Cu	4	95.5	11.8	100.0	89.0	102.0	59.7	7.4	62.5	55.6	63.8
	320 mg/kg Cu	3	184.7	23.2	196.0	158.0	200.0	57.7	7.2	61.3	49.4	62.5
	640 mg/kg Cu	4	349.5	34.4	360.0	327.0	372.0	54.6	5.4	56.3	51.1	58.1
	960 mg/kg Cu	1	540.0	0.0	540.0	540.0	540.0	56.3	0.0	56.3	56.3	56.3
A. trapezoides	Control	4	2.0	2.8	1.0	0.0	4.0					
	20 mg/kg Cu	4	12.5	1.0	12.0	12.0	13.0	62.5	5.0	60.0	60.0	65.0
	80 mg/kg Cu	4	47.0	3.5	48.0	45.0	49.0	58.8	4.3	60.0	56.3	61.3
	160 mg/kg Cu	4	87.0	5.3	88.0	83.0	91.0	54.4	3.3	55.0	51.9	56.9
	320 mg/kg Cu	4	168.0	11.3	172.0	160.0	176.0	52.5	3.5	53.8	50.0	55.0
	640 mg/kg Cu	4	320.0	19.5	315.0	305.0	335.0	50.0	3.1	49.2	47.7	52.3
Chilota sp.	Control	3	2.0	0.0	2.0	2.0	2.0					
	20 mg/kg Cu	3	16.0	0.0	16.0	16.0	16.0	80.0	0.0	80.0	80.0	80.0
	80 mg/kg Cu	3	50.7	5.0	50.0	46.0	56.0	63.3	6.3	62.5	57.5	70.0
	160 mg/kg Cu	3	108.0	8.0	108.0	100.0	116.0	67.5	5.0	67.5	62.5	72.5
	320 mg/kg Cu	3	189.3	9.5	186.0	182.0	200.0	59.2	3.0	58.1	56.9	62.5
	640 mg/kg Cu	3	412.7	53.3	434.0	352.0	452.0	64.5	8.3	67.8	55.0	70.6
E. andrei (A)	Control	5	0.0	0.0	0.0	0.0	0.0					
	20 mg/kg Cu	5	15.2	1.1	16.0	14.0	16.0	76.0	5.5	80.0	70.0	80.0
	80 mg/kg Cu	5	53.6	4.6	54.0	54.0	56.0	67.0	5.7	67.5	67.5	70.0
	160 mg/kg Cu	5	104.0	9.7	106.0	104.0	108.0	65.0	6.1	66.3	65.0	67.5
	640 mg/kg Cu	5	360.0	10.3	364.0	350.0	368.0	56.3	1.6	56.9	54.7	57.5
	960 mg/kg Cu	5	592.0	33.5	600.0	580.0	620.0	61.7	3.5	62.5	60.4	64.6
E. andrei (B)	Control	4	1.5	1.0	2.0	1.0	2.0					
	20 mg/kg Cu	4	15.0	1.2	15.0	14.0	16.0	75.0	5.8	75.0	70.0	80.0
	80 mg/kg Cu	4	52.5	2.5	52.0	51.0	54.0	65.6	3.1	65.0	63.8	67.5
	160 mg/kg Cu	4	106.5	7.5	109.0	101.0	112.0	66.6	4.7	68.1	63.1	70.0
	320 mg/kg Cu	4	195.5	7.0	195.0	190.0	201.0	61.1	2.2	60.9	59.4	62.8
	640 mg/kg Cu	4	367.0	32.9	366.0	340.0	394.0	57.3	5.1	57.2	53.1	61.6
P. excavatus	Control	4	2.5	1.0	2.0	2.0	3.0					
	20 mg/kg Cu	4	14.0	1.6	14.0	13.0	15.0	70.0	8.2	70.0	65.0	75.0
	80 mg/kg Cu	4	48.5	7.0	52.0	45.0	52.0	60.6	8.8	65.0	56.3	65.0
	160 mg/kg Cu	4	86.0	8.2	86.0	81.0	91.0	53.8	5.1	53.8	50.6	56.9
	320 mg/kg Cu	4	179.5	23.2	177.0	164.0	195.0	56.1	7.2	55.3	51.3	60.9
	640 mg/kg Cu	4	326.0	23.4	318.0	311.0	341.0	50.9	3.7	49.7	48.6	53.3

Table 7 (data): Corrected summarised Cu concentrations measured after the exposure period in OECD soil used for exposing five earthworm species to Cu in the form of copper oxychloride for 14 days. In this table, the Cu values from Table 6 in this Appendix were used and were corrected using the 71% mean recovery from AAS measurements of OECD soil samples spiked with a Cu standard (from Table 1 in this Appendix). The Corrected % Recovery is the corrected Cu concentration calculated as a percentage of the nominal concentration. The nominal Cu concentrations are given in the Treatment column (Control = 0 mg/kg Cu).

			Corrected Cu (mg/kg)					Corrected % Recovery				
Experiment with	Treatment	n	Mean	Std Dev	Me-dian	Q25	Q75	Mean	Std Dev	Me-dian	Q25	Q75
A. diffringens	Control	4	2.1	1.4	2.8	1.4	2.8					
	20 mg/kg Cu	4	17.0	2.3	17.0	15.6	18.4	84.9	11.6	84.9	77.8	92.0
	80 mg/kg Cu	4	65.8	1.4	65.1	65.1	66.5	82.3	1.8	81.4	81.4	83.1
	160 mg/kg Cu	4	135.1	16.7	141.5	125.9	144.3	84.5	10.5	88.4	78.7	90.2
	320 mg/kg Cu	3	261.3	32.8	277.4	223.6	283.0	81.7	10.3	86.7	69.9	88.4
	640 mg/kg Cu	4	494.6	48.7	509.4	462.7	526.4	77.3	7.6	79.6	72.3	82.3
	960 mg/kg Cu	1	764.1	0.0	764.1	764.1	764.1	79.6	0.0	79.6	79.6	79.6
A. trapezoides	Control	4	2.8	4.0	1.4	0.0	5.7					
	20 mg/kg Cu	4	17.7	1.4	17.0	17.0	18.4	88.4	7.1	84.9	84.9	92.0
	80 mg/kg Cu	4	66.5	4.9	67.9	63.7	69.3	83.1	6.1	84.9	79.6	86.7
	160 mg/kg Cu	4	123.1	7.5	124.5	117.5	128.8	76.9	4.7	77.8	73.4	80.5
	320 mg/kg Cu	4	237.7	16.0	243.4	226.4	249.1	74.3	5.0	76.1	70.8	77.8
	640 mg/kg Cu	4	452.8	27.6	445.8	431.6	474.1	70.8	4.3	69.6	67.4	74.1
Chilota sp.	Control	3	2.8	0.0	2.8	2.8	2.8					
	20 mg/kg Cu	3	22.6	0.0	22.6	22.6	22.6	113.2	0.0	113.2	113.2	113.2
	80 mg/kg Cu	3	71.7	7.1	70.8	65.1	79.2	89.6	8.9	88.4	81.4	99.1
	160 mg/kg Cu	3	152.8	11.3	152.8	141.5	164.2	95.5	7.1	95.5	88.4	102.6
	320 mg/kg Cu	3	267.9	13.4	263.2	257.5	283.0	83.7	4.2	82.3	80.5	88.4
	640 mg/kg Cu	3	584.0	75.4	614.1	498.1	639.6	91.2	11.8	96.0	77.8	99.9
E. andrei (A)	Control	5	0.0	0.0	0.0	0.0	0.0					
	20 mg/kg Cu	5	21.5	1.6	22.6	19.8	22.6	107.5	7.8	113.2	99.1	113.2
	80 mg/kg Cu	5	75.8	6.5	76.4	76.4	79.2	94.8	8.1	95.5	95.5	99.1
	160 mg/kg Cu	5	147.2	13.7	150.0	147.2	152.8	92.0	8.6	93.7	92.0	95.5
	640 mg/kg Cu	5	509.4	14.6	515.1	495.3	520.8	79.6	2.3	80.5	77.4	81.4
	960 mg/kg Cu	5	837.7	47.4	849.1	820.8	877.4	87.3	4.9	88.4	85.5	91.4
E. andrei (B)	Control	4	2.1	1.4	2.8	1.4	2.8					
	20 mg/kg Cu	4	21.2	1.6	21.2	19.8	22.6	106.1	8.2	106.1	99.1	113.2
	80 mg/kg Cu	4	74.3	3.6	73.6	72.2	76.4	92.9	4.5	92.0	90.2	95.5
	160 mg/kg Cu	4	150.7	10.7	154.2	142.9	158.5	94.2	6.7	96.4	89.3	99.1
	320 mg/kg Cu	4	276.6	9.9	275.9	268.9	284.4	86.5	3.1	86.2	84.0	88.9
	640 mg/kg Cu	4	519.3	46.5	517.9	481.1	557.5	81.1	7.3	80.9	75.2	87.1
P. excavatus	Control	4	3.5	1.4	2.8	2.8	4.2					
	20 mg/kg Cu	4	19.8	2.3	19.8	18.4	21.2	100.1	10.0	99.1	94.0	106.1
	80 mg/kg Cu	4	68.6	9.9	73.6	63.7	73.6	86.3	12.8	92.0	79.6	93.0
	160 mg/kg Cu	4	121.7	11.6	121.7	114.6	128.8	77.7	8.0	79.4	71.6	83.8
	320 mg/kg Cu	4	254.0	32.8	250.5	232.1	275.9	79.8	11.0	78.3	72.5	87.1
	640 mg/kg Cu	4	461.3	33.1	450.0	440.1	482.5	73.5	7.9	70.3	68.8	78.2

Table 8 (data): Summarised earthworm body Cu concentrations and BCFs for five earthworm species, measured after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. BCF = bioconcentration factor, calculated by dividing the body Cu concentration by the corresponding measured (corrected) soil Cu concentration from Table 7 in this Appendix. For the control of E. andrei (A), no BCF could be calculated because no Cu could be detected in the OECD soil; the cell is thus filled with a dash.

		Cu (mg/kg)						
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75	BCF
A. diffringens	Control	8	2.99	0.67	3.03	2.50	3.57	2.0
	20 mg/kg Cu	8	8.14	1.64	8.09	7.12	9.16	0.7
	80 mg/kg Cu	8	15.74	3.61	14.46	14.20	15.37	0.3
	160 mg/kg Cu	8	22.39	4.50	23.82	17.78	26.49	0.2
	320 mg/kg Cu	6	38.81	7.57	37.59	31.93	42.84	0.2
	640 mg/kg Cu	2	44.31	16.48	44.31	32.66	55.97	0.1
	960 mg/kg Cu	1	43.18	0.00	43.18	43.18	43.18	0.1
A. trapezoides	Control	8	1.49	0.33	1.50	1.33	1.73	0.7
	20 mg/kg Cu	8	3.67	1.16	3.79	2.70	4.37	0.3
	80 mg/kg Cu	8	8.86	1.73	9.00	7.31	10.31	0.2
	160 mg/kg Cu	8	12.78	3.07	12.98	9.84	15.18	0.1
	320 mg/kg Cu	8	11.13	3.37	10.39	9.11	11.95	0.1
	640 mg/kg Cu	8	17.08	4.96	17.97	12.02	21.63	0.1
Chilota sp.	Control	6	2.29	0.40	2.40	1.89	2.62	1.1
	20 mg/kg Cu	6	4.25	0.53	4.23	4.03	4.57	0.3
	80 mg/kg Cu	6	9.20	1.74	9.21	8.10	10.57	0.2
	160 mg/kg Cu	6	18.26	4.53	17.64	14.21	20.48	0.2
	320 mg/kg Cu	6	22.23	5.21	21.43	19.45	26.82	0.1
	640 mg/kg Cu	4	28.10	4.51	28.97	24.60	31.61	0.1
E. andrei (A)	Control	10	3.25	0.86	3.04	2.64	4.18	-
	20 mg/kg Cu	10	4.31	1.99	4.15	3.39	5.64	0.3
	80 mg/kg Cu	10	6.74	1.28	6.60	5.96	7.82	0.1
	160 mg/kg Cu	10	8.79	3.38	7.99	6.49	10.06	0.1
	640 mg/kg Cu	10	17.38	7.08	15.33	11.67	22.02	0.0
	960 mg/kg Cu	10	21.27	10.17	17.68	14.85	20.11	0.0
E. andrei (B)	Control	8	4.28	3.37	3.47	2.47	5.97	2.9
	20 mg/kg Cu	8	4.21	1.49	3.83	3.02	5.21	0.3
	80 mg/kg Cu	8	7.67	1.81	6.88	6.52	9.08	0.1
	160 mg/kg Cu	8	8.41	1.40	8.38	7.03	9.65	0.1
	320 mg/kg Cu	8	17.17	4.96	18.22	11.98	21.22	0.1
	640 mg/kg Cu	8	17.19	6.19	15.77	12.92	22.05	0.0
P. excavatus	Control	8	6.21	1.21	5.97	5.48	7.00	2.5
	20 mg/kg Cu	8	6.65	1.11	6.42	6.31	7.39	0.5
	80 mg/kg Cu	8	8.49	1.57	7.96	7.44	9.79	0.2
	160 mg/kg Cu	8	9.84	1.59	9.64	8.65	10.79	0.1
	320 mg/kg Cu	8	15.80	2.70	15.53	14.85	17.42	0.1
	640 mg/kg Cu	6	12.62	7.74	9.26	8.72	14.50	0.0

Table 9 (data): The number of cocoons found in OECD soil after exposing five earthworm species to Cu in the form of copper oxychloride and benomyl (positive control) for 14 days. The values given are for all replicates combined for each species.

Species	Treatment	No. of replicates	No. worms at end of exposure period	Total cocoons	Cocoons / worm	Cocoons / worm / day
A. diffringens	Control	2	14	15	1.88	0.13
	20 mg/kg Cu	2	15	15	2.14	0.15
	80 mg/kg Cu	2	16	24	3.00	0.21
	160 mg/kg Cu	2	14	5	0.63	0.04
	320 mg/kg Cu	1	9	0	0	0
	640 mg/kg Cu	2	2	0	0	0
	960 mg/kg Cu	1	1	0	0	0
	Benomyl	2	14	14	1.75	0.13
A. trapezoides	Control	4	16	6	0.38	0.03
	20 mg/kg Cu	4	16	6	0.38	0.03
	80 mg/kg Cu	4	16	2	0.13	0.01
	160 mg/kg Cu	4	16	0	0	0
	320 mg/kg Cu	4	16	0	0	0
	640 mg/kg Cu	4	16	0	0	0
	Benomyl	4	16	0	0	0
Chilota sp.	Control	3	12	0	0	0
	20 mg/kg Cu	3	12	0	0	0
	80 mg/kg Cu	3	12	0	0	0
	160 mg/kg Cu	3	13	0	0	0
	320 mg/kg Cu	3	11	0	0	0
	640 mg/kg Cu	3	8	0	0	0
	Benomyl	3	12	0	0	0
E. andrei (A)	Control	5	37	58	1.57	0.11
	20 mg/kg Cu	5	37	63	1.70	0.12
	80 mg/kg Cu	5	37	60	1.62	0.12
	160 mg/kg Cu	5	37	41	1.11	0.08
	640 mg/kg Cu	5	36	6	0.17	0.01
	960 mg/kg Cu	5	34	4	0.12	0.01
	Benomyl	5	37	3	0.08	0.01
E. andrei (B)	Control	4	16	7	0.44	0.03
	20 mg/kg Cu	4	16	7	0.44	0.03
	80 mg/kg Cu	4	16	6	0.38	0.03
	160 mg/kg Cu	4	16	2	0.13	0.01
	320 mg/kg Cu	4	16	2	0.13	0.01
	640 mg/kg Cu	4	16	0	0	0
	Benomyl	4	16	2	0.13	0.01
P. excavatus	Control	4	16	3	0.75	0.05
	20 mg/kg Cu	4	16	7	1.75	0.13
	80 mg/kg Cu	4	16	4	1.00	0.07
	160 mg/kg Cu	4	16	3	0.75	0.05
	320 mg/kg Cu	4	16	0	0	0
	640 mg/kg Cu	4	9	0	0	0
	Benomyl	4	16	0	0	0

Table 10 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman Rank correlations) between the number of cocoons produced per earthworm and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) after 14 days exposure of four earthworm species to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = cocoons produced per worm. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs cocoons				Body Cu vs cocoons			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.01x + 2.11	-0.69	0.03	10	y = -0.05x + 2.36	-0.70	0.03	10
A. trapezoides	y = -0.001x + 0.26	-0.63	< 0.05	24	y = -0.03x + 0.38	-0.60	< 0.05	24
E. andrei (A)	y = -0.002x + 1.54	-0.74	< 0.05	30	y = -0.08x + 1.79	-0.74	< 0.05	30
E. andrei (B)	y = -0.001x + 0.40	-0.66	< 0.05	24	y = -0.02x + 0.43	-0.46	0.02	24
P. excavatus	y = -0.001x + 0.29	-0.47	0.02	24	y = -0.027x + 0.45	-0.37	0.08	23

Table 11a (statistical results): Results of the Kruskal-Wallis ANOVA by ranks test to determine significant differences between treatments for the number of cocoons produced per earthworm in three earthworm species exposed to Cu in the form of copper oxychloride in OECD artificial soil for 14 days. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 11b. Only treatments where cocoons were produced were included in these analyses.

Species	n	H	*P*	df
A. trapezoides	12	2.07	0.35	2
E. andrei (A)	35	25.68	**< 0.001**	6
E. andrei (B)	24	6.73	0.24	5
P. excavatus	16	2.35	0.50	3

Table 11b (statistical results): Results of the Kruskal-Wallis ANOVA by ranks Post hoc multiple comparison tests for cocoon production, for *E. andrei* (A) (from Table 11a in this Appendix). The z' values are above the diagonal and the corresponding *P* values are below the diagonal. K = negative control, B = positive control (benomyl). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

E. andrei (A)		z'						
		K	Cu20	Cu80	Cu160	Cu640	Cu960	B
	K		0.15	0.32	0.46	2.48	2.75	2.78
	Cu20	1.00		0.17	0.62	2.64	2.90	2.93
	Cu80	1.00	1.00		0.79	2.81	**3.07**	**3.10**
P	Cu160	1.00	1.00	1.00		2.02	2.28	2.31
	Cu640	0.27	0.17	0.10	0.91		0.26	0.29
	Cu960	0.13	0.08	**0.04**	0.47	1.00		0.03
	B	0.12	0.07	**0.04**	0.43	1.00	1.00	

Table 12 (data): Summarised start mass of specimens of five earthworm species (g wet weight) before exposure to different treatments of Cu (in the form of copper oxychloride) and a positive control (10 mg/kg benomyl) in OECD soil. In the last section of the table, summaries are given for all replicates and treatments combined for each species.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	16	1.00	0.21	1.04	0.85	1.11
	20 mg/kg Cu	16	1.01	0.25	1.00	0.82	1.21
	80 mg/kg Cu	16	0.98	0.28	0.91	0.77	1.12
	160 mg/kg Cu	16	1.09	0.32	1.05	0.84	1.34
	320 mg/kg Cu	12	0.94	0.23	0.91	0.78	1.08
	640 mg/kg Cu	16	0.95	0.23	0.92	0.75	1.11
	960 mg/kg Cu	4	1.18	0.35	1.12	0.90	1.46
	Benomyl	16	0.96	0.24	0.87	0.79	1.18
A. trapezoides	Control	16	0.77	0.14	0.75	0.65	0.88
	20 mg/kg Cu	16	0.80	0.15	0.79	0.67	0.89
	80 mg/kg Cu	16	0.78	0.16	0.78	0.65	0.87
	160 mg/kg Cu	16	0.78	0.13	0.76	0.67	0.85
	320 mg/kg Cu	16	0.79	0.17	0.78	0.68	0.90
	640 mg/kg Cu	16	0.78	0.15	0.75	0.69	0.82
	Benomyl	16	0.79	0.10	0.79	0.73	0.86
Chilota sp.	Control	12	2.75	0.71	2.84	2.47	3.27
	20 mg/kg Cu	12	2.20	0.54	2.06	1.75	2.66
	80 mg/kg Cu	12	2.72	0.84	2.68	2.19	3.36
	160 mg/kg Cu	13	2.28	0.62	2.31	1.84	2.63
	320 mg/kg Cu	11	2.29	0.60	2.07	1.77	2.76
	640 mg/kg Cu	12	2.54	0.68	2.54	1.95	3.03
	Benomyl	12	2.31	0.55	2.34	1.96	2.74
E. andrei (A)	Control	37	0.50	0.07	0.49	0.44	0.55
	20 mg/kg Cu	37	0.48	0.08	0.49	0.41	0.55
	80 mg/kg Cu	37	0.49	0.07	0.49	0.45	0.54
	160 mg/kg Cu	37	0.49	0.07	0.49	0.44	0.54
	640 mg/kg Cu	37	0.47	0.06	0.47	0.42	0.52
	960 mg/kg Cu	37	0.49	0.07	0.48	0.45	0.52
	Benomyl	37	0.49	0.06	0.49	0.43	0.54
E. andrei (B)	Control	16	0.40	0.08	0.37	0.34	0.45
	20 mg/kg Cu	16	0.39	0.07	0.40	0.35	0.43
	80 mg/kg Cu	16	0.38	0.07	0.39	0.34	0.43
	160 mg/kg Cu	16	0.39	0.09	0.40	0.31	0.45
	320 mg/kg Cu	16	0.37	0.09	0.35	0.33	0.40
	640 mg/kg Cu	16	0.36	0.09	0.35	0.31	0.43
	Benomyl	16	0.37	0.06	0.37	0.33	0.41
P. excavatus	Control	16	0.45	0.07	0.44	0.41	0.50
	20 mg/kg Cu	16	0.44	0.07	0.44	0.38	0.50
	80 mg/kg Cu	16	0.46	0.06	0.47	0.44	0.49
	160 mg/kg Cu	16	0.47	0.07	0.49	0.43	0.51
	320 mg/kg Cu	16	0.44	0.09	0.41	0.38	0.49
	640 mg/kg Cu	16	0.47	0.06	0.47	0.41	0.51
	Benomyl	16	0.43	0.08	0.41	0.36	0.49
A. diffringens	All	112	1.00	0.26	0.96	0.79	1.18
A. trapezoides	All	112	0.78	0.14	0.78	0.68	0.87
Chilota sp.	All	84	2.44	0.67	2.44	1.87	2.89
E. andrei (A)	All	259	0.49	0.07	0.43	0.49	0.54
E. andrei (B)	All	112	0.38	0.08	0.33	0.38	0.43
E. andrei (both)	All	371	0.46	0.09	0.45	0.40	0.52
P. excavatus	All	112	0.45	0.07	0.46	0.40	0.50

Table 13 (statistical results): Results of the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the mass before experimentation (start mass) for five earthworm species. None of the *P*-values were significant ($P < 0.05$).

ANOVA (parametric data)				
Species	n	F	*P*	df
Chilota sp.	84	1.43	0.21	6
P. excavatus	112	0.73	0.62	6
E. andrei (B)	112	0.41	0.87	6

KW ANOVA (nonparametric data)				
Species	n	H	*P*	df
A. diffringens	112	4.62	0.71	7
A. trapezoides	112	0.63	1.00	6
E. andrei (A)	259	3.20	0.78	6

Table 14 (data): Summarised earthworm mass (g wet weight) of specimens of five earthworm species after 14 days exposure (end mass, measured on day 14) to Cu in the form of copper oxychloride and a positive control (10 mg/kg benomyl) in OECD soil.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	14	1.11	0.27	1.06	0.91	1.40
	20 mg/kg Cu	15	1.04	0.30	1.00	0.78	1.29
	80 mg/kg Cu	16	1.05	0.31	1.04	0.82	1.21
	160 mg/kg Cu	14	1.11	0.29	1.12	0.86	1.35
	320 mg/kg Cu	9	0.80	0.16	0.78	0.75	0.82
	640 mg/kg Cu	2	0.79	0.14	0.79	0.69	0.89
	960 mg/kg Cu	1	0.51	0.00	0.51	0.51	0.51
	Benomyl	14	1.05	0.27	1.03	0.86	1.17
A. trapezoides	Control	16	0.75	0.16	0.77	0.64	0.87
	20 mg/kg Cu	16	0.85	0.19	0.83	0.75	0.96
	80 mg/kg Cu	16	0.72	0.16	0.72	0.59	0.82
	160 mg/kg Cu	16	0.62	0.13	0.60	0.54	0.70
	320 mg/kg Cu	16	0.59	0.12	0.58	0.51	0.65
	640 mg/kg Cu	16	0.51	0.12	0.50	0.42	0.55
	Benomyl	4	0.49	0.05	0.50	0.46	0.52
Chilota sp.	Control	12	2.83	0.69	2.97	2.64	3.03
	20 mg/kg Cu	12	2.27	0.59	2.20	1.74	2.76
	80 mg/kg Cu	12	2.82	0.77	2.85	2.31	3.37
	160 mg/kg Cu	13	2.38	0.64	2.28	1.91	2.69
	320 mg/kg Cu	11	2.07	0.59	1.85	1.70	2.73
	640 mg/kg Cu	8	1.78	0.50	1.63	1.42	2.23
	Benomyl	12	2.35	0.54	2.31	1.91	2.70
E. andrei (A)	Control	37	0.49	0.07	0.51	0.43	0.55
	20 mg/kg Cu	37	0.46	0.07	0.46	0.43	0.48
	80 mg/kg Cu	37	0.47	0.06	0.46	0.43	0.50
	160 mg/kg Cu	37	0.48	0.08	0.46	0.42	0.53
	640 mg/kg Cu	36	0.39	0.08	0.35	0.33	0.45
	960 mg/kg Cu	34	0.37	0.09	0.36	0.31	0.44
	Benomyl	37	0.42	0.07	0.41	0.37	0.47
E. andrei (B)	Control	16	0.40	0.10	0.36	0.34	0.45
	20 mg/kg Cu	16	0.42	0.10	0.43	0.36	0.48
	80 mg/kg Cu	16	0.38	0.07	0.38	0.35	0.42
	160 mg/kg Cu	16	0.41	0.09	0.41	0.32	0.49

Table 14 continued (Earthworm mass after exposure)

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
E. andrei (B)	320 mg/kg Cu	16	0.39	0.13	0.36	0.30	0.43
	640 mg/kg Cu	16	0.33	0.09	0.33	0.26	0.42
	Benomyl	16	0.35	0.07	0.34	0.30	0.41
P. excavatus	Control	16	0.44	0.07	0.45	0.40	0.50
	20 mg/kg Cu	16	0.44	0.07	0.44	0.39	0.48
	80 mg/kg Cu	16	0.46	0.07	0.47	0.42	0.50
	160 mg/kg Cu	16	0.46	0.07	0.47	0.42	0.50
	320 mg/kg Cu	16	0.44	0.08	0.42	0.38	0.50
	640 mg/kg Cu	9	0.38	0.08	0.40	0.33	0.43
	Benomyl	16	0.43	0.08	0.43	0.36	0.49

Table 15 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between earthworm mass at the end of the 14 day exposure period (end mass) and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = end mass. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman $R > 0.8$ or < -0.8).

	Soil Cu vs End mass				Body Cu vs End mass			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.001x + 1.10	-0.21	0.08	71	y = -0.0053x + 1.10	-0.28	0.07	41
A. trapezoides	y = -0.0009x + 0.77	-0.58	< 0.01	96	y = -0.02x + 0.86	-0.57	< 0.01	48
Chilota sp.	y = -0.002x + 2.65	-0.39	< 0.01	68	y = -0.03x + 3.00	-0.53	< 0.01	34
E. andrei (A)	y = -0.002x + 0.48	-0.45	< 0.01	218	y = -0.006x + 0.52	-0.45	< 0.01	60
E. andrei (B)	y = -0.0002x + 0.42	-0.22	0.03	96	y = -0.002x + 0.41	-0.15	0.30	48
P. excavatus	y = -0.0001x + 0.45	-0.09	0.40	89	y = -0.002x + 0.45	-0.05	0.75	46

Table 16a (statistical results): Results of the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for earthworm mass after 14 days (end mass, measured on day 14) for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 16b.

ANOVA (parametric data)					Reference to
Species	n	F	*P*	df	Table 16b
Chilota sp.	80	3.76	**0.003**	6	I
P. excavatus	105	1.61	0.15	6	

KW ANOVA (nonparametric data)					
Species	n	H	*P*	df	
A. diffringens	85	14.06	0.05	7	
A. trapezoides	100	42.25	**< 0.0001**	6	II
E. andrei (A)	255	59.73	**< 0.0001**	6	III
E. andrei (B)	112	10.53	0.10	6	

Table 16b (statistical results): Results of the ANOVA Fisher LSD post hoc test (for *Chilota sp.*) and the Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons tests (for *A. trapezoides* and *E. andrei* (A)) for end mass (from Table 16a in this Appendix). For the ANOVA post-hoc tests, the *P*-values are presented below the diagonal, and for the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P* values are below the diagonal. K = negative control, B = positive control (benomyl). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

I *Chilota sp.* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	**0.03**						
	Cu80	0.97	**0.03**					
P	Cu160	0.07	0.67	0.08				
	Cu320	**0.005**	0.45	**0.01**	0.24			
	Cu640	**< 0.001**	0.09	**< 0.001**	**0.04**	0.32		
	B	0.06	0.75	0.07	0.92	0.29	**0.05**	

II *A. trapezoides* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K		1.08	0.55	2.04	2.61	**4.14**	**3.07**
	Cu20	1.00		1.63	**3.11**	**3.69**	**5.22**	**3.75**
	Cu80	1.00	1.00		1.48	2.06	**3.59**	2.72
P	Cu160	0.88	**0.04**	1.00		0.58	2.11	1.78
	Cu320	0.19	**0.005**	0.83	1.00		1.53	1.42
	Cu640	**0.001**	**< 0.001**	**0.01**	0.74	1.00		0.45
	B	**0.04**	**0.004**	0.14	1.00	1.00	1.00	

III *E. andrei* (A) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu640	Cu960	B
	K		1.58	1.16	1.11	**5.34**	**5.68**	**3.51**
	Cu20	1.00		0.42	0.48	**3.77**	**4.13**	1.93
	Cu80	1.00	1.00		0.05	**4.19**	**4.54**	2.36
P	Cu160	1.00	1.00	1.00		**4.24**	**4.59**	2.41
	Cu640	**< 0.001**	**0.003**	**0.001**	**< 0.001**		0.41	1.85
	Cu960	**< 0.001**	**0.001**	**< 0.001**	**< 0.001**	1.00		2.24
	B	**0.01**	1.00	0.39	0.34	1.00	0.53	

Table 17 (statistical results): Pairwise comparisons (Mann-Whitney U Test for nonparametric data or Student's t-test for parametric data) between earthworm body mass at each treatment before (start mass) and after (end mass) 14 days exposure to Cu in the form of copper oxychloride, and a positive control, benomyl, in OECD soil. Significant *P*-values ($P < 0.05$) are in bold. nd = not done due to insufficient number of data points.

Mann-Whitney U test (nonparametric data)

Species	Treatment	U	Z	*P*	Start mass n	End mass n
A. diffringens	Control	88.00	-0.98	0.33	16	14
	20 mg/kg Cu	113.00	-0.26	0.80	16	15
	80 mg/kg Cu	104.00	-0.89	0.38	16	16
	160 mg/kg Cu	105.00	-0.27	0.79	16	14
	320 mg/kg Cu	30.00	1.67	0.09	12	9
	640 mg/kg Cu	10.00	0.77	0.44	16	2
	960 mg/kg Cu	nd	nd	nd	4	1
	Benomyl	89.00	-0.94	0.35	16	14
A. trapezoides	Control	123.00	0.17	0.87	16	16
	20 mg/kg Cu	107.00	-0.77	0.44	16	16
	80 mg/kg Cu	105.00	0.85	0.40	16	16
	160 mg/kg Cu	41.00	3.26	**0.001**	16	16
	320 mg/kg Cu	42.00	3.22	**0.001**	16	16
	640 mg/kg Cu	16.00	4.20	< **0.001**	16	16
	Benomyl	0.00	2.98	**0.003**	16	4
E. andrei (A)	Control	658.00	0.28	0.78	37	37
	20 mg/kg Cu	601.00	0.90	0.37	37	37
	80 mg/kg Cu	512.00	1.86	**0.06**	37	37
	160 mg/kg Cu	617.00	0.72	0.47	37	37
	640 mg/kg Cu	273.00	4.33	< **0.001**	37	36
	960 mg/kg Cu	188.00	5.07	< **0.001**	37	34
	Benomyl	357.00	3.54	< **0.001**	37	37
E. andrei (B)	Control	121.00	0.24	0.81	16	16
	20 mg/kg Cu	100.00	-1.04	0.30	16	16
	80 mg/kg Cu	128.00	0.00	1.00	16	16
	160 mg/kg Cu	111.00	-0.62	0.53	16	16
	320 mg/kg Cu	128.00	0.00	1.00	16	16
	640 mg/kg Cu	101.00	1.00	0.32	16	16
	Benomyl	113.00	0.55	0.58	16	16

Student's t-test (parametric data)

Species	Treatment	t	df	*P*	Start mass n	End mass n
Chilota sp.	Control	-0.28	22	0.78	12	12
	20 mg/kg Cu	-0.31	22	0.76	12	12
	80 mg/kg Cu	-0.32	22	0.75	12	12
	160 mg/kg Cu	-0.38	24	0.70	13	13
	320 mg/kg Cu	0.86	20	0.40	11	11
	640 mg/kg Cu	2.72	18	**0.01**	12	8
	Benomyl	-0.17	22	0.86	12	12
P. excavatus	Control	0.20	30	0.84	16	16
	20 mg/kg Cu	0.39	30	0.70	16	16
	80 mg/kg Cu	0.11	30	0.91	16	16
	160 mg/kg Cu	0.30	30	0.77	16	16
	320 mg/kg Cu	-0.15	30	0.88	16	16
	640 mg/kg Cu	3.07	23	**0.01**	16	9
	Benomyl	0.19	30	0.85	16	16

Table 18 (data): Summarised earthworm mass changes during exposure (exposure mass change, which is the difference between the mass after exposure and the mass before exposure, calculated as a percentage of the mass before exposure) of five earthworm species after 14 days exposure to Cu in the form of copper oxychloride and a positive control (10 mg/kg benomyl) in OECD soil.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	4	8.48	10.99	9.43	0.33	16.63
	20 mg/kg Cu	4	3.07	12.89	2.77	-6.15	12.28
	80 mg/kg Cu	4	7.67	7.64	7.85	1.06	14.27
	160 mg/kg Cu	4	2.49	16.85	-1.31	-9.90	14.89
	320 mg/kg Cu	3	-16.41	4.04	-15.83	-20.71	-12.70
	640 mg/kg Cu	2	-22.65	19.49	-22.65	-36.43	-8.87
	960 mg/kg Cu	1	-56.50	0.00	-56.50	-56.50	-56.50
	Benomyl	4	7.88	8.04	5.70	3.03	12.74
A. trapezoides	Control	4	-2.17	3.19	-1.83	-4.70	0.36
	20 mg/kg Cu	4	7.11	4.35	7.33	3.42	10.81
	80 mg/kg Cu	4	-7.59	12.55	-8.58	-18.35	3.17
	160 mg/kg Cu	4	-20.48	7.32	-19.44	-25.83	-15.13
	320 mg/kg Cu	4	-24.86	4.55	-26.42	-27.51	-22.21
	640 mg/kg Cu	4	-33.96	4.64	-33.52	-37.12	-30.81
	Benomyl	1	-36.41	0.00	-36.41	-36.41	-36.41
Chilota sp.	Control	3	2.70	2.36	2.96	0.22	4.92
	20 mg/kg Cu	3	2.88	3.63	0.82	0.76	7.07
	80 mg/kg Cu	3	3.78	1.65	4.36	1.92	5.06
	160 mg/kg Cu	3	3.86	4.92	1.89	0.24	9.46
	320 mg/kg Cu	3	-9.35	10.37	-13.29	-17.16	2.41
	640 mg/kg Cu	3	-32.94	9.17	-30.74	-43.01	-25.06
	Benomyl	3	1.54	2.63	0.09	-0.04	4.58
E. andrei (A)	Control	5	0.21	9.94	-3.09	-6.72	2.70
	20 mg/kg Cu	5	-3.77	5.44	-3.50	-7.43	-0.99
	80 mg/kg Cu	5	-4.10	8.27	-8.38	-10.21	2.55
	160 mg/kg Cu	5	-1.77	4.08	-2.27	-4.64	-0.64
	640 mg/kg Cu	5	-17.63	7.78	-19.19	-20.01	-13.12
	960 mg/kg Cu	5	-25.14	5.33	-26.12	-28.88	-21.58
	Benomyl	5	-12.28	8.24	-14.87	-18.18	-8.77
E. andrei (B)	Control	4	1.04	5.23	1.20	-3.43	5.51
	20 mg/kg Cu	4	9.09	3.24	8.45	6.91	11.28
	80 mg/kg Cu	4	0.86	4.29	2.00	-1.94	3.67
	160 mg/kg Cu	4	5.22	3.32	5.82	2.68	7.76
	320 mg/kg Cu	4	5.35	4.35	4.49	1.99	8.71
	640 mg/kg Cu	4	-8.91	12.93	-5.85	-17.21	-0.61
	Benomyl	4	-3.71	11.03	-8.30	-9.98	2.57
P. excavatus	Control	4	-0.97	3.40	-2.14	-3.23	1.28
	20 mg/kg Cu	4	-1.99	2.51	-2.77	-3.51	-0.48
	80 mg/kg Cu	4	-0.45	4.35	0.69	-3.19	2.29
	160 mg/kg Cu	4	-1.43	5.48	-1.78	-5.30	2.44
	320 mg/kg Cu	4	1.05	4.67	1.84	-2.21	4.31
	640 mg/kg Cu	3	-19.58	7.23	-22.76	-24.68	-11.30
	Benomyl	4	-1.22	0.83	-1.27	-1.88	-0.55

Table 19 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the change in earthworm mass during the 14 day exposure period (exposure mass change, which is the difference between the mass after exposure and before exposure, calculated as a percentage of the mass before exposure) and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = exposure mass change. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. Significant correlations (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8) are in bold.

	Soil Cu vs Exposure mass change				Body Cu vs Exposure mass change			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.10x + 8.76	-0.63	< 0.01	22	y = -0.93x + 15.89	-0.67	< 0.01	22
A. trapezoides	y = -0.12x - 1.41	**-0.83**	**< 0.01**	24	y = -2.08x + 5.42	-0.68	< 0.01	24
Chilota sp.	y = -0.09x + 6.47	-0.58	0.01	18	y = -0.91x + 9.50	-0.59	0.01	17
E. andrei (A)	y = -0.04x - 0.79	-0.64	< 0.01	30	y = -0.94x + 1.03	-0.56	< 0.01	30
E. andrei (B)	y = -0.03x + 0.615	-0.28	0.18	24	y = -0.48x + 6.85	-0.30	0.15	24
P. excavatus	y = -0.04x + 1.10	-0.22	0.31	23	y = -0.38x + 0.52	0.01	0.98	23

Table 20a (statistical results): Results for the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the mass change during exposure (exposure mass change, which is the difference between the mass after exposure and the mass before exposure, calculated as a percentage of the mass before exposure) for five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 20b.

ANOVA (parametric data)					Reference to
Species	n	F	*P*	df	Table 20b
A. trapezoides	25	18.53	**< 0.01**	6	I
E. andrei (A)	35	8.41	**< 0.01**	6	II

KW ANOVA (nonparametric data)				
Species	n	H	*P*	df
A. diffringens	26	13.43	0.06	7
Chilota sp.	21	11.01	0.09	6
E. andrei (B)	28	12.02	0.06	6
P. excavatus	27	9.40	0.15	6

Table 20b (statistical results): Results of the ANOVA Fisher LSD post hoc test (for *A. trapezoides* and *E. andrei* (A)) for exposure mass change (from Table 20a in this Appendix). For the ANOVA post-hoc tests, the *P*-values are presented below the diagonal. K = negative control, B = positive control (benomyl). Significant *P*-values ($P < 0.05$) are in bold.

I *A. trapezoides* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.07						
	Cu80	0.28	**0.01**					
P	Cu160	< **0.01**	< **0.01**	**0.02**				
	Cu320	< **0.01**	< **0.01**	< **0.01**	0.38			
	Cu640	< **0.01**	< **0.01**	< **0.01**	**0.01**	0.08		
	B	< **0.01**	< **0.01**	< **0.01**	0.05	0.15	0.75	

II *E. andrei* (A) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.39						
	Cu80	0.36	0.94					
P	Cu160	0.67	0.67	0.62				
	Cu640	< **0.01**	**0.01**	**0.01**	< **0.01**			
	Cu960	< **0.01**	< **0.01**	< **0.01**	< **0.01**	0.11		
	B	**0.01**	0.07	0.09	**0.03**	0.25	**0.01**	

Table 21 (data): Summarised earthworm mass (g wet weight) of five earthworm species after depuration (depurated mass) of 24 h (48 h for *E. andrei* (A)) on moist filter paper, after 14 days exposure to Cu in the form of copper oxychloride and a positive control (10 mg/kg benomyl) in OECD soil.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	14	1.00	0.21	0.98	0.86	1.19
	20 mg/kg Cu	15	0.95	0.28	0.96	0.68	1.19
	80 mg/kg Cu	16	0.97	0.27	0.95	0.77	1.12
	160 mg/kg Cu	14	1.04	0.30	1.04	0.81	1.20
	320 mg/kg Cu	9	0.78	0.17	0.76	0.68	0.82
	640 mg/kg Cu	2	0.87	0.13	0.87	0.78	0.97
	960 mg/kg Cu	1	0.56	0.00	0.56	0.56	0.56
	Benomyl	14	0.95	0.24	0.88	0.77	1.14
A. trapezoides	Control	16	0.68	0.13	0.70	0.59	0.77
	20 mg/kg Cu	16	0.73	0.13	0.70	0.63	0.83
	80 mg/kg Cu	16	0.66	0.12	0.67	0.56	0.77
	160 mg/kg Cu	16	0.59	0.10	0.57	0.53	0.68
	320 mg/kg Cu	16	0.59	0.15	0.58	0.49	0.63
	640 mg/kg Cu	16	0.50	0.12	0.47	0.42	0.55
	Benomyl	4	0.53	0.08	0.51	0.47	0.58
Chilota sp.	Control	12	2.64	0.72	2.65	2.44	2.91
	20 mg/kg Cu	12	2.25	0.64	2.20	1.66	2.70
	80 mg/kg Cu	12	2.67	0.72	2.70	2.27	3.03
	160 mg/kg Cu	13	2.31	0.62	2.22	1.77	2.64
	320 mg/kg Cu	11	2.10	0.60	1.99	1.66	2.85
	640 mg/kg Cu	6	1.94	0.51	1.74	1.59	2.48
	Benomyl	12	2.19	0.55	2.13	1.78	2.54

Table 21 continued (Earthworm mass after depuration)

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
E. andrei (A)	Control	36	0.42	0.06	0.42	0.37	0.46
	20 mg/kg Cu	37	0.40	0.07	0.39	0.34	0.44
	80 mg/kg Cu	37	0.41	0.05	0.40	0.37	0.45
	160 mg/kg Cu	37	0.42	0.08	0.41	0.36	0.48
	640 mg/kg Cu	36	0.38	0.06	0.37	0.33	0.42
	960 mg/kg Cu	33	0.37	0.09	0.34	0.32	0.42
	Benomyl	21	0.38	0.06	0.38	0.33	0.43
E. andrei (B)	Control	16	0.36	0.08	0.34	0.31	0.37
	20 mg/kg Cu	16	0.38	0.09	0.39	0.32	0.43
	80 mg/kg Cu	16	0.35	0.06	0.35	0.30	0.37
	160 mg/kg Cu	16	0.38	0.08	0.38	0.32	0.45
	320 mg/kg Cu	16	0.36	0.11	0.33	0.29	0.41
	640 mg/kg Cu	16	0.32	0.07	0.33	0.27	0.37
	Benomyl	16	0.34	0.06	0.35	0.29	0.39
P. excavatus	Control	15	0.44	0.06	0.45	0.41	0.50
	20 mg/kg Cu	16	0.44	0.07	0.43	0.38	0.49
	80 mg/kg Cu	16	0.47	0.07	0.48	0.44	0.52
	160 mg/kg Cu	16	0.47	0.07	0.48	0.41	0.50
	320 mg/kg Cu	16	0.43	0.12	0.42	0.39	0.50
	640 mg/kg Cu	8	0.36	0.12	0.37	0.27	0.46
	Benomyl	16	0.44	0.08	0.44	0.35	0.50

Table 22 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between earthworm mass (depurated mass) after 24 h (48 h for *E. andrei* (A)) on moist filter paper after a 14 day exposure period and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = depurated mass. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs Depurated mass				Body Cu vs Depurated mass			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.0006x + 1.00	-0.13	0.26	71	y = -0.006x + 1.07	-0.30	0.05	41
A. trapezoides	y = -0.0006x + 0.69	-0.49	< 0.01	96	y = -0.009x + 0.71	-0.40	0.01	48
Chilota sp.	y = -0.002x + 2.52	-0.29	0.02	66	y = -0.02x + 2.68	-0.35	0.04	34
E. andrei (A)	y = -0.00008x + 0.41	-0.23	< 0.01	216	y = -0.00005x + 0.40	-0.08	0.51	60
E. andrei (B)	y = -0.0001x + 0.37	-0.12	0.24	96	y = -0.001x + 0.38	-0.16	0.27	48
P. excavatus	y = -0.0002x + 0.46	-0.07	0.50	87	y = -0.002x + 0.47	0.01	0.93	46

Table 23a (statistical results): Results for the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the mass after 24 h (48 h for *E. andrei* (A)) depuration on moist filter paper (Depurated mass) after 14 days exposure of five earthworm species to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 23b.

ANOVA (parametric data)					Reference to
Species	n	F	*P*	df	Table 23b
Chilota sp.	78	1.80	0.11	6	
P. excavatus	103	1.94	0.08	6	
E. andrei (A)	237	2.94	**0.01**	6	I

KW ANOVA (nonparametric data)					
Species	n	H	*P*	df	
A. diffringens	85	10.02	0.19	7	
A. trapezoides	100	29.45	**0.001**	6	II
E. andrei (B)	112	6.31	0.39	6	

Table 23b (statistical results): Results of the ANOVA Fisher LSD post hoc test (for *E. andrei* (A)) and Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons (for *A. trapezoides*) for Depurated mass (from Table 23a in this Appendix). For the ANOVA post-hoc tests, the *P*-values are presented below the diagonal, and for the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, B = positive control (benomyl). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

I *E. andrei* (A) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.29						
	Cu80	0.51	0.70					
P	Cu160	0.81	0.19	0.36				
	Cu640	**0.02**	0.17	0.08	**0.01**			
	Cu960	**0.003**	0.05	**0.02**	**0.001**	0.54		
	B	0.10	0.44	0.27	0.06	0.69	0.35	

II *A. trapezoides* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K		0.73	0.26	1.74	2.00	**3.77**	2.13
	Cu20	1.00		0.99	2.47	2.73	**4.50**	2.59
	Cu80	1.00	1.00		1.48	1.74	**3.50**	1.97
P	Cu160	1.00	0.28	1.00		0.26	2.02	1.03
	Cu320	0.95	0.13	1.00	1.00		1.76	0.87
	Cu640	**0.003**	**< 0.001**	**0.01**	0.90	1.00		0.25
	B	0.69	0.20	1.00	1.00	1.00	1.00	

Table 24 (statistical results): Pairwise comparisons (Mann-Whitney U Test for nonparametric data or Student's t-test for parametric data) between earthworm body mass after 14 days exposure (end mass, measured on day 14) to Cu in the form of copper oxychloride in OECD soil and earthworm mass after 24 h (48 h for *E. andrei* (A)) depuration on moist filter paper (Depurated mass, measured on day 15 or 16 for *E. andrei* (A)). Significant *P*-values ($P < 0.05$) are in bold. nd = not done due to insufficient number of data points.

Mann-Whitney U test (nonparametric data)

Species	Treatment	U	Z	*P*	End mass n	Depurated mass n
A. diffringens	Control	81.00	0.76	0.45	14	14
	20 mg/kg Cu	89.00	0.95	0.34	15	15
	80 mg/kg Cu	100.00	1.04	0.30	16	16
	160 mg/kg Cu	78.00	0.90	0.37	14	14
	320 mg/kg Cu	35.00	0.44	0.66	9	9
	640 mg/kg Cu	1.00	-0.39	0.70	2	2
	960 mg/kg Cu	nd	nd	nd	1	1
	Benomyl	74.00	1.08	0.28	14	14
A. trapezoides	Control	97.00	1.15	0.25	16	16
	20 mg/kg Cu	72.00	2.09	**0.04**	16	16
	80 mg/kg Cu	102.00	0.96	0.34	16	16
	160 mg/kg Cu	111.00	0.62	0.53	16	16
	320 mg/kg Cu	122.00	0.21	0.84	16	16
	640 mg/kg Cu	119.00	0.32	0.75	16	16
	Benomyl	6.00	-0.43	0.67	4	4
E. andrei (A)	Control	292.50	4.12	< **0.001**	37	36
	20 mg/kg Cu	349.00	3.62	< **0.001**	37	37
	80 mg/kg Cu	284.00	4.32	< **0.001**	37	37
	160 mg/kg Cu	432.50	2.72	**0.01**	37	37
	640 mg/kg Cu	626.00	0.24	0.81	36	36
	960 mg/kg Cu	557.50	0.04	0.97	34	33
	Benomyl	255.50	2.14	**0.03**	37	21
E. andrei (B)	Control	87.00	1.53	0.13	16	16
	20 mg/kg Cu	92.00	1.34	0.18	16	16
	80 mg/kg Cu	87.00	1.53	0.13	16	16
	160 mg/kg Cu	101.00	1.00	0.32	16	16
	640 mg/kg Cu	107.00	0.77	0.44	16	16
	960 mg/kg Cu	115.00	0.47	0.64	16	16
	Benomyl	117.00	0.40	0.69	16	16

Student's t-test (parametric data)

Species	Treatment	t	df	*P*	End mass n	Depurated mass n
Chilota sp.	Control	0.66	22	0.52	12	12
	20 mg/kg Cu	0.07	22	0.95	12	12
	80 mg/kg Cu	0.52	22	0.61	12	12
	160 mg/kg Cu	0.25	24	0.80	13	13
	320 mg/kg Cu	-0.10	20	0.92	11	11
	640 mg/kg Cu	-0.58	12	0.57	8	6
	Benomyl	0.72	22	0.48	12	12
P. excavatus	Control	0.05	29	0.96	16	15
	20 mg/kg Cu	-0.03	30	0.97	16	16
	80 mg/kg Cu	-0.50	30	0.62	16	16
	160 mg/kg Cu	-0.30	30	0.76	16	16
	320 mg/kg Cu	0.39	30	0.70	16	16
	640 mg/kg Cu	0.38	15	0.71	9	8
	Benomyl	-0.29	30	0.77	16	16

Table 25 (data): Summarised earthworm mass changes during depuration for 24 h (or 48 h for *E. andrei* (A)) on moist filter paper (depuration mass change, which is the difference between the mass after depuration and the mass before depuration, calculated as a percentage of the mass before depuration) of five earthworm species after 14 days exposure to Cu in the form of copper oxychloride and a positive control (10 mg/kg Benomyl) in OECD soil.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	4	-9.18	6.41	-6.96	-13.47	-4.89
	20 mg/kg Cu	4	-8.90	1.16	-8.93	-9.90	-7.90
	80 mg/kg Cu	4	-8.21	1.54	-8.40	-9.31	-7.11
	160 mg/kg Cu	4	-8.11	3.83	-8.30	-10.47	-5.75
	320 mg/kg Cu	3	-5.08	6.21	-6.01	-10.77	1.54
	640 mg/kg Cu	2	10.58	2.24	10.58	8.99	12.16
	960 mg/kg Cu	1	8.87	0.00	8.87	8.87	8.87
	Benomyl	4	-9.80	6.62	-9.07	-14.28	-5.31
A. trapezoides	Control	4	-9.75	0.94	-10.04	-10.35	-9.15
	20 mg/kg Cu	4	-14.89	2.03	-14.80	-16.38	-13.40
	80 mg/kg Cu	4	-7.45	4.71	-7.01	-11.12	-3.77
	160 mg/kg Cu	4	-4.14	7.67	-4.77	-10.14	1.85
	320 mg/kg Cu	4	-0.13	6.43	-0.10	-5.15	4.88
	640 mg/kg Cu	4	-1.95	2.53	-0.96	-3.58	-0.31
	Benomyl	1	8.16	0.00	8.16	8.16	8.16
Chilota sp.	Control	3	-7.09	3.87	-7.71	-10.61	-2.95
	20 mg/kg Cu	3	-1.22	4.97	-0.79	-6.40	3.52
	80 mg/kg Cu	3	-5.62	5.81	-5.08	-11.69	-0.10
	160 mg/kg Cu	3	-3.01	3.45	-2.96	-6.48	0.42
	320 mg/kg Cu	3	1.22	2.56	0.11	-0.60	4.15
	640 mg/kg Cu	2	4.75	0.07	4.75	4.70	4.80
	Benomyl	3	-6.57	4.82	-5.89	-11.69	-2.12
E. andrei (A)	Control	5	-16.13	4.53	-15.45	-18.27	-12.59
	20 mg/kg Cu	5	-14.11	4.42	-14.11	-14.95	-13.76
	80 mg/kg Cu	5	-13.34	5.83	-10.53	-16.11	-9.93
	160 mg/kg Cu	5	-11.23	5.44	-10.62	-12.07	-7.17
	640 mg/kg Cu	5	-2.87	4.80	0.10	-5.24	0.32
	960 mg/kg Cu	5	-0.09	7.60	-0.57	-1.52	5.45
	Benomyl	3	-8.66	3.18	-6.84	-12.33	-6.80
E. andrei (B)	Control	4	-11.11	2.21	-11.60	-12.57	-9.66
	20 mg/kg Cu	4	-11.19	4.05	-11.67	-14.16	-8.22
	80 mg/kg Cu	4	-9.57	1.70	-9.78	-10.74	-8.40
	160 mg/kg Cu	4	-7.36	3.72	-8.77	-9.80	-4.91
	320 mg/kg Cu	4	-6.51	2.59	-7.35	-8.15	-4.87
	640 mg/kg Cu	4	-2.91	5.68	-3.30	-7.58	1.76
	Benomyl	4	-3.28	3.55	-2.02	-5.56	-1.01
P. excavatus	Control	4	0.22	4.70	-1.02	-2.70	3.13
	20 mg/kg Cu	4	0.08	2.55	-0.69	-1.63	1.78
	80 mg/kg Cu	4	2.66	3.92	1.51	-0.16	5.47
	160 mg/kg Cu	4	1.72	4.59	1.46	-1.87	5.31
	320 mg/kg Cu	4	-3.46	7.35	-0.56	-7.88	0.96
	640 mg/kg Cu	3	-5.81	21.79	4.93	-30.88	8.53
	Benomyl	4	1.79	1.84	2.42	0.60	2.98

Table 26 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the change in earthworm mass during a 24 h (48 h for *E. andrei* (A)) depuration period on moist filter paper after a 14 day exposure period (depuration mass change, which is the difference between the mass after depuration and the mass before depuration, calculated as a percentage of the mass before depuration) and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for five earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = exposure mass change. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman $R > 0.8$ or < -0.8).

	Soil Cu vs Depuration mass change				Body Cu vs Depuration mass change			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = 0.04x - 10.29	0.43	0.05	22	y = 0.34x - 12.48	0.48	0.02	22
A. trapezoides	y = 0.03x - 10.00	0.70	< 0.01	24	y = 0.52x - 11.17	0.51	0.01	24
Chilota sp.	y = 0.02x - 4.92	0.60	0.01	17	y = 0.30x - 6.04	0.56	0.02	17
E. andrei (A)	y = 0.03x - 14.70	0.70	< 0.01	30	y = 0.56x - 15.39	0.60	< 0.01	30
E. andrei (B)	y = 0.02x - 11.05	0.69	< 0.01	24	y = 0.34x - 11.43	0.57	< 0.01	24
P. excavatus	y = - 0.02x + 1.47	0.10	0.65	23	y = -0.49x + 0.42	-0.06	0.80	23

Table 27a (statistical results): Results from the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the mass change during depuration (depuration mass change) for 24 h (48 h for *E. andrei* (A)) on moist filter paper for five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 27b.

ANOVA (parametric data)

Species	n	F	*P*	df	Reference to Table 27b
A. trapezoides	25	5.96	**< 0.01**	6	I
Chilota sp.	20	2.74	0.06	6	
E. andrei (A)	35	6.17	**< 0.01**	6	II
E. andrei (B)	28	3.69	**0.01**	6	III

KW ANOVA (nonparametric data)

Species	n	H	*P*	df
A. diffringens	27	8.47	0.29	7
P. excavatus	27	3.58	0.73	6

Table 27b (statistical results): Results of the ANOVA Fisher LSD post hoc test (for *A. trapezoides* and *E. andrei* (A)) for depuration mass change (from Table 27a in this Appendix). For the ANOVA post-hoc tests, the *P*-values are presented below the diagonal. K = negative control, B = positive control (benomyl). Significant *P*-values ($P < 0.05$) are in bold.

I *A. trapezoides* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.14						
	Cu80	0.50	**0.04**					
P	Cu160	0.11	< **0.01**	0.34				
	Cu320	**0.01**	< **0.01**	**0.04**	0.25			
	Cu640	**0.03**	< **0.01**	0.12	0.52	0.59		
	B	< **0.01**	< **0.01**	**0.01**	**0.03**	0.13	0.07	

II *E. andrei* (A) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.56						
	Cu80	0.42	0.82					
P	Cu160	0.16	0.41	0.54				
	Cu640	< **0.01**	< **0.01**	**0.01**	**0.02**			
	Cu960	< **0.01**	< **0.01**	< **0.01**	< **0.01**	0.42		
	B	0.07	0.18	0.25	0.52	0.15	**0.04**	

III *E. andrei* (B) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K							
	Cu20	0.98						
	Cu80	0.55	0.53					
P	Cu160	0.15	0.14	0.39				
	Cu320	0.08	0.08	0.24	0.74			
	Cu640	< **0.01**	< **0.01**	**0.02**	0.09	0.17		
	B	**0.01**	**0.01**	**0.02**	0.12	0.22	0.88	

Table 28 (data): Summarised earthworm coelomic fluid protein content (µg/µl) of five earthworm species after exposure to Cu in the form of copper oxychloride in OECD soil for 14 days. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2. The number of cells per µl, calculated from the equation in Figure 2.7, is indicated in the last column. The negative numbers for *A. trapezoides* indicate that the protein content in the coelomic fluid was too low to enable calculation of the cell number.

Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75	Cells/µl
A. diffringens	Control	14	4.30	1.68	4.15	3.47	5.27	697
	20 mg/kg Cu	15	5.36	2.67	5.71	3.88	7.78	993
	80 mg/kg Cu	16	4.37	2.09	4.27	2.89	5.47	720
	160 mg/kg Cu	14	4.93	2.47	4.30	3.65	6.71	726
	320 mg/kg Cu	9	4.25	1.54	4.18	4.00	4.92	704
	640 mg/kg Cu	2	2.62	2.57	2.62	0.81	4.44	410
	Positive control	7	4.14	1.63	3.78	3.11	5.53	629
A. trapezoides	Control	6	0.90	1.03	0.35	0.27	1.57	-20
	20 mg/kg Cu	6	0.80	1.12	0.14	0.04	1.92	-59
	80 mg/kg Cu	6	1.08	1.59	0.11	0.02	2.74	-66
	160 mg/kg Cu	6	0.62	0.72	0.29	0.06	1.41	-32
	320 mg/kg Cu	2	2.47	1.02	2.47	1.75	3.20	381
	640 mg/kg Cu	6	0.85	1.22	0.25	0.10	1.27	-38
	Positive control	3	0.69	0.88	0.21	0.16	1.71	-47
Chilota sp.	Control	12	3.59	2.38	3.77	1.56	5.39	626
	20 mg/kg Cu	12	4.26	2.34	4.42	2.58	6.40	748
	80 mg/kg Cu	12	5.06	1.43	5.12	4.14	5.88	881
	160 mg/kg Cu	13	4.96	2.32	5.00	3.81	6.01	858
	320 mg/kg Cu	11	4.61	2.03	4.48	2.98	6.40	760
	640 mg/kg Cu	4	2.42	3.51	0.83	0.46	4.39	70
	Positive control	6	3.38	1.03	3.69	2.89	4.05	610
E. andrei (A)	Control	16	3.39	1.40	2.98	2.05	4.74	477
	20 mg/kg Cu	16	3.85	1.22	3.87	3.04	4.50	644
	80 mg/kg Cu	16	3.57	1.49	3.80	2.46	4.36	633
	160 mg/kg Cu	16	3.55	1.73	3.36	2.26	4.60	549
	640 mg/kg Cu	16	3.39	1.44	3.00	2.74	4.22	481
	960 mg/kg Cu	16	3.28	1.60	3.36	2.09	4.14	549
	Positive control	8	3.05	1.25	2.82	2.12	3.67	447
E. andrei (B)	Control	16	3.82	0.97	3.76	3.12	4.66	624
	20 mg/kg Cu	16	2.98	0.88	2.93	2.41	3.52	467
	80 mg/kg Cu	16	3.32	1.00	3.33	2.37	4.10	543
	160 mg/kg Cu	16	3.29	1.00	3.10	2.93	3.55	500
	320 mg/kg Cu	16	3.27	1.23	3.32	2.43	3.91	541
	640 mg/kg Cu	16	3.07	1.29	3.04	2.01	3.70	489
	Positive control	8	5.06	1.35	4.95	3.96	5.68	849
P. excavatus	Control	16	6.17	2.83	5.54	4.36	8.06	961
	20 mg/kg Cu	16	5.50	3.51	5.93	2.08	8.24	1034
	80 mg/kg Cu	16	7.11	3.25	7.81	4.50	10.06	1388
	160 mg/kg Cu	16	6.78	3.62	7.95	3.08	10.09	1415
	320 mg/kg Cu	16	7.96	2.62	8.76	6.23	10.21	1569
	640 mg/kg Cu	8	3.60	2.65	3.13	1.28	5.70	506
	Positive control	8	5.74	2.66	5.19	3.82	7.65	894

Table 29 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the protein content of earthworm coelomic fluid after a 14 day exposure period and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for four earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = Protein content. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs Protein content				Body Cu vs Protein content			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.003x + 4.86	-0.04	0.76	70	y = -0.02x + 5.17	-0.09	0.59	40
Chilota sp.	y = -0.002x + 4.56	0.08	0.52	64	y = -0.05x + 3.92	0.02	0.20	32
E. andrei (A)	y = -0.0007x + 3.63	-0.10	0.34	96	y = -0.009x + 2.66	-0.10	0.96	24
E. andrei (B)	y = -0.001x + 3.41	-0.13	0.22	96	y = -0.02x + 3.45	-0.02	0.90	48
P. excavatus	y = -0.002x + 6.63	0.02	0.83	88	y = -0.02x + 6.35	0.12	0.43	46

Table 30a (statistical results): Results of the Kruskal-Wallis ANOVA by ranks test (for nonparametric data) to determine significant differences between treatments for the protein content of earthworm coelomic fluid in five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 30b.

KW ANOVA (nonparametric data)					Reference to
Species	n	H	*P*	df	Table 30b
A. diffringens	77	4.42	0.62	6	
Chilota sp.	70	7.34	0.29	6	
E. andrei (A)	104	2.54	0.86	6	
E. andrei (B)	104	18.36	**< 0.01**	6	I
P. excavatus	96	11.62	0.07	6	

Table 30b (statistical results): Results of the Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons for *E. andrei* (B) for protein content of earthworm coelomic fluid (from Table 30a in this Appendix). For the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, PK = positive control (subsamples of the negative controls, spiked with 1 mM H_2O_2). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

I *E. andrei* (B) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	B
	K		2.31	1.34	1.55	1.44	2.05	1.75
	Cu20	0.44		0.97	0.76	0.87	0.26	**3.64**
	Cu80	1.00	1.00		0.21	0.10	0.71	2.85
P	Cu160	1.00	1.00	1.00		0.11	0.50	3.01
	Cu320	1.00	1.00	1.00	1.00		0.61	2.93
	Cu640	0.85	1.00	1.00	1.00	1.00		**3.43**
	PK	1.00	**0.01**	0.09	0.05	0.07	**0.01**	

Table 31 (data): Summarised results of the neutral red retention (NRR) assay performed on coelomocytes (NRR (corrected) = photometric readings (absorption values) corrected for background noise and divided by protein content) in coelomic fluid of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2.

		NRR (corrected)					
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	13	0.06	0.02	0.06	0.05	0.08
	20 mg/kg Cu	13	0.04	0.01	0.04	0.03	0.05
	80 mg/kg Cu	14	0.05	0.02	0.05	0.04	0.06
	160 mg/kg Cu	12	0.05	0.02	0.04	0.03	0.06
	320 mg/kg Cu	8	0.04	0.02	0.04	0.03	0.05
	640 mg/kg Cu	1	0.08	0.00	0.08	0.08	0.08
	Positive control	6	0.00	0.00	0.00	0.00	0.01
Chilota sp.	Control	9	0.04	0.01	0.04	0.04	0.05
	20 mg/kg Cu	9	0.04	0.02	0.03	0.03	0.05
	80 mg/kg Cu	12	0.06	0.02	0.05	0.03	0.08
	160 mg/kg Cu	12	0.04	0.02	0.04	0.03	0.05
	320 mg/kg Cu	10	0.04	0.02	0.04	0.02	0.06
	640 mg/kg Cu	1	0.02	0.00	0.02	0.02	0.02
	Positive control	5	0.03	0.01	0.02	0.02	0.03
E. andrei (A)	Control	13	0.04	0.02	0.05	0.03	0.05
	20 mg/kg Cu	15	0.05	0.01	0.05	0.04	0.05
	80 mg/kg Cu	13	0.05	0.02	0.05	0.03	0.05
	160 mg/kg Cu	14	0.05	0.02	0.05	0.03	0.06
	640 mg/kg Cu	13	0.04	0.01	0.04	0.03	0.05
	960 mg/kg Cu	12	0.04	0.01	0.04	0.03	0.05
	Positive control	7	0.02	0.01	0.01	0.01	0.03
E. andrei (B)	Control	15	0.08	0.02	0.08	0.07	0.09
	20 mg/kg Cu	13	0.08	0.03	0.08	0.06	0.09
	80 mg/kg Cu	14	0.08	0.03	0.08	0.06	0.10
	160 mg/kg Cu	14	0.10	0.03	0.11	0.07	0.12
	320 mg/kg Cu	14	0.07	0.03	0.07	0.05	0.08
	640 mg/kg Cu	12	0.07	0.03	0.06	0.05	0.08
	Positive control	8	0.02	0.00	0.02	0.02	0.02
P. excavatus	Control	15	0.03	0.01	0.03	0.02	0.03
	20 mg/kg Cu	12	0.02	0.01	0.02	0.02	0.03
	80 mg/kg Cu	14	0.02	0.01	0.02	0.02	0.03
	160 mg/kg Cu	14	0.02	0.01	0.02	0.02	0.03
	320 mg/kg Cu	15	0.02	0.01	0.02	0.01	0.03
	640 mg/kg Cu	5	0.05	0.02	0.04	0.04	0.05
	Positive control	8	0.02	0.01	0.01	0.01	0.02

Table 32 (data): Summarised results of the neutral red retention (NRR) assay performed on coelomocytes (NRR (% of control) = NRR (corrected) (from Table 32 in this Appendix) for each individual calculated as a percentage of the mean NRR (corrected) from the negative control in its replicate) in coelomic fluid of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2.

		NRR (% of control)					
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	13	100.00	25.77	101.79	76.57	127.20
	20 mg/kg Cu	13	69.28	20.77	71.41	56.38	74.03
	80 mg/kg Cu	14	79.28	23.03	79.55	62.51	95.21
	160 mg/kg Cu	12	77.31	31.96	79.03	48.04	105.38
	320 mg/kg Cu	8	67.29	19.15	66.22	53.45	81.59
	640 mg/kg Cu	1	130.61	0.00	130.61	130.61	130.61
	Positive control	6	7.42	5.96	7.67	2.04	12.86
Chilota sp.	Control	9	100.00	27.79	86.52	82.64	114.07
	20 mg/kg Cu	9	95.65	42.67	106.04	57.20	116.19
	80 mg/kg Cu	12	128.87	44.40	125.32	95.33	157.87
	160 mg/kg Cu	12	98.92	44.24	81.67	62.53	137.07
	320 mg/kg Cu	10	98.37	43.92	95.25	66.32	130.91
	640 mg/kg Cu	1	40.26	0.00	40.26	40.26	40.26
	Positive control	5	64.79	27.26	49.34	48.39	75.85
E. andrei (A)	Control	13	100.00	19.78	94.58	92.24	114.31
	20 mg/kg Cu	15	123.00	62.54	120.24	71.73	171.82
	80 mg/kg Cu	13	113.65	53.41	99.03	78.20	134.27
	160 mg/kg Cu	14	121.09	49.51	111.08	90.21	143.51
	640 mg/kg Cu	13	113.31	53.36	100.04	88.79	159.17
	960 mg/kg Cu	12	121.35	52.95	117.07	75.18	162.03
	Positive control	7	36.01	9.75	38.85	26.25	44.19
E. andrei (B)	Control	15	100.00	23.15	98.18	86.71	109.95
	20 mg/kg Cu	13	103.01	45.35	88.76	67.40	130.37
	80 mg/kg Cu	14	100.52	36.14	93.51	75.10	107.08
	160 mg/kg Cu	14	120.72	35.43	121.19	91.24	151.31
	320 mg/kg Cu	14	89.52	38.72	91.84	57.72	126.61
	640 mg/kg Cu	12	86.68	33.21	80.84	70.81	90.23
	Positive control	8	23.90	8.25	21.52	17.74	29.50
P. excavatus	Control	15	100.00	21.92	102.83	90.18	110.94
	20 mg/kg Cu	12	78.55	30.27	81.36	50.88	100.47
	80 mg/kg Cu	14	86.13	25.97	91.59	70.37	102.55
	160 mg/kg Cu	14	92.00	29.48	80.53	72.46	110.45
	320 mg/kg Cu	15	73.51	25.31	72.28	54.19	95.88
	640 mg/kg Cu	5	186.61	69.14	188.30	128.39	198.15
	Positive control	8	57.89	21.26	49.41	40.81	80.28

Table 33 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the neutral red retention (NRR) of earthworm coelomocytes after a 14 day exposure period and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for four earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = NRR. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs NRR				Body Cu vs NRR			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = -0.003x + 80.61	-0.22	0.09	61	y = -0.37x + 87.75	-0.21	0.21	38
Chilota sp.	y = -0.09x + 111.34	-0.10	0.49	53	y = -0.36x + 112.21	0.04	0.87	25
E. andrei (A)	y = 0.01x + 113.84	0.04	0.73	80	y = -0.36x + 88.18	-0.40	0.12	17
E. andrei (B)	y = -0.05x + 106.45	-0.19	0.09	82	y = -0.01x + 102.75	-0.11	0.47	48
P. excavatus	y = 0.17x + 78.65	0.02	0.89	75	y = -2.73x + 125.37	-0.24	0.13	40

Table 34a (statistical results): Results of the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the neutral red retention (NRR) of earthworm coelomocytes in coelomic fluid from five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 34b.

ANOVA (parametric data)					Reference to
Species	n	F	*P*	df	Table 34b
A. diffringens	67	11.69	< **0.01**	6	I
Chilota sp.	58	2.01	0.08	6	
E. andrei (A)	87	3.19	**0.01**	6	II
P. excavatus	83	8.47	< **0.01**	6	III

KW ANOVA (nonparametric data)					
Species	n	H	*P*	df	
E. andrei (B)	90	28.55	<**0.01**	6	IV

Table 34b (statistical results): Results of the ANOVA Fisher LSD post hoc tests (for *A. diffringens*, *E. andrei* (A) and *P. excavatus*) and Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons (for *E. andrei* (B)) for neutral red retention (NRR) of earthworm coelomocytes (from Table 34a in this Appendix). For the ANOVA post hoc results, the *P*-values are below the diagonal. For the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, PK = positive control (subsamples of the negative controls, spiked with 1 mM H_2O_2). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold. nd = not done due to insufficient number of data points.

I *A. diffringens* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K							
	Cu20	**< 0.01**						
	Cu80	**0.03**	0.28					
P	Cu160	**0.02**	0.40	0.83				
	Cu320	**< 0.01**	0.85	0.26	0.36			
	Cu640	nd	nd	nd	nd	nd		
	PK	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	

II *E. andrei* (A) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu640	Cu960	PK
	K							
	Cu20	0.22						
	Cu80	0.48	0.62					
P	Cu160	0.27	0.92	0.69				
	Cu640	0.49	0.60	0.99	0.68			
	Cu960	0.28	0.93	0.69	0.99	0.68		
	PK	**0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	

III *P. excavatus* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K							
	Cu20	0.17						
	Cu80	0.47	0.49					
P	Cu160	0.58	0.40	0.87				
	Cu320	0.07	0.75	0.29	0.22			
	Cu640	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**		
	PK	**0.02**	0.27	0.08	0.06	0.39	**< 0.01**	

IV *E. andrei* (B) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		0.40	0.37	0.99	1.02	1.50	**4.23**
	Cu20	1.00		0.04	1.36	0.59	1.07	**3.78**
	Cu80	1.00	1.00		1.34	0.64	1.13	**3.87**
P	Cu160	1.00	1.00	1.00		1.98	2.41	**5.01**
	Cu320	1.00	1.00	1.00	1.00		0.51	**3.32**
	Cu640	1.00	1.00	1.00	0.33	1.00		2.78
	PK	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**0.02**	0.11	

Table 35 (data): Summarised results of MTT assay performed on coelomocytes (MTT (corrected) = photometric readings (absorption values) corrected for background noise and divided by protein content) in coelomic fluid of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2.

		MTT (corrected)					
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	13	0.05	0.01	0.05	0.05	0.05
	20 mg/kg Cu	13	0.04	0.01	0.04	0.04	0.05
	80 mg/kg Cu	14	0.05	0.01	0.05	0.04	0.06
	160 mg/kg Cu	12	0.05	0.01	0.04	0.04	0.05
	320 mg/kg Cu	8	0.05	0.01	0.04	0.03	0.06
	640 mg/kg Cu	1	0.07	0.00	0.07	0.07	0.07
	Positive control	6	0.04	0.01	0.04	0.03	0.04
Chilota sp.	Control	9	0.05	0.01	0.05	0.04	0.05
	20 mg/kg Cu	9	0.04	0.01	0.04	0.04	0.04
	80 mg/kg Cu	12	0.05	0.02	0.05	0.04	0.06
	160 mg/kg Cu	12	0.06	0.02	0.05	0.04	0.08
	320 mg/kg Cu	10	0.04	0.01	0.04	0.04	0.05
	640 mg/kg Cu	1	0.05	0.00	0.05	0.05	0.05
	Positive control	5	0.03	0.01	0.03	0.03	0.04
E. andrei (A)	Control	13	0.05	0.01	0.04	0.03	0.06
	20 mg/kg Cu	15	0.06	0.02	0.05	0.04	0.07
	80 mg/kg Cu	13	0.05	0.02	0.05	0.04	0.06
	160 mg/kg Cu	14	0.06	0.01	0.05	0.05	0.07
	640 mg/kg Cu	13	0.06	0.02	0.06	0.05	0.07
	960 mg/kg Cu	12	0.05	0.02	0.05	0.04	0.06
	Positive control	7	0.03	0.00	0.03	0.03	0.03
E. andrei (B)	Control	15	0.08	0.02	0.08	0.06	0.09
	20 mg/kg Cu	13	0.09	0.03	0.10	0.07	0.11
	80 mg/kg Cu	14	0.09	0.04	0.08	0.06	0.10
	160 mg/kg Cu	14	0.10	0.02	0.11	0.10	0.12
	320 mg/kg Cu	14	0.08	0.02	0.08	0.07	0.09
	640 mg/kg Cu	12	0.08	0.02	0.07	0.06	0.09
	Positive control	8	0.05	0.02	0.04	0.03	0.07
P. excavatus	Control	15	0.02	0.01	0.02	0.01	0.03
	20 mg/kg Cu	12	0.02	0.01	0.02	0.02	0.03
	80 mg/kg Cu	14	0.03	0.01	0.03	0.02	0.04
	160 mg/kg Cu	14	0.03	0.01	0.03	0.02	0.04
	320 mg/kg Cu	15	0.03	0.01	0.03	0.02	0.04
	640 mg/kg Cu	5	0.03	0.00	0.03	0.03	0.03
	Positive control	8	0.01	0.01	0.01	0.01	0.02

Table 36 (data): Summarised results of the MTT assay performed on coelomocytes (MTT (% of control) = MTT (corrected) (from Table 36 in this Appendix) for each individual calculated as a percentage of the mean MTT (corrected) from the negative control in its replicate) in coelomic fluid of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2.

		MTT (% of control)					
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	13	100.00	14.42	102.19	92.02	108.88
	20 mg/kg Cu	13	80.72	18.95	80.61	69.04	90.99
	80 mg/kg Cu	14	99.08	14.42	100.72	91.63	105.99
	160 mg/kg Cu	12	90.54	13.47	93.10	79.50	97.13
	320 mg/kg Cu	8	80.33	16.21	83.42	67.20	89.30
	640 mg/kg Cu	1	158.91	0.00	158.91	158.91	158.91
	Positive control	6	74.90	18.49	75.29	57.75	92.43
Chilota sp.	Control	9	100.00	19.02	106.48	94.32	113.54
	20 mg/kg Cu	9	85.22	27.19	84.01	73.71	93.50
	80 mg/kg Cu	12	107.79	38.15	98.01	85.81	126.60
	160 mg/kg Cu	12	127.46	36.61	124.58	90.75	160.58
	320 mg/kg Cu	10	92.64	19.87	98.71	85.45	109.01
	640 mg/kg Cu	1	94.64	0.00	94.64	94.64	94.64
	Positive control	5	70.92	13.63	71.34	59.88	75.16
E. andrei (A)	Control	13	100.00	23.76	95.06	82.95	116.33
	20 mg/kg Cu	15	118.00	26.23	116.42	100.21	141.62
	80 mg/kg Cu	13	111.45	23.22	115.96	106.33	125.34
	160 mg/kg Cu	14	119.45	24.92	114.06	103.83	130.92
	640 mg/kg Cu	13	128.80	34.92	127.88	106.48	156.13
	960 mg/kg Cu	12	123.99	25.31	125.56	104.25	142.15
	Positive control	7	64.71	8.78	64.90	57.66	70.58
E. andrei (B)	Control	15	100.00	17.07	100.46	82.05	113.55
	20 mg/kg Cu	13	117.75	39.55	102.82	88.49	150.31
	80 mg/kg Cu	14	116.42	53.23	103.89	84.37	127.68
	160 mg/kg Cu	14	141.49	34.44	142.22	120.39	174.11
	320 mg/kg Cu	14	109.76	20.91	115.43	101.98	122.10
	640 mg/kg Cu	12	106.89	31.61	96.50	92.48	111.80
	Positive control	8	64.42	29.24	58.21	44.49	86.70
P. excavatus	Control	15	100.00	28.12	102.55	81.13	120.06
	20 mg/kg Cu	12	97.47	30.92	103.79	77.19	112.89
	80 mg/kg Cu	14	124.89	41.32	128.64	95.71	130.85
	160 mg/kg Cu	14	118.38	36.80	122.86	87.73	140.78
	320 mg/kg Cu	15	146.19	37.24	154.63	115.25	164.83
	640 mg/kg Cu	5	120.96	26.31	109.89	105.84	122.65
	Positive control	8	57.59	18.11	52.65	46.68	72.47

Table 37 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the MTT conversion to formazan in earthworm coelomocytes after a 14 day exposure period and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for four earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = MTT. For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs MTT				Body Cu vs MTT			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = 0.04x + 89.46	-0.15	0.26	61	y = 0.02 + 92.71	-0.14	0.41	38
Chilota sp.	y = 0.01x + 103.08	0.09	0.51	53	y = 1.38x + 87.73	0.34	0.10	25
E. andrei (A)	y = 0.03x + 111.90	0.25	0.02	80	y = 0.66x + 98.90	0.21	0.42	17
E. andrei (B)	y = -0.02x + 117.05	0.05	0.63	82	y = 0.09x + 114.07	-0.02	0.90	48
P. excavatus	y = 0.13x + 107.46	0.40	< 0.01	75	y = 2.48x + 92.70	0.30	0.06	40

Table 38a (statistical results): Results of the ANOVA (for parametric data) and Kruskal-Wallis ANOVA by ranks (nonparametric data) tests to determine significant differences between treatments for the MTT conversion to formazan blue in earthworm coelomocytes from coelomic fluid of five earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 38b.

ANOVA (parametric data)					Reference to
Species	n	F	*P*	df	Table 38b
A. diffringens	67	6.92	**< 0.01**	6	I
Chilota sp.	58	3.19	**0.01**	6	II
E. andrei (A)	87	6.08	**< 0.01**	6	III

KW ANOVA (nonparametric data)					
Species	n	H	*P*	df	
E. andrei (B)	90	23.32	**< 0.01**	6	IV
P. excavatus	83	30.51	**< 0.01**	6	V

Table 38b (statistical results): Results of the ANOVA Fisher LSD post hoc tests (for *A. diffringens*, *Chilota sp.* and *E. andrei* (A)) and Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons (for *E. andrei* (B) and *P. excavatus*) for the MTT conversion to formazan in earthworm coelomocytes (from Table 38a in this Appendix). For the ANOVA post hoc results, the *P*-values are below the diagonal. For the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, PK = positive control (subsamples of the negative controls, spiked with 1 mM H_2O_2). Significant *P*-values ($P < 0.05$) and corresponding z' values are in bold. nd = not done due to insufficient number of data points.

I *A. diffringens* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K							
	Cu20	**< 0.01**						
	Cu80	0.88	**< 0.01**					
P	Cu160	0.14	0.13	0.18				
	Cu320	**0.01**	0.96	**0.01**	0.16			
	Cu640	nd	nd	nd	nd	nd		
	PK	**< 0.01**	0.46	**< 0.01**	0.05	0.53	**< 0.01**	

Table 38b continued (post hoc tests for MTT)

II *Chilota sp.* (ANOVA)

		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K							
	Cu20	0.29						
	Cu80	0.55	0.09					
P	Cu160	**0.04**	**< 0.01**	0.11				
	Cu320	0.59	0.58	0.23	**0.01**			
	Cu640	nd	nd	nd	nd	nd		
	PK	0.08	0.39	**0.02**	**< 0.01**	0.18	0.46	

III *E. andrei* (A) (ANOVA)

		K	Cu20	Cu80	Cu160	Cu640	Cu960	PK
	K							
	Cu20	0.07						
	Cu80	0.26	0.50					
P	Cu160	0.05	0.88	0.42				
	Cu640	**0.01**	0.27	0.09	0.35			
	Cu960	**0.02**	0.55	0.23	0.66	0.64		
	PK	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	

IV *E. andrei* (B) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		1.12	0.59	2.98	0.99	0.23	2.13
	Cu20	1.00		0.52	1.78	0.14	0.84	3.02
	Cu80	1.00	1.00		2.34	0.39	0.34	2.60
P	Cu160	0.06	1.00	0.40		1.95	2.59	**4.60**
	Cu320	1.00	1.00	1.00	1.00		0.71	2.94
	Cu640	1.00	1.00	1.00	0.20	1.00		2.24
	PK	0.70	0.05	0.19	**< 0.01**	0.07	0.53	

V *P. excavatus* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		0.13	1.65	1.33	**3.14**	1.07	2.41
	Cu20	1.00		1.69	1.39	**3.10**	1.13	2.20
	Cu80	1.00	1.00		0.31	1.44	0.12	**3.76**
P	Cu160	1.00	1.00	1.00		1.75	0.10	**3.50**
	Cu320	**0.04**	**0.04**	1.00	1.00		1.16	**5.03**
	Cu640	1.00	1.00	1.00	1.00	1.00		2.82
	PK	0.33	0.58	**< 0.01**	**0.01**	**< 0.01**	0.10	

Table 39 (data): Summarised measurements of DNA damage (Tail DNA % (raw), which is the Tail DNA % data from individual comet structures used per treatment per species) measured with the comet assay in coelomocytes of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2.

		Tail DNA % (raw)					
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75
A. diffringens	Control	1699	14.26	19.29	5.68	1.55	19.66
	20 mg/kg Cu	2047	18.34	21.66	9.20	2.58	25.80
	80 mg/kg Cu	1857	23.98	26.70	12.32	3.05	38.06
	160 mg/kg Cu	1574	26.77	26.78	17.01	4.19	42.40
	320 mg/kg Cu	1127	23.61	26.07	12.55	3.42	36.21
	640 mg/kg Cu	318	32.51	31.50	18.46	5.23	59.22
	Positive control	811	60.11	20.86	61.35	44.90	75.22
Chilota sp.	Control	634	44.10	24.57	39.93	23.72	63.39
	20 mg/kg Cu	570	39.00	25.48	33.93	17.30	56.55
	80 mg/kg Cu	821	51.72	21.87	49.94	34.68	67.36
	160 mg/kg Cu	639	66.28	22.74	71.16	50.99	83.33
	320 mg/kg Cu	720	61.94	21.51	63.57	45.11	78.09
	640 mg/kg Cu	184	55.59	26.00	57.54	35.10	77.52
	Positive control	320	83.81	20.56	90.19	80.39	99.03
E. andrei (A)	Control	974	29.65	28.92	17.72	3.57	55.34
	20 mg/kg Cu	1272	27.07	26.54	17.62	4.02	44.18
	80 mg/kg Cu	1461	27.53	26.16	17.41	6.30	43.22
	160 mg/kg Cu	1256	33.64	27.04	27.87	8.80	55.10
	640 mg/kg Cu	1173	31.05	28.64	21.66	5.05	54.15
	960 mg/kg Cu	1364	31.80	26.85	25.04	8.17	51.55
	Positive control	669	64.38	21.94	66.31	49.93	80.19
E. andrei (B)	Control	1122	31.13	23.89	27.07	10.31	49.08
	20 mg/kg Cu	1032	41.19	25.33	39.22	20.87	61.70
	80 mg/kg Cu	1165	42.61	26.18	39.76	20.28	63.62
	160 mg/kg Cu	1322	39.34	26.25	33.94	18.27	58.38
	320 mg/kg Cu	1116	40.42	28.56	35.71	15.38	61.92
	640 mg/kg Cu	1194	37.18	26.78	33.52	13.95	55.74
	Positive control	693	78.31	14.07	79.75	70.44	87.73
P. excavatus	Control	672	48.39	25.43	49.18	27.29	68.81
	20 mg/kg Cu	692	47.58	24.32	47.99	27.13	65.75
	80 mg/kg Cu	797	53.00	26.56	52.70	32.58	75.28
	160 mg/kg Cu	845	53.00	22.54	52.73	35.09	69.70
	320 mg/kg Cu	970	51.19	24.65	50.56	31.04	69.93
	640 mg/kg Cu	520	53.77	25.41	52.07	33.54	74.68
	Positive control	353	83.02	14.68	85.32	74.46	95.00

Table 40 (data): Summarised measurements of DNA damage (Tail DNA % (median), which are the Tail DNA % values from individual comet assay structures summarised (median) for each specimen and then used per treatment per species) measured with the comet assay in coelomocytes of four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD artificial soil. The positive controls are subsamples of the negative controls, spiked with 1 mM H_2O_2. The relative increase of Tail DNA % in each treatment was calculated by dividing the Tail DNA % in each treatment by that of the control.

		Tail DNA % (median)						Relative increase
Species	Treatment	n	Mean	Std Dev	Median	Q25	Q75	in Tail DNA %
A. diffringens	Control	8	6.78	2.73	7.82	3.66	9.00	1.00
	20 mg/kg Cu	8	10.07	3.08	10.03	7.89	11.90	1.28
	80 mg/kg Cu	8	13.90	6.26	12.90	9.02	17.06	1.65
	160 mg/kg Cu	7	16.77	5.25	14.36	13.55	23.10	1.84
	320 mg/kg Cu	6	14.76	8.19	11.50	9.85	15.53	1.47
	640 mg/kg Cu	2	18.84	6.22	18.84	14.44	23.24	2.41
	Positive control	7	59.05	11.33	56.88	49.90	63.20	7.27
Chilota sp.	Control	6	47.55	20.57	46.49	31.27	60.50	1.00
	20 mg/kg Cu	6	38.52	20.49	40.97	17.78	46.18	0.88
	80 mg/kg Cu	6	49.10	7.49	51.71	40.96	53.92	1.11
	160 mg/kg Cu	5	69.42	15.00	78.11	65.06	78.15	1.68
	320 mg/kg Cu	6	60.39	11.26	56.17	54.31	73.96	1.21
	640 mg/kg Cu	2	41.94	26.43	41.94	23.25	60.63	0.90
	Positive control	6	91.24	4.18	91.21	87.56	92.90	1.96
E. andrei (A)	Control	10	28.75	25.12	21.25	6.12	50.49	1.00
	20 mg/kg Cu	10	23.50	19.09	19.38	6.43	30.91	0.91
	80 mg/kg Cu	10	25.75	17.69	18.22	12.73	44.00	0.86
	160 mg/kg Cu	10	30.18	14.67	30.22	19.56	40.21	1.42
	640 mg/kg Cu	10	32.90	23.86	19.85	15.13	54.78	0.93
	960 mg/kg Cu	10	29.36	13.89	26.30	21.00	32.97	1.24
	Positive control	9	66.38	10.78	64.93	57.45	76.07	3.06
E. andrei (B)	Control	8	29.35	17.04	29.59	15.97	43.75	1.00
	20 mg/kg Cu	8	41.32	14.16	42.27	31.90	51.68	1.43
	80 mg/kg Cu	8	41.72	15.73	48.04	24.84	55.45	1.62
	160 mg/kg Cu	8	37.22	18.25	40.95	22.10	51.21	1.38
	320 mg/kg Cu	8	44.40	23.59	44.24	27.39	60.87	1.50
	640 mg/kg Cu	8	35.44	18.71	33.56	22.35	47.46	1.13
	Positive control	8	79.40	3.52	78.66	77.16	82.70	2.66
P. excavatus	Control	7	49.67	16.84	49.37	31.08	68.90	1.00
	20 mg/kg Cu	8	50.00	18.44	49.06	34.80	63.31	0.99
	80 mg/kg Cu	8	51.18	17.97	51.16	42.98	61.91	1.04
	160 mg/kg Cu	8	57.23	11.59	53.74	52.87	64.42	1.09
	320 mg/kg Cu	8	50.93	14.17	48.06	42.67	60.02	0.97
	640 mg/kg Cu	6	51.16	9.26	49.52	42.80	56.24	1.00
	Positive control	8	89.56	6.80	92.30	84.36	94.00	1.87

Table 41 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the DNA damage in earthworm coelomocytes measured by the comet assay (Tail DNA % (median), which is the median value for all the Tail DNA % data from all the measured comets in a specimen) after a 14 day exposure period and both (a) soil Cu content (mg/kg) and (b) earthworm body Cu content (mg/kg) for four earthworm species exposed to Cu in the form of copper oxychloride in OECD soil. For both Functions: y = Tail DNA % (median). For Function a, x = soil Cu content and for Function b, x = earthworm body Cu content. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Soil Cu vs Tail DNA % (median)				Body Cu vs Tail DNA % (median)			
Species	Function a	Spearman R	*P*	n	Function b	Spearman R	*P*	n
A. diffringens	y = 0.03x + 10.48	0.61	< 0.01	39	y = 0.24x + 8.44	0.61	< 0.01	39
Chilota sp.	y = 0.022x + 49.66	0.33	0.07	31	y = 0.96x + 43.00	0.39	0.05	26
E. andrei (A)	y = 0.009x + 26.81	0.22	0.09	60	y = -0.22x + 40.63	-0.09	0.60	36
E. andrei (B)	y = 0.008x + 37.3	0.09	0.53	48	y = 0.69x + 31.48	0.23	0.11	48
P. excavatus	y = 0.022x + 51.60	0.13	0.39	45	y = 0.53x + 50.84	0.05	0.77	33

Table 42a (statistical results): Results of the Kruskal-Wallis ANOVA by ranks test (for nonparametric data) to determine significant differences between treatments for the DNA damage (Tail DNA % (raw), which are Tail DNA % data from individual comets from all the specimens used per treatment) as measured with the alkaline comet assay in earthworm coelomocytes in four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 42b.

KW ANOVA (nonparametric data)					Reference to
Species	n	H	*P*	df	Table 42b
A. diffringens	9433	1537.11	**< 0.001**	6	I
Chilota sp.	3888	878.51	**< 0.001**	6	II
E. andrei (A)	8169	837.50	**< 0.001**	6	IV
E. andrei (B)	7644	1270.06	**< 0.001**	6	VI
P. excavatus	4849	528.02	**< 0.001**	6	VIII

Table 42b (statistical results): Results of the Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons for the DNA damage (Tail DNA % (raw)) in earthworm coelomocytes (from Table 42a in this Appendix). For the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, PK = positive control (subsamples of the negative controls, spiked with 1 mM H_2O_2). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

I *A. diffringens* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		**6.76**	**11.30**	**14.77**	**10.36**	**10.73**	**37.39**
	Cu20	**< 0.001**		**4.92**	**8.80**	**4.75**	**7.20**	**33.11**
	Cu80	**< 0.001**	**< 0.001**		**4.01**	0.49	**4.55**	**28.90**
P	Cu160	**< 0.001**	**< 0.001**	**0.001**		**3.04**	2.26	**24.96**
	Cu320	**< 0.001**	**< 0.001**	1.00	**0.05**		**4.06**	**26.01**
	Cu640	**< 0.001**	**< 0.001**	**< 0.001**	0.50	**0.001**		**14.21**
	PK	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	

Table 42b continued (post hoc tests for KW ANOVA for Tail DNA %(raw))

II *Chilota sp.* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		**3.07**	**5.23**	**15.08**	**12.37**	**5.24**	**22.14**
	Cu20	**0.04**		**8.32**	**17.75**	**15.18**	**7.26**	**24.28**
	Cu80	**< 0.001**	**< 0.001**		**10.78**	**7.78**	1.99	**18.84**
P	Cu160	**< 0.001**	**< 0.001**	**< 0.001**		**3.16**	**4.86**	**9.83**
	Cu320	**< 0.001**	**< 0.001**	**< 0.001**	**0.03**		2.85	**12.57**
	Cu640	**< 0.001**	**< 0.001**	0.99	**< 0.001**	0.09		**11.67**
	PK	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	

IV *E. andrei* (A) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu640	Cu960	PK
	K		1.42	0.04	**4.89**	1.68	**3.31**	**23.56**
	Cu20	1.00		1.53	**6.76**	**3.29**	**5.12**	**26.04**
	Cu80	1.00	1.00		**5.46**	1.90	**3.73**	**25.38**
P	Cu160	**<0.001**	**<0.001**	**<0.001**		3.35	1.78	**20.36**
	Cu640	1.00	**0.02**	1.00	**0.02**		1.66	**22.92**
	Cu960	**0.02**	**<0.001**	**0.004**	1.00	1.00		**22.12**
	PK	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

VI *E. andrei* (B) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		**8.76**	**10.14**	**7.47**	**7.67**	**5.25**	**33.53**
	Cu20	**<0.001**		1.09	1.79	1.23	**3.75**	**25.29**
	Cu80	**<0.001**	1.00		3.01	2.38	**5.00**	**24.92**
P	Cu160	**<0.001**	1.00	0.05		0.52	2.13	**28.08**
	Cu320	**<0.001**	1.00	0.36	1.00		2.55	**26.79**
	Cu640	**<0.001**	**0.004**	**<0.001**	0.71	0.23		**29.35**
	PK	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

VIII *P. excavatus* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		0.78	**3.24**	**3.16**	1.90	**3.17**	**20.20**
	Cu20	1.00		**4.08**	**4.01**	2.77	**3.92**	**20.95**
	Cu80	**0.03**	**<0.001**		0.13	1.55	0.27	**18.11**
P	Cu160	**0.03**	**0.001**	1.00		1.44	0.39	**18.37**
	Cu320	1.00	0.12	1.00	1.00		1.64	**19.82**
	Cu640	**0.03**	**0.002**	1.00	1.00	1.00		**16.57**
	PK	**<0.001**	**<0.001**	<0.001	<0.001	<0.001	<0.001	

Table 43a (statistical results): Results from the Kruskal-Wallis ANOVA by ranks test (for nonparametric data) to determine significant differences between treatments for the DNA damage (Tail DNA % (median), which is the median value for all the Tail DNA % data from all the comets in a specimen, used per treatment) as measured with the alkaline comet assay in earthworm coelomocytes in four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. Significant *P*-values ($P < 0.05$) are in bold, and results for the post-hoc tests for these species are presented in Table 43b.

KW ANOVA (nonparametric data)					Reference to
Species	n	H	*P*	df	Table 43b
A. diffringens	46	30.12	**< 0.001**	6	I
Chilota sp.	37	21.68	**0.001**	6	II
E. andrei (A)	69	20.29	**0.003**	6	IV
E. andrei (B)	56	22.01	**0.001**	6	VI
P. excavatus	53	21.07	**0.002**	6	VIII

Table 43b (statistical results): Results of the Kruskal-Wallis ANOVA by ranks Post hoc multiple comparisons for the DNA damage (Tail DNA % (median)) in earthworm coelomocytes (from Table 43a in this Appendix). For the KW ANOVA post hoc results, the z' values are above the diagonal, and the corresponding *P*-values are below the diagonal. K = negative control, PK = positive control (subsamples of the negative controls, spiked with 1 mM H_2O_2). Significant *P*-values ($P < 0.05$) and the corresponding z' values are in bold.

I *A. diffringens* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		1.21	2.31	2.98	2.20	2.29	**5.07**
	Cu20	1.00		1.10	1.81	1.07	1.52	**3.90**
	Cu80	0.44	1.00		0.75	0.06	0.82	2.84
P	Cu160	0.06	1.00	1.00		0.64	0.33	2.03
	Cu320	0.59	1.00	1.00	1.00		0.76	2.59
	Cu640	0.47	1.00	1.00	1.00	1.00		1.02
	PK	**< 0.001**	**0.002**	0.09	0.89	0.20	1.00	

II *Chilota sp.* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		0.67	0.05	1.64	1.09	0.04	**3.31**
	Cu20	1.00		0.61	2.28	1.76	0.43	**3.97**
	Cu80	1.00	1.00		1.69	1.15	0.00	**3.36**
P	Cu160	1.00	0.48	1.00		0.60	1.23	1.51
	Cu320	1.00	1.00	1.00	1.00		0.81	2.21
	Cu640	0.04	0.43	0.00	1.23	0.81		2.38
	PK	**0.02**	**0.001**	**0.02**	1.00	0.56	0.37	

IV *E. andrei* (A) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu640	Cu960	PK
	K		0.31	0.14	0.52	0.49	0.69	**3.56**
	Cu20	1.00		0.17	0.84	0.80	1.00	**3.86**
	Cu80	1.00	1.00		0.67	0.64	0.84	**3.70**
P	Cu160	1.00	1.00	1.00		0.03	0.17	**3.05**
	Cu640	1.00	1.00	1.00	1.00		0.20	**3.08**
	Cu960	1.00	1.00	1.00	1.00	1.00		2.88
	PK	**0.01**	**0.002**	**0.005**	**0.05**	**0.04**	0.08	

Table 43b continued (post hoc tests for KW ANOVA for Tail DNA %(median))

VI *E. andrei* (B) (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		1.15	1.32	0.80	1.36	0.51	**4.20**
	Cu20	1.00		0.17	0.35	0.21	0.64	**3.05**
	Cu80	1.00	1.00		0.52	0.05	0.81	2.88
P	Cu160	1.00	1.00	1.00		0.57	0.29	**3.40**
	Cu320	1.00	1.00	1.00	1.00		0.86	2.84
	Cu640	1.00	1.00	1.00	1.00	1.00		**3.69**
	PK	**< 0.001**	**0.05**	0.08	**0.01**	0.10	**0.005**	

VIII *P. excavatus* (KW ANOVA)

		z'						
		K	Cu20	Cu80	Cu160	Cu320	Cu640	PK
	K		0.07	0.21	0.90	0.11	0.01	**3.45**
	Cu20	1.00		0.29	1.00	0.03	0.08	**3.64**
	Cu80	1.00	1.00		0.71	0.32	0.18	**3.35**
P	Cu160	1.00	1.00	1.00		1.04	0.84	2.64
	Cu320	1.00	1.00	1.00	1.00		0.11	**3.67**
	Cu640	1.00	1.00	1.00	1.00	1.00		**3.29**
	PK	**0.01**	**0.01**	**0.02**	0.17	**0.01**	**0.02**	

Table 44 (statistical results): Functions and correlation coefficients for correlations (nonparametric Spearman rank-order correlations) between the exposure mass change (the difference between the mass after exposure and before exposure, calculated as a percentage of the mass before exposure) and the number of cocoons produced per earthworm for four earthworm species after 14 days exposure to Cu in the form of copper oxychloride in OECD soil. For the Function, y = cocoons per earthworm and x = exposure mass change. None of the correlations were significant (where both $P < 0.05$ and Spearman R > 0.8 or < -0.8).

	Exposure mass change vs Cocoons per worm			
Species	Function	Spearman R	*P*	n
A. diffringens	y = 0.05x + 1.39	0.70	0.03	10
A. trapezoides	y = 0.01x + 0.29	0.69	< 0.01	25
E. andrei (A)	y = 0.03x + 1.16	0.51	< 0.01	35
E. andrei (B)	y = 0.002x + 0.23	0.07	0.71	28
P. excavatus	y = 0.002x + 0.16	-0.11	0.60	27

-- fin --