MODELLING THE EFFECTS OF TOXIC METAL MIXTURES ON THE REPRODUCTION OF *EISENIA VENETA* IN DIFFERENT TYPES OF SOIL

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ABSTRACT

Previously, toxicity studies have mainly focused on the responses of organisms to single toxicants; however the importance of studying mixtures of toxicants is now being recognised, along with the importance of speciation as a modifier of toxic effect. The aim of this study was to improve our understanding of the toxic response of the compost worm *Eisenia veneta* to cadmium, copper and zinc by integrating understanding of speciation effects into existing mixture models. Adult earthworm tests were carried out in four soils with different pH and organic matter contents, spiked with varying single and mixed doses of Cu/Zn and Cd/Zn. The reproduction rate of the earthworms after a four week exposure was studied. The three metals had different levels of toxicity in different soils. There were clear differences in the responses of the earthworms to the toxic metals in the different soils, and the extent of metal mixture interaction found depended on the form of metal (total metal, free ion (M^{2+}) or diffusive gradient in thin film (DGT) effective concentrations) used to express the dose to the organisms. Mixture interactions were different when expressed in different metal forms and in the four soils. Detailed analysis of the different chemical species provides further insight into how the soil environment instigates the observed mixture effects. The results from this study support the need to consider bioavailability and mixture effects in toxicity studies.

DECLARATION

I hereby declare that the dissertation, submitted for the degree of Doctorate of Philosophy and entitled "Modelling the effects of toxic metal mixtures on the reproduction of *Eisenia veneta* in different types of soil", represents my own work and has not been previously submitted to this or any other institution for any degree, diploma or other qualification.

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Chapter One

General Introduction

1.1 Heavy metals in the soil environment

Soils are a vital part of the terrestrial environment. They provide a habitat for the growth of micro-organisms, invertebrates and plants which in turn sustain higher organisms and provide essential ecosystem services. Soils are heterogeneous mixtures of organic and mineral solids that incorporate aqueous and gaseous mediums. The organic matter fraction includes the decomposed fragments of litter as well as living organisms such as bacteria. Mineral solids in soil can be divided, according to their size, into clay (>2µm), silt (2µm – 60µm) and sand (60µm – 2mm). The mineral fraction mainly consists of weathered rock material, such as oxides, as well as larger rock fragments (Cooke, 2007).

Weathering of parent rock can lead to the release of heavy metals into the soil (Newman, 2003). Heavy metals exist in relatively low concentration in the natural environment in comparison to the concentration that can be associated with industrialised sites. Natural sources of heavy metals include weathering of parent rock and atmospheric deposition (e.g. windblown dust or from volcanic activity), the residence time of metals in soils is controlled by processes such as leaching (Alloway, 1995; Ure and Davidson, 1995). Heavy metals are widely spread and some, such as (Cu, Fe, Mn, Ni, Zn and V), are known to be important micronutrients needed for biochemical process in many organisms (Demuynck et al., 2007). Generally the biogeochemical cycles governing the release of heavy metals into the environment are at a steady state. However, anthropogenic inputs have disrupted this balance (Fergusson, 1990).

Human activities have left many parts of the environment with elevated concentrations of heavy metals in soils. Anthropogenic sources resulting of heavy metals include the deposition of industrial emissions, application of sewage sludge to the land (Koptsik et al., 2005) as well as mining activity. Some sources are by-products (secondary sources) such as the release of mercury due to the smelting and mining of copper and zinc as well as the burning of fossil fuel (Lock and Janssen, 2001).

Heavy metals originating from anthropogenic sources, as opposed to weathered material from parent rocks, tend to be in a more mobile reactive form than the metals native to the soil (Iskandar and Kirkham, 2001). As noted, concentrations of heavy metals in soil have increased in recent decades due to their intensified use in industry, application of sewage sludge to the soil (Koptsik et al., 2005), and indirectly through secondary sources, such as mining activity and the burning of fossil fuels (Lock and Janssen, 2001a). The numerous sources, natural and anthropogenic, of heavy metals result in almost all soils containing mixtures of metals (Demuynck et al., 2007). Continuous industrial and agricultural activity, and the persistent nature of the metals, is worsening the issue of heavy metal contamination especially at this time in the developing region of the world.

Heavy metal contamination begun to draw political and economical attention due to the decline in ‘clean’ land available for development and the consequent re-use of many contaminated field sites (Davies et al., 2003). Further risks involved with heavy metal contamination present the possibility of groundwater contamination and the accumulation of the heavy metals through the food chain (biomagnification), which may eventually cause adverse human health effects (Alloway, 1995; Loureiro et al., 2005). There are a number of policy regimes in operation within the UK and European Union member states involved in monitoring and regulating heavy metal contamination within the environment. European Union directives are in place in association with the metal industries governing Cu and Zn risk assessment procedures. The United Nations Economic Commission for Europe (UNECE) 1979 Convention on Long Range Transboundary Air Pollution includes the 1998 protocol on heavy metals within a total of 8 protocols. European Food Safety Authority (EFSA) provides scientific support and advice for risk assessment of uranium, cadmium, mercury, lead and arsenic in food. The UK based Contaminated Land Exposure Assessment (CLEA) project, operating under the Environmental Protection Act of 1990, makes available tools to estimate risk to people from soil contaminants, including heavy metals.

1.2 Earthworms

Earthworms constitute a large proportion of the biomass of soil; effects on earthworm communities will largely affect the fertility of the terrestrial environment (Spurgeon et al., 2003). Earthworms maintain soil fertility by fulfilling numerous vital functions to the ecosystem such as mixing of soil layers, organic matter degradation and soil aeration (Arnold and Hodson, 2007). Earthworms are an example of soil-dwelling organisms at risk from heavy metal contamination, and are among the groups thought to be more at risk than many other groups of soil invertebrates (Demuynck et al., 2007). This is because earthworms are in intimate contact with the solid soil phase, and soil solution (Spurgeon et al., 2006). Their body walls steadily exchange fluids with the surrounding soil through a moist film maintained over the body (Brusca and Brusca, 2002).

Observed toxic effects of metals on earthworms in laboratory studies include mortality (Spurgeon and Hopkin, 1995, 1996a; Spurgeon et al., 1994, 2000), reduced cocoon production (Spurgeon and Hopkin, 1996a; Spurgeon et al., 2000), reduced cocoon viability (Spurgeon and Hopkin, 1996b; Van Gestel et al., 1992), decrease in growth rates (Van Gestel et al., 1991; Khalil et al., 1996) and a range of biochemical responses to trace metal exposure such as gene expression or metabolic functions. Adverse effects of heavy metals in earthworms have also been observed in field conditions at the landscape scale. For example, heavy metal contamination has been found to reduce species richness from six to one in a study carried out on the alluvial plain of Scarpe-Escault in northern France (Nahmani et al., 2003). Further, effects on population density and distribution of earthworms have also been observed in field situations associated with specific known point sources. A study by Spurgeon and Hopkin (1996a) studied the effects of metal concentrations of the population density and species distribution at 22 sites around a smelting works at Avonmouth, UK. They found that total earthworm density decreased with increasing metal concentrations and some earthworm species (e.g. *Lumbricus rubellus*) were more tolerant than other species (e.g. *Aporrectodea rosea*) and therefore present at more contaminated areas. Another study in three different areas around metal industries in Finland found that earthworm diversity, density, and biomass decreased with increasing proximity to the emission sources (Lukkari et al., 2004).

Clearly as outlined, heavy metal contamination of soils presents a widespread problem. Accumulation of the metals in soil organisms and plants growing in the soil may cause damage to organisms further along the food chain (Alloway, 1995). Earthworms (Oligochaetes) are detritivores and tend to accumulate residues such as heavy metals, therefore there is a large potential for the transfer of the pollutants up the food chain (Spurgeon et al., 2003). The survival and condition of birds and small mammals that feed on earthworms have been linked to metal accumulation in earthworms (Demuynck et al., 2007); this is also known as secondary poisoning (Spurgeon et al., 2003). Soil dwelling organisms are at highest risk from the toxic effects of heavy metals, as they come into direct contact with the contaminated soil (Demuynck et al., 2007), and the metals are readily absorbed into living tissue (Landis and Yu, 2004).

As a result of the role of earthworms in the terrestrial environment, they are regarded as important environmental indicators and play an essential role in ecotoxicological risk assessment. Their avoidance behaviour from heavily contaminated areas is regarded as an early warning sign for polluted soils (Natal da Luz et al., 2004). They are ideal organisms for toxicity assessment as their route of exposure to heavy metal in soils is representative of a number of other soil-dwelling organisms being in direct contact with the soil solid phase and soil pore-water, which are the main routes of exposure for most species in the soil (Spurgeon et al., 2006).

1.3 Metal availability

The classical viewpoint of environmental toxicology divides toxicological studies into three stages: the interaction between the pollutant and environment, the interaction of the pollutant at the site of uptake into the organism and the disruption of the functioning of the organism through interactions with biological receptors (Landis and Yu, 2004). The interaction between the organism and the polluting metal is dependent on rates of metal uptake (toxicokinetics) and then metal binding at the site of action (toxicodynamics). The extent to which the metal can interact with the organism is related to the toxic effect caused (Campbell, 1995; Spurgeon et al., 2003). Results from various studies show that a certain level of contamination (concentration of metal) causes different levels of effect under different chemical and physical conditions (Sauvé et al., 2000). This indicates that environmental condition can control the rate of supply (availability) of the metal to the organisms. There are a number of factors controlling metal availability to organisms, defining the amount of bioavailable metal is a compound concept of chemical and biological processes (Hamelink et al., 1994). Observations concerning the effect of soil type and pH on metal toxicity to organisms have been documented in a number of cases. An early study by Reuther et al. (1953) found the toxicity of copper to citrus seedlings was linked to soil pH and soil characteristics. In a study on the toxicity, and bioavailability, of Cd to *Folsomia candida*, in artificial soils with different pH and different organic matter content, the sensitivity of the species was found to be determined by the internal concentrations and the different soil characteristics (Crommentuijn et al., 1997).

1.4 Chemical influences on metal bioavailability

The availability of metals is determined by both physical and chemical processes governing the distribution of metals in the soil. The total metal content of the soil is divided between the solid and solution phases (Fig 1.1).

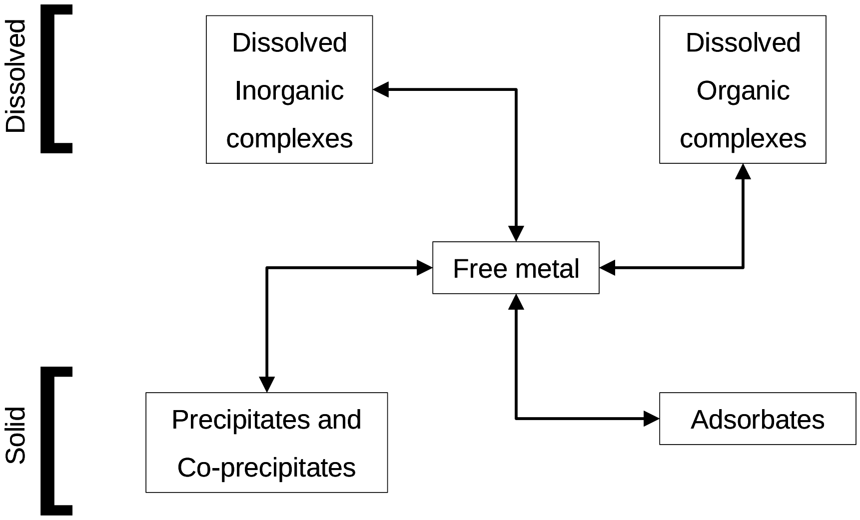

Figure 1.1: A simplification of the geochemical speciation in soils (Salomons et al., 1995 *simplified*).

Metals in the solid phase exist mainly as precipitates, adsorbed onto soil surfaces and occluded into soil particles (Ure and Davidson, 1995; Solomons et al., 1995). Metals in the solid form are less mobile then those in the solution phase. Heavy metals in soils move from more mobile phases in soil solution and exchange sites to less mobile phases the longer they remain in the soil (Davies et al., 2003). As the metals bind to the soil a proportion becomes in-extractable and is immobilised (Cappuynus and Swennen, 2006; Ford et al., 1997). This process is known as ageing and has been found to be an important driver of temporal changes in metal toxicity in laboratory and field studies. The processes of precipitation of metals and occlusion into microporous material, which ultimately control ageing, are slow processes that not only lead to a decrease in the concentration of metal available but also cause changes in metal speciation with time. These slow processes that contribute to the persistence of heavy metals in soils (Selim and Sparks, 2001). Extensive binding of metal to soil ligands in the solid phase is important in controlling the concentration of free metal ion in the soil solution.

In the soil solution metals may be present in the soluble form, exchangeable form or complexed with organic and inorganic ligands (Ure and Davidson, 1995). Organic ligands include organic matter (e.g. dissolved organic carbon), which has been recognized as an important binding phase in many soils (Almas et al., 2007), and inorganic ligands include hydrous oxides and carbonates (Ure and Davidson, 1995; Allen et al., 1995). The solution phase governs the downward and lateral movement of metal through the soil (Almas et al., 2007). The mobility of metals in soils is important in determining the ultimate concentrations and positions within the soil profile. Soil properties such as pH, clay content, organic matter content, and iron containing minerals, the nature of parent material as well as rainfall and micro-organisms are all factors controlling the soil profile of a given metal (Fergusson, 1990).

The solid-solution partitioning of a metal in soil is controlled primarily by complexation, ion exchange and precipitation-dissolution processes (Salomons et al., 1995; Kopstik et al., 2005).

- Complexation denotes the binding of an ion to a ligand. This is done by the formation of a chemical bond which can include the formation of chemical bonds to the soil solid surface and complexations with dissolved ligands. The process can be summarized (1);

$$_{x}M + _{y}L = M_{x}L_{y} \qquad (1)$$

Where M is the heavy metal and L is the ligand or surface (Kopstik et al., 2005).

- Ion exchange refers to the process where ions in solution build up against a surface due to the charges on the surface and that of the ion. No chemical reaction occurs to make a chemical bond. Charged surfaces can include clays and humic substances (Ure and Davidson, 1995; Kopstik et al., 2005).

The processes that control the solid-solution partitioning of the metals are affected by soil conditions. These soil conditions include pH and the cation exchange capacity (CEC). Increasing the pH or the CEC increases the number of sorption sites available on clay minerals, hydrous oxides or organic matter and may lead to a larger amount of metal adsorbed to the soil component (Ure and Davidson, 1995), alternatively increase the proportion of complexes that are mobile in the soil solution (Almas et al., 2007). The CEC is the ability of the soil to exchange and fix ions. Organic matter and clays increase the CEC of the soil as they are good ion exchangers. The strength of sorption of the cations is determined by either their charge $M^{3+}>M^{2+}>M^{+}$ or the ionic radius of the hydrated cation (Fergusson, 1990). The pH is a measure of the acidity of the soil. Soil pH is influenced by cations released from clay component in the soil. This is controlled by a number of other processes, for example the degree of mineral weathering, the nature of the parent material, atmospheric inputs and biological activity. The pH changes the overall charge of the surfaces in soils due to of the exchange of cations, e.g. exchanging Mg^{2+} for Al^{3+}. Increases in pH values make the charge more negative, and decreases in pH make the charge more positive. These changes are caused by the protonation and de-protonation of the functional groups on the surfaces and changes of the amount of bound H^{+} (Allen et al., 1995).

Dissolved organic matter may ultimately control metal solubility in two further ways: by sorption of the organic ligand bound to metal oxide surfaces, for example, to the soil which results in increased sorption to the solid phase; alternatively by competition with the metal for the soil surface which results in a decrease in metal sorption to the solid phase (Koptsik et al., 2005).

Clearly soil chemistry has a large role to play in determining the behaviour of metals, since soil characteristics have a great influence on chemical speciation and ultimately bioavailability (Campbell, 1995; Lock and Janssen, 2002). When assessing the toxicity of metals it is important to understand the processes controlling the speciation of metals in soil. This is not only important with regards to the solid and solution phase, but also the species of the metal in the dissolved phase. Many studies in the literature report that one species (or form) of metal is more available to cause toxic effects than other forms. For example, copper has been shown to be taken up in larger quantities by Italian ryegrass and red clover when it is found in positively charged forms as opposed to the elemental or negatively charged forms (Ure and Davidson, 1995), indicating the importance of not only the concentration of metal in the soil but also the metal species present. Consequently during the assessment of metal effects it is important to consider the amount of soil bound metal that would be available to exchange with the soil solution and determine the concentration of the free metal ion (Lofts et al., 2004).

1.5 Biological processes influencing bioavailability

Physiochemical properties of the soil environment have an important role in controlling metal availability to soil dwelling organisms, as discussed in section 1.4. These organisms also have an effect on the metal availability due to the biological processes they control. For example microbial communities degrading organic matter will subsequently release the organically bound heavy metal into the soil solution (Rada et al., 1996). Evidence has shown that the introducing earthworms to heavy metal contaminated soils leads to changes in metal fractionation (Li et al., 2009).

Earthworms as soft-bodied organisms are able to absorb soluble metal ions through dermal exposure routes as well as through ingestion, though studies have shown that the former largely contributes to the uptake of heavy metals (Saxe et al., 2001; Vijver et al., 2003). Uptake of the free metal ion (M^{2+}) into the organism can also be limited by competition from protons and other cations in solution, which results in a protective effect to the organism (Steenbergen et al., 2005). Biochemical mechanisms enable the earthworm to regulate the reactive metal concentrations both of essential (Cu and Zn) and non-essential (Cd) metals, through various detoxification methods. These include the production of metallothioneins and metal-binding proteins and peptides, increased excretion (Kostera et al., 2006) and storage in inorganic granules located within the chlorogogenous tissue (Morgan et al., 2002). Different mechanisms are known to dominate the process involved in detoxification depending on characteristics of the metal to which the species is exposed (essential or non- essential; hard or soft etc.).

Earthworm excretions in the form of urine or casts have different composition in terms of metal content and microbial population to the rest of the soil environment (Li et al., 2009). Consequently, earthworms appear to increase the availability of heavy metals in soils by altering soil pH, dissolved organic matter content, microbial activity as well physical changes to the soil, for example improved aeration (Sizmur et al., 2009).

1.6 Predicting bioavailability

Recent studies have attempted to measure, or model, the available fraction of metals by studying processes that are known to influence metal availability. Different approaches were used.

Dissolved metal in the soil solution exists in part as the free metal ion. The free metal ion is thought to be the best index of bioavailability (Di Toro et al., 2001; Becquer et al., 2005). As bioavailability is related to the speciation of the metal it has been suggested that it can be predicted using soil characteristics. The cation exchange capacity, pH and organic matter content along with total metal concentration have been used to predict metal bioavailability in the soil (Lock and Janssen, 2001b; Van Gestel and Koolhaas, 2004; Peijnenburg et al., 1997). It is often proposed that the crucial measure of bioavailability is the uptake of metal into the organism since this rate governs the overall internal concentration which is linked to toxicity effect (see below). Consequently, many studies have used the concentration of metal in the organism, the body burden (Newman, 2003), as a measure of the bioavailable fraction (Van Straalen et al., 2005). Metal concentrations calculated through chemical extractions that have been shown to correlate well with the total body burden of the earthworm have also been used. Examples are Diethylenetriaminepentaacetic acid (DTPA) multi-element soil extraction (Dai et al., 2004) and a weak electrolyte soil extraction technique (i.e. acetic acid) (Weljte, 1998; Posthuma et al, 1997; Lukkari et al., 2004). Although the results of some of these approaches correlate well with effects of specific metals, the methods do not provide an overall approach to measuring bioavailability.

The Critical body residue (CBR) concept is an accepted approach for the assessment of the adverse effects on organisms, independent of species, where the CBR is the highest internal concentration of toxicant that will not cause an effect on the organism (McCarty and MacKay, 1993). While it was found to be useful for organic compounds (Parkerton and Konkel, 2000) it was not a suitable tool for the assessment of metals (Indeherberg et al., 1999). Further, it has been widely questioned whether the concept of a CBR can really be justified on the basis of what is known about the nature of the link between external concentration and effect. While rising internal metal concentrations are known to be associated with increased effect (Baas et al., 2007), it is likely that these effects in individuals are the result of relative stochastic processes rather than truly representing differences in the CBR within individuals. Thus, in a population exposed at an LC_{50} concentration the actual individuals within the population that die can do so largely by chance rather than because they have an inherently lower threshold of critical body residues for lethal effects.

An alternative to measuring uptake directly into organisms is through the use of bio-mimicking techniques. A prominent example widely used for metals is DGT (diffusive gradients in thin films) (Harper et al., 1999). DGT uses a chelex-resin layer to accumulate metals, the concentration of metal accumulated can then be related to the concentration of metal available to a sink from the solution phase (by diffusion) as well as release from the solid phase. This concentration is known as the effective concentration of labile metal (C_E) (Zhang et al., 2001). DGT mimics the main mechanism of plant uptake, making a dynamic measurement that takes into account the rate of transport and release of the metal. DGT was successful in correlating with the toxic effects observed to plants (Zhang et al., 2001). As previously mentioned the main route of metal uptake for earthworms is thought to be through dermal exposure, which relies on the same principles as plant root uptake. DGT may therefore be a useful principle for the quantification of metal bioavailability to earthworms.

1.7 The FIAM and biotic ligand (BLM) models

The biotic ligand model (BLM) has been proposed as a tool to evaluate the effects of chemistry on speciation and bioavailability of metals in aquatic environments. It is a chemical equilibrium based model that takes into consideration the importance of bioavailability on the potential toxic effects on the organism (Fig 1.2) (Santore et al., 2001; Paquina et al., 2002). The metal-organism interaction part of the BLM is based on the free ion activity model (FIAM) (Tessier and Turner, 1995).

The FIAM is a conceptual model that illustrates how the extent of adverse effects on the organism can be related to the metal speciation and its interaction with the organism (Paquina et al., 2002). The FIAM assumes that the amount of metal bound to the biotic ligand is proportional to the biological response, and that the solution is in equilibrium (Tessier and Turner, 1995)(2). Where;

$$M^{Z+} + L \longleftrightarrow ML \qquad (2)$$

For the solution equilibrium, where **M** is the metal cation and **L** is the dissolved ligand in solution (3).

$$M^{Z+} + {}^{-}X\text{-Cell} \longleftrightarrow M\text{-}X\text{-cell} \qquad (3)$$

For the surface reaction, where **X-cell** is the binding site at the organism cell surface.

The key assumptions are that the plasma membrane is the primary site for metal interaction with the organism, the reaction (ML + $^{-}$X-cell) is possible and moreover that the reactions are not the rate limiting step and the biological surface remains unchanged during the exposure (Tessier and Turner, 1995). The biological part of the BLM is based on the gill surface interaction model (GSIM); this model supposes that the concentration of metal bound to the fish gill is proportional to the acute toxicity observed in the fish (Pagenkopf, 1983). The BLM takes into account the competition of protons and other cations in solution at the site of action which leads to a protective effect on the level of toxicity to the organism (Pagenkopf, 1983; Di Toro et al., 2001). The site of action is treated as a biotic ligand to which metals bind (Di Toro et al., 2001). As long as the identity of the metal is not significant but only the fact that it is binding to the biotic ligand, and thereby disrupting ion regulation, then any toxic metal would exhibit a similar effect and equal toxicity at the site of action. While this is true for the site of action the identity, and in turn chemistry, of the metal will lead to differences in interaction strengths. The implication is different levels of effect for each metal per unit of concentration.

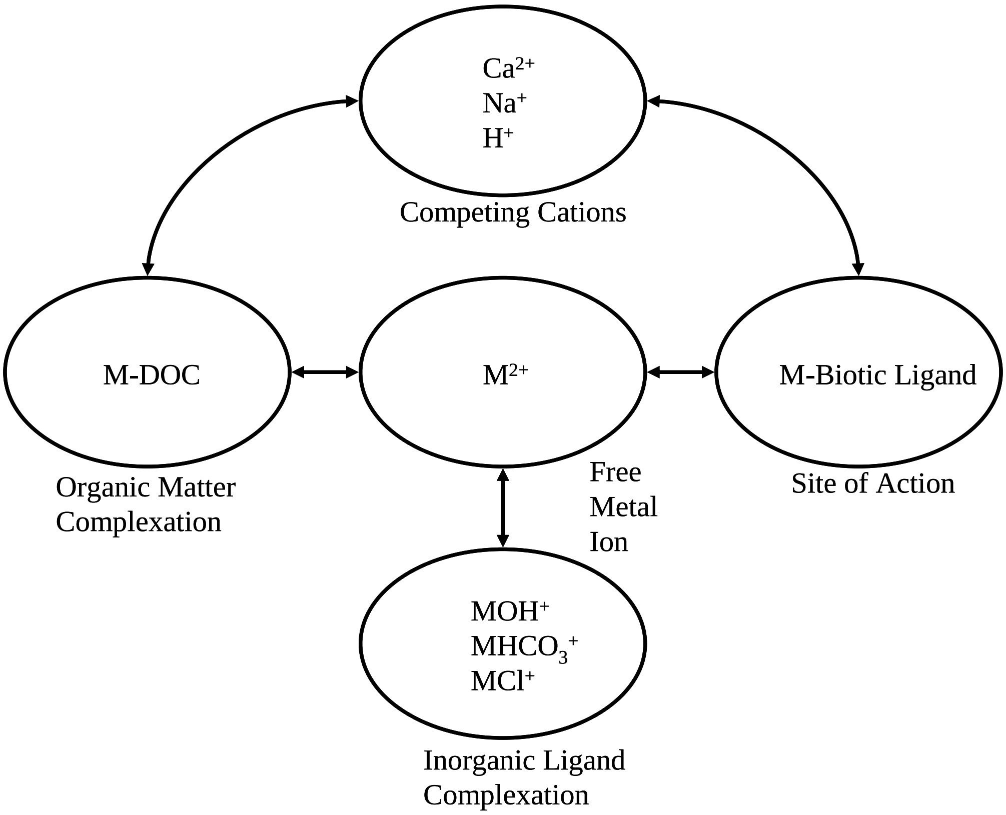

Figure 1.2: Diagram of the biotic ligand model. Processes governing the amount of free metal ion binding to the biotic ligand (Di Toro et al., 2001).

1.8 The BLM in the terrestrial environment

A vital assumption in toxicology is that metal toxicity occurs as a consequence of free metal ions reacting with the biotic ligand at the organism-solution interface. In turn, toxicity mechanisms are assumed to be similar for aquatic and terrestrial organisms. This is due to the similarity between the binding sites for H^+ and Na^+ in aquatic organisms and earthworms. Thus it has been postulated that the BLM can be applied to terrestrial organisms, even though the routes of uptake in soil are more complex than water (Steenbergen et al., 2005). Working on this hypothesis, the Terrestrial BLM (TBLM) has been developed. Like the BLM, the TBLM relies on the assumption that the soil bound metal is in equilibrium with the metal in solution. Metal ions react at the receptor site with the biotic ligand and the reaction is limited by competition from other cations. The toxicity is therefore related to the fraction of biotic ligands that are occupied by the divalent metal (free metal ion) (Di Toro et al., 2001; Thakali et al., 2006a).

Even though it is thought that the BLM is not valid for sub-lethal effects (Kostera et al., 2006), the TBLM was able to predict within a factor of two of the observed values, the EC_{50} (concentration of the metal causing a 50% effect) values for two metals (Cu and Ni) corresponding to six biological endpoints (including earthworm cocoon production (*Eisenia fetida*) and springtail juvenile production (*Folsomia candida*)) for soil invertebrates, plants and microbes in different soils (Thakali et al., 2006a; Thakali et al., 2006b).

1.9 Mixture toxicity

Soil organisms inhabiting contaminated field sites are usually exposed to mixtures of toxic metals (Weltje, 1998), however, soil based toxicological studies have mainly focused on the effects and bioavailability of single metals. Current risk assessment procedures also rely on the effects of single metals (Spurgeon et al., 2005). There is a body of evidence that suggests that joint toxic effects of metals differ from the toxic effect that would be expected given single metal toxicity (Van Gestel and Hensbergen, 1997; Posthuma et al., 1997). In the soil environment mixture effects will be subject to chemical interaction in the soil, competition at the stage of metal uptake as well as at the site of action (Calamari and Alabaster, 1980) for metals with the same site of action. Although attempts to classify the combined effects of toxicants started at the beginning of the 20th century, information on the interactions among metals, and their combined effects, is limited.

Mixture toxicity is usually analysed by comparing the observed combined effect of the metal mixture with the predicted effects of a model. Many approaches have been developed to assess joint mixture effects; one approach concerning two key concepts has gained popularity (Greco et al., 1992; Cassee et al., 1998). This is that the joint effects of multiple chemicals are additive. The additive approach relies on two models which describe the observed mixture effects as a function of the toxicity of the individual components in the mixture. These are known as the Concentration Addition (CA) and Independent Action (IA) models (Lock and Janssen, 2002; Jonker et al., 2005). Theoretically, if the metals have similar modes of action then their joint toxicity should be better described using the CA model, and if they have different modes of action then the IA model can be used. The ability to fit data to either model, however, is not indicative of the modes of action functioning and in reality it is often difficult to conceptually choose which model should be applied given that chemical may have multiple and/or unknown modes of action in the studied species.

1.10 Mixture toxicity reference models of non-interaction

Loewe and Muischnek (1926) are accredited as the first to introduce a model for describing mixtures where the components have similar modes of action, the CA model. This model assumes that the overall effect of the mixture can be expressed as the sum of the individual components in the mixture (4).

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad (4)$$

Where n is the number of components in the mixture and c_i is the concentration of the i^{th} component which results in x% effect. ECx_i corresponds to the concentration of that component which causes an x% effect and the term c_i/ ECx_i, also known as the toxic unit, scales the concentration of the component in the mixture for its relative toxicity (Jonker, 2003). Thus using known fractions of a known response value (for example the EC_{50} values) of the single metals it is possible to make up the toxic unit of a mixture (Khalil et al., 1996; Posthuma et al., 1997). The toxic unit scales the relative toxicity of each component so that if the toxicity of the mixture equals the sum of the individual fractions the sum is equal to one, and CA holds. The total effect is considered ‘additive’. The EC_{50} concentration is considered more reliable compared to concentrations denoting other level effects (e.g. EC_{10}) (Rombke et al., 2006). It is commonly used to express the toxic unit, however any other concentrations (e.g. EC_{10}, EC_{25} for sub-lethal effects, LC_{10}, LC_{50} for lethal effects) can be used.

The other model for non-interaction is the IA model first proposed by Bliss (1939). The IA model is based on the suggestion that the components in the mixture have different modes of action, and will act dissimilarly (5).

$$Y = u_0 \prod_{i=1}^{n} q_i\,(c_i) \qquad (5)$$

Where Y is the measured biological response and u_0 is the either the maximum response (e.g. mortality) or the control response (e.g. reproduction). For a mixture of n components, $q_i(c_i)$ is the probability no response will be observed functionally related to the concentration c of component i (Jonker, 2003).

The relevance of the CA and IA models to eco-toxicological studies has been demonstrated for chemicals with similar and dissimilar modes of action (Backhaus et al., 2000; Faust et al., 2001). The use of the CA concept can be traced back to the early version of the BLM, the GSIM, which assumes an additive effect, and describes the relationship between the concentration of the pollutant bound to the gill surface and the concentration of the same pollutant species in the surrounding solution (Pagenkopf, 1983). CA is perceived as the obvious choice when considering mixtures of chemicals that have a similar mode of action and little interaction between them (Posthuma et al., 1997), it has also been suggested that the use of CA is a reasonable worst-case scenario for metal mixtures in soils (Lock and Janssen, 2002). This is because effects predicted using this model are usually greater than those made using IA and so are precautionary in a risk assessment context. The study by Backhaus et al. (2000) on chemicals with different modes of action proved that IA is a useful tool for predicting mixture effects of dissimilarly acting toxicants. As is often the case in toxicological studies, it is not always possible to know the modes of action for the different chemicals, which is why the use of both reference models is valuable when assessing environmentally relevant mixtures.

1.11 Deviation from mixture toxicity reference models

Although both CA and IA are useful tools in describing mixture toxicity, there are some scenarios when the mixture effect cannot be fully explained with either reference model. Indeed there have been studies that showed clear patterns of interaction between metals (Chardon, 1984; Ireland and Fischer, 1978; Luo and Rimmer, 1995). Significant deviations from the reference models include (Jonker, 2003):

- Absolute synergism or antagonism: where the observed mixture effects were more severe (synergistic) or less severe (antagonistic) than the mixture effects calculated by either reference model.

- Dose ratio dependant deviation: where the deviation pattern (synergistic or antagonistic) from the predicted mixture effect is dependent on the relative amount of one of the toxicants in the mixture.

- Dose level dependant deviation: where low level or high level effects show different deviation patterns from hose predicted by the reference models.

Different deviation patterns have been reported from a number of studies (Van Gestel and Hensbergen, 1997; Lock and Janssen, 2002; Khalil et al., 1996). Jonker et al. (2004) have developed an approach to describe deviations from CA and IA reference models in a statistically well-founded way. This approach not only includes the synergistic and antagonistic deviations from the CA and IA reference models, but also the more complex deviations such as dose level and dose ratio dependant response, using log-logistic dose response models as the foundation. Although this study greatly supports our understanding of mixture effects, there is still a pressing need for more research into the joint action of chemical mixtures (Spurgeon et al., 2003; Baas et al., 2007).

1.12 The BLM and mixtures

A study by DiToro et al. (2001) provides a possible application of the BLM to mixture toxicity. For the purposes of simplicity it is assumed that if the identity of the individual metal, and therefore its chemistry, is not as important as the fact that it causes disruption at the same biotic ligand (site of action) then it will exert a similar toxic effect. If the metals bound at the site of action are assumed to cause an equal effect then the sum of metals bound to the biotic ligand would be comparable to the 'critical concentration' (C^*_M) corresponding to a given percentage effect on the test species (6);

$$C^*_M = [M_1L_b^+] + [M_2L_b^+] \qquad (6)$$

Where M_1^{2+} and M_2^{2+} are the two metals in the mixture, and $[M_1L_b^+]$ and $[M_2L_b^+]$ represent the concentration of each metal bound to the biotic ligand (Di Toro et al., 2001). The equation given corresponds to a combination of the BLM and mixture model concepts, but will not cover instances when the metals in the mixture have different modes of action. The terms of the equation 6 will hold true when the metals have the same potency. This is a possible demonstration of the application of the BLM concept to mixture studies.

1.13 Research aims

Recent developments in toxicity testing have revealed shortfalls in our understanding of the effects of toxicants on organisms in the natural environment. These issues are just as valid for the effects of metals on invertebrates in soils as they are for other areas of the environment. The main problems highlighted were (i) being able to identify the true amount and form of metal causing toxic effects on test species, and in turn to be able to use soil chemistry and biological factors to predict the bioavailability of metals to organisms, and (ii) assessing the joint effects of mixtures of metals to an organism – since this represents realistic field scenarios. In this study toxicity tests have been conducted using the earthworm species *Eisenia veneta* to generate a range of log-logistic dose response curves for metal mixtures in order to generate specifically designed data-sets from which to address the above issues. Adult earthworm toxicity tests were carried out to observe the effects of two mixtures (Cu/Zn and Cd/Zn) on the reproduction of the earthworm across four different soils. Chemical analysis was carried out on the soil and a number of different metal forms were measured.

The aim of the study was to ascertain which metal form best correlated with effects on reproduction in both single and joint metal doses; to assess biological responses by combining existing mixture models with bioavailability concepts in order to illustrate whether or not a unified concept can be generated to describe the effects of metals on an organism in different soils.

1.14 Thesis outline

Chapter one: provided an overall introduction and background to the current status of toxicological experimentation. The main issues were highlighted and ways in which to tackle the knowledge gaps identified.

Chapter two: details the methods for soil preparation, adult earthworm toxicity testing and the analysis and extraction of soil solution. Information on the modelling techniques used is also included.

Chapter three: reports the results found for the preliminary studies performed. This includes the overall performance of the earthworms in the control treatments, the biological effects of changes in soil solution pH and nitrate salt concentrations and finally the temporal effects on earthworm reproduction.

Chapter four: describes the effects of the single metal doses on the reproduction of *Eisenia veneta* in four different soils. Toxicity results are described in each soil with the metal dose expressed as total, dissolved and free metal ion.

Chapters five: describes the modelling procedures and results from the joint mixture effects as described by mixture toxicity reference models CA and IA, for mixtures of Cu/Zn in four soils. The mixture effects observed in different soils were expressed in different metal forms and compared in order to draw relationships between the deviations from mixture models observed and the different metal forms, and soil properties.

Chapter six: as above describes the modelling procedures and results from the joint mixture effects as described by mixture toxicity reference models CA and IA, for mixtures of Cd/Zn in four soils.

Chapter seven: illustrates the role of DGT (diffusive gradients in thin films) in describing the effects of Cu, Cd and Zn as single metals and as mixtures, in four different soils. The DGT effective concentration (C_E) is used as a metal dose to describe the effects observed on reproduction.

Chapter eight: presents the summarising discussion on the results reported in Chapters 4-7 and discusses briefly areas of further work.

Chapter Two

Experimental design and source material

2.1 Introduction

The experimental designs used during the study were designed to be able to derive as much information as possible from the earthworm reproduction rates observed. The methods also aimed at standardising the execution of the experiments as much as possible in order to ensure the behaviour observed was due to the experimental conditions and not external interference. The following section covers all parts of experimental design and material sourcing.

2.2 Soil Preparation

Four soils were used with different chemical and physical properties (Table 2.1). For the purposes of soil classification particle size distribution was determined using the laser diffraction method. All pH readings were measured at 17°C using a combined pH electrode and meterlab© pH/ion meter (Radiometer analytical). $\mathrm{pH}_{(H_2O)}$ was calculated by method of water extraction to the ratio of $10_{\text{deionised water}}$: $1_{\text{dry weight soil}}$, the samples were sieved to 4mm, and oven dried before extracting with water. Extractions were left to shake overnight, the supernatant is then extracted and filtered to 0.2µm. $\mathrm{pH}_{\text{solution}}$ was measured in soil solution samples extracted using Rhizon soil solution samplers as described in section 2.7. $\mathrm{pH}_{\text{solution}}$

value for each soil is the average of the measurements made in the eight controls (Thetford soil, Acidic compost) or 16 controls from two experiments (Kettering Loam, Neutral compost). The maximum water holding capacity (by mass) of the soils was calculated using the ‘European method’, with no applied pressure, and a modification of the laboratory apparatus. In this method, the soil sample is saturated with water and placed on an absorbent membrane until the excess water is drained by gravity. When equilibrium is reached, the maximum water holding capacity is calculated based on the weight of the water held in the soil sample and the dry weight of the soil. In order to calculate the % loss on ignition a dried and ground soil sample was weighed into a crucible then placed in a furnace at 550^{o}C for 5 hours. Once removed from the furnace the sample was re-weighed and the %LOI calculated.

Kettering loam was bought commercially and used as a low organic matter neutral soil (Broughton Loams, Kettering, UK). Neutral pH, high organic matter soil was acquired as commercially available general purpose compost (Redwood Garden Centre, Lancaster, UK). Neutral compost obtained from the same source was also used to obtain a high organic matter acidic medium. This was achieved by acidifying the neutral compost with 1M nitric acid and leaching to remove excess salts after acidification. As an acidic soil with low organic matter content, a field soil was obtained from Thetford, Cambridgeshire, UK (TL853815)(Fig 2.1).

Figure 2.1: Field site at Thetford, Cambridgeshire, UK (TL853815), image captured by author.

Table 2.1: Physical and chemical characteristics of test soils used during adult earthworm exposures. Kettering loam (KL), Neutral compost (NC), Thetford soil (TH), Acidic compost (AC).

Soil	**pH** $_{(H_2O)}$	**pH**$_{(solution)}$	**% LOI***	**%Clay/silt /sand**	**MWHC****
KL	7.6	7.7	5.7	14/62/24	36%
NC	6.8	7.6	62	1.3/28/8.5	290%
TH	6.4	6.7	12	<0.1/5.7/82	27%
AC	5.2	5.2	62	0.5/16/22	290%

* Loss on ignition **Water holding capacity (g H_2O/g dry weight soil)

The Kettering loam and Thetford soil were sieved to 2mm and the compost was sorted to remove any large fragments. The processed soil was transferred to plastic boxes (2.0L volume- 9x14x16 cm) and the soil mixed with the required amount of 1M cadmium nitrate ($Cd(NO_3)_2.4H_2O$), 1M copper nitrate ($Cu(NO_3)_2.3H_2O$) and zinc nitrate ($Zn(NO_3)_2.6H_2O$) (VWR, Portsmouth, UK) solutions and distilled water to give a total moisture content of 56% of the soil water holding capacity.

Each experiment consisted of 8 single metal doses (for each metal) in duplicates, 24 mixture doses where only odd numbered doses were duplicated and a total of eight control replicates. This design provides a large number of treatments to support the fitting of regression models that describe the relationships between exposure concentration and toxic effect. The soils were dosed to within specific metal concentrations during all experiments (Table 2.2). The arrays of metal concentrations were selected to cover a range of ratios which would enable the study of mixture doses throughout the experiment. The soils were dosed to ratios of 0:1, 1:0, 1:1, 1:3 and 3:1 (Fig 2.4).

Table 2.2: Concentration ranges of dosed metal in each of the four soils (mg metal/ kg dry weight soil).

	Cadmium	**Copper**	**Zinc**
Kettering loam	0-800	0-740	0-2820
Neutral compost	0-1200	0-1800	0-7050
Acidic compost	0-1440	0-1320	0-5080
Thetford soil	0-800	0-740	0-2820

Following addition of metal salt solutions, all treatments were left to equilibrate in continuous light at 17°C for two weeks after dosing. A two week period was used because this has been shown to be sufficient to allow metals added to the soil to reach an initial stable equilibrium (Smit and van Gestel, 1998).

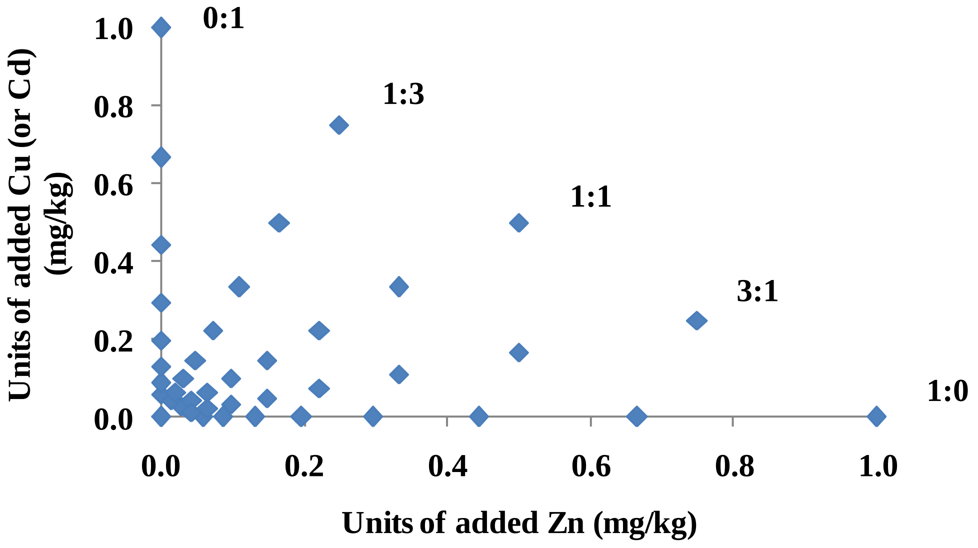

Figure 2.2: The ranges of metal additions showing the ratios of metal used in the mixture experiments where a unit of 1.0 for any of the metals represents the maximum concentration of that metal which is used in the experiment. The units of metal are therefore relative to the maximum concentration of the metal which is assigned according to their toxicity. The ratios quoted are in the form Zn:Cu (or Cd).

2.3 Test species

The test organism used was *Eisenia veneta* (Lumbricidae: Oligochaeta) (Michaelsen, 1889). Earthworms were obtained from a commercial source (Ecology Earthworms, Ipswich, UK) and subsequently cultured in a combination of compost and topsoil (2:1). During culturing, the earthworms were fed using horse manure acquired from an animal grazing on uncontaminated land and not exposed to recent medication. At the start of the experiment individual adult worms were removed from the culture and identified as adults by the presence of a fully formed clitellum. Chosen worms were then acclimated at a constant temperature of 17^{o}C in the culture medium for two weeks at a storing density appropriate to that used in the experiment.

2.4 Toxicity testing

At the start of the exposure (Day 0) eight randomly chosen earthworms were washed and weighed then introduced to each of the test boxes in random order. The average individual weight of the worms was 1.03 g ± 0.38. For food during the test 20g of dried manure per test box was spiked to Cd, Cu and Zn concentration equivalent to those of the test soil and the manure was wetted to 80% water content. The manure was then added to the surface of the soil. Lids were then placed on the boxes, which were then weighed and stored under constant light at 17^{o}C.

Two weeks later (Day 14) the boxes were weighed and de-ionised water was used to replace any water lost. Manure remaining on the surface was removed, dried in an oven at 105^{o}C and weighed with the difference between the initial weights used to determine feeding. The test boxes were then tipped out and the surviving worms were weighed, counted and returned. Test soil was then returned to the boxes and 20g of dry weight manure wetted to 80% water content was added to the soil surface. At the end of the exposure (Day 28) the remaining manure was removed, dried and weighed as previously. Surviving worms were removed from the soil, counted, weighed and depurated overnight to remove the gut content and stored in a freezer at -40^{o}C. Soils were sieved through a 2mm mesh and the number of cocoons in each container recorded, the neutral and acidic compost soils were sorted by hand. Cocoon production was expressed as cocoons per surviving worm per week of exposure.

With accordance to the recently published study by De Laender et al. (2009) all single metal exposures and their corresponding mixtures in this study were tested simultaneously. The experiments (6 in total) were performed successively over an 18 months period. In order to disregard the possible temporal effects over time on earthworm performance, a set of six separate test units were included within all experiments conducted. These repeated treatments included replicates of treatments containing 370 and 840 mg Zn/Kg dry weight soil in Kettering loam soil, three tests of each concentration.

2.5 Biological effects of nitrate salts and pH

In addition to the metal exposure studies, two further preliminary studies were carried out in order to assess the absolute effects of decrease in pH and increase in nitrate salts, as a result of the metal dosing procedure, on the rate of earthworm reproduction. These tests were designed to reflect the effects of changes in soil nitrate and pH values on earthworm rates of reproduction. In order to achieve this test soils were acidified using nitric acid to a range corresponding to those observed during the studies, and in order to test the effects of nitrate salt addition a separate set of the test soils were dosed using $CaNO_3$ to a range corresponding to that which would have been achieved as a result of metal spiking. The concentration of nitrate salt added to the soil was calculated using the volume of metal solution used (at a concentration of 1M) to dose each soil. These additional studies were conducted using the same adult earthworm reproduction methodology outlined in section 2.4.

2.6 Total metal concentrations in soil

Aqua regia digestions were performed on the dosed soils to determine total metal concentrations. The aqua regia solution was made using 39% ‘Analar’ grade HCl and 22.5% ‘Analar’ grade HNO_3 (VWR, UK). Dry, ground soil (1.5g) was digested in the aqua regia solution (15ml), and refluxed for 2 hours at 110^{o}C in a fume cupboard. The final solutions were diluted using 12.5% ‘Analar’ HNO_3 and analysed by an Optima 4300DV ICP-OES. The total metal concentrations were correct within 16% of the nominal doses. Background Cd, Cu and Zn concentrations (mg/kg dry weight soil) in control soil were respectively 0.8, 12.5, 73.1 for Kettering loam, 0.6, 16.1, 78.8 for Neutral compost, 7.6, 3.8, 46.1 for Thetford soil and 1.8, 31.1, 107.1 for Acidic compost. Earthworm tissue was dried in the oven overnight prior to digestion, and the above specified method was followed. As a quality check, digestion of 1.5 g of standard soil reference (SR3 and SR8) was used. Results indicated metal concentrations within range of the specified values.

Total Cu, Cd and Zn concentrations were measured on 15% of doses and the standard deviation from the nominal concentrations were calculated. The standard deviation was then used in conjunction with a bootstrapping model to estimate the total metal concentrations of the doses used during the experiments.

2.7 Soil solution separation and analysis

Soil solution was removed from the test soils at the end of the experiment (i.e. after earthworm removal) after a period of 24 hour equilibration at 100% of the water holding capacity. The soil solution was removed using Rhizon soil solution samplers (Eijkelkamp, UK)(Fig 2.3). An aliquot of the recovered solution was kept refrigerated and un-acidified to measure pH and dissolved organic carbon (DOC) concentration. Soil solution DOC was measured using a DC-190 Dohrmann TOC Analyser and pH values measured at 17^{o}C using a combined pH electrode and meterlab$^{©}$ pH/ion meter (Radiometer analytical). The remainder of the soil solution was acidified to a concentration of 1% nitric acid, using 'Analar' grade nitric acid (VWR, UK) and analysed for major cation (Mg, Ca, K, Fe, Al, Na) concentrations and dissolved Cd, Cu and Zn. The major cations were measured by an Optima 4300DV ICP-OES and the dissolved heavy metal concentrations measured by a Thermo X series ICP-MS.

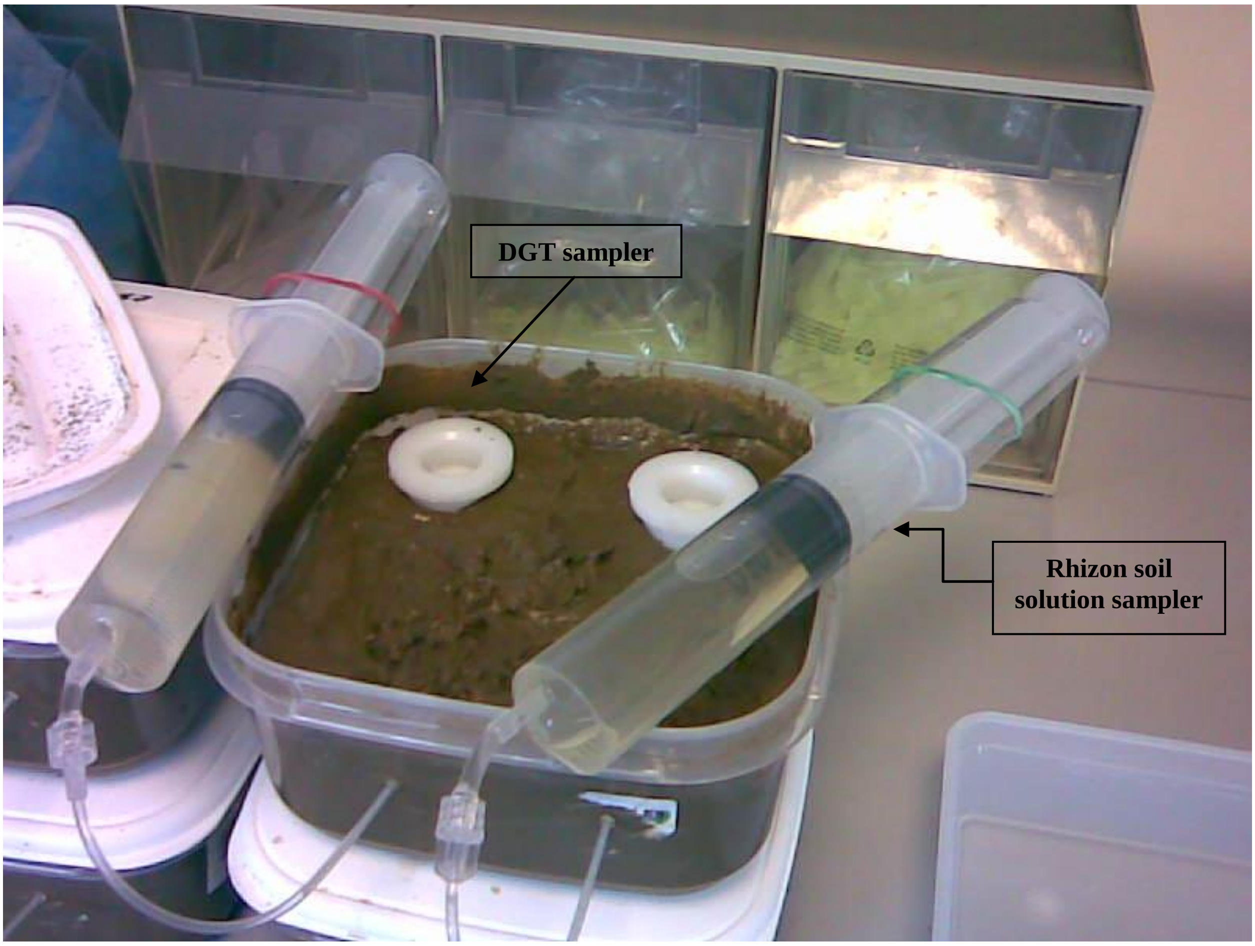

Figure 2.3: Deployment of Rhizon soil solution sampler and diffusive gradient in thin films devices (DGT) into Kettering loam soil, image captured by author.

2.8 Chemical speciation modelling

Free metal ion concentrations (M) in the soil solution were calculated using the speciation model *W*indermere *H*umic *A*queous *M*odel VI (WHAM VI) (Tipping, 1998). The measured parameters for the soil solution including DOC, major cation concentrations, dissolved Cd, Cu and Zn concentrations and pH values were used as model inputs. In addition to the model inputs it was assumed that, for the purposes of modelling the ion-binding properties of DOC, the DOC consisted of 65% active fulvic acid, concentration of humic acid was set to 10^{-10} M and $Al(OH)_3$ was allowed to precipitate out of solution (Tipping, 1998).

2.9 Data analysis and statistics

The dose-response relationships for the effect of metals in each soil on cocoon production were analysed with respect to all metal dose concentrations by least-square fitting of a log-logistic model to the experimental data (7)

$$R = R_0 \Big/ 1 + e^{\beta(\log_{10} EC_R - \log_{10} EC_{50})} \qquad (7)$$

Where R_0 is the maximum reproduction rate and was set to 1, EC_R is the concentration of metal corresponding to that value expressed as total soil metal (mg/kg), dissolved metal (M), free metal ion in solution (M) and DGT effective concentration (ppb). Finally β is the gradient of the curve. Uncertainties in the EC_{50} values were estimated by bootstrapping, this was achieved though random sampling of the dataset with replacement 5000 times. The median, 95%ile and

5%ile of the distribution of the EC_{50} values were calculated. Prior to fitting the dose-response equation each observation was scaled to mean control response, in order to allow the comparison of earthworm reproduction rates in the four soils.

2.10 Overview of experimental design

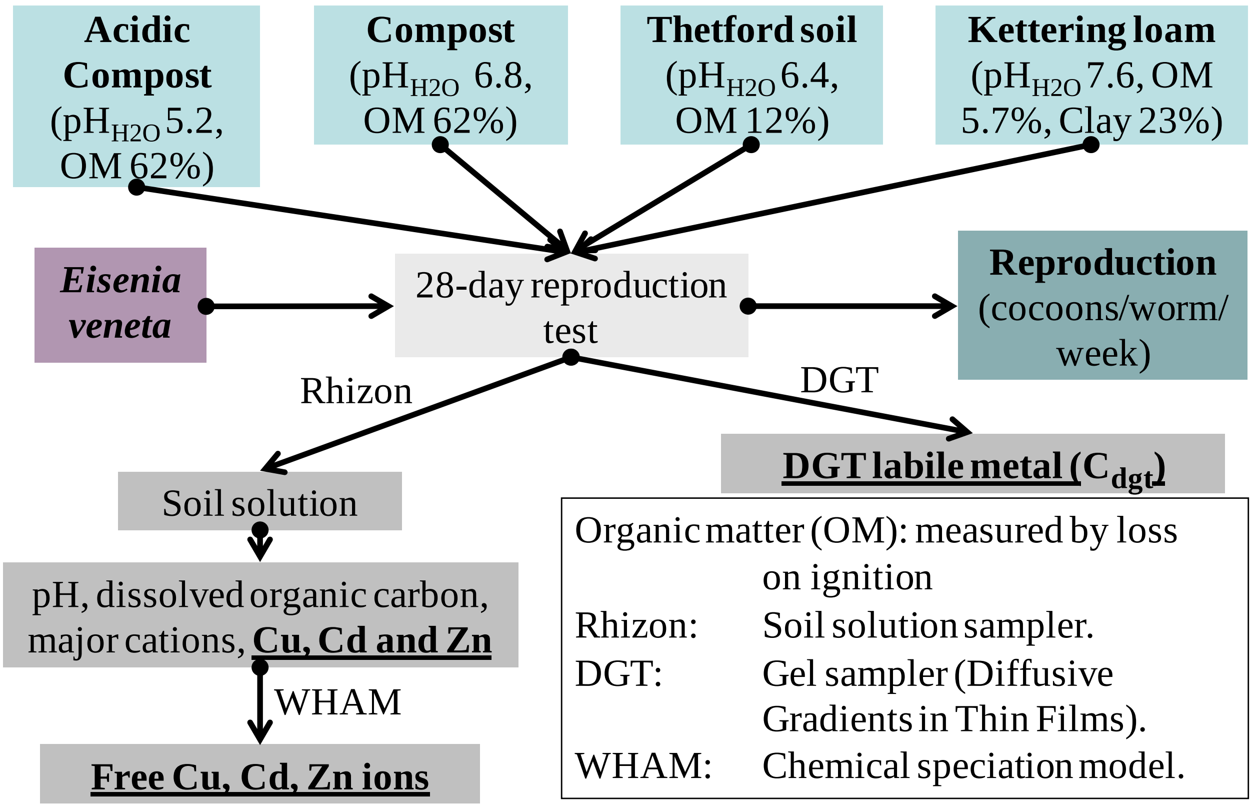

Figure 2.4: An overview of the main points involved in the experimental design of the study.

Chapter Three

Experimental results

3.1Introduction

The following chapter outlines the experimental results following the earthworm toxicity tests detailed in chapter 2 and the viability of the tests are discussed. Further results relating to the individual chapter aims are focused at a later stage within chapters 4-7. Results from further experimentation to test the effects of changes in soil solution pH and increasing salt concentration on cocoon production rates are also outlined in addition to temporal effects on biological performance and finally a brief introduction to mixture toxicity results.

3.2Initial results

Earthworm mortality during all experiments was at minimum rates, <5% mortality for all control treatments. This is within the guidelines set for earthworm toxicity tests which state that control mortality rate must ≤10%, it was concluded all the adult earthworm tests performed were valid (OECD, 1984; ISO, 1997; Mangala et al., 2009). The average weight loss in control earthworm was less than 30% in all experiments, also in agreement with the recommended criteria (Frund et al., 2009). 100% mortality was only observed in the highest Zn doses for all four soils. The highest Cu and Cd doses in the Acidic compost also caused 100% mortality, however this level of effect was not observed for the remaining soils. Earthworms successfully reproduced in the control tests for all four soils in conjunction with the high survival rates. The soils used varied predominantly in organic matter content and pH (Table 2.1). Maximum earthworm reproduction rates were different in each of the soils due to the variation in these properties. Although the maximum reproduction rate of the earthworms in the control soils varied, it was consistently high enough to represent a reasonable maximum level that supported the validity of the results. In the control soils, the highest cocoon production rate was observed in the neutral compost with an average reproduction rate of 1.55 ± 0.20 (cocoons/worm/week) and minimum reproduction recorded in the acidic compost with an average reproduction rate of 0.60 ± 0.13 (cocoons/worm/week). The $pH_{solution}$ values of the control treatments varied by ± 0.12 of a pH unit in all four soils.

3.3 The biological effects of $pH_{solution}$ and nitrate salt

Metal additions did result in a decrease in soil solution pH in all test soils. The largest decrease in pH values from soil control values was observed in the highest single Zn doses for all soils with an average decrease of 1.03 ± 0.26 pH units. The lowest drop in pH values was observed in the Kettering Loam and the largest drop in pH values was observed in Thetford soil. The difference in pH values between the average control pH values in soil solution and soil solution pH of the highest Zn doses, indicating the largest drop in pH due to metal salt addition can be ordered as follows:

Thetford soil (1.31 ± 0.04)> Neutral compost (1.19 ± 0.08)>
Acidic compost (0.83 ± 0.11)> Kettering loam (0.78 ± 0.09)

Cocoon production in earthworms is known to be pH dependant and this effect was seen during the earthworm exposures (Fig 3.1). A series of independent test units with acidified soil and no metal addition allowed the quantification of the effect of pH alone on the reproduction rate of the earthworms. The results of the preliminary study revealed the following relationships expressed as linear equations ($y = mx + c$) where y is the earthworm reproduction rate, x is the soil solution pH, m is the gradient and c is the intercept (8-10).

Kettering loam: $y = 0.18x - 0.49$ ($r^2 = 0.72$) (8)

Thetford soil: $y = 0.16x - 0.26$ ($r^2 = 0.57$) (9)

Compost: $y = 0.37x - 1.37$ ($r^2 = 0.82$) (10)

The same relationship was used for acidic and neutral compost as they are the same soil with the exception of acidification. Correction for the direct effect of soil pH on cocoon production was achieved by using the measured pH values at each metal dose, and the effect on cocoon production corresponding to the measured pH value to estimate an alternative maximum or pH corrected ‘mean control response’ for that dose (Crommentuijin et al., 1997; Spurgeon et al., 2006).

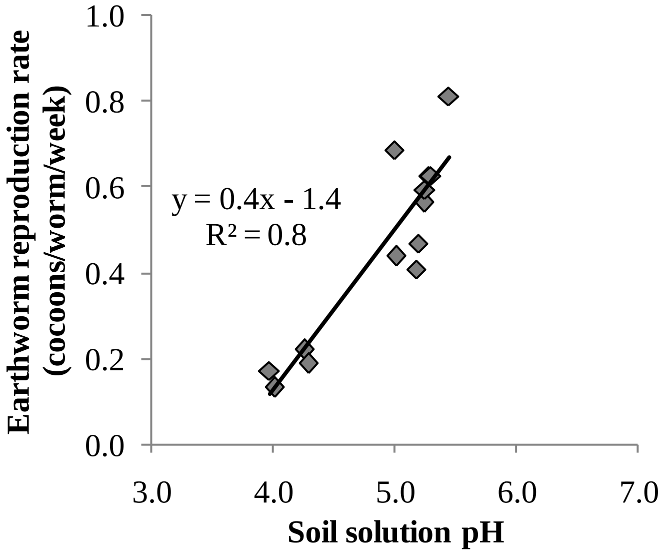

Figure 3.1: Effect of pH on earthworm reproduction rates in Acidic compost soil. The straight line represents the linear relationship between soil solution pH and rate of earthworm reproduction after 28 day exposure in the soil. Acidic compost is used as an example of the relationships observed between soil solution pH and the rate of earthworm reproduction (cocoons/worm/week).

The estimated maximum cocoon production was then used to standardise the observed cocoon production for each soil. The absolute effect of pH on the cocoon production was calculated using the linear relationship observed between cocoon reproduction rates and the pH of the soil calculated from preliminary tests described previously. The response (R) is normalised against the experimental control (11) and then standardised against the 'alternative maximum' (12) which is calculated using one of the aforementioned equations (8-10) giving the corrected response R'.

$$R = R_{observed} / R_{control} \qquad (11)$$

$$R' = R / R_{pH} \qquad (12)$$

The effect of pH was significant at *p*-value <0.05 (Student's t-test). There was no correction for the effect of increasing salt concentrations in the soil as the preliminary experiment did not reveal a significant effect of increasing nitrate concentrations (when applied as $CaNO_3$) on the rate of cocoon production (*p*-value >0.09) (see Appendix 1). Prior to fitting the dose-response equation each observation was scaled to mean control response, in order to allow the comparison of earthworm reproduction rates in the four soils. The soils used varied predominantly in organic matter content and pH (Table 2.1).

Maximum earthworm reproduction rates were different in each of the soils due to the variation in these properties. By standardising the observed cocoon production rates to the control means adjusted for pH effects, we assume the effects of the soil properties on the reproduction rates are normalised, thus comparing the response of the earthworms as a function of metal dose is well founded.

3.4 Temporal effects on cocoon production

The temporal effects in earthworm reproduction rates were analysed in order to determine if there was a need to correct for the control response of the earthworm population used for the series of toxicity tests. However, it was found that the average change in cocoon production was not significantly different from the average reproduction rates observed for the low and high Zn additions (0.64 ± 0.15 and 0.45 ± 0.07 cocoons/worm/week respectively) in the first experiment. The *p* value of the Student's t-test for the series analysis was *p*>0.10 and the lowest *p* value for the pair-wise comparison was *p*>0.37(Fig 3.2). Accordingly no correction for temporal effects was needed meaning that the patterns of response could be assessed across the full data-set.

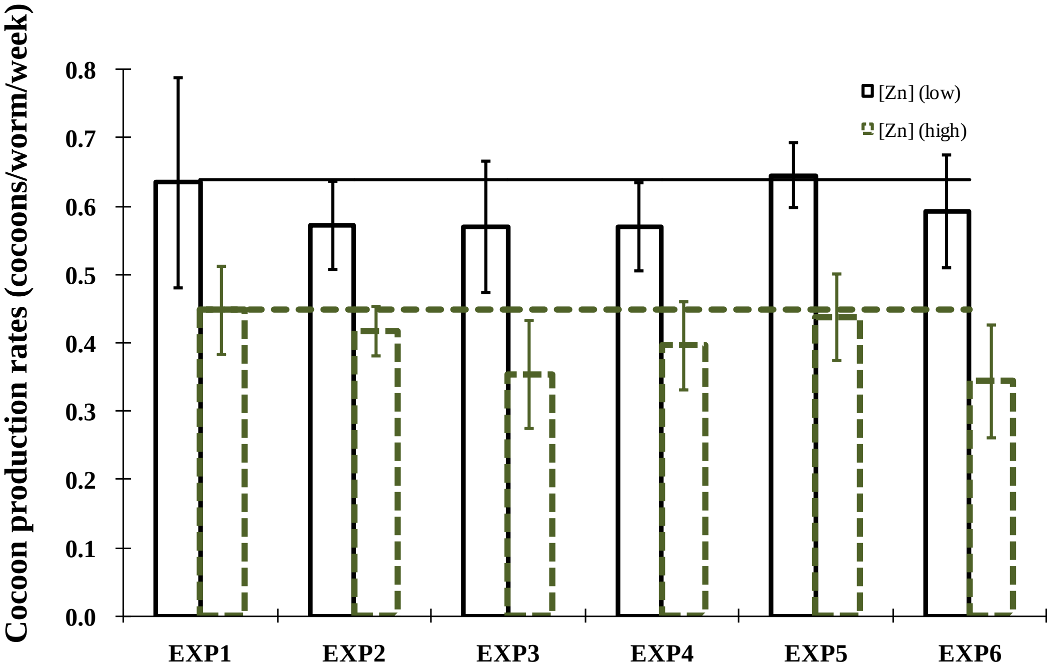

Figure 3.2: Average cocoon production rates (from three replicates) for two Zn doses low (370 mg Zn/Kg dry weight soil) and high (840 mg Zn/Kg dry weight soil), performed alongside each of the 6 experiments. The straight lines indicate the mean cocoon reproduction value from the first exposure.

3.5 Mixture exposure results

A review of the reproducibility of past mixture studies has revealed that in some cases small variations occur that are difficult to repeat. This is due to the fact that the variations are on a small scale and will be difficult to identify statistically. Such synergistic and antagonistic deviation patterns from the CA model may in turn negatively affect the replication potential of mixture studies (Cedergreen et al., 2007). With accordance to the study by De Laender et al. (2009) all single metal exposures and their corresponding mixtures in this study were tested simultaneously. The duration for the completion of all the mixture experiments was 18 months. The possible effect of time on the performance of the earthworms was monitored as previously discussed (Fig 3.2).

The results of the mixture experiments of this study supported the assumption that the same conditions were sustained throughout the time frame required to execute the experiments. In addition the use of CA as well as the IA models as descriptors of the observed mixture data may minimise the probability of wrongly identifying deviation patterns form the reference models. Both models are similar in their underlying principles with regards to the assumption of joint toxic effect of the chemicals and the use of different models may increase the probability of correctly identifying mixture patterns.

Chapter Four

Effects of soil and soil solution characteristics on the toxicity of Cd, Cu and Zn to *Eisenia veneta* in diverse soils

4.1 Introduction and background

Heavy metal contamination of land is a widespread environmental problem in many parts of the world (Fergusson, 1990). The associated potential risks include groundwater contamination (Loureiro et al., 2005) and effects on organisms exposed to heavy metals in the soil (Demuynck et al., 2007). Earthworms are an example of soil invertebrates at high risk from the toxic effects of heavy metals. They play a vital role in maintaining the fertility of the terrestrial environment and the health of organisms at higher levels of the food chain (Kostera et al., 2006; Spurgeon et al., 2006; Arnold and Hodson, 2007).

The methods of heavy metal uptake and exposure in earthworms are representative of methods applicable to a number of other soil dwelling species (Spurgeon et al., 2006). Due to their role in sustaining various important functions in the ecosystem (Schuurmann and Markert, 1998) and their exposure routes to heavy metals, they are considered ideal species for toxicity assessment of the detrimental effects of soil borne contaminants (Spurgeon et al., 2006).

Laboratory toxicity testing underpins the development of environmental risk assessments of metal toxicity (Lock and Janssen, 2001). From numerous past toxicity studies performed, it has become apparent that laboratory based studies overestimate the effects of chemicals on organisms in the field (Spurgeon and Hopkin, 1995). This is a consequence of most laboratory toxicity tests not taking into account the soil ageing process which occludes metals into finer micropores in the solid phase causing them to be less available to be released into the soil solution (Selim and Sparks, 2001; Ford et al., 1997). Metal toxicity in soils is affected by the chemical and physical properties of the soil (Almas et al., 2007). For a metal to cause adverse effects on an organism there needs to be an interaction between the organism and the polluting metal. This interaction is thought to depend on the metal binding at surface sites. The amount of metal available for this reaction is the bioavailable metal (Campbell, 1995). Bioavailability has become widely accepted as the main factor determining metal toxicity in soil (Spurgeon and Hopkin, 1996; Lock et al., 2002; Rombke et al., 2006). Evidence strongly suggests that the speciation of metal in the soil solution controls the amount of metal available to the organism and in turn toxicity of metals to soil dwelling species (Amaral et al., 2006). Soil properties such as pH, organic matter and clay content, as well as physical properties such as temperature are all identified as having an effect on the concentration of available metal in soil (Hankard et al., 2005; Amaral et al., 2006).

Previously it has been shown that, under varying soil conditions, earthworms respond differently to the same concentration of chemicals (Spurgeon et al., 2006). There have been many attempts at quantifying the concentration of available metal in order to assess the metal species responsible for causing toxic effects. Some studies have used different extraction techniques, representing different chemical fractions, to estimate available metal fractions (Lukkari et al., 2004). The available chemical species is mainly concerned with the soil solution and soluble metal species (Koptsik et al., 2005), as supported by the significant correlation between metal porewater concentrations and earthworm body burdens (Hobbelen et al., 2006). A number of studies have focused on the ‘non-complexed’ or free ion metal form as being the fraction of metal available for uptake by biota (Lofts et al., 2004; Becquer et al., 2005). The effect of pH on metal speciation is thought to be one of the most important chemical factors determining metal uptake and toxicity (Van Gestel and Hensbergen, 1997). This applies both to the effect of pH on metal speciation and also the potential protective effect of protons which is in accordance with the biotic ligand model (Playle, 1998; Meyer et al., 1999; Spurgeon et al., 2006).

Changes in soil solution pH values can not only cause a change in the rates of reproduction, but also have an effect on the speciation of cationic metal species. The removal of metal from the soil solution is due to sorption by solid phases such as clays and organic matter. Competition from H^+ ions, as well as other solution cations, for dissolved ligands (e.g. DOC) affects the distribution of the metal between the soil and solution phases (Ure and Davidson, 1995), changing the concentration of environmentally available metal. More recently models such as the terrestrial biotic ligand model (TBLM) have been established to take into account the competition between metal free ions and H^+ ion as well as major cations in solution (Thakali et al., 2006a). The TBLM assumes the interface between the organism and the soil solution can be treated like a biotic ligand, and that the number of occupied sites is related to the toxic effect observed. Consequently by competing for binding sites, on the biotic ligand, solution cations, e.g. H^+, will provide a protective effect (Thakali et al., 2006b).

4.2 Study outline

In order to quantify the effect of soil chemistry on metal toxicity in diverse soils, this study investigated the variability in metal toxicity causing toxic effects on the reproduction rate of a single earthworm species, *Eisenia veneta*, in different soils. Worms were exposed to four soils with different physical and chemical properties, dosed with a range of concentrations of Cd, Cu and Zn (applied singly). In each soil different forms of available metal were measured (total and dissolved), the chemical speciation model WHAM VI was used to model the concentration of the free metal ion species, with metal effects on rates of cocoon production described as a function of the total and free metal ion forms. Using soil properties the patterns of metal toxicity in the different soils should be explained. When expressing the metal dose as total metal the soil pH and complexation capacity will be important, similarly the soil solution pH when expressing the metal dose in the free metal ion form.

4.3 Methods

The adult earthworm reproduction experiments, chemical analysis and data preparation were performed in accordance with the methods outlines in Chapter 2. In addition, the following procedures were followed to support the aims of this chapter.

4.3.1 The 'information-theoretic' approach

The 'information-theoretic' approach was used to select the model which best describes the data (Gonzalez-Megias et al., 2008). This approach compares the fits of a set of different models using Akaike's information criterion (AIC). If (13)

$$n / k < 40 \qquad (13)$$

Where n is the sample size and k is the number of fitting parameters in the model, then it is recommended that a second order of AIC, AICc, be used (Hurvich and Tsai, 1989) (14),

$$AICc = AIC + \frac{2k(k+1)}{n-k-1} \qquad (14)$$

The model best describing the data is the model scoring the lowest AICc value. This technique was used when using additional parameters to explain model fits.

4.4 Results

The response of earthworms to Cd, Cu and Zn under different soil conditions was tested. Toxicity effects were apparent at different concentrations of Cd, Cu and Zn (Tables 4.1, 4.2 and 4.3). The dosed metal concentration ranges for all three metals were able to illustrate the complete range of effects on cocoon reproduction (from control to maximum effect) (Fig 4.1).

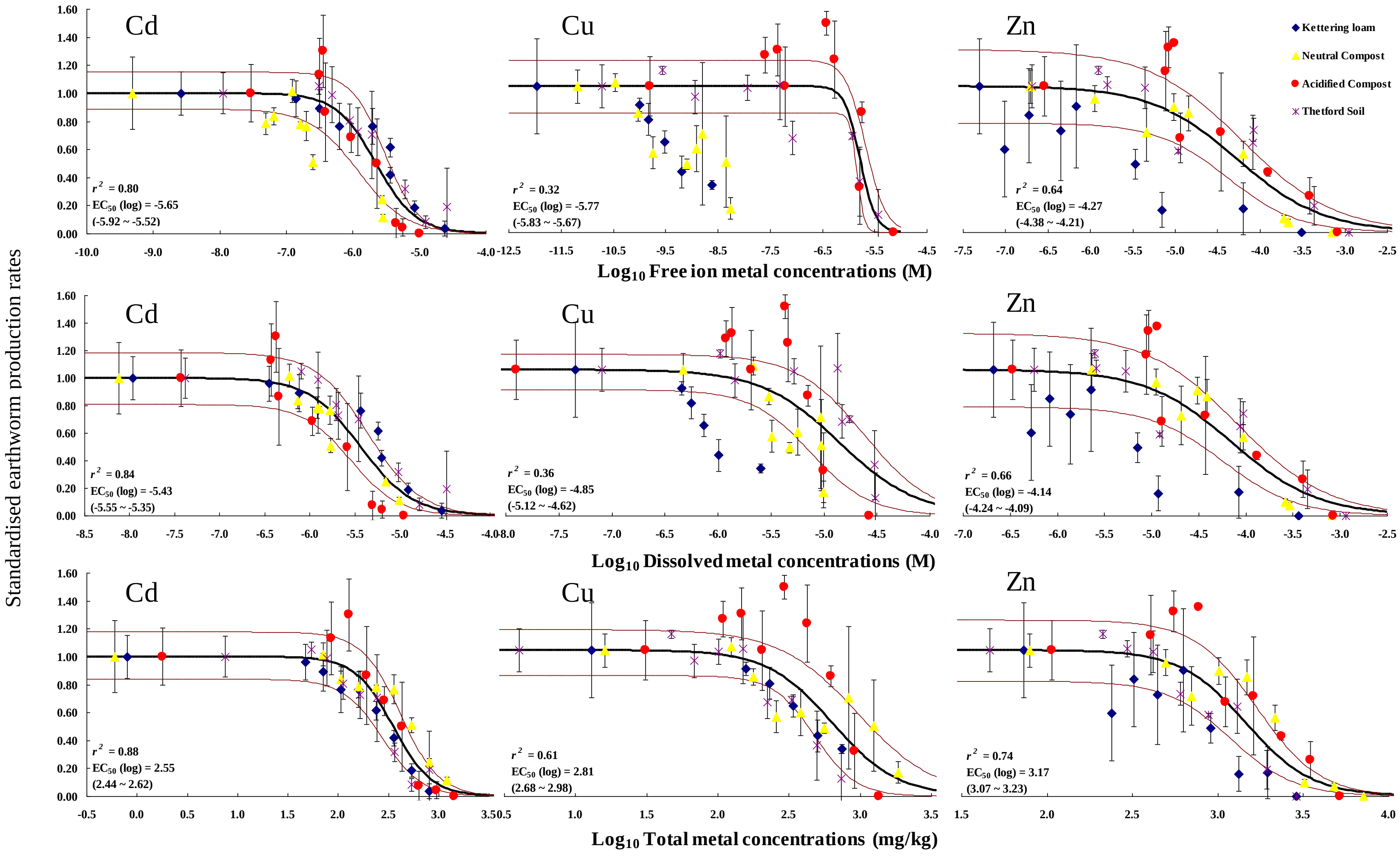

Figure 4.1: Fitted dose response curves of cocoon production for Cd, Cu and Zn. Each graph represents the log-logistic relationship between the cocoon production rates from all four soils against the metal dose (*cont.*).

For Figure 4.1 (*cont.*) the Log_{10} (EC_{50}) values and r^2 values correspond to the optimized log-logistic model fits of the cocoon production data against the associated metal dose, range values and upper and lower confidence interval lines correspond to the 95% confidence intervals. The metal doses expressed are total, dissolved and free ion concentrations. Individual points on the graphs represent the average cocoon production rate for the particular dose; the error bars signify the standard deviation of the observations.

When expressing the metals as total metal, the order in which EC_{50} values lie is in support of the theory that metal toxicity decreases with increasing pH and particularly the organic matter content (Spurgeon and Hopkin, 1996). Cd, Cu and Zn were always most toxic in low organic matter soils Kettering loam or Thetford soils (often with overlapping EC_{50} value ranges) and least toxic in Neutral compost for Cd and Zn, and Neutral compost and Acidic compost for Cu (again with very similar EC_{50} ranges)(Tables 4.1, 4.2 and 4.3). When metal dose is expressed as total soil concentrations, the order of metals in terms of their toxicity follows the same pattern in all four soils starting with Zn as the least toxic, followed by Cu then the most toxic being Cd (Tables 4.1, 4.2 and 4.3).

Table 4.1: (EC_{50}) and (EC_{10}) values of Cd expressed as total metal and dissolved and free metal ion (as Log_{10}) doses. The range values correspond to the 95% confidence intervals, and were calculated by method of bootstrapping.

		Cadmium					
		[Total metal (mg/kg)]		Log_{10}[Dissolved metal (M)]		Log_{10}[Free metal ion (M)]	
Soil		EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}
Kettering loam *Low OM, Neutral pH*	*Median*	**275**	**82.6**	**-5.20**	**-5.75**	**-5.41**	**-6.19**
	Range	(191- 388)	(30.2- 192)	(-5.38- -5.07)	(-6.44- -5.39)	(-5.67- -5.21)	(-6.97- -5.64)
Neutral compost *High OM, Neutral pH*	*Median*	**500**	**145**	**-5.58**	**-6.24**	**-6.21**	**-7.08**
	Range	(339- 714)	(46.6- 380)	(-5.75- -5.39)	(-6.56- -5.93)	(-6.25- -5.87)	(-7.84- -6.50)
Thetford soil *Low OM, Acidic pH*	*Median*	**263**	**89.1**	**-5.29**	**-6.00**	**-5.50**	**-6.30**
	Range	(188- 363)	(38.1- 188)	(-5.46- -5.12)	(-6.36- -5.69)	(-5.71- -5.29)	(-6.78- -5.87)
Acidic compost *High OM, Acidic pH*	*Median*	**346**	**165**	**-5.73**	**-6.26**	**-5.79**	**-6.27**
	Range	(265- 461)	(100- 267)	(-5.89- -5.58)	(-6.53- -5.99)	(-5.97- -5.62)	(-6.60- -5.98)

Table 4.2: (EC_{50}) and (EC_{10}) values of Cu expressed as total metal and dissolved and free metal ion (as Log_{10}) doses. The range values correspond to the 95% confidence intervals, and were calculated by method of bootstrapping.

		Copper					
		[Total metal (mg/kg)]		Log_{10}[Dissolved metal (M)]		Log_{10}[Free metal ion (M)]	
Soil		EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}
Kettering loam *Low OM, Neutral pH*	*Median*	**446**	**132**	**-6.02**	**-6.61**	**-9.28**	**-10.47**
	Range	(320- 604)	(45.6- 266)	(-6.21- -5.83)	(-7.40- -6.28)	(-9.66- -8.96)	(-11.83- -9.80)
Neutral compost *High OM, Neutral pH*	*Median*	**512**	**37**	**-5.31**	**-6.16**	**-9.61**	**-12.29**
	Range	(115- 1060)	(0.69- 152)	(-7.02- -5.10)	(-9.73- -5.70)	(-20.0- -8.65)	(-26.4- -9.97)
Thetford soil *Low OM, Acidic pH*	*Median*	**381**	**144**	**-4.67**	**-4.94**	**-6.06**	**-7.40**
	Range	(293- 487)	(75.0- 245)	(-4.74- -4.59)	(-5.15- -4.80)	(-6.51- -5.73)	(-8.61- -6.17)
Acidic compost *High OM, Acidic pH*	*Median*	**821**	**560**	**-5.08**	**-5.20**	**-5.72**	**-6.02**
	Range	(698- 953)	(446- 700)	(-5.14- -5.01)	(-5.29- -5.13)	(-5.89- -5.52)	(-6.29- -5.80)

Table 4.3: (EC_{50}) and (EC_{10}) values of Zn expressed as total metal and dissolved and free metal ion (as Log_{10}) doses. The range values correspond to the 95% confidence intervals, and were calculated by method of bootstrapping.

		Zinc					
		[Total metal (mg/kg)]		Log_{10}[Dissolved metal (M)]		Log_{10}[Free metal ion (M)]	
Soil		EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}
Kettering loam *Low OM, Neutral pH*	*Median*	**967**	**487**	**-5.17**	**-5.93**	**-5.48**	**-6.26**
	Range	(458- 1370)	(54.1- 940)	(-7.11 - -4.98)	(-9.88 - -5.25)	(-6.75- -5.20)	(-9.54- -5.60)
Neutral compost *High OM, Neutral pH*	*Median*	**2280**	**1210**	**-4.00**	**-4.56**	**-4.17**	**-4.82**
	Range	(1580- 3030)	(378- 2140)	(-4.22- -3.83)	(-5.18- -4.14)	(-4.52- -3.96)	(-5.80- -4.31)
Thetford soil *Low OM, Acidic pH*	*Median*	**1080**	**372**	**-4.01**	**-5.28**	**-4.07**	**-5.42**
	Range	(790- 1450)	(187- 680)	(-4.81- -3.69)	(-6.94- -4.31)	(-4.74- -3.71)	(-6.89- -4.36)
Acidic compost *High OM, Acidic pH*	*Median*	**1850**	**825**	**-4.13**	**-4.98**	**-4.15**	**-4.98**
	Range	(1430- 2400)	(508- 1230)	(-4.38- -3.91)	(-5.42- -4.65)	(-4.24- -3.91)	(-5.45- -4.61)

In order to describe the observed toxicity on earthworm reproduction expressed as total soil metal concentrations in terms of the soil physiochemical properties, regression analysis was performed on the total metal as a function of the soil clay content, the % loss on ignition and the soil solution pH (the average soil solution pH of all the control in that particular soil) (Fig 4.2).

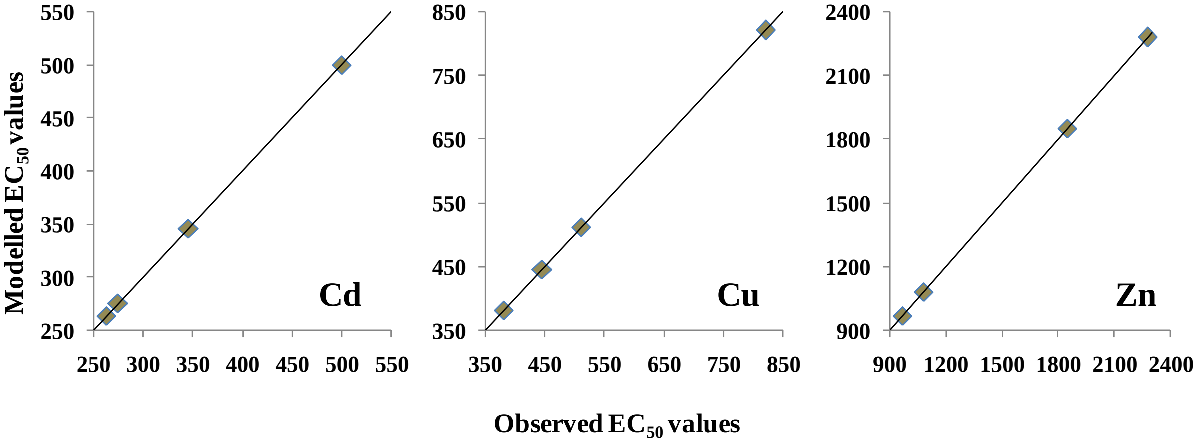

Figure 4.2: EC_{50} values in four soils expressed as a function of the total metal concentrations plotted against the EC_{50} values of Cd, Cu and Zn modeled using a regression including the %clay, %loss on ignition and the soil solution pH values. The straight line indicated a 1:1 line.

The parameters fitted for the three metals are described in equations 15, 16 and 17; while the use of 4 points in the regression model which contains 3 parameters is not statistically reliable the models provide a good description of the relationship between soil characteristics and metal toxicity.

$$\mathrm{LogEC}_{50}\ (\mathbf{Cd}\ \text{total metal}) = 1.72 + 0.06(\mathrm{pH}_{\text{solution}}) + 0.27(\log\%LOI) + 0.01(\log\%Clay) \quad (15)$$

$$\mathrm{LogEC}_{50}\ (\mathbf{Cu}\ \text{total metal}) = 3.21 - 0.10(\mathrm{pH}_{\text{solution}}) + 0.15(\log\%LOI) + 0.10(\log\%Clay) \quad (16)$$

$$\mathrm{LogEC}_{50}\ (\mathbf{Zn}\ \text{total metal}) = 2.41 + 0.04(\mathrm{pH}_{\text{solution}}) + 0.37(\log\%LOI) + 0.01(\log\%Clay) \quad (17)$$

EC_{50} values for Zn expressed as total soil concentrations observed were compared to estimated EC_{50} values calculated using the parameters reported by Munn et al. (2010), the equation of which is as follows (18) was derived from a number of species and not only *Eisenia veneta* the species used in this study.

$$\mathrm{Log}EC_{50}\,(\mathrm{Zn}) = 1.9 + 0.79(\log CEC) \qquad (18)$$

where *CEC* is the cation exchange capacity of the soil in meq/100g (= cmol/kg), this was calculated using the %loss on ignition and % clay content of the soil (19) (UMN, 2010; and references therein). The input values for the individual soils can be found in Table 2.1.

$$CEC = 2(\%\mathrm{OM}) + 0.5(\%\mathrm{Clay}) \qquad (19)$$

The calculated and observed values are comparable in all four soils (Kettering loam, Thetford soil, Neutral compost and Acidic compost). The largest variation between values where the estimated value does not lie within the range of the observed values is found in the case of the soil with high organic matter and acidic pH (Fig 4.3).

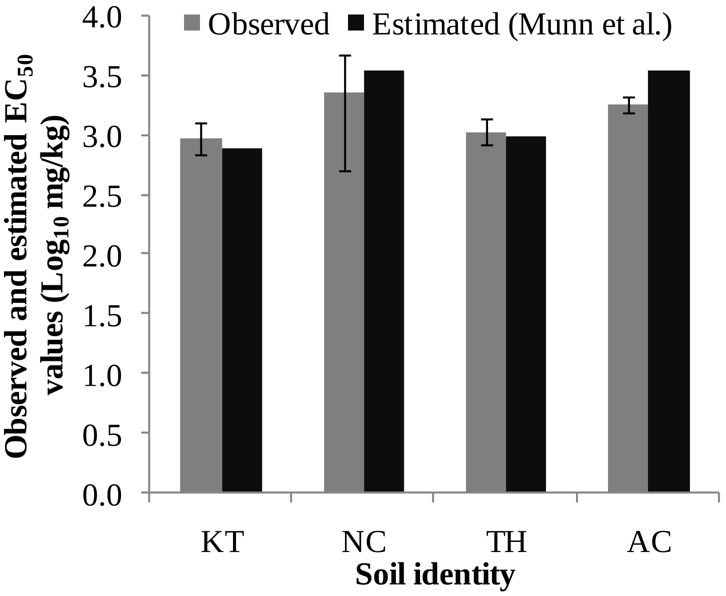

Figure 4.3: EC_{50} values expressed as Log_{10} total soil Zn concentrations observed and estimated using parameters reported by Munn et al. (2010) in four soils with varied physiochemical properties (Table 2.1). Error bars represent the 95% confidence intervals of observed Zn EC_{50}.

EC_{50} values for Cu expressed as total soil concentrations observed were compared to EC_{50} values calculated using the parameters reported by Criel et al. (2008) for *Eisenia fetida*, the equation of which is as follows (20)

$$\mathrm{LogEC_{50}}\,(\mathbf{Cu}) = 1.4 + 0.296(\log \mathit{OrgC}) + 076(\log \%\mathrm{C}\mathit{lay}) \qquad (20)$$

where OrgC is the %loss on ignition of the soil multiplied by 0.5, the input values for the equation inputs can be found in Table 2.1. The difference between the observed and calculated EC_{50} values in the soils with neutral pH (Kettering loam and Acidic compost) was smaller than the difference in EC_{50} values for the soils with more acidic pH (Thetford soil and Acidic compost) (Fig 4.4).

The comparison between the earthworms *Eisenia veneta* and *fetida* is suitable as the two species have similar traits and are closely related.

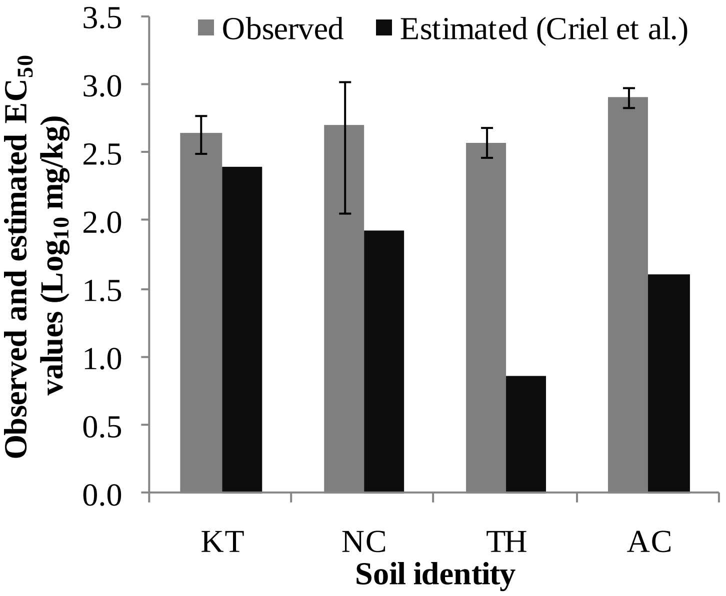

Figure 4.4: EC_{50} values expressed as Log_{10} total soil Cu concentrations observed and estimated using parameters reported by Criel et al. (2008) in four soils with different physiochemical properties (Table 2.1). The error bars represent the 95% confidence intervals of observed EC_{50} values for Cu.

When expressing the metal dose in dissolved soil solution concentrations the same order of metal toxicity continues for all soils (Zn the least toxic, Cd the most toxic) apart from in the Kettering loam (low OM, neutral pH). The EC_{50} (and EC_{10}) expressed as dissolved concentrations for Zn in Kettering loam are lower than the EC_{50} (and EC_{10}) values in the other soils (Table 4.3). In comparison to the other soils Zn appears to be much more complexed in Kettering loam (Fig 4.5). When metal EC_{50} values are expressed in dissolved metal form for Cu, the order in which Cu is most toxic (Table 4.2) does not follow the order in which Cu is most available in soil solution (Fig 4.5).

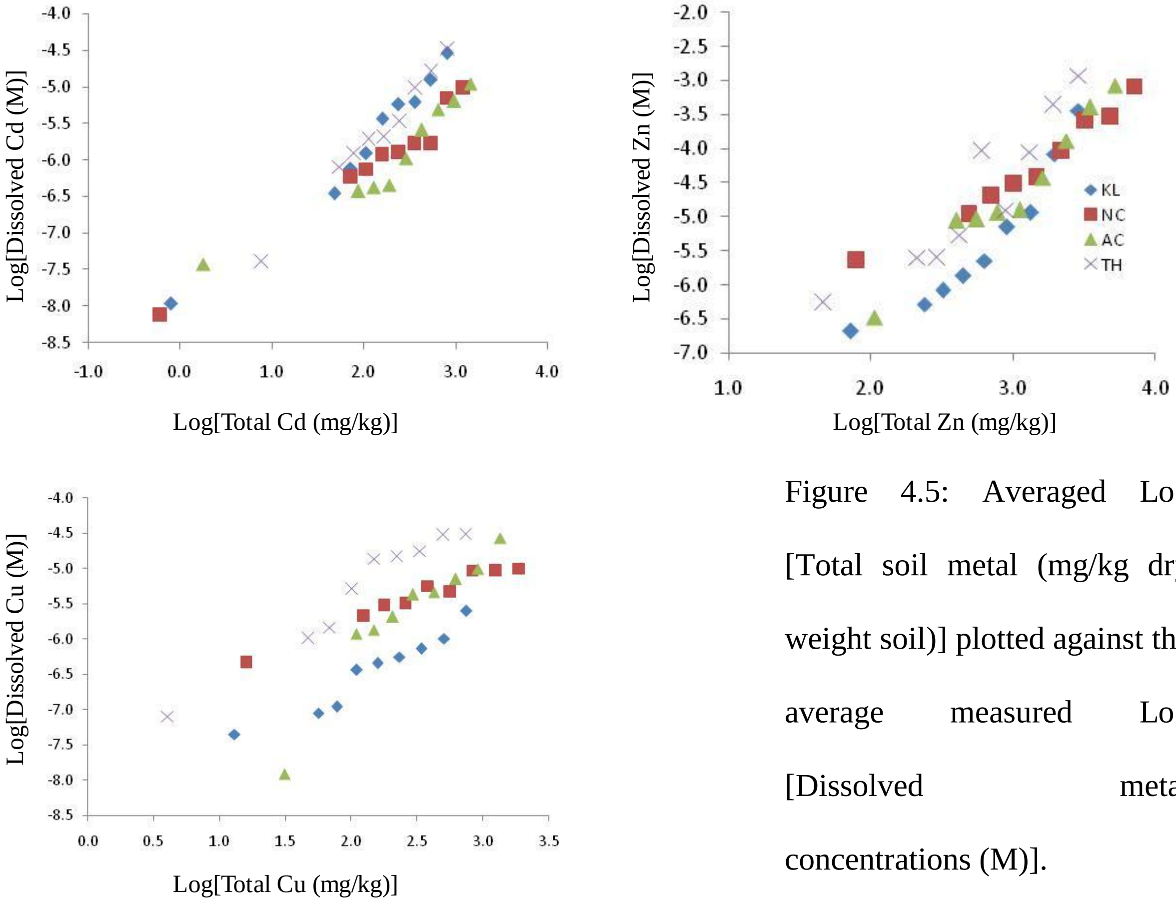

Figure 4.5: Averaged Log [Total soil metal (mg/kg dry weight soil)] plotted against the average measured Log [Dissolved metal concentrations (M)].

The EC_{50} (and EC_{10} values) for Cd and Zn expressed as dissolved metal in Kettering loam are very similar, this is not found in the other soils. Possible reasons as to why the EC_{50} (and EC_{10}) value of Cd in Kettering loam is higher, relatively speaking, than what is expected from observations in the other types of soil are discussed later. The same inconsistency applies to the EC_{50} (and EC_{10}) value of Cd when expressed as free metal ion in Kettering loam. This is not unexpected as the free metal ion concentrations were calculated using the dissolved metal concentrations as input values in the chemical speciation model.

The dissolved metal concentration relationship with total metal concentration for Cu and Zn (Fig 4.5) do not explain the order of EC_{50} (EC_{10}) values observed (Tables 4.2 and 4.3). This is also true for Cd where the EC_{50} values expressed as dissolved metal (Table 4.1) do not represent the metal availability in soil solution (Fig 4.5). This may be a factor of the narrow range covered by the EC_{50} values, alternatively as it has been previously reported earthworms respond in a different manner to essential and non-essential metals.

Expressing the metal dose as free metal ion for Neutral compost, Thetford soil and Acidic compost Zn is the least toxic. In the Kettering loam the EC_{50} (and EC_{10}) value of Cd appear to be very similar to the EC_{50} (and EC_{10}) value for Zn. The reasons as to why Cd appears to have low toxicity in Kettering loam is discussed at a later stage. Interestingly in the soils with lower pH values (Thetford soil and Acidic compost) Cd appeared to be more toxic than Cu. In the soils with circum-neutral pH Cu is more toxic than Cd, and the difference in EC_{50} values is much greater.

Previously, the toxicity of Cu, expressed as free metal ion (or Cu^{2+} activity), as a function of soil pH has been discussed (DiToro et al., 2001; DeSchamphelaere and Janssen, 2002; Lofts et al., 2004). The competition between protons and free metal ions for binding, at the site of uptake (or site of action) of the metal, results in a protective effect of the protons (and major cations) to the organism. Although this relationship between pH and metal ions is observed for all three metals used in this study (with one exception) it appears to be most prominent for Cu (Fig 4.6).

As formerly mentioned the EC_{50} value of Cd in Kettering loam expressed the free metal ion does not follow the trend of the other soils with respect to decreasing toxicity with decreasing soil solution pH as the protective effect of the protons becomes apparent. On analysis of the soil solution in Kettering loam, it was found that the for the single Cd doses the Ca concentrations (in the dissolved form) were on average a factor of >2.4 higher than those found in the single Zn doses and mixture doses for this soil. Possible reasons could be contamination of Ca in the soil or the result of an anomaly during the use of Rhizons to extract soil solution, causing complexed Ca and Cd to diffuse into the extracted solutions and consequently be detected during analysis. While the reasons for the high Ca concentrations cannot be determined, it was deemed necessary to investigate the effect on Cd toxicity.

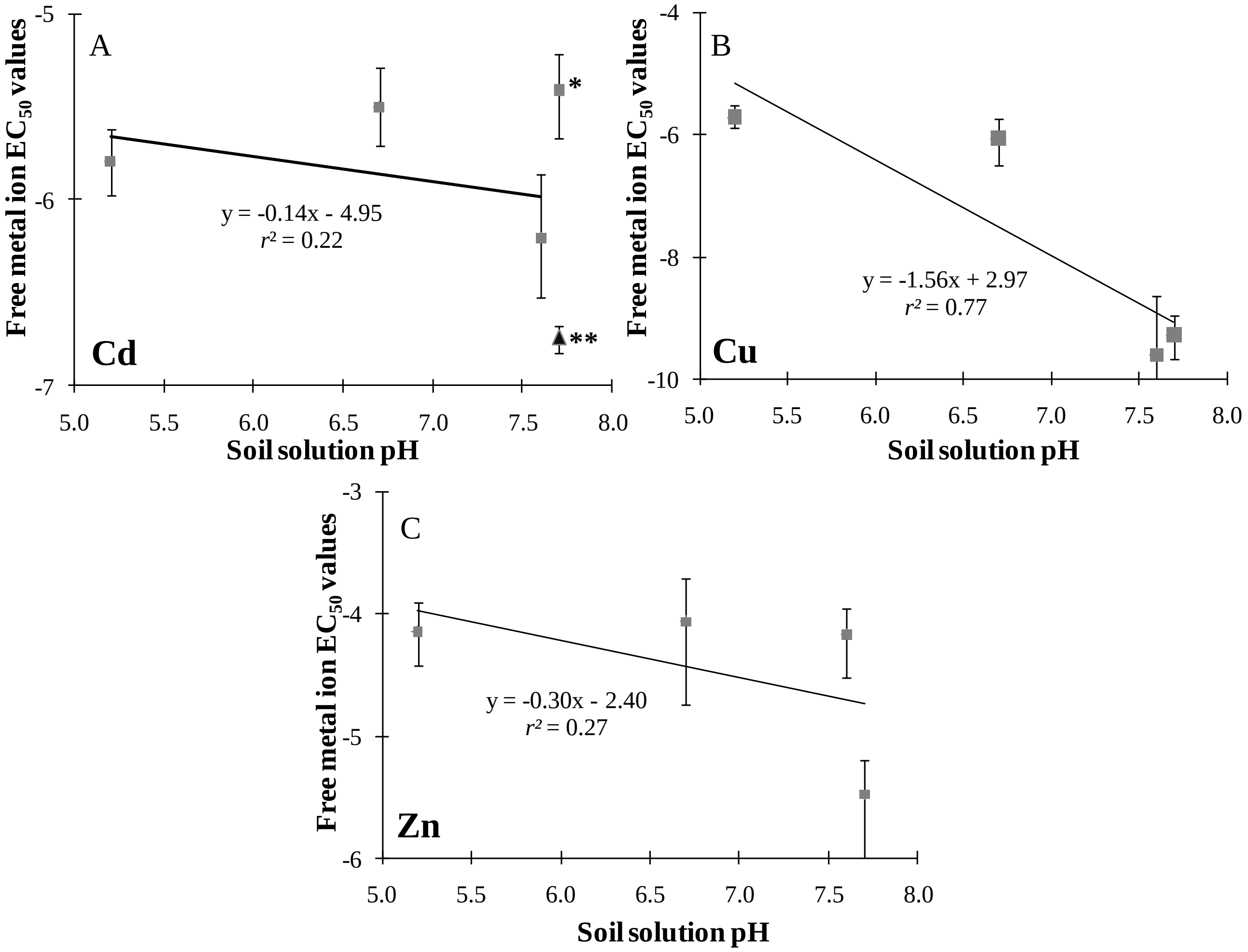

Figure 4.6: Soil solution pH (average measured pH values in control soils) plotted against $\log_{10}EC_{50}$ values of Cd, Cu and Zn expressed as free metal ion concentrations (M). The error bars represent the 95% confidence intervals (Tables 4.1, 4.2 and 4.3). Graph A: *EC_{50} value of Cd from experiment, not included in linear fit, ** EC_{50} value of Cd after refitting to include Ca concentration, not included in linear fit.

Body burdens of the earthworms exposed to Cd in Kettering loam were analysed in order to assess the effects of soil properties and experimental conditions on metal uptake. As an initial basis of comparison assessment, earthworm tissue from the Cd and Zn exposures were analysed for both the Kettering loam and the Neutral compost. The bioaccumulation factor (BAF), defined as the Cd or Zn concentration in earthworm tissue (mg metal/kg dry tissue) divided by the metal concentration in soil (mg metal/kg dry soil) (An, 2004) were calculated in Kettering loam and Neutral compost for the Cd and Zn bioassays. The ranges of BAF found for Cd were (0.52 – 5.76) and (0.36 – 2.66) and for Zn were (0.52 – 1.81) and (0.18 - 0.63) for Kettering loam and Neutral compost respectively. These values are comparable to the BAF ranges reported by Nahmani et al. (2009) for three field soils Shipham, Glenridding and Avonmouth (Nahmani et al., 2007) with respective BAF values of 1.42, 1.14, 4.47 for Cd and 0.06, 0.16, 0.09 for Zn for the species *Eisenia fetida*.

As a measure of comparison between the BAF values calculated for the two soils, the ratio of Zn BAFs to Cd BAFs in the range of soil metal concentrations were evaluated and it was found that the ratios in Neutral compost were not significantly different from the ratio of BAF in Kettering loam ($p>0.76$). It was therefore concluded that the high EC_{50} value found for Cd (expressed as dissolved and free ion form) could be explained through chemical factors and not the biological response of the earthworms.

The concentration of Ca^{2+} ions (M) were calculated using WHAM (the chemical speciation technique mentioned above) and were in turn used to calculate a new 'dose' corrected for the concentration of Ca^{2+} ions scaled using an extra parameter (α =-0.98). This 'corrected dose' (21) was used to fit a dose response curve for Cd expressed in the free metal ion form (M) (Fig 4.7). The 'corrected dose' was labelled as the effective dose ($D_{effective}$) (M). The value of α was fitted using the method of least squares.

$$\mathrm{Log}_{10}[D_{effective}] = \log_{10}[\mathrm{Cd}^{2+}] - \alpha(\log_{10}[\mathrm{Ca}^{2+}]) \qquad (21)$$

This approach was used as the concentration of Ca^{2+} ions changed with increasing Cd^{2+} dose and therefore taking an average Ca^{2+} concentration to use for the correction would not have been acceptable.

The 'information-theoretic' approach was used to select the model which best describes the data (Gonzalez-Megias et al., 2008) for the Ca correction of Cd data.

The model best describing the data is the model scoring the lowest AICc value. The model using the additional parameter which includes the [Ca^{2+}] had an AICc value of -11.1 (r^2= 0.95), and the free ion model fitting had an AICc value of -14.0 (r^2= 0.95). The differences in AICc values indicate that the additional model parameter does not improve the model fit.

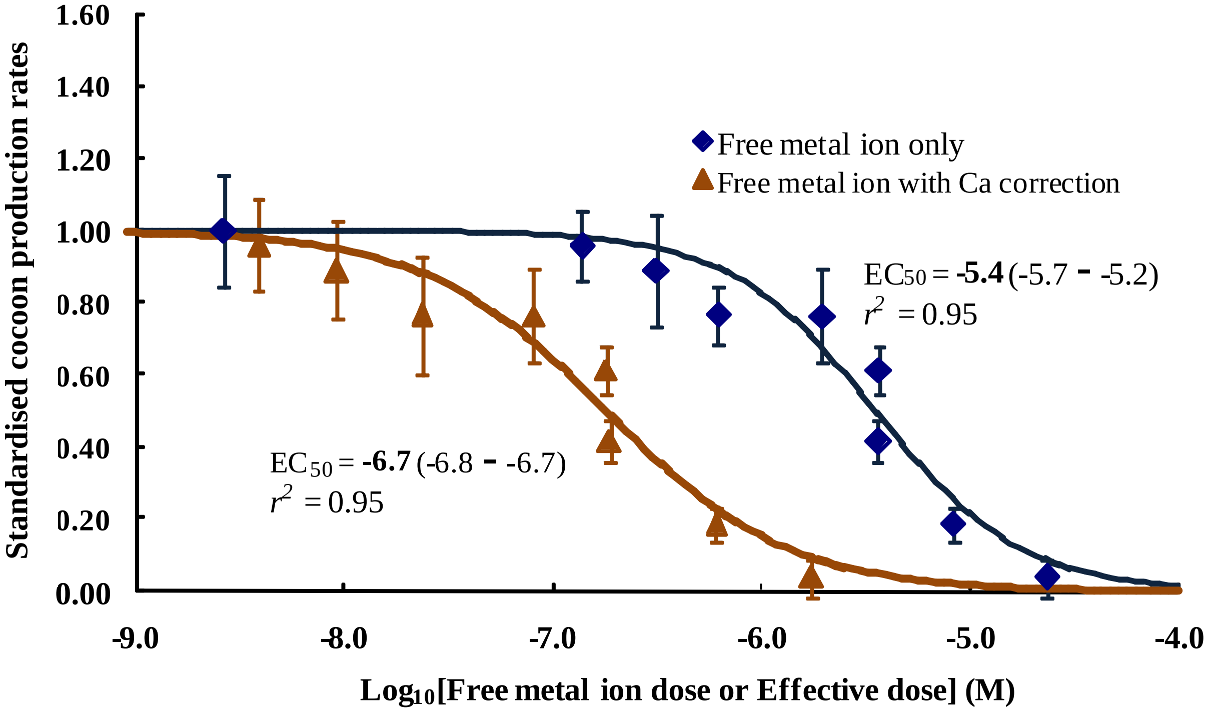

Figure 4.7: Fitted dose response curves of cocoon production for Cd in Kettering loam showing the observed Cd effects and the corrected Ca model ($D_{effective}$). Each data set represents the log-logistic relationship between the cocoon production rates against the metal dose expressed as the free metal ion or the effective dose ($D_{effective}$)(i.e. free metal ion dose corrected using a coefficient and concentration of Ca^{2+} (M)) . Log_{10} (EC_{50}) values and r^2 values correspond to the optimized log-logistic model fits of the cocoon production data against the associated metal dose, range values correspond to the 95% confidence intervals. Individual points on the graphs represent the average cocoon production rate for the particular dose; the error bars signify the standard deviation of the observations.

4.5 Discussion

Metal toxicity is closely related to metal speciation because it has an important role in controlling metal availability. The main soil properties influencing metal speciation and therefore metal toxicity are pH, soil organic carbon content, oxide content, dissolved organic matter as well as cation exchange capacity (Criel et al., 2008). Spurgeon and Hopkin (1996) found that when expressing the metals as total metal, metal toxicity decreases with increasing pH and organic matter content. The order in which EC_{50} values lie are in support of this theory. The metals appeared to be most toxic in Kettering loam and Thetford soil and least toxic in Neutral compost.

Criel et al. (2008) studied the effects of soil properties on the toxicity of copper to *Eisenia fetida* in 19 European field soils. Figure 4.4 shows good correlation between the observed EC_{50} values for Cu and those calculated using parameters reported by Criel et al. 2008 for two soils with circumneutral pH. The parameters described by the authors do not include a function for the inclusion of the pH of the soil solution. This may explain why there is such a discrepancy between the observed and calculated values for Cu EC_{50} values expressed as total metal.

The reproduction results used in this study have been corrected for the absolute effect of pH on earthworm reproduction. The pH of the soil will still cause an effect on the reproduction rates of the earthworm by influencing metal speciation and through the competition of protons with the metal ion at the site of uptake. The observed EC_{50} values were higher (less toxic) than the calculated EC_{50} values

(Fig 4.4), as the effect of pH which accounts for a proportion of the toxicity has been accounted for by correcting for pH effects. In the case of Zn (Fig 4.3), where the observed EC_{50} values were compared to calculated values a high correlation was found in every case. In the acidic compost the estimated value was not in the range of the observed EC_{50} values for Cu, the reason for the disagreement may be that at those conditions simply using the cation exchange capacity as a fitting parameter is not sufficient to account for all effects due to the low values of these parameters. The observed EC_{50} values (Figures 4.4) vary much less than the EC_{50} values predicted using either model. The Cu model used in Figure 4.4 relies on toxicity data relating to the earthworm species *Eisenia fetida*, not *Eisenia veneta* which is the earthworm used in this study. Given the range in EC_{50} values it may be suggested that metal toxicity expressed as total metal for *Eisenia veneta* is much less dependent on soil chemistry than *Eisenia fetida*.

The fit of the regression analysis performed using the % clay content, % loss on ignition and soil solution pH yielded very good results (Fig 4.2). Including more fitting parameters did not increase the power of the model. As soil toxicology is greatly dependant on the physiochemical properties of the soil it may be favourable to include a number of soil properties during analysis of toxicity results.

Previously, the toxicity of Cu, expressed as free metal ion (or Cu^{2+} activity), and a function of soil pH has been discussed (DiToro et al., 2001; DeSchamphelaere and Janssen, 2002; Lofts et al., 2004). The competition between protons and free metal ions for binding, at the site of uptake (or site of action) of the metal, results in a protective effect of the protons (and major cations) to the organism. Although this relationship between pH and metal ions is observed for all three metals used in this study (with one exception) it appears to be most prominent for Cu (Fig 4.6). In the case of Cd, only one of the soils (Kettering loam) did not to follow the expected pattern. A correction was made using the soil solution Ca^{2+} concentrations (Fig 4.7).

A number of studies have previously used Ca concentrations in conjunction with Cd to describe metal behaviour (van Gestel and Koolhaas, 2004; Temminghoff et al., 1995; Li et al., 2009). The two cations have ionic radii of similar sizes and therefore do compete for the same binding sites (Li et al., 2008). The ameliorating effect of Ca^{2+} ions through competition with Cd^{2+} at the site of uptake will cause an increase in the EC_{50} value expressed as free metal ion. The re-fitted EC_{50} value (from the re-fitted dose response curve)(Fig 4.7) correlates well with the expected trend of decreasing EC_{50} values with increasing pH for free metal ion, however the improvement of data fit is not significant when compared to the fit achieved through the ‘un-corrected’ free metal ion dose, it does help support the theory for the relationship between pH and free metal ion EC_{50} and therefore it may be biologically significant even if the model is not a better fit than the previous.

The three metals used in this study showed different levels of toxicity in the different soils. The differences in the toxicity of Cd, Cu and Zn could be due to several factors affecting the interaction between the metal and the organisms at various levels i.e. at the site of action or at an intracellular level. This may be related to the different response of earthworms to essential and non-essential metals. The relationship between total metal and soil solution metal has been plotted in Figure 4.5. The order of metal toxicity in each of the soils can be related to the availability of soil solution metal

Once a metal has entered the organism, the toxic effects can be countered in a number of ways including altering the metal absorption efficiency or the excretory rate (Marino and Morgan, 1999). Cu and Zn are both important micronutrients for animals, whereas Cd is known to be a non-essential element (Landis and Yu, 2004). Essential metals such as Cu are regulated within the earthworm body and excreted, whereas non-essential metals such as Cd are detoxified. This is achieved through sequestration within inorganic matrices or through binding to organic ligands (Spurgeon and Hopkin, 1999). These differences may cause the response of earthworms to the individual metals to differ (Sizmur and Hodson, 2009). A study conducted by Spurgeon et al. (2004) on the effects of Cd and Cu on the juvenile growth and development of the earthworm *Lumbricus rubellus* concluded that the differences observed in the effects of the two metals may be due to the different mechanisms for coping with high concentrations of the two metals.

The importance of including soil properties in the analysis of metal toxicity in soils has been demonstrated previously. The toxicity patterns observed in this study, as well as a number of past studies, has revealed that the metal form which is used can also be an important aspect for understanding metal toxicity in soils.

The range of EC_{50} values found for Cd in the four soils when fitted independently (Table 4.1) were all within the range of the EC_{50} value fitted for all soils collectively (Fig 4.1). This was not true for the other two metal forms. For Zn,

EC_{50} dissolved and free metal ion ranges overlapped with all but the EC_{50} value calculated for Kettering loam individually (Fig 4.1, Table 4.3). For Cu the free metal ion provided the most overlap in term of EC_{50} values for single soils and all soils in combination (Table 4.2). However the model fit when using the free metal ion dose had the lowest r^2 value (Fig 4.1). Comparing the EC_{50} values observed for *Eisenia veneta* for Zn and Cu to modelled values we have already noted that the variation in values is less than for the species *Eisenia fetida* (Fig 4.3 and 4.4). These results support the fact that gut uptake may be an important aspect of metal toxicity. However, more detailed work into directly studying the role of gut uptake would need to be done to understand the mechanisms involved.

Earthworms can be exposed to metals by direct dermal contact through the soil solution or by ingestion of soil, contaminated food or pore water (Lanno et al., 2004). Lock and Janssen, (2001) found that dietary exposure of Zn to *Folsomia candida* may be an important uptake route as effects on reproduction were underestimated when using just the porewater metal dose.

Various studies have assessed the relative contribution of the dermal and gut route to metal uptake in earthworms and found differences in the means of uptake of different metals. One study used a model to estimate the percentage of metal uptake accounted for by the dermal route and found that it explained >96% of the overall uptake of Cd and Cu in the earthworm *Eisenia andrei*, and 82% of total Zn uptake (Saxe et al., 2001). Vijver et al. (2003) used a technique where the mouths of the earthworms were sealed using glue. They found that for the earthworm *Lumbricus rubellus* Cu uptake could be fully explained using the dermal route, but found that it explained 83% of Cd uptake and 79% of Zn uptake, when compared to internal concentrations of earthworm in the same soil conditions with unsealed mouths. In order to find a metal fraction that best describes sublethal effects of all three metals individually in different soils; we must take into account all exposure routes of the metals and study the body burdens of the metal in the earthworms. This introduces a new pool of metal that may not be explained by the dissolved or free metal ion concentrations alone, and this may account for the high r^2 values (Fig 4.1) when expressing the metal dose as total metal.

The cumulative analytical error associated with the dissolved and free ion metal forms which is not present to the same extent in total metal extractions may cause a bias in the goodness of fit when using the total metal dose. Furthermore the differences in the goodness of fit of a single relationship describing the effects on reproduction of Cd, and Cu and Zn could be due to several factors affecting the interaction between the metal and the organisms at a various levels i.e. at the site of action or at an intracellular level, which can be related to the different response of earthworm to essential and non-essential metals.

4.6 Conclusions

Regression models with parameters that include organic matter content and soil solution pH were shown to describe toxicity data in the form of total metal EC_{50} values for Zn, Cd and Cu. This supports models found in the literature but also supports the relationship between the physiochemical soil properties and toxicity to earthworms. Including toxicity data into regression models from more soils of different physiochemical properties would further enhance the predictive power of the model fits.

Further to the observed results, it can be concluded that EC_{50} values expressed in the free metal ion form for all three metals display a strong relationship with soil solution pH. This relationship is altered in the presence of elevated concentrations of major cations in the soil namely calcium.

The results also support the argument of studying gut uptake as an exposure route through which Zn, Cd and Cu can exert toxicity.

Chapter Five

Integrating chemical parameters to mixture modelling techniques: Joint toxic effects of Cu and Zn to the earthworm *Eisenia veneta*

5.1 Introduction and background

Heavy metal contamination in the environment is often characterized by mixtures of different metals, and therefore soil organisms inhabiting contaminated field sites are usually exposed to mixtures of toxic metals at varying concentrations (Weltje, 1998; Posthuma et al., 1997). There are concerns that exposure of organisms to mixtures of chemicals may lead to unexpected effects at low concentrations (Baas et al., 2007), and over recent years there has been a drive to research the joint action of chemical mixtures (Spurgeon et al., 2003; Baas et al., 2007).

Thus far, an insufficient amount of information has been available on the behaviour of mixtures in the environment to confidently predict the effects they may cause in different types of soil. This has implications to risk assessment objectives for soils as toxicity tests in the terrestrial environment have predominantly referred to the effects of metals applied singly (Spurgeon et al., 2005; Baas et al., 2007). In contrast, a considerable number of metal mixture studies have been performed in the aquatic environment (e.g. Paulsson and Lundbergh, 1991; Backhaus et al., 2000; Otitoloju et al., 2002; Barata et al., 2006; Cooper et al., 2009; Vandenbrouck et al., 2009).

Solid-liquid equilibrium partitioning coefficients have been used with aquatic toxicity data in an attempt to derive soil quality standards, but this method has been criticised due to a number of issues including the variability in behaviour of chemicals in different soils, and in uptake routes for soil organisms (Janssen et al., 1997). Soil toxicology relies heavily on controlled laboratory experiments when investigating the effects of different environmental pollutants on soil organisms, and earthworm species have been widely regarded as model organisms (Frund et al., 2009). While results from laboratory based studies are an invaluable part of soil toxicology, issues exist in relating such findings to the field where mixture interaction and bioavailability are known to be important (Lock and Janssen, 2001; Posthuma et al., 1997; Koptsik et al., 2005).

Soil properties influence the speciation of metals in soils through ion exchange, complexation and precipitation-dissolution processes (Koptsik et al., 2005). There is strong evidence to suggest that the soil characteristics that determine metal speciation over solid phase and soil pore water are the same characteristics that influence the concentration of metal available to the organism (Janssen et al., 1997).

Reliable prediction of mixture toxicity requires the knowledge of the relationships between the single chemical toxicities and those of their mixtures, which can only be achieved through appropriate testing procedures (Barata et al., 2006). A number of authors have begun investigating ways of quantifying the effects of mixtures in soil ecotoxicology. Among the most popular methodologies are the utilization of mathematical models previously developed and used in pharmaceutical research, namely the Concentration Addition (CA) (Loewe and Muischnek, 1926) and Independent Action (IA) models (Bliss, 1939). Concentration Addition is a conceptual model that assumes that toxicants have similar modes of action and that their joint action can be defined as the sum of individual component concentrations scaled to their relative potency (Drescher and Boedeker, 1995). The Independent Action model is thought to hold true for chemicals with different modes of action and makes a statement about the probability of an observed effect. A number of studies have relied on the guiding principles of these two conceptual models to describe the effects of mixtures (Backhaus et al., 2000; Altenburger et al., 2004; Jonker et al., 2004, 2005; Barata et al., 2006; Baas et al., 2007). The models can be extended to describe synergism or antagonism using deviation functions (Jonker et al., 2005).

Deviations from the CA and IA reference models can also be considered. Some chemicals can interrelate synergistically showing a higher toxic effect that would otherwise be expected from the toxicity of the single chemicals within the mixture, similarly chemicals within a mixture can interact antagonistically by showing a lower toxic effect than that expected. Mixtures may also enhance the probability of effect of one of the chemicals in the mixture. The difference being that on occasion when a toxic effect is not predicted from the toxicity of the individual chemicals in the mixture, the presence of the combination of chemicals will cause a toxic effect to be observed.

Synergistic/antagonistic deviations may be dependent on the mixture dose level or on dose ratio (Jonker et al., 2005). The dose level-dependency means that observed deviations from the reference models are dissimilar at low dose levels and at high dose levels. The dose ratio-dependency means that the fraction of the chemicals in the mixture is a compounding factor of the deviation from the reference model, with one of the chemicals being the dominant stressor for the observed deviation (Jonker et al., 2005; Loureiro, 2009). There is therefore a need to understand the complex nature of mixture toxicity in order to advance the predictive powers of terrestrial toxicology.

Given that Cu and Zn are both essential metals and therefore have dedicated biochemical pathways through which they are dealt with, interaction between the two metals would not be expected. The mixture effects however are expected to be affected by the physiochemical properties of the different soils, making the use of EC_{50} values in standard soils to predict mixture effects ineffective.

5.2 Study outline

The experimental design used in this study was designed to describe how changes in soil properties affect the joint toxicity of binary mixtures of copper and zinc on the compost worm *Eisenia veneta*. Adult earthworm reproduction experiments were performed in four soils with different physical and chemical properties, dosed to a range of concentrations of Cu and Zn. The observed effects on the rate of reproduction were then described in each soil/mixture combination using the mixture reference models. All experiments were designed to allow the identification of deviation from CA and IA models, should these exist including absolute synergism or antagonism. As well as synergistic/antagonistic interactions at different dose levels and dose ratios. Each soil metal dose was expressed as total soil metal and free metal ion. Patterns of mixture behaviour were described in terms of each of the two metal forms.

If there is no interaction between the metals in the mixture, the effect of the mixtures would be additive. This would imply that the reference models CA and IA would describe the toxicity data in the free metal ion form without the inclusion of an extra parameter to describe synergism and antagonism, regardless of the results obtained when using the total metal dose.

5.3 Methods

The adult earthworm reproduction experiments, chemical analysis and data preparation were performed in accordance with the methods outlines in Chapter 2. In addition, the following procedures were followed to support the aims of this chapter.

5.3.1 Mixture modelling and statistics

To tackle the toxic effects in the mixtures experiments, the observed effect on earthworm reproduction was compared to the expected effect of mixtures calculated from the single metal exposure toxicities by applying the computational framework proposed by Jonker et al. (2003). The model is based on the reference models concentration addition (CA) and independent action (IA). The CA model is defined by equation 4 and the IA model by equation 5 in Chapter 1. The model allows further quantification of deviation from the reference models (Absolute synergism or antagonism (S/A), dose ratio dependant S/A, dose level dependant S/A) (Table 5.1). The model fits are compared to evaluate whether or not the extra parameter would present a significant improvement. A significance level of $p < 0.05$ was chosen as the threshold value. The maximum (or control) response was set to 1.0. The calculations include the slopes of the individual dose-response curves for the metals in the mixture, EC_{50} values for the two chemicals in the mixture, and finally the correlation coefficient (R^2).

Table 5.1: Record of additional model fitting parameters denoting the form of deviation pattern. Chemical B represents Cu in the respective mixtures, as Zn is the common metal in both mixtures tested. Adapted from Jonker et al. (2005).

Deviation pattern	Parameter ***a***	Parameter ***b***
Synergism/antagonism (S/A)	***a***>0 antagonism (ant) ***a***<0 synergism (syn)	
Dose ratio (DR)	***a***>0 ant, except where -ve ***b*** indicates syn. ***a***<0 syn, except where +ve ***b*** indicates ant.	***b***>0 ant where toxicity of mixture mainly caused by Chemical B ***b***<0 syn where toxicity of mixture mainly caused by Chemical B
Dose level (DL)	***a***>0 low DL (ant),high DL(syn) ***a***<0 low DL (syn),high DL(ant)	***b***=1 change at EC_{50} 0<***b***<1 change at higher DL ***b***>1 change at lower DL

5.4 Results

The results of model fitting for observed earthworm responses and relevant deviation fits for the mixture of copper and zinc are shown in Table 5.2. Significant model fits were possible for all datasets using the CA and IA models, in four soils for all metal forms. Fitting parameters and EC_{50} values were different in each metal form and in the different soils. Similarly deviation fits were also specific to each set of conditions. No absolute antagonism was observed in any of the mixtures, significant synergism was observed and more complex dose level and dose ratio deviations were not observed. In three of the four soils (excluding Neutral compost), expressing the metal dose as total metal yielded synergism deviation patterns. Only in one of the cases with free metal ion as the dose is synergism observed, in the Acidic compost.

Table 5.2 (appears on page 84): the best descriptive model, the fitting parameters and the relationship between the observed and the modelled data (after CA and IA model fit) for Cu and Zn expressed as total, and free ion Cu and Zn concentrations in four soils. Concentration units are total (mg/kg dry weight), and free ion (µM). The cocoon reproduction maximum was set to 1.0, and the values highlighted in red are set values. β is the gradient of the dose response curves, CA is the concentration addition model, IA is the independent action model and S/A is synergism or antagonism. The significance of the fits was expressed as $p=0.05$ *, $p<0.05$ **, $p<0.01$ ***.

Table 5.2 : Mixture patterns of Cu/Zn mixtures.

Soil	Form	Model	Cu(β)	Cu(EC_{50})	Zn(β)	Zn(EC_{50})	r^2	*a*	Interpretation
Kettering loam	Total	CA***	3.05	469	1.41	619	0.68		
	Total	**S/A****	2.83	703	2.01	1070	0.75	-3.13	Sig synergism
	Total	**IA*****	1.09	394	1.45	668	0.68		
	Total	**S/A*****	3.31	591	2.26	1280	0.78	-7.06	Sig synergism
	Free M^{2+}	**CA*****	1.18	9.90x10^{-4}	0.67	1.50	0.77		
	Free M^{2+}	**IA*****	1.16	1.85x10^{-3}	0.62	1.59	0.77		
Neutral compost	Total	**CA*****	0.84	819	4.29	1950	0.73		
	Total	**IA*****	0.79	757	2.83	1890	0.73		
	Free M^{2+}	**CA*****	0.48	8.30x10^{-4}	6.89	145	0.73		
	Free M^{2+}	**IA*****	0.78	2.96x10^{-3}	0.45	39.37	0.72		
Thetford soil	Total	**CA*****	1.83	314	1.79	847	0.84		
	Total	**S/A*****	2.73	615	1.91	1158	0.91	-3.48	Sig synergism
	Total	**IA*****	1.42	300	1.45	811	0.83		
	Total	**S/A*****	2.42	567	2.41	1217	0.90	-6.21	Sig synergism
	Free M^{2+}	CA***	2.51	2.09	0.81	61.3	0.86		
	Free M^{2+}	**IA*****	8.50	2.12	0.83	57.0	0.86		
Acidic compost	Total	**CA*****	4.68	678	2.92	2720	0.80		
	Total	**IA*****	4.72	536	2.58	2390	0.78		
	Total	**S/A***	4.46	776	4.34	3210	0.80	-6.27	Sig synergism
	Free M^{2+}	**CA*****	3.62	2.06	7.14	133	0.68		
	Free M^{2+}	**S/A****	4.00	2.56	1.55	338	0.74	-6.95	Sig synergism
	Free M^{2+}	**IA*****	2.59	1.98	1.51	125	0.68		
	Free M^{2+}	**S/A****	3.77	2.24	2.22	355	0.74	-9.28	Sig synergism

5.5 Demonstration of mixture behaviour patterns

In order to demonstrate the differences in compliance with additivity and deviation from additivity Figures 5.1 and 5.2 each show one of the mixture behaviour patterns observed for the Cu/Zn mixture (Table 5.2). ECx is the x% effect observed on the population (ie EC_{10} is where 10% of the population is affected). The ECx values do not present concentrations but proportions of the population affected. The horizontal (x-axis) represents the ECx value predicted by the IA or CA model and the vertical (y-axis) represents the observed ECx value or fitted values. The IA or CA predicted dataset are the values that would be predicted by the model given the single metal fits only. The IA fit (or CA fit) shows the model fit given the entire dataset including the mixture data, and Fit S/A includes the parameter allowing for synergistic/antagonistic deviation. In Kettering loam when the metal is expressed in the total metal form at the maximum point of deviation the IA model predicts an EC_{39} whereas the actual value is EC_{78} (Fig 5.1). In the Neutral compost at the maximum point of deviation for CA model with metal dose expressed in the total metal form the difference in values is much lower and statistically not significant where the predicted value is EC_{65} and actual value EC_{69} (Fig 5.2).

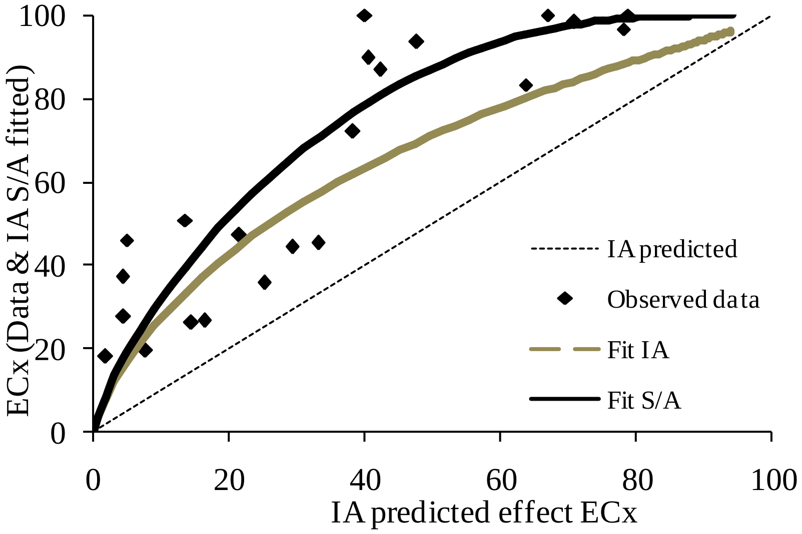

Figure 5.1: Predicted ECx values against fitted ECx values for Cu/Zn mixture in Kettering loam, expressed as total metal concentrations. Synergism was significant at *p*< 0.01.

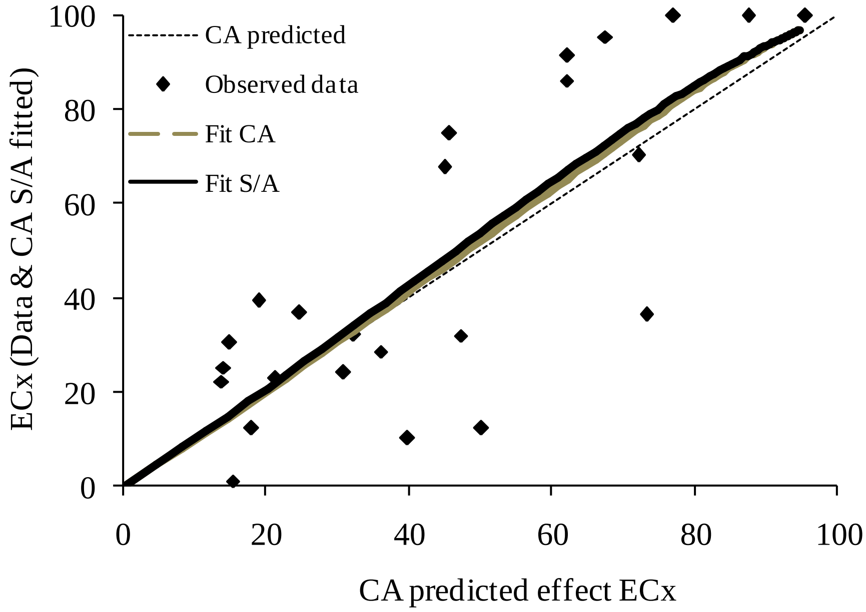

Figure 5.2: Predicted EC_x values against fitted EC_x values for Cu/Zn mixture in Neutral compost, expressed as total metal concentrations. No deviation from additivity was observed.

5.6 Discussion

Different models have been observed to give different categorization of mixture effects between pollutants. In order to achieve more reliable classification of mixtures, analysis of mixture data must utilize more than one model when describing the types of interactions existing between pollutant mixtures exposed to test organisms (Parrott and Sprague, 1993). CA and IA reference models used to analyse data from this study have been previously shown to fit toxicity data equally well (Drescher and Boedeker, 1995), although CA is usually considered appropriate for worst-case scenario for terrestrial environments (Lock and Janssen, 2002).

Differences in the mathematical formulation of CA and IA can be a factor in the dissimilarities in the extent of absolute synergism and antagonism observed in the interpretation of the mixture data by the two models. The slopes of the dose response curves relating to the chemicals in the mixtures can be an indication of the discrepancies, for example, for low slopes synergistic properties are more pronounced in CA in comparison to IA, the opposite is true at high slopes (Drescher and Boedeker, 1995). Given the slopes found in this study range between moderate to high slopes, this relationship will not be important and in fact both reference model fit show similar deviation patterns (Table 5.2).

CA and IA models make specific assumptions on the modes of action of chemicals; CA assumes similar modes of action and IA assumes different modes of action for the chemicals in the mixture. The information about the modes of action of metals on *Eisenia veneta* is not available. Using both models for Cu and

Zn adds to the argument of using more than one model, but also important as the metals may have more than one site of action. The site of action is an important stage in considering the toxic effect of metals on organisms.

Chemical interaction of metals and the surrounding environments can be important at three stages when studying toxic effects of metals on organisms. These are the interaction between the metal and the soil particles, in other words metal speciation in the soil solution, the competition at the site of uptake and finally interactions within the organism (Janssen et al., 1997; Posthuma 1997). The toxicity of metals to organisms is assumed to occur as the result of free metal ion reacting with the physiologically active binding sites at the site of action (Di Toro et al., 2001). Mixture effects expressed as free metal ion showed no deviation from additivity apart from in the acidic compost soil. The amount of metal binding to the site of uptake may be a good representation for dermal uptake however, may not represent uptake at other routes, namely gut uptake. This may explain the difference in mixture patterns when expressed as total and free metal ion (Table 5.2). There has been evidence to suggest some metal uptake may be through the earthworm gut (Jager, 2004; Vijver et al., 2003; Becquer et al., 2005). Earthworm feeding activity is complex (Jager et al., 2003) and consequently there will be variation in the levels of exposure to toxic metals through the gut. The feeding pattern of the earthworms in low organic matter soil may be different than in high organic matter soil. In a general sense, or as is the case for the IA reference model, there was no difference in the mixture behaviour for free ion and total metal, but this was not the case in the low organic matter soils, Kettering loam and Thetford soil (Table 5.2).

Observed mixture toxicity and metal free ion concentrations (Table 5.2) correlate well with high r^2 values. Total metal concentrations show equally good fits with mixture data, and that could be a function of the fact that total metal concentrations will encompass bioavailable metal concentrations but also be associated with lower levels of experimental error. Total metal concentrations are verified using one analytical technique (aqua regia, Chapter 2) while free metal ion concentrations are modelled using results of three analytical techniques (pH analysis, DOC analysis and dissolved metal analysis, Chapter 2). This will ultimately imply that free metal ions will be affected with compound experimental error and while these results are still viable for toxicity assessment they carry larger scatter than total metal concentrations.

An important observation of the mixture patterns is that for most of the datasets there was no deviation from the CA and IA reference models. There was no absolute antagonistic deviation and in the neutral compost additivity was found for both free metal ion and total metal concentrations.

Synergism was observed in all soils, apart from the neutral compost, when the metal dose was expressed as total metal but only in acidic compost when expressed as the free metal ion (Table 5.2). This may therefore suggest that the mixture interactions seen in all four soils are related to the speciation effect to a greater extent than an inherent toxicological interaction in the organism. A toxicological interaction would have given a consistent interaction for all external exposure forms.

In a study by Baas et al. (2007) six binary mixtures of Cd, Cu, Pb and Zn were tested for their lethal effects on *Folsomia candida* in loamy sand. The same modelling approach using CA and IA reference models was used and synergism was also observed for Cu/Cd mixtures. In another study on the lethal effects of Zn/Cu, Zn/Cd and Cd/Cu mixtures on *Tympanotonus fuscatus,* the effects of Cu/Zn mixtures were synergistic, however only in specific ratios of 1:1, 2:3 and 1:4 (Otitoloju et al., 2002). A possible reason for synergistic properties of Cu/Zn mixtures in this dataset is interaction between the two metals which is not included in the reference models. This may imply that interactions seen are not toxicological but instead are the results of effects on bioavailability. Some studies have observed interaction between Cu and Zn in the terrestrial environment.

In a study of the effects of Cu and Zn on the reproduction of *Enchytraeus crypticus* possible interactions were found between the two metals during uptake (Posthuma et al., 1997), likewise Lou and Rimmer (1995) found that copper increased the concentration of zinc in the plant *Hordeum vulgare*.

5.7 Conclusions

The results from this study show the importance of the chemical and physical properties of the soil on the behaviour of mixtures and their effects on the reproduction of *Eisenia veneta*. CA and IA reference models were equally useful at describing observed toxic effects, deviation pattern observed was absolute synergism for all metal forms which is in agreement with studies in the literature, and no dose-ratio and dose-level deviation patterns were established.

Analysis of the mixture data by total metal and free metal ion concentrations indicated that speciation to free metal ion explained the mixture interaction in three of the four soils. To gain generality and thus predictive power, it can be concluded that a greater understanding of the availability of various operationally defined chemical forms to earthworms and their mechanism of toxicity is needed.

Chapter Six

Mixture effects of Cd and Zn mixtures on the compost worm *Eisenia veneta*

6.1 Introduction and background

Organisms in contaminated environments are seldom exposed to single chemical but instead to a host of different chemicals, as part of a complex mixture (Altenburger et al., 2004). Accordingly, it has been widely recognised that it is important to incorporate the toxicity of chemical mixtures into environmental quality objectives and assessment procedures (De Zwart and Posthuma, 2005; McCarty and Borgert, 2006; Spurgeon et al., 2005; Baas et al., 2007; Nursita et al., 2009). Given the potential importance of chemical mixture effects for human health and environmental protection, toxicologists have developed a number of approaches to quantify mixture toxicity. Two of the most widely used models for chemicals with a similar mode of toxic action are the concentration addition (CA) model and for chemicals with a dissimilar mode of action the independent action (IA) model is proposed (Greco et al., 1992; Altenburger et al., 2004; Loureiro, 2009).

Although useful as default model, there are cases where CA and IA fail to correctly describe joint effects. Examples are where effects may be more severe than expected from the single chemical toxicities (synergism) or less severe (antagonism). Studies into the joint action of metal mixtures reveal these types of ‘interactive’ relationships for different chemical (e.g. metal) combinations and in different species. Kohler and Eckwert (1997) found synergistic patterns when investigating the effects of Zn and Cd on the production of stress proteins in the isopod *Oniscus asellus*. The mixture effects of Cu and Pb were shown to be antagonistic when studying lethal effects of the metals on *Folsomia candida* in loamy sand (Baas et al., 2007). Recent data have shown that as well as overall synergistic or antagonistic effects, more complex deviation patterns of mixture effects compared to prediction based on single chemical toxicity can also be observed. These patterns are dose ratio-dependent deviation, where the toxic effects depend on the ratios in each of the metals, and dose level-dependent deviation, where the effects are reliant on the concentration at which the chemicals are present (Jonker et al., 2005).

The natures of the chemicals forming a mixture play an important role in determining the patterns that may be observed in the toxicity of the mixture. However, the properties of the soil and the speciation of the chemical are vital factors in determining the toxic effects exerted by mixtures. The overall joint effect of chemicals is a result of interactions at various levels both outside and within the organism (Posthuma et al., 1997). Speciation of metals in soil is governed by complexation, ion exchange and precipitation-dissolution processes, where the amount of metal available to the organism could be interpreted as a function of the total soil metal concentration moderated by soil characteristics and the presence of other metals (Janssen et al., 1997; Hankard et al., 2005; Kopstik et al., 2005; Amaral et al., 2006).

In order to take account of the possible effects of soil properties on sub-lethal toxicity of Cd and Zn mixtures this study has aimed to describe observed patterns of mixture behaviour as a function of different metal fractions. While it is possible to use existing mixture models and single metal toxicity to predict mixture behaviour, this does not allow for the inclusion of possible metal interactions due to soil properties and at the site of uptake.

6.2 Study outline

To understand how speciation in different soils may affect the nature of a species response to a metal mixture, adult earthworm reproduction experiments were performed on the compost worm *Eisenia veneta* in four soils with different physical and chemical properties, dosed to a range of concentrations of Cd and Zn. The series of mixture experiments was designed with the intention of being able to describe absolute synergism/antagonism as well as dose level and dose ratio deviation patterns from CA and IA reference models. In each different soil the resulting mixture effects were expressed as total metal concentrations and free metal ion concentrations modelled using the chemical speciation model WHAM VI. The rate of earthworm cocoon production was used as the biological endpoint.

The mixture toxicity data when expressed as free metal ion would be best described by the reference models IA and CA if there is no interaction between the metals, independent of the patterns of toxicity that may be found when the metal dose is expressed in the total metal form. However if there is interaction between the metals, deviation parameters will be needed to describe the data in the free metal ion form.

6.3 Methods

The adult earthworm reproduction experiments, chemical analysis and data preparation were performed in accordance with the methods outlines in Chapter 2. In addition, the following procedures were followed to support the aims of this chapter.

6.3.1 Mixture modelling and statistics

To tackle the toxic effects in the mixtures experiments, the observed effect on earthworm reproduction was compared to the expected effect of mixtures calculated from the single metal exposure toxicities by applying the computational framework proposed by Jonker et al. (2003). The model is based on the reference models concentration addition and independent action. The concentration addition model (CA) is defined by equation 4 and the independent action model (IA) by equation 5 in Chapter 1.

The model allows further quantification of deviation from the reference models (Absolute synergism or antagonism (S/A), dose ratio dependant S/A, dose level dependant S/A) (Table 6.1). The model fits are compared to evaluate whether or not the extra parameter would present a significant improvement. A significance level of $p < 0.05$ was chosen as the threshold value. The maximum (or control) response was set to 1.0. The calculations include the slopes of the individual dose-response curves for the metals in the mixture, EC_{50} values for the two chemicals in the mixture, and finally the correlation coefficient (r^2).

6.4 Results

The results of model fitting for observed earthworm responses and relevant deviation fits for the Cd/Zn mixture are shown in Table 6.1. Significant model fits were possible for all datasets using the CA and IA models, in four soils for all metal forms, with one exception. In the Thetford soil when the metal dose is expressed as free metal ion, the *p* value for the best fit IA model was 0.05 (0.0503 to four decimal places) which does not comply with the pre-determined criteria of a significant fit being established when a *p* value is less than 0.05. However as the value is within a reasonable range of the statistical criterion set, the results found will be considered during the result discussion. Fitting parameters and EC_{50} values were different in each metal form and in the different soils. Similarly deviation fits were also specific to each set of conditions. Absolute antagonism and synergism was observed, however, no dose level and dose ratio deviations were found.

Table 6.1(table appears on page 99): The best descriptive model, the fitting parameters and the relationship between the observed and the modelled data (after CA and IA model fit) for Cd and Zn expressed as total, and free ion Cd and Zn concentrations in four soils. Concentration units are total (mg/kg dry weight), and free ion (µM). The cocoon reproduction maximum was set to 1.0, and the values highlighted in red are set values. β is the gradient of the dose response curves, CA is the concentration addition model, IA is the independent action model and S/A is synergism or antagonism. The significance of the fits was expressed as $p=0.05$ *, $p<0.05$ **, $p<0.01$ ***, $p<0.001$ ****.

Table 6.1: Mixture patterns for Cd/Zn mixtures.

Soil	Form	Model	Cd(β)	Cd(EC_{50})	Zn(β)	Zn(EC_{50})	r^2	*a*	Interpretation
Kettering loam	Total	**CA******	1.93	262	2.46	842	0.92		
	Total	**S/A*****	2.16	364	2.39	985	0.94	-1.65	Sig Synergism
	Total	**IA******	1.51	225	1.92	767	0.91		
	Total	**S/A******	2.39	387	2.43	983	0.94	-4.40	Sig Synergism
	Free M^{2+}	**CA******	1.87	8.78	0.56	607	0.56		
	Free M^{2+}	**S/A******	0.83	$8.40x10^{-1}$	1.42	68.0	0.77	11.4	Sig Antagonism
	Free M^{2+}	**IA******	2.10	11.7	0.61	495	0.56		
	Free M^{2+}	**S/A******	0.58	$7.69x10^{-1}$	1.15	77.2	0.77	8.40	Sig Antagonism
Neutral compost	Total	**CA******	1.33	342	4.64	1920	0.89		
	Total	**S/A*****	1.57	484	3.08	2240	0.90	-1.84	Sig Synergism
	Total	**IA******	1.14	294	3.05	1850	0.86		
	Total	**S/A******	2.14	553	3.46	2190	0.91	-5.56	Sig Synergism
	Free M^{2+}	**CA******	0.61	7.21	10.7	548	0.69		
	Free M^{2+}	**IA******	0.74	2.48	10.0	534	0.72		
Thetford soil	Total	**CA******	1.88	294	1.98	809	0.80		
	Total	**S/A*****	2.16	407	2.54	1330	0.84	-2.72	Sig Synergism
	Total	**IA******	1.45	263	1.67	756	0.79		
	Total	**S/A******	2.88	423	2.93	1370	0.84	-7.09	Sig Synergism
	Free M^{2+}	**CA******	0.71	6.91	10.0	167	0.68		
	Free M^{2+}	**S/A******	0.50	1.24	10.0	86.2	0.76	12.6	Sig Antagonism
	Free M^{2+}	**IA******	0.77	4.59	9.49	155	0.73		
	Free M^{2+}	**S/A***	0.54	1.77	8.57	145	0.75	2.99	Sig Antagonism
Acidic compost	Total	**CA******	2.21	567	2.38	3780	0.60		
	Total	**S/A******	3.26	296	3.10	2320	0.75	4.31	Sig Antagonism
	Total	**IA******	3.37	427	5.68	3240	0.69		
	Total	**S/A*****	2.69	279	5.92	2440	0.75	6.43	Sig Antagonism
	Free M^{2+}	**CA****	0.71	12.1	2.74	100	0.26		
	Free M^{2+}	**S/A*****	0.67	1.28	0.80	31.0	0.41	15.3	Sig Antagonism
	Free M^{2+}	**IA****	0.96	6.85	3.00	100	0.28		
	Free M^{2+}	**S/A*****	0.73	1.59	0.43	33.7	0.41	8.19	Sig Antagonism

6.5 Demonstration of mixture behaviour patterns

In order to demonstrate the differences in deviation from additivity Figures 6.1 and 6.2 each show one of the mixture behaviour patterns observed for the Cd/Zn mixture (Table 6.1). ECx is the x% effect observed on the population (i.e. EC_{10} is where 10% of the population is affected). The ECx values do not present concentrations but proportions of the population affected. The horizontal (x-axis) represents the ECx value predicted by the IA model and the vertical (y-axis) represents the observed ECx value or fitted values. The IA predicted dataset are the values that would be predicted by the model given the single metal fits only. The IA fit shows the model fit given the entire dataset including the mixture data, and Fit S/A includes the parameter allowing for synergistic/antagonistic deviation. In Kettering loam when the metal is expressed in the total metal form at the maximum point of deviation the IA model predicts an EC_{47} whereas the actual value is EC_{72} (Figure 6.1). In the Acidic compost at the maximum point of deviation for the metal dose expressed as the total metal form the predicted value is EC_{81} and actual value EC_{31} (Figure 6.2).

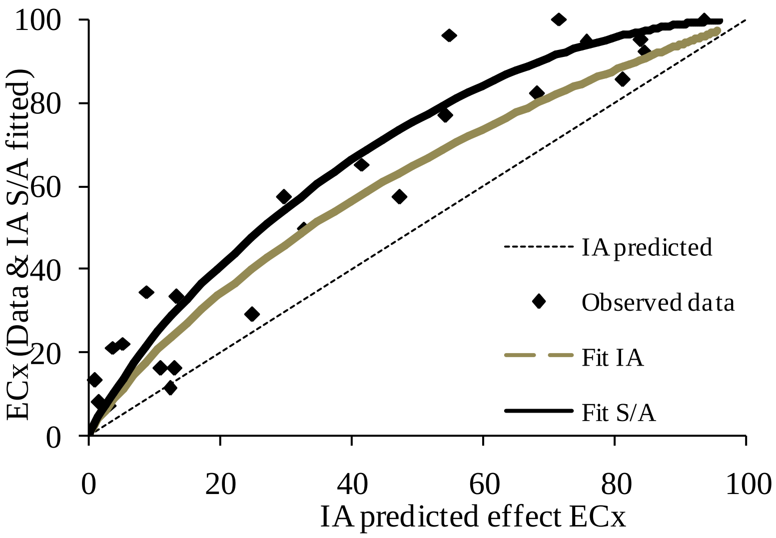

Figure 6.1: Predicted ECx values against fitted ECx values for Cd/Zn mixture in Kettering loam, expressed as total metal concentrations. Synergism was significant at *p* <0.01.

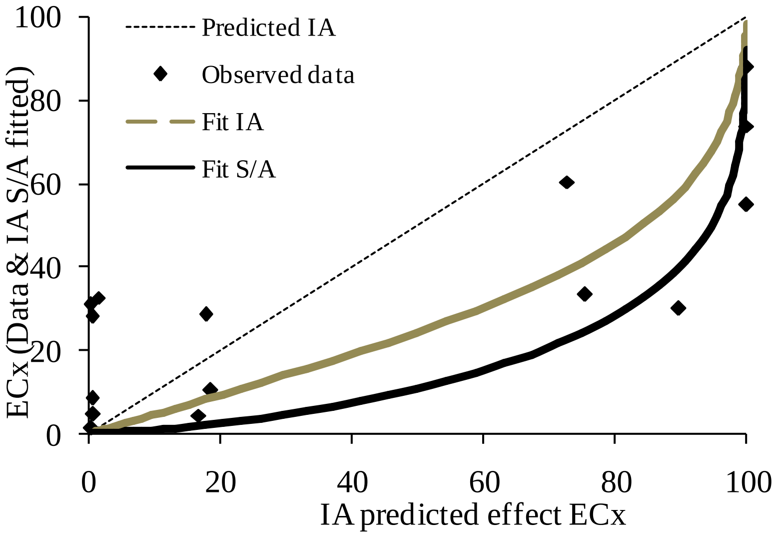

Figure 6.2: Predicted ECx values against fitted ECx values for Cd/Zn mixture in Acidic compost, expressed as total metal concentrations. Antagonism was significant at *p* <0.01.

6.6 Discussion

This study provided insight into the joint effect of cadmium and zinc mixtures on the reproduction of *Eisenia veneta*. Comparison of the experimental results for the mixtures to the effects predicted using the models CA and IA, that are commonly used for assessing mixture effects from single chemical toxicities indicate different response patterns. The level of mixture toxicity was different in the four different soils. This supports the initial expectation that, as is the case in studies with single metals (See Chapter 4), the exact nature of joint effect will be affected by the chemical and physical properties of the soil. Synergistic deviation patterns of deviation from CA and IA predictions were observed when the metal concentration was expressed as total soil metal. The exception was for the acidic compost, with high organic matter content and low pH, for which antagonistic interactions dominated (Table 6.1).

The goodness-of-fit assessed as the correlation coefficient for the IA reference model was not significantly different from that for CA. This was true when data was expressed in both metal forms. IA and CA are often found to fit equally well to mixture data (Drescher and Boedeker, 1995). In another study on the avoidance behaviour of two species by Loureiro et al. (2009) the effects of a mixture of zinc and cadmium to *Enchytraeus albidus* and *Porcellionides pruinosus,* the observed mixture effects showed no significant deviation for both species, and it was concluded the metals were acting similarly, as fits to the IA model were not consistent for both species. Both Cd and Zn have similar chemistries (Posthuma et al., 1997), and although Cd is not an essential metal to earthworms and Zn is an essential metal (Alloway, 1995), the biochemical pathways for Cd and Zn may use similar pathways due to similarities in their chemistry. This is turn may raise the possibility of interaction occurring between the two metals.

The differences found for mixture effects in soil with different properties could explain the apparent inconsistencies that exist in the literature in relation to the joint effects of Zn and Cd. Van Gestel and Hensbergen (1997) found antagonistic mixture behaviour for Cd and Zn when testing the toxicity of the mixture to the springtail *Folsomia candida*. Similarly the same antagonistic pattern was found by Khalil et al. (1996) when testing the effects of tertiary mixtures of Cd, Cu and Zn on the reproduction of the worm *Aporrectodea caliginosa*.

In a separate study, exposure to a mixture of Cd and Zn at high concentrations was found to increase cytosolic metallothioneins levels in *Eisenia fetida*. Metallothioneins are a family of proteins that is able to bind to metals in the body, an increase in its concentration would allow worms to regulate body Zn concentrations and also to limit Cd toxicity (Demuynck et al., 2007). Possible implications would be that observed toxicity of Cd/Zn mixtures is lower than the expected toxicity. Expressing the metal form as free metal ion does show antagonistic interactions in the soils Kettering loam, Thetford soil and Acidic compost. The same interaction patterns are not observed when the metal dose is expressed in the total metal form. The free metal ion would be the species interacting with the metallothionein and therefore it is not unexpected that antagonism is only observed with the free metal ion if an increase in metallothionein concentration is responsible for the antagonistic effect.

In the case of the Cu and Zn mixture discussed in the previous section (see Chapter 5), remodelling of the effect data on the basis of metal species for the two metals indicated additivy based on assessment using metal speciation (Table 5.2). For the Cd and Zn mixture tests, only in the case of the Neutral compost was the deviation seen for models based on total metal absent when modelled using the free metal ion. In two cases, Kettering loam and Thetford soil different patterns of mixture deviation were found when modelled based on total or free metal ion concentrations. Synergistic deviation was evident when the metal was expressed in the total metal form and antagonistic effects when expressed as the free metal ion. Both the soils have lower organic matter contents than the other two compost soils studied suggesting that interactions with organic matter are important in determining this shift in overall response. In the acid compost soils with high organic matter, the deviation patterns observed were similar for models based on both soil metal pools.

As outlined in the acidic compost antagonism was observed when the metal concentration was expressed as both total and free metal ion concentrations while in the neutral compost synergism was observed when the metal form was expressed as total metal (Table 6.1). This may imply that at high organic matter content, which closely matches the natural environment of *Eisenia veneta*- a compost worm, the soil pH plays a larger part in determining the mixture patterns observed for the Cd/Zn metal mixture then organic matter. The mixture patterns observed in the high organic matter soil will be due to the change in pH between the neutral and acidic compost as the organic matter content might not be influencing the earthworm response to metals in the mixture. On the other hand, the $pH_{solution}$ difference between the two soils with high organic matter content is much larger at ~2.4 pH units than the difference in $pH_{solution}$ between the soils with lower organic matter content which equals ~1.0 pH unit. Given this information metal speciation may play as vital a role in determining the effects of mixture deviation patterns as does the biochemical interaction of the metals within earthworms.

6.7 Conclusion

Cd/Zn mixture tested in this study showed reasonable fits to the reference models CA and IA while including deviation parameters, but also that the fits varied among soils and when describing the metal in different forms. Deviation patterns observed were generally synergistic when the total metal concentrations were used and mostly antagonistic where deviations from additivity were found for free metal ion concentrations. For total metal concentrations the Acidic compost soil resulted in the only antagonistic deviation in comparison to synergistic patterns for all other soils. Describing the mixture pattern data in the free metal ion form in neutral compost showed no deviation from additivity while antagonism was expressed in the other soils.

The metal mixture shows interactions when expressed as both the total and the free ion concentrations and therefore may suggest the behaviour observed is due to both in the soil affecting the speciation and also a biochemical interaction arising because two metals may share a common pathway.

Chapter Seven

On the application of DGT to describing the toxicity of Cadmium, Copper and Zinc to *Eisenia veneta*

7.1 Introduction and background

Improving our understanding of the effects of heavy metals on terrestrial organisms is important for maintaining the health and fertility of soils. In the terrestrial environment chemical speciation of heavy metals is controlled by the physiochemical properties of soil. These properties influence the biological availability and in turn the toxicity of heavy metals in soils (Meers et al., 2005; Vijver et al., 2007; Degryse et al., 2009). In order to assess the hazards resulting from heavy metal contamination in soil, it is important to be able to use an analytical technique that is able to represent the amount of metal available to cause toxic effects to soil organisms. In contaminated soils, organisms are often exposed to a mixture of contaminants, and as a result in recent years there has been a drive to include the joint effects of chemicals into toxicity studies in order to improve our understanding of heavy metal toxicity (Weltje, 1998; Posthuma et al., 1997; Spurgeon et al., 2003; Baas et al., 2007).

Earthworms are in intimate contact with the soil solution, and are thought to be model organisms in terms of exposure routes for other soil dwelling invertebrates (Spurgeon et al., 2006). A number of studies have suggested that the main route of uptake of metals into earthworms is through dermal exposure (Saxe et al., 2001; Lanno et al., 2004). This would imply that like plant routes, metal uptake into earthworm tissue may be dependent on diffusion. Another possible path of metal uptake into earthworms is via ingested soil particles through the gut wall where both passive and active mechanisms could be involved (Jager, 2004; Vijver et al., 2003; Becquer et al., 2005). It can be rationalised that both methods of exposure could ultimately contribute to toxic metals entering the organism; however, it is the significance of each pathway that is yet to be explained in terms of toxic effects observed at organism level.

Depending on the kinetics of the uptake (slow or fast) by earthworms, metals in soil solution may be depleted. This would induce metal desorption from the solid phase and resupply to solution. Integrating the kinetics of heavy metals into hazard assessment in contaminated soils has been restricted by the lack of straightforward procedures that respond to the rate at which metals can be released from the solid phase and be available to organisms (Zhang et al., 2001). The recently developed dynamic technique of DGT (diffusive gradient in thin film) may offer a way of assessing availability and toxicity of metals to organisms in soils. The DGT method relies on a layer of Chelex resin which acts as a binding layer where metals are accumulated during deployment of DGT in a water, sediment or soil (Zhang et al., 1998). Metal chelating resins have previously been used directly in soil (Skogley and Dobermann, 1996; Lee et al., 1996), DGT further inserts a diffusion layer at the chelating resin and soil interface which limits the uptake of metals to the resin layer and permits the calculation of metal concentrations from measured fluxes using Fick's law of diffusion (Zhang et al., 2001).

The DGT technique is therefore based on kinetic principles as opposed to equilibrium principles (Davidson and Zhang, 1994). Further development of DGT theory and its application to soils has led to the concept and calculation of the effective concentrations, C_E, which represents the concentration of metal that is available from both the solid phase labile pool and the soil solution (Zhang et al., 2004).

Diffusive gradients in thin films (DGT) has been suggested as a useful tool for measuring the bioavailable fraction of metal in soil (Zhang et al., 2001). In the case where diffusion is the main transport mechanism, uptake of metal from soil solution will locally lower the metal concentration in the immediate surrounding environment. This is known to occur in the plant rhizosphere and is mimicked by DGT (Zhang et al., 2004). DGT can then measure the supply of the metal from the soil environment. The flux of metal available for measurement by DGT is influenced by the overall concentration in soil solution, the available metal in the solid phase and the kinetics controlling the fractionation between the solid and solution compartments in the soil (Harper et al., 1997; Zhang et al., 1998).

Considering the exchange kinetics of trace metals between the solid and solution phase is crucial when trying to estimate their bioavailability in soil (Lehto et al, 2008; Ruello et al., 2008). With the focus of assessing bioavailability of metals, DGT is a technique that can be used to interpret how soil properties influence the speciation and toxicity of metals in tests conducted with single metals and mixtures.

7.2 Study outline

In order to gauge the suitability of DGT effective concentrations as a measure of biologically available metal, as single metals and in mixtures, to the compost worm species *Eisenia veneta* a series of adult earthworm reproduction tests were performed. The effects on earthworm reproduction were observed in four physiochemically different soils dosed to a range of cadmium, copper and zinc applied singly and in mixtures (Cd/Zn and Cu/Zn). The reproduction rates were then expressed as a function of the DGT effective concentrations of the metals in the soil.

If DGT is able to mimic earthworm metal uptake then describing the toxic effects of the metals (in mixtures and singly) on earthworm reproduction using C_E will be possible in all soil forms.

7.3 Methods

The adult earthworm reproduction experiments, chemical analysis and data preparation were performed in accordance with the methods outlines in Chapter 2. In addition, the following procedures were followed to support the aims of this chapter.

7.3.1 DGT soil deployment

DGT cylindrical devices were obtained from DGT Research Limited (Lancaster, UK). The DGT devices comprise of a plastic base and cap, a layer of Chelex resin embedded in a gel (resin layer), a layer of diffusive gel and a protective outer filter membrane. At the end of the experiment (i.e. after earthworm removal) test soils were wetted to 100% of the water holding capacity, mixed thoroughly and left to equilibrate for 24 hours (Fig 2.3). DGT devices were then deployed following the procedure described in Zhang et al., 2004. Deployment times for all soils and metal doses were between 20-22 hours (recorded more precisely for each deployment), with the exception of the two highest Zn additions, and three highest mixture doses in experiments (2-6), where the deployment time was 8 hours in order to avoid saturation of the resin gel. Deployment temperature was also recorded with an average of 17.5 ± 1.4^{o}C. On retrieval of the DGT devices, the resin layer was removed and stored in test tubes containing 1 ml of 1M ‘Analar’ grade nitric acid (VWR, UK). After a minimum of 24 hours the concentrations of the three metals Cu, Cd and Zn in the acid solution were measured using a Thermo X series ICP-MS, after appropriate dilution.

7.3.2 Calculating DGT effective concentrations

DGT data interpretation was carried following the procedure outlined in Zhang et al., (2004) to find the DGT effective concentrations (C_E). The amount of metal accumulated on the resin gel was calculated using the equation 22

$$M = C\,(V_{acid} + V_{gel})/\,f_e \qquad (22)$$

C is the concentration of metal in the samples measure by ICP-MS, V_{gel} is the volume of the resin gel (V_{gel} = 0.15 ml), V_{acid} is the volume of the acid used to store the gels (V_{acid} = 1 ml) and f_e is the elution factor (f_e = 0.8). Using M, $\mathrm{C_{DGT}}$ can be calculated. $\mathrm{C_{DGT}}$ is the ‘time-averaged’ concentration of metal at the soil/device interface in the soil (23).

$$C_{DGT} = M\Delta g/\,(DAt) \qquad (23)$$

Δg is the thickness of the filter membrane (0.14 mm) and the diffusion gel layer (0.8 mm) (Δg = 0.94 mm), D is the diffusion coefficient of the metal in the diffusion gel (at a specific temperature), as calculated in a previous study by Zhang and Davison (1999). A is the surface area of the DGT device sampling window and t is the deployment time in seconds.

In order to calculate effective concentrations (C_E), the following equation 24 was used:

$$C_E = C_{DGT} / R_{diff} \qquad (24)$$

R_{diff} is the ratio of the theoretical metal concentration at the DGT surface not taking into account supply from the solid phase to the soil solution concentration. R_{diff} was calculated using the freely available DGT induced fluxes in soils and sediments model in two dimensions (2D DIFS) (Sochaczewski et al., 2007). The R_{diff} values used for the purposes of this study were within the range (0.05 – 0.1). The 2D DIFS model is used to calculate R_{diff} by inputting the deployment time, diffusion layer thickness, porosity, diffusion coefficients, assuming distribution coefficient (Kd) is infinitly small and the response time (Tc) is infantly larger (Degryse et al., 2009).

7.4 Results

The earthworm reproduction rates were fitted to dose response curves as a function of C_E concentrations calculated for each soil. The single metal dose response curves were plotted for each metal in the individual soils, and also for all soils collectively. The EC_{50} and EC_{10} values for the single metals in the separate soils are listed in Table 7.1. The order of toxicity of the metals with regards to the soils was Neutral compost < Kettering loam < Thetford soil < Acidic compost from least toxic to most toxic for cadmium and zinc, and Kettering loam < Acidic compost < Neutral compost < Thetford soil for copper.

Table 7.1: EC_{50} and EC_{10} values of Cd, Cu and Zn expressed C_E (ppb), in four different soils. The range values correspond to the 95% confidence intervals, and were calculated by the method of bootstrapping.

		Kettering loam	**Neutral compost**	**Thetford soil**	**Acidic compost**
Cadmium	EC_{50}	**363**	**865**	**341**	**170**
		(243 - 556)	(629 - 1204)	(243 - 523)	(118 - 238)
	EC_{10}	50	163	87	45
		(22 - 95)	(63 - 389)	(42 - 158)	(19 - 97)
Copper	EC_{50}	**2570**	**532**	**468**	**869**
		(1720 - 5860)	(354 - 880)	(250 - 870)	(609 - 1340)
	EC_{10}	312	41	33	682
		(32 - 971)	(8 - 103)	(13 - 79)	(447 - 1062)
Zinc	EC_{50}	**28400**	**43800**	**12900**	**6900**
		(18000 - 40600)	(21400 - 109000)	(8670 - 17900)	(6320 - 7500)
	EC_{10}	2330	1220	1650	5070
		(475 - 6810)	(243 - 26100)	(634 - 4730)	(4200 - 5900)

Figure 7.1 shows the dose response relationships for each of the three metals in the four soils jointly. The order of toxicity is reflected in the order of EC_{50} values. Zinc is the least toxic of the three metals, followed by copper and finally cadmium is the most toxic. The r^2 values reveal that the correlations for cadmium and zinc (r^2 = 0.82 and 0.72 respectively) explain more of the data (and variation in the data) than for copper (r^2 = 0.48). Metal EC_{50} values generally increased with increasing $pH_{solution}$ values (Fig 7.1). Metal toxicity decreased in less acidic soils. This is similar to the trend observed with total metal EC_{50} values, but contrary to the relationship between free metal ion EC_{50} values and $pH_{solution}$ (Chapter 4).

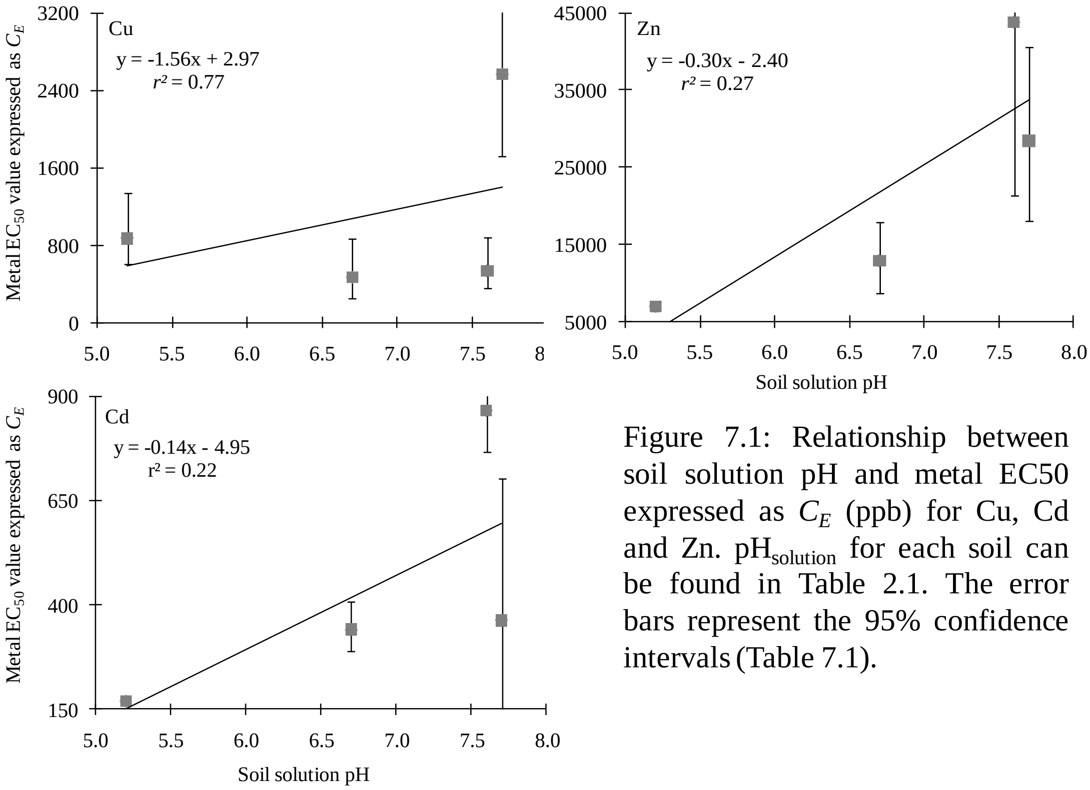

Figure 7.1: Relationship between soil solution pH and metal EC50 expressed as C_E (ppb) for Cu, Cd and Zn. $pH_{solution}$ for each soil can be found in Table 2.1. The error bars represent the 95% confidence intervals (Table 7.1).

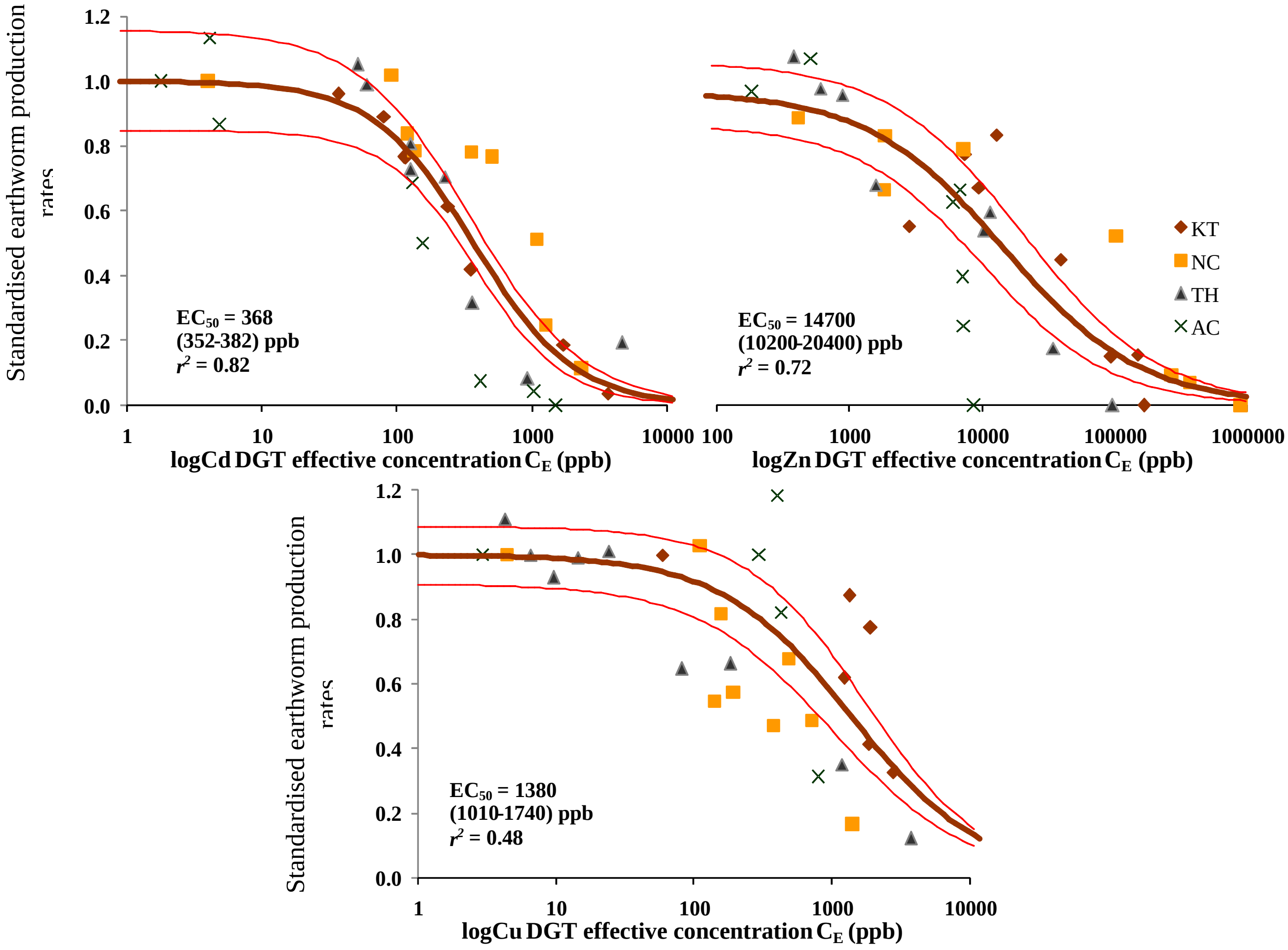

Figure 7.2: Fitted dose response curves of cocoon production for Cd, Cu and Zn. Each graph represents the log-logistic relationship between the cocoon production rates from all four soils against the metal dose expressed as C_E. EC_{50} values and r^2 values correspond to the optimized log-logistic model fits of the cocoon production data against C_E. Range values and upper and lower confidence interval lines correspond to the 95% confidence intervals. Individual points on the graphs represent the average cocoon production rate for the particular dose.

Tables 7.2 and 7.3 show the parameters relating to the mixture model fitting for observed earthworm responses and relevant deviation fits. Significant model fits were possible for all datasets using the concentration addition and independent action reference models. Fitting parameters and EC_{50} values were different for the different soils. Similarly deviation fits were also specific to each soil.

The results of exposing the earthworms to mixtures of copper and zinc are summarised in table 7.2. It emerged that zinc was less toxic than copper, in all soils, as was apparent with the single metal exposures (Table 7.1). The characterisation of the mixture effects differed only in the Kettering loam from the other soils. No deviation from additivity was detected in the Neutral compost, Thetford soil or the Acidic compost. The fitting parameter *a* for reference models, concentration addition and independent action for Cu/Zn mixtures in Kettering loam is negative indicating synergism.

Table 7.2: Summary of mixture modelling outcomes for Cu/Zn combinations expressed as C_E (ppb). After fitting the observed data using the reference models CA and IA, the *p* value relates to the significance of the observed data fit to the reference models (for CA and IA) and in the case of deviation patterns the significance of the improved fit having included the extra parameter. S/A (absolute synergism or antagonism), β is the gradient and r^2 refers to the fit of the observed data. *a* is the extra fitting parameters, and the maximum reproduction rate was set to 1. The explanation relates to the best descriptive model.

Soil	Model	Cu- β	Cu- EC$_{50}$	Zn- β	Zn- EC$_{50}$	*p*value	r^2	a	Deviation pattern from reference model
Kettering loam	**CA**	3.2	2016	1.0	15754	$9.95x10^{-11}$	0.79		Significant synergism
	S/A	1.5	2490	1.5	31830	$2.72x10^{-2}$	0.81	-2.8	
	IA	1.3	2185	1.1	16820	$1.05x10^{-10}$	0.79		Significant synergism
	S/A	1.6	2428	1.5	31840	$5.22x10^{-3}$	0.83	-3.3	
Neutral compost	**CA**	1.0	600	0.7	33774	$1.83x10^{-10}$	0.75		No deviation
	IA	0.8	760	0.8	48515	$1.12x10^{-10}$	0.76		No deviation
Thetford soil	**CA**	0.8	1114	4.8	11765	$5.06x10^{-13}$	0.82		No deviation
	IA	0.8	926	2.7	11879	$3.76x10^{-13}$	0.82		No deviation
Acidic compost	**CA**	8.6	759	2.7	6250	$2.16x10^{-8}$	0.68		No deviation
	IA	5.6	685	2.2	5433	$2.73x10^{-8}$	0.68		No deviation

Table 7.3 shows the results of analysing the effect of cadmium and zinc on earthworm reproduction. The EC_{50} values indicate cadmium was more toxic than zinc in all soils. In the case of neutral compost, there was no deviation found from additivity. There were dose ratio effects found in Kettering loam and Thetford soil. This implies that the synergistic or antagonistic deviation observed is influenced by the relative amount of one of the two metals in the mixture. In Kettering loam, the toxicity of the mixture is dependent on the relative amount of cadmium in the mixture. Cadmium dominance in the mixture will cause an increase in toxicity and vice versa. For Thetford soil, mixture toxicity is influenced by the amount of zinc in the mixture. There is an increase in toxicity when zinc dominated the mixture. Antagonism was observed in the Acidic compost. Absolute antagonism was detected in relation to the independent action model; however in the concentration addition model, deviation was dependant on the dose level. Antagonism was identified at low dose levels and synergism at high dose levels, the change occurring at levels above the EC_{50} value.

Table 7.3: Summary of mixture modelling outcomes for Cd/Zn combinations expressed as C_E (ppb). After fitting the observed data using the reference models CA and IA, the *p* value relates to the significance of the observed data fit to the reference models (for CA and IA) and in the case of deviation patterns the significance of the improved fit having included the extra parameter. S/A (absolute synergism or antagonism), DR (dose ratio) and DL (dose level), β is the gradient and r^2 refers to the fit of the observed data. *a* and *b* are the extra fitting parameters, and the maximum reproduction rate was set to 1. The explanation relates to the best descriptive model. Values highlighted in red were set during fitting.

Soil	**Model**	**Cd- β**	**Cd- EC$_{50}$**	**Zn- β**	**Zn- EC$_{50}$**	***pvalue***	**r^2**	**a**	**b**	**Deviation pattern from reference model**
Kettering loam	**CA**	0.9	174	1.5	21411	8.84x10^{-6}	0.54			Toxicity mainly caused by Cd
	S/A	5.3	377	0.5	7022	3.59x10^{-5}	0.70	-5.11		
	DR	3.6	394	0.9	14070	1.87x10^{-5}	0.81	10.99	-26.53	
	IA	1.5	262	0.4	21223	2.05x10^{-6}	0.58			Toxicity mainly caused by Cd
	S/A	2.2	334	0.6	7021	4.99x10^{-3}	0.65	-2.48		
	DR	4.1	357	5.8	14345	1.94x10^{-8}	0.84	26.30	-59.76	
Neutral compost	**CA**	1.1	603	2.5	48266	1.89x10^{-11}	0.78			No deviation
	IA	1.1	638	1.2	45026	1.43x10^{-11}	0.79			No deviation
Thetford soil	**CA**	0.8	1451	1.8	17914	9.59x10^{-9}	0.69			Toxicity mainly caused by Zn
	S/A	1.1	576	1.5	7185	3.05x10^{-4}	0.77	5.52		
	DR	1.2	447	2.2	12220	1.18x10^{-3}	0.83	-6.71	19.84	
	IA	0.9	1309	2.6	18377	6.14x10^{-9}	0.70			Toxicity mainly caused by Zn
	S/A	0.8	533	3.2	10369	1.64x10^{-5}	0.81	4.82		
	DR	1.0	368	2.4	12130	2.75x10^{-3}	0.85	-6.37	18.52	
Acidic compost	**CA**	0.4	1381	5.2	7336	1.31x10^{-2}	0.29			Ant (low DL), Syn (High DL), change above EC$_{50}$
	S/A	0.6	528	12.6	6868	5.70x10^{-3}	0.41	6.76		
	DL	0.4	976	10.9	6825	3.55x10^{-2}	0.47	28.82	0.40	
	IA	0.7	1381	12.3	7146	2.07x10^{-3}	0.37			Significant antagonism
	S/A	0.4	521	6.3	7016	5.01x10^{-3}	0.48	5.45		

7.5 Discussion

Bioavailable metal has been described as the chemical fraction of metal that is available to be taken up by the organism and react at the site of action to cause a toxic effect (Meers et al., 2005). A unified definition of the specifics of bioavailability and of the methods of measuring the bioavailable fraction of metal in soil does not yet exist and consequently there is intensive discuss around the application of the concept within the ecotoxicological literature. A study by Van Straalen et al. (2005) suggested that in order to be able to use the concept of bioavailability in a predictive sense, the rate of uptake of metals into the organism needs to be estimated. They studied the accumulation of zinc in the isopod *Porcellio scaber* and it was found that sublethal toxicity may depend on the rate of metal uptake into the organism as opposed to the total body burden concentration. The implication of this work was that when considering metal bioavailability it is important to consider fluxes as well as total body burdens. As for isopods, the kinetics of heavy metals in earthworms has been found to be poorly correlated with total body burdens (Van Gestel et al., 1993; Lanno et al., 2004). In earthworms it is well known that metals can be detoxified and excreted in a number of different ways. These include excretion via the nephridia (kidney like organ), or storage in granules in the chloragogenus tissue (Andersen and Laursen, 1982; Vivjer et al., 2004). The latter can be of a number of different types, including sulphur rich granules that are well known to bind cadmium and phosphate rich granules that incorporate lead.

Because the DGT method measures the concentration of metal influenced by fluxes that are available to biota, DGT measurements may represent the rate of uptake to the earthworm if the uptake is purely dependant on diffusion through the skin or through the gut wall. Earthworms are able o detoxify metals to a certain extent within their tissue and this is not mimicked by DGT. Also earthworms are mobile in soil, whereas plant roots and the DGT devices are not. This may in turn imply that the rate of porewater metal reduction and replenishment from the solid phase due to metal uptake may differ between DGT and an earthworm.

In a study of the accumulation of zinc it was found that DGT effective concentrations (C_E) correlated well with zinc accumulation in grass and lettuce, but not so well with the accumulation of this metal in the hard bodied soil dwelling isopod *Oniscus asellus* (Koster et al., 2005) for which the rates of uptake (e.g. via preferential litter feeding) may not be the same as for the plants (diffusion from soil solution). It is important to note that the toxicity of metals to earthworms has not been tested as a function of DGT effective concentrations previously. The only available data relates to other species, as mentioned earlier. Correlations between the effects on reproduction of earthworms in each soil and C_E indicate that the dynamic factors affecting uptake to DGT in the studied soils may also affect uptake by earthworms.

These conclusions must be made cautiously as they do not conclusively show that diffusion is the main control of metal uptake, or of exposure to the earthworms to each metal. Expressing the observed single metal effects on reproduction from all four soils as a function of total soil metal (Fig 4.1; Chapter 4) provided r^2 values of 0.88, 0.61 and 0.74 for cadmium, copper and zinc respectively. When the effects of each metal on cocoon production were fitted against $C_{E,}$ similar values of

r^2 were obtained to those estimated based on total metal. In contrast, fits based on the estimated free metal ion concentration resulted in fits with lower explanatory power than for either total metal or C_E, with r^2 values of 0.80, 0.32 and 0.64 for cadmium, copper and zinc respectively. There was a clear relationship between EC_{50} values of Cd and Zn, expressed as C_E and the pH of the soil solution (Fig 7.1). As pH values decrease in the soil (becomes more acidic), the EC_{50} value decreases (the metal becomes more toxic). This is in contrast to the relationship observed for the free metal ion EC_{50} values (Fig 4.6).

The results are promising for the application of DGT as a measure of bioavailable metal. In view of the fact that total metal and C_E concentrations correlate well with the observed toxicity data for single metals, inclusion of the solid phase can be seen to be an important step in understanding heavy metal toxicity to earthworms.

Copper and zinc are known to be essential metals for earthworms, whereas cadmium is a non-essential metal (Alloway, 1995). Earthworms are known to regulate essential and non-essential metals differently and therefore their responses to the different categories of metals are different (Sizmur and Hodson, 2009). The pattern of toxicity in the different soils is similar for single metal cadmium and zinc when expressed as C_E, but different for copper. This may suggest that C_E values are a reflection of the chemistries of the metals in the soils and not the regulation within the earthworms. The chemical properties of cadmium and zinc in soils are similar. This is reflected in the order of the EC_{50} values observed in the single soils (Table 7.1 and Fig 7.2). The order of toxicity in the different soils is also similar to that observed for the toxicity of metals when expressed as total metal concentrations; the toxicity of metal decreases with increasing pH and organic matter content (Spurgeon and Hopkin, 1996; Chapter 4). Cadmium and zinc are present mainly in the free metal ion form at low pH, unless there is a high concentration of ligands, implying cadmium and zinc may be most available in the acidic compost. At low pH the effect of the high free metal ion concentration would be expected to be reduced by the competition with protons at the site of uptake, leading to a protective effect on the organism. However, as the metals are more toxic in the Acidic compost (with the lowest pH) it can be concluded that the high free metal ion concentration is not completely counteracted by the protective effect of the protons. A large fraction of cadmium and zinc will be complexed by dissolve organic ligands at high pH values > 6.5 (Nolan et al., 2003; Meers et al., 2005) suggesting they are less available for uptake. The chemistry of Cd and Zn is similar, and different from that of Cu.

There appears to be no distinct pattern in the order of toxicity for copper with regards to soil pH and total organic content (Table 7.1). Copper is least toxic in Kettering loam which may be a consequence of metal being bound more strongly to the large clay content in the soil in comparison to the other soils (Table 2.1). Copper in solution is complexed strongly by organic matter (Nolan et al., 2003) and this makes it less DGT labile (Yapici et al., 2008). Neutral compost and Acidic compost contain the highest organic matter contents which may explain their order of toxicity in between Thetford soil and Kettering loam. Cu was most toxic in Thetford soil which is a low organic matter soil, with no clay content meaning copper may be least bound and therefore most available to cause a toxic effect.

7.6 Using DGT to investigate the basis of synergism and antagonism in mixtures

Describing mixture effects of cadmium/zinc (Table 7.3) and copper/zinc (Table 7.2) on *Eisenia veneta* as a function of C_E values revealed different behaviour patterns and deviations from additivity. Correlation between the reference models and observed mixture results were significant. As is the case with the single metal toxicity, if C_E is reflective of the uptake into the organism, then the significant relationship between C_E and mixture effect may be representative of mixture interactions at the site of uptake. This would suggest the mixture patterns or additivity observed represent interaction between the metals at the site of uptake and not in the soil solution.

The site of uptake of chemicals may provide a likely site of interaction between the mixture components (Spurgeon et al., 2010). The deviation patterns found were specific to each mixture and each soil. Copper/zinc mixtures were additive in all soils tested with the exception of Kettering loam. Significant synergism in Kettering loam was also found for *Eisenia veneta* when expressing the metal dose as total soil metal (Chapter 5). Kettering loam soil contains the lowest organic matter content of the four soils (Table 2.1). For the Cu/Zn mixture the low organic matter content appears to cause a deviation from additivity. Although Cu and Zn are both essential metals, they have different chemistries. They can be expected to have different systems for handling the metals within the organisms and so may not react at the same receptors. This would mean their mixtures should be additive.

Other studies that have reported instances of synergism between Cu and Zn include a study by Baas et al. (2007) where six binary mixtures of Cd, Cu, Pb and Zn were tested for their lethal effects on *Folsomia candida* in loamy sand. Synergism was observed for the copper/cadmium mixture. An aquatic mixture study found synergism when testing a mixture of metals (cadmium and copper) and pesticides on the survival and enzyme activity of marine crustacean *Tigriopus brevicornis* (Forget et al., 1999). In a study on the lethal effects of copper/zinc mixtures on *Tympanotonus fuscatus*, synergism was observed when the mixtures were in the ratios of 1:1, 2:3 and 1:4 (Otitoloju et al., 2002).

In the cadmium/zinc mixture, the observed patterns of mixture toxicity were also soil specific. More complex deviation patterns were observed in the form of dose level (Acidic compost) and dose ratio (Kettering loam and Thetford soil) dependencies. Kettering loam and Thetford soil are the two low organic matter media. Dose ratio deviation was observed in both these soils; however the preference as to the dominant metal in the mixture is different. Cadmium is the dominant metal in Kettering loam where the pH is higher, and in the Thetford soil, with lower pH, the toxicity of the mixture is dependent on the ratio with relation to zinc. Dose ratio deviation has been reported in a study on *Caenorhabditis elegans* using the mixture cadmium/copper (Jonker et al., 2004).

There was no deviation from additivity observed in the Neutral compost for the cadmium/zinc mixture. In the Acidic compost antagonism was illustrated in both reference models. Absolute antagonism was seen using the IA model. Using the

CA model a more complex pattern was found: antagonism at low doses and synergism at high doses. This is consistent with the theory that the CA model represents a worst case scenario for mixture effects (Lock and Janssen, 2002), as it describes patterns of synergism which are not found by the IA model. Antagonistic patterns have previously been observed in the growth patterns of the collembolan *Folsomia candida* in response to a cadmium/zinc mixture (Van Gestel and Hensbergen, 1997). Dose level dependency has also been reported in the literature in a study on the effects of the mixture carbendazim/copper on *Caenorhabditis elegans* (Jonker et al., 2004).

The mixture patterns observed for cadmium/zinc on *Eisenia veneta* when expressing the metal as total soil metal and calculated free metal ion concentrations are different to those observed for C_E. The only similarities are in the Acidic compost where antagonism was also observed in total metal form, and in Kettering loam where antagonism was observed but no dose ratio deviation patterns were in the free metal ion form (Chapter 6). Interaction between the metals is found when expressing the mixture doses as C_E.

The similarity in the chemistries between Cd and Zn will results in the metals binding to the same locations. This is observed in the enzyme carbonic anhydrase in marine diatoms (Xu et al., 2008). This ultimately means that the two metals will interact toxicokinetically (during uptake) and toxicodynamically (during absorption, metabolism, distribution and excretion). In comparison to the previous patterns observed for mixtures when metal is expressed in total or free metal ion form, where Zn was behaving differently in both mixtures (Chapters 5 and 6), antagonism is also evident when the metal dose is expressed as C_E concentrations.

With increased understanding of the uptake of metals by DGT, further study into the mechanisms of mixtures is possible. DGT is therefore potentially, an important diagnostic tool when investigating mixture toxicity.

7.7 Conclusions

Effective concentrations (C_E) measured by DGT correlated well with observed effects on earthworm reproduction rates due to exposure to heavy metals. When expressed as a function of C_E, single metal exposures as well as mixture toxicity were well explained. As a simple method of analysis DGT offers a convenient means of studying soil contamination, and affords an understanding of the metal kinetics in soil. Patterns of toxicity observed using C_E values cannot be directly compared to conventional means of dose expression (i.e. total metal concentrations and free ion concentrations) as DGT provides a dynamic measurement of soil metal concentration. Until the mechanisms of toxicity in earthworms are better understood, results from this study and other similar studies, can only provide a means of describing observed behavior that affects the bioavailability of metals for uptake. Studies that investigate metal toxicokinetics and toxicodynamics are needed to fully interpret mechanisms associated with mixture effects.

Chapter Eight

Further discussion and concluding remarks

In order to effectively predict the effects of toxicants on organisms in the natural environment a comprehensive understanding of the mechanisms of toxicity is needed. The complexity of the soil environment presents a barrier of identifying where it is best to focus the research in order to effectively bridge the gaps in understanding between chemical exposure and resulting effect. *This study aimed to assess biological responses of a reference species Eisenia veneta to the metals Cd, Cu and Zn and mixtures of Cu/Zn and Cd/Zn in a range of widely differing soils. By combining existing mixture models with bioavailability concepts it has been possible to investigate whether a unified concept can be generated to describe the effects of metals on an organism in different soils that integrates these two differing concepts.*

The results of this study improve the knowledge and understanding of the effects of the heavy metals (Cu, Cd and Zn) and their mixtures.

8.1 Physiochemical soil properties and effects on metal toxicity

Experimental design included the use of four different soils and different metal forms. The effect of soil chemistry on metal toxicity would in turn be incorporated into the study of the metal toxicity on the reproduction rate of *Eisenia veneta*.

Further to the discussions on single metal toxicity of Cd, Cu and Zn (Chapter 4) it was shown that relationships between soil properties and specific metal forms could be drawn. Namely the relationship between pH and free metal ion EC_{50} and well as the fit of regression models as a function of total metal dose using parameters for % clay content, % loss on ignition and soil solution pH.

With regards to mixture behaviour it is generally more difficult to draw general conclusions due to the specificity of deviation patterns in terms of the metal mixture and soil being tested. Synergism was found for both Cu/Zn and Cd/Zn mixtures when the metal was expressed in the total metal form in Kettering loam and Thetford soils. Mixture deviation patterns were otherwise different in other soils and metal forms.

While an integrated interpretation of mixture behaviour is not possible (Chapters 5 and 6) the deviation patterns clearly show that the use of single metal toxicity to predict mixture behaviour is not an accurate method of assessing mixture toxicity.

The understanding of mixture toxicity must extend from the ‘bulk’ environmental conditions to include the amount of metal available to the organisms, the effects of the soil on the rate of supply of the metals to the organism and the methods of uptake and site of action within the organism. A comprehensive review of these conditions would be both complex and would require a multi-disciplinary approach to invertebrate ecotoxicology, but would allow the differences in toxicity between mixtures to be explained on a smaller and ultimately more sensitive scale.

8.2 Metal properties affecting toxicity

In both mixtures Zn was the common metal and in turn differences in the toxicity of Zn can be used to investigate potential interaction between the metals in the two mixtures. An example of this would be to compare the EC_{50} values of Zn in all four soils for the two mixtures (as determined by IA and CA best fits).

Table 8.1 Comparison of Zn EC_{50} values in two mixtures expressed as total and free ion concentrations. Values in () refer to total metal concentrations expressed as mg/kg dry weight; the other value refers to free metal ion concentration expressed as μM.

	Cu/Zn mixture		**Cd/Zn mixture**	
Soil	*CA*	*IA*	*CA*	*IA*
Kettering loam	1.50 (1070)	1.59 (1280)	68.0 (985)	77.2 (983)
Neutral compost	145 (1950)	39.4 (1890)	548 (2240)	534 (2190)
Thetford soil	61.3 (1158)	57.0 (1217)	86.2 (1330)	145 (1370)
Acidic compost	338 (2720)	355 (3210)	31.0 (2320)	33.7 (2440)

There were clear differences between the behaviour of zinc in the two mixtures. Although both reference models assume no interaction among the chemicals in the mixture the differences in zinc behaviour indicate there may be interactions between the metals as previously discussed in Chapters 5 and 6. The chemistries of Cd and Zn metals are thought to be similar so, as previously discussed, this may be a factor in the interpretation of mixture behaviour. Cu and Zn are both essential metals and in turn have a specific biochemical pathway which implies that there is less potential for interaction between the metals.

In terms of free metal ion concentrations the implication to earthworms is that the presence of Cu increases the toxicity of Zn in three of the four soils. Only in the Acidic compost is Zn less toxic with the presence of Cu that in the Cd/Zn mixture (Table 8.1).

8.3 Metal form and the role of speciation in explaining metal toxicity

Diffusive gradients in thin films (DGT) devices were used as a measure of possibly predicting the concentration of bioavailable metal. As DGT is able to mimic the rate of metal uptake by plants through diffusion the correlation between the toxicity data and the DGT effective concentrations C_E show that diffusion may be an important process determining the uptake rates of Cd, Cu and Zn into *Eisenia veneta*.

All the metal forms used in this study revealed reasonable correlation between toxicity data and metal concentrations. The metal forms used are innately interrelated and therefore it is not unexpected that the toxicity data correlate with the metal concentrations in all forms. The specific patterns of interaction observed for each metal form does provide further insight into the details governing the toxicity of the metals in each of the soils. As Cu, Cd and Zn have different levels of toxicity in each of the soils it is not possible to state that the free metal ion, total metal or C_E is a measure of the soil metal correlating with toxicity independent of soil physiochemical properties.

8.4 Further work and future direction

- The results of this study provided a clear indication of the importance of testing a variety of soil types when studying metal toxicity both singly and in mixtures. While the four soils selected do cover a wide range of soil properties and their combination, the use of a greater number of soils varying in pH and organic matter content in toxicity studies will provide a more comprehensive overview of the relationship between metal toxicity and soil properties.

- Studies by Ireland and Fisher (1978) and the review by Beeby (1991) have reported the various interactions and non-interactions between different chemicals in various organisms. While this study was limited to two mixtures, it is clear that the components of a mixture play an important role in the overall mixture toxicity. Further work into different combinations of metals and the inclusion of lesser studied metals would provide much more insight into the components needed to build comprehensive risk assessment techniques for the field. A short study into the toxicity of mercury to *Eisenia veneta* was performed in order to assess the toxicity of inorganic mercury (Appendix 2). Such studies would provide a starting point required to successfully design mixture studies for the particular metal.

- Various analysis techniques are being developed to study the toxicity of chemicals to living organisms. An example of which is the use of metabolomics to study the sublethal effects of pesticides on *Eisenia fetida* (McKelvie et al., 2008). Others have not been applied to earthworms however may provide valuable insight into the mechanisms of toxicity in earthworms. A possible example has been outlined in Appendix 3.

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Appendices

Appendix 1: *Testing the effects of nitrate salt on the reproduction of the earthworm Eisenia veneta*

Appendix 2: *Toxicity of inorganic mercury to Eisenia veneta*

Appendix 3: *Applying 'fingerprint' profiling techniques to study the effects of toxic chemicals on earthworms*

Appendix one

Testing the effects of nitrate salt on the reproduction of the earthworm *Eisenia veneta*

A preliminary study was carried out in order to assess the absolute effects of increase in nitrate salts, as a result of the metal dosing procedure, on the rate of earthworm reproduction. A set of the test soils were dosed using 2M $CaNO_3$ to a range corresponding to that which would have been achieved as a result of metal spiking. The concentration of nitrate salt added to the soil was estimated using the volume of metal solution used (at a concentration of 1M) to dose each soil. The toxicity experiments were carried out according to the method outlined in Chapter 2.

The nitrate concentrations ranges added to each of the soils in mg/kg are as follows:

Kettering loam	0.00, 500, 1700, 5600
Neutral compost	0.00, 1200, 4200, 14000
Acidic compost	0.00, 900, 3000, 10200
Thetford soil	0.00, 500, 1700, 5600

The results of the study are outlined below. There were three doses for each soil (in triplicate). The standard response was set as the average response of the earthworms to each of the control soils.

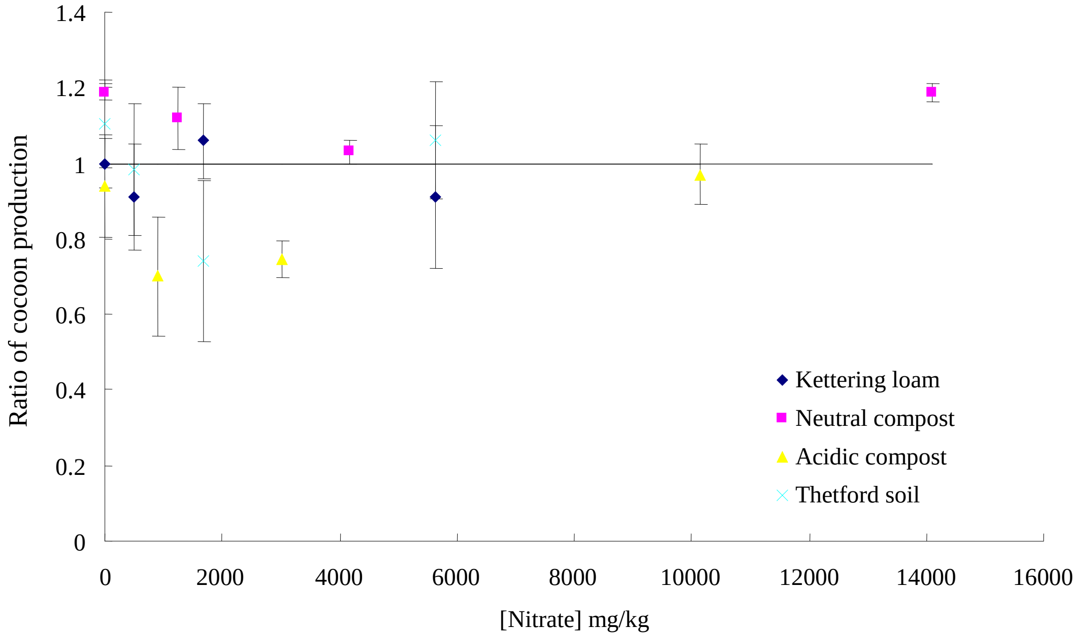

Figure two: Cocoon production rates for experiment set up to monitor the effects of increasing nominal nitrate concentrations on earthworm reproduction. The different colours represent different soils. The Y-error bars denote standard deviation for three replicates. The straight lines represent the cocoon production for the null hypothesis (there is no effect of increasing salt concentration, ie the mean cocoon production for the control response).

Statistical analysis was performed using Student's t-test. There was no significant difference between the results of the increasing salt doses (soil dosed with $CaNO_3$) and the control reproduction. The *p*-values for Kettering loam, neutral compost, acidic compost and Thetford soil were 0.37, 0.09, 0.15 and 0.16 respectively.

The significant *p* value was set at 0.05 and therefore accordingly no correction was made to the metal toxicity reproduction data.

Appendix Two

Toxicity of inorganic mercury to *Eisenia veneta*

Introduction

Elevated mercury concentrations have been observed throughout the world due to increased anthropogenic outputs over the past decades. Mercury mainly occurs naturally in the elemental state or as volatile forms (Alloway, 1995; Ure and Davidson, 1995). Anthropogenic Hg contamination of soil can be a by-product of historic mining activities, fossil fuel burning and various industrial processes. Hg is also used in chlorine production and can be found in high concentrations in sewage sludge applied to some agricultural soils (Lock and Janssen, 2001). Anthropogenic inputs of mercury (Hg) are generally in ionic forms e.g Hg (I), Hg (II) that are mobile and reactive. Hg (II) shows high acute toxicity for most biota, with a variety of damaging effects, and so poses a great risk to ecosystems (Iskandar and Kirkham, 2001). Organisms living in such contaminated field sites are therefore at risk from exposure to high levels of Hg.

Earthworms are important organisms in the terrestrial environment. Their role in the food chain and their ability to transfer a number of toxic metals, including Hg, into higher trophic levels has led to their incorporation as a key organism in ecotoxicity testing (Fischer and Koszorus, 1992; Lawrence et al., 1999; Spurgeon et al., 2003). The survival and fitness of birds and small mammals that feed on earthworms have been related to metal accumulation in the earthworms (Demuynck et al., 2007). These soil dwelling organisms are at high risk from the toxic effects of heavy metals, as they come into direct contact with the contaminated soil (Demuynck et al., 2007), allowing the metals to be readily absorbed into living tissue by diffusion (Landis and Yu, 2004). Anthropogenic emissions causing increased Hg contamination of soil have been observed in numerous sites around the world, with the concentrations varing greatly (Ure and Davidson, 1995).

Despite the highly toxic effects of Hg, relatively few studies have considered the effects of Hg on earthworms (Lock and Janssen, 2001). Lock and Janssen (2001) looked at the toxic effects of Hg on *Eisenia fetida* and concluded that the effects of soil chemistry are important when studying the toxicity of Hg to organisms. Gudbrandsen et al. (2007) tested the effects of earthworm pre-exposure on the lethal toxicity of Hg where he found a significant increase in the tolerance of earthworms to Hg after pre-exposure. Other studies included the effects of feeding behaviour on Hg accumulation in earthworms. Fischer and Koszorus (1992) studied the sublethal effects and accumulation capacities of Hg in the manure worm *Eisenia fetida*. They observed an enhancement in cocoon production at sublethal concentrations of Hg (100 mg/kg) compared to the control, and no accumulation of Hg in the earthworms. In contrast to this study, Ernst and Frey (2007) found that Hg was concentrated in the earthworms and the accumulation rates not only depended on the source oh Hg but also on the species The present study is aimed at quantifying Hg concentrations in a clayey soil causing significant lethal and sublethal effects (weight change and cocoon production) to the compost worm *Eisenia veneta,* in a different soil from the above mentioned studies and at a wider concentration range.

Materials and Methods

Soil Preparation

The soil medium used for all experimental work was a commercially bought clay loam soil (Broughton Loam, Kettering, UK) with a pH of 7.1 (soil:water 1:1) (Spurgeon et al., 2003). Soil was sieved to 2 mm and the organic matter content increased from 5% to 10% using composted bark (circumneutral pH). The water content of the soil was 2% w/w. The sieved soil was transferred to small plastic boxes (2.0 L volume) and the soil mixed with the required amount of 1 M Hg chloride (VWR, UK) solution and deionised water to give a total moisture content of 56% of the water holding capacity. Twelve Hg amendments corresponding to final concentrations of 6, 9, 13, 20, 30, 44, 66, 99, 148, 222, 333, 500 mg/kg were made. There were eight control replicates, one replicate of the amendments 6, 13, 20, 44, 66, 148, 222, 500 mg/kg nominal added and five replicates of the amendments 9, 30, 99, 333 mg/kg nominal added. The test boxes were left to equilibrate in continuous light at 17^{o}C for two weeks after dosing.

Test species

The test organism used was *Eisenia veneta* (Lumbricidae: Oligochaeta) (Michaelsen, 1889) cultured in a combination of compost and topsoil (2:1), and fed using horse manure. At the start of the experiment individual adult worms were removed from the culture. Worms with a fully formed clitellum were chosen and acclimated at a constant temperature of 17^{o}C in the culture medium for two weeks. They were kept in large plastic boxes containing culture medium and horse manure as food to ensure they were fully reproducing when introduced into the test boxes.

Toxicity testing

Experiments were principally conducted following OECD guidelines (OECD, 1984) for soil preparation and ISO (1997) with some adjustments (i.e. soil used, earthworm species). 20 g of dried manure per test box was spiked to a concentration equivalent to those of the test soil and the manure water content was set to four times the dry weight. At the start of the exposure (day 0) eight worms were washed and weighed then introduced to each of the test boxes in random order. The average individual weight of the worms was 900 mg. Sufficient manure for two weeks (5 g of dry manure per container per week) was added to the surface of the soil. The test boxes were weighed. Two weeks later (day 14) the test boxes were weighed again. De-ionised water was used to replace any water loss. Manure remaining on the surface was removed, dried in an oven at 105°C and weighed.

The test boxes were then tipped out and the surviving worms weighed and counted. The soil was then replaced into the boxes and more manure was added to the surface. The boxes were weighed. At the end of the exposure (day 28) the remaining manure was also removed, dried and weighed. The surviving worms were removed from the soil and weighed. The worms were then transferred to smaller containers and frozen at -60°C. The soil was wet sieved and the number of cocoons in each container recorded. Cocoon production was expressed as cocoons per surviving worm per week of exposure.

Data analysis

The dose-response relationships were analysed with respect to added Hg concentrations using the log-logistic model for the rate of cocoon production

$$R = R_0 \Big/ 1 + e^{\beta(\log_{10} EC_R - \log_{10} EC_{50})}$$

Where R_0 is the outcome (cocoons produced), EC_R is the concentration of Hg_{added} corresponding to that value and β is the gradient of the curve. The EC_{50} value for the rates of reproduction per worm/ week and the Probit analysis for mortality response as well as the estimated LC_{50} and LC_{10} were estimated using Genstat (Microsoft, USA).

Results and Discussion

The range of tested Hg concentrations showed effects on cocoon production, worm weight and mortality. The control mortality was below 10%. The LC_{10} and LC_{50} for mortality was 68.0 ± 15.4 (s.e) and 220 ± 18.6 (s.e) mg Hg_{added} / kg dry wt respectively (Fig. 1). At concentrations above 333 mg Hg_{added} /kg dry wt some earthworms survived however there was no observed cocoon production. The 28-day EC_{50} value for cocoon production was 71.8± 16.2 (s.e) mg Hg_{added} / kg dry wt (Fig. 2). The 28-day EC_{10} value could not be defined. The standard deviation of the EC_{10} value prevented the accurate measure of the value itself.

The results reported by this study, as well as the three previous studies demonstrate the significant difference in values of the toxic endpoints (rate of reproduction and mortality) used for the same species. This supports work done by Spurgeon et al. (1994) on the effects of Cd, Cu, Pb and Zn on *Eisenia fetida* in the OECD standard artificial soil where they showed that the no effect concentration of metal (NOEC) with respect to reproduction was an order of magnitude lower than the NOEC for mortality. There are also substantial differences between the toxic effects reported for the same species by different workers when the same test was used. For example the 28-day EC_{50} value for cocoon production in this study was 71.8± 16.2 (s.e) mg Hg_{added} / kg dry wt. For an organism of the same genus but different species (*Eisenia fetida*) Lock and Janssen (2001) reported the 21-day EC_{50} value for cocoon production was 9.16 mg Hg / kg dry wt, although this study was conducted in a different soil.

Fischer and Koszorus (1992) found that Hg had less toxic lethal and sublethal effects than arsenic and selenium. Conversely EC_{50} values for cocoon production of other frequently studied metals in a soil with pH 6.1 and an organic matter content of 5% have been reported for a similar earthworm species (*Eisenia fetida*) as 295 mg/Kg dry wt soil for cadmium, 716 mg/ kg dry wt soil for copper and 357 mg/ kg dry wt soil for zinc (Spurgeon and Hopkin, 1995). By comparison with the 21-day EC_{50} value of reproduction in *Eisenia veneta* for Hg of 72 mg/kg it appears that Hg is 5 times more toxic than Zn, 10 times more toxic than Cu and 4 times more toxic than Cd. The toxic endpoints of the different metals can only be compared directly if we speculate that the soil can complex Hg^{2+},and in turn reduce its potential to cause a toxic effect, to a similar extent to that of the other cationic divalent metals.

Different species are expected to show different sensitivities to the same concentration of toxicant. However, the considerable differences in the endpoints for the same species also reflect the role of the soil properties on the toxic nature of the metal. Therefore deriving a standard methodology to include the soil parameters that influence metal behaviour in soils (Smit et al., 1998; Lock and Janssen, 2001) may help draw together the results from different toxicity studies.

Consideration of the speciation of Hg in different types of soil is an important issue when attempting to assess the risks involved with Hg contamination, due to the different toxicity levels of organometallic (methylmercury) and inorganic (Hg^{2+}) forms (Lock and Janssen, 2001). Information on the possible production of the more toxic organometallic forms of Hg (Lock and Janssen, 2001) may greatly benefit the analysis of the reproduction/mortality data from this study.

Conclusion

The range of concentrations of Hg in the amended soil produced sufficient effects to fit complete dose-response curves. While the effects of soil properties and uptake mechanisms of Hg by organisms are still poorly understood, the findings of this paper provide further evidence to support the highly toxic nature of Hg to *Eisenia veneta*. Further studies aimed at describing observed sublethal effects on *Eisenia veneta* using the concentrations of the different species of Hg in the soil may allow the effects of speciation to be assessed.

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Figures

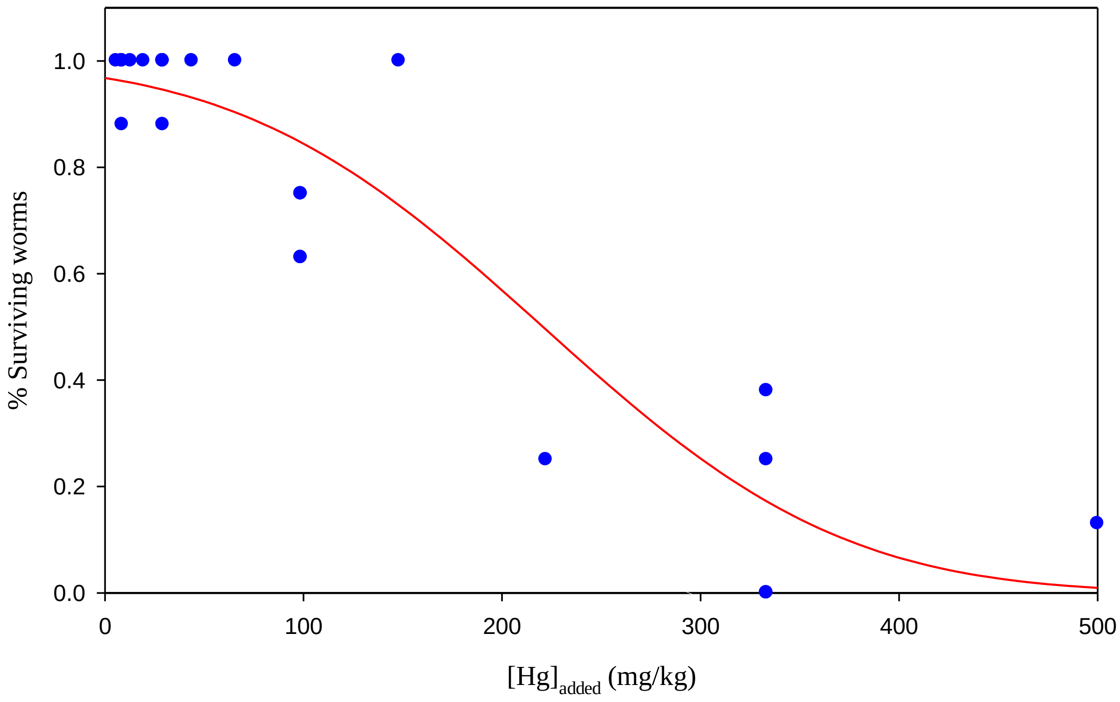

Figure 1: Effects of Hg on the survival rate of *Eisenia veneta* in Kettering loam after 28-days exposure. The points represent individual measurements.

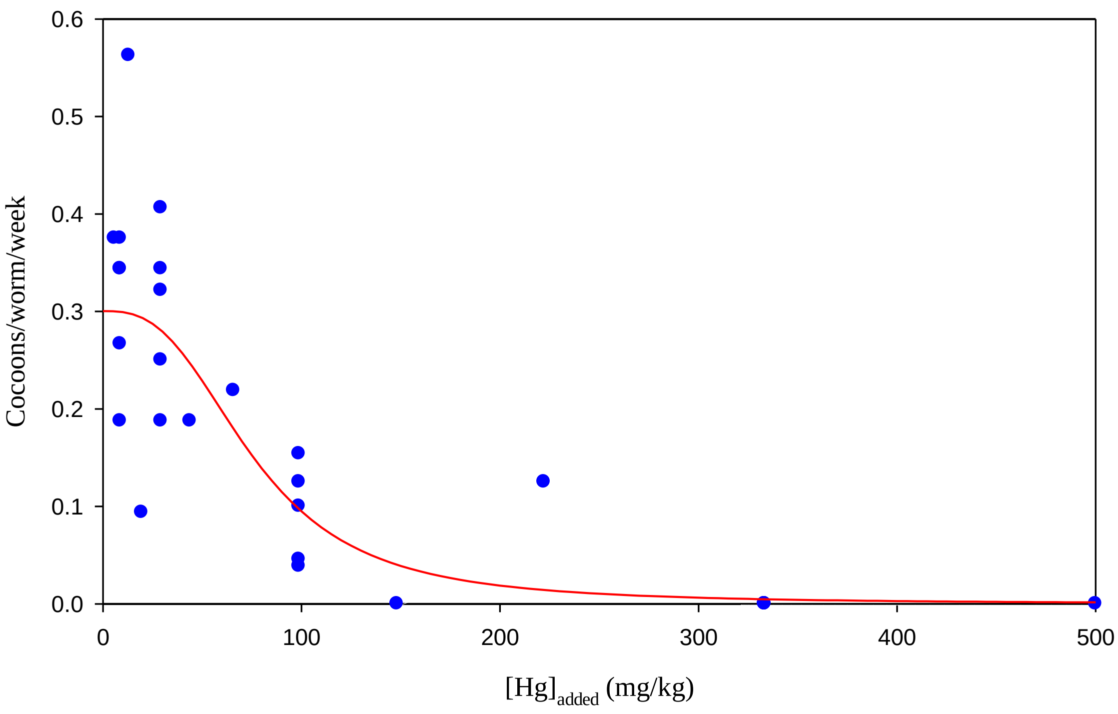

Figure 2: Effects of Hg on the reproduction rate of *Eisenia veneta* in Kettering loam following the exposure period of 28-days. The points represent individual measurements.

Table one: Summary of mercury toxicity studies performed on soil invertebrates, where study A (Lock and Janssen, 2001), study B (Fischer and Kozorus, 1992), study C (Gudbrandsen et al., 2007) and study D (Abbasi and Soni, 1983).

Species	**Soil**	**pH**	**OM%**	**Exposure duration**	**Endpoint**	**Value (mg Hg/kg soil)**	**Additional notes**	**Study**
Folsomia candida	OECD artificial soil	6.0	10	28	EC_{50} **	3.3	$HgCl_2$	A
Eisenia fetida	OECD artificial soil	6.0	10	21	EC_{50} **	9.16	$HgCl_2$	A
Enchytraeus albidus	OECD artificial soil	6.0	10	42	EC_{50} **	22.0	$HgCl_2$	A
Folsomia candida	OECD artificial soil	6.0	10	28	NOEC	1.8	$HgCl_2$	A
Folsomia candida	OECD artificial soil	6.0	10	28	LOEC	3.2	$HgCl_2$	A
Eisenia fetida	OECD artificial soil	6.0	10	21	NOEC	10.0	$HgCl_2$	A
Eisenia fetida	OECD artificial soil	6.0	10	21	LOEC	18.0	$HgCl_2$	A
Enchytraeus albidus	OECD artificial soil	6.0	10	42	NOEC	18.0	$HgCl_2$	A
Enchytraeus albidus	OECD artificial soil	6.0	10	21	LC_{50}	26.1	$HgCl_2$	A
Enchytraeus albidus	OECD artificial soil	6.0	10	42	LOEC	32	$HgCl_2$	A
Eisenia fetida	Peaty soil			56	No mortality	500	$HgCl_2$	B
Eisenia fetida	Norwegian field soil	6.2	1.6 (TCC)*	28	EC_{50} **	9.6	2 weeks pre-exposure in clean test soil	C
Eisenia fetida	Norwegian field soil	6.2	1.6 (TCC)*	28	EC_{50} **	9.2	no pre-exposure	C
Eisenia fetida	Norwegian field soil	6.2	1.6 (TCC)*	28	EC_{50} **	16.5	2 week pre-exposure in 22 mg Hg/kg	C
Octochaetus pattoni				60	LC_{50}	0.79	$HgCl_2$	D

*Total carbon content (TCC) **For reproduction

Appendix Three

Applying 'fingerprint' profiling techniques to study the effects of toxic chemicals on earthworms

Rationale

Laboratory based toxicity testing has long been employed as a vital technique for studying the effects of chemicals on soil dwelling species. While these results have been an invaluable commodity, there is a significant debate among the scientific community as to their application to real environmental situations. Standardised testing techniques also involve a degree of compromise between what is practically feasible and the number of test units needed to make the experiment statistically robust. Recently, a study from Lancaster University has exploited the infrared microspectroscopy for the characterisation of effects associated with chemicals in the tissue of predatory birds (Llabjani et al., 2009). Fingerprinting methods produce a profiles for each sample analysed which can be compared to other such profile using statistical testing. As the effects of the chemicals would be observed at a molecular level, the biological endpoint would be more sensitive and in turn provide a more protective assessment of the chemicals to the environment. Other fingerprinting methods involving the extraction of enzymes have been applied to some degree to test the effects of chemicals on earthworm species and in terrestrial environments (Song et al., 2009), work into molecular biomarkers has also been performed (Ricketts et al., 2004). Application of the IR method to a cross-sectional layer of tissue from an entire earthworm exposed to a specific chemical, and subsequently comparing it to an earthworm of the same species may provide evidence to not only show if there is an effect detectable using IR microspectroscopy, but also for each chemical of mixture of chemicals where that effect would occur.

Objectives

The main aim of this project would be to test the validity of fingerprinting techniques to common earthworm species. This would involve the development of a technique to extract a viable tissue sample, and determining a ‘control’ sample for comparison. This would be followed by the development of an initial database containing a number of profiles for the same species of worm. The earthworms can be field collected and along with soil samples form the same location for chemical analysis. Each profile would then be associated with a comprehensive review of the soil properties.

The main advantages of developing this method for earthworms would be as follows:

1) A greater number of chemicals could be tested with faster response times.
2) A more sensitive endpoint would be employed, increasing the strength of the test.
3) Assessing the degree of contamination in field sites would be more effective.

The results from this study would benefit researchers from the wider scientific community and not only ecotoxicologists interested in the study of invertebrates. The findings would provide a new technique for tissue extraction and analysis; this could be applied to other species but also a technique that can be used when developing risk assessments.

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