INFLUENCE OF CLAY CONTENT AND SALINITY ON THE BIOAVAILABILITY AND TOXICITY OF METALS (COPPER AND ZINC) TO SOIL ORGANISMS

BY

OLUGBENGA J. OWOJORI

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PROMOTER: PROF A.J. REINECKE (US)
CO-PROMOTER: DR A.B. ROZANOV (US)

DECLARATION

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

Signature……. Date…….

ABSTRACT

Metal pollution is a problem of increasing global concern. It could arise from industrial activities, as well as pesticide use in agriculture, among other sources. For adequate protection of the soil ecosystem from metal toxicity, the bioavailability of metals must be properly evaluated. A plethora of soil factors affect the bioavailability of metals to soil organisms. These include pH, clay and organic matter contents, salinity among others. While much is known about the influence of some of these parameters, little is known on how clay content and salinity modify the bioavailability of metals to soil organisms.

This study investigated the influence of clay content and salinity on partitioning, uptake and toxicity of two essential metals (Cu and Zn) to the earthworm *Eisenia fetida* in separate laboratory trials. Partitioning of the metals was evaluated with 0.01 M $CaCl_2$, DTPA (di-ethylene-triamine-penta acetic acid), and nitric acid extractions. The metal content of worms was determined by acid digestion, while growth, cocoon production, and mortality were used as endpoints showing toxicity to metals and/or salinity. To test the validity of some of the laboratory results, a field study was undertaken, using the earthworm *Aporrectodea caliginosa*. Further, the study assessed the effect of salinity, using a battery of laboratory tests (acute, chronic and avoidance tests) with natural and/or artificial soils on four species of organisms (a collembolan *Folsomia candida*, a potworm *Enchytraeus doerjesi* and two earthworm species *E. fetida* and *A. caliginosa*), representing different feeding patterns and ecological roles in soil.

Results showed that with increased clay content, there was increased availability of Cu in the substrate, and increased toxicity to *E. fetida* as shown by data for mortality and growth. The situation with Zn was less significant at sub-lethal concentrations but much so at lethal concentrations. DTPA and $CaCl_2$ extracted metals revealed changes in partitioning of Cu and Zn with changes in clay content, but this trend was not always consistent. Both DTPA and $CaCl_2$ revealed increased availability of Zn in substrates with increased salinity. Salinity had an additive to synergistic effect with Zn in toxicity to *E. fetida*. When combined with Cu, salinity also increased the availability of Cu as shown by $CaCl_2$ extracted fraction, and had additive effect on toxicity of Cu to the earthworm. The field study did not succeed in

confirming the results of the laboratory study due to confounding role of flooding after heavy rainfall and subsequent leaching of salts and Cu. The results of the experiment on acute and chronic toxicity tests for NaCl on *E. fetida* showed LC50 of 5436 mg/kg NaCl and EC50 for growth and cocoon production of 4985 and 2020 mg/kg NaCl. These values showed that earthworms might be negatively affected in many soils containing fairly moderate concentrations of salts. Similarly, *A. caliginosa* could not survive in natural soil containing relatively low salt concentrations (EC = 1.62 dS/m) while reproduction was severely affected at lower EC value of 0.52 dS/m. *F. candida* and *E. doerjesi* could survive in the highest salinity soil (EC = 1.62 dS/m) used in this study but their reproduction was severely affected from 1.03 dS/m. Overall, it appears that of all the taxa used, earthworm species were the most sensitive to saline stress and could proof useful in determining 'safe levels' of salt in contaminated soils. The results of the avoidance test showed that *A. caliginosa* avoided both natural and artificial saline soil containing concentrations lower than those avoided by *E. fetida.*

The conclusion is that the influence of clay content and salinity on the bioavailability of Cu and Zn depends largely on the metal in question, but generally speaking, bioavailability and toxicity of the metals were reduced with increased clay content while the opposite was true for salinity. If the species used in this study can be seen as fairly representative of a wide range of soil organisms, the conclusion is that salinisation of soil will be detrimental to most soil organisms at relatively low saline concentrations. Given the role of beneficial soil organisms in several soil processes which in turn contribute to soil fertility and sustainable use of land, it is recommended that any farming practices that may lead to an increase in salt content of agricultural soils should be discouraged.

OPSOMMING

Metaalbesoedeling is ‘n probleem wat toenemende globale kommer veroorsaak. Dit kan ontstaan as gevolg van industriële aktiwiteite sowel as van plaagmiddelgebruik in die landbou en ander bronne.Ten einde die grondekostelsel genoegsaam te beskerm, moet die biobeskikbaarheid van metale ge-evalueer word. ‘n Verskeidenheid van grondfaktore be-invloed die biobeskikbaarheid van metale vir grondorganismes Hulle sluit onder andere in pH, klei, organiese inhoud en soutgehalte. Hoewel heelwat bekend is oor die rol van sommige van hierdie parameters, is min bekend oor hoe klei en soutgehalte die biobeskikbaarheid van metale vir grondorganismes kan modifieer. Hierdie studie het die invloed van klei-inhoud and soutgehalte op die verdeling/partisie, opname en toksisiteit van twee essensiële metale (Cu en Zn) vir die erdwurm *Eisenia fetida* in afsonderlike laboratoriumproewe ondersoek. Kompartementele verdeling van die metale is ge-evalueer deur middel van ekstraksie-metodes met 0.01 M $CaCl_2$, DTPA (di-etileen-triamien-penta asysnsuur), en salpertersuur ekstraksies. Die metaalinhoud van wurms is bepaal deur suurverterings en spektrofotometriese analises te doen terwyl groei, kokonproduksie en mortaliteit van organismes gebruik is as gevoeligheidseindpunte om toksisiteit van metale en soutgehalte aan te toon. Om die geldigheid van somige van die laboratoriumresultate te toets, is ‘n veldstudie ook onderneem met die erdwurm *Aporrectodea caliginosa*. Die effek van soutgehalte is verder ondersoek deur ‘n battery van laboratoriumtoetse met vier spesies (‘n kollembool *Folsomia candida*,’n potwurm *Enchytraeus doerjesi* en twee erdwurmspesies *E. fetida* en *A. caliginosa*), wat verskillende voedingspatrone verteenwoordig.

Die resultate het getoon dat met toenmende klei-inhoud was daar ‘n toename in die beskikbaarheid van Cu vir opname vanuit die substraat, asook ‘n toename in toksisiteit vir *E. fetida* soos deur die gegewens vir mortaliteit en groei uitgewys. Die situasie met Zn was minder betekenisvol by subletale konsentrasies en selfs baie minder so by letale konsentrasies. DTPA en $CaCl_2$ ge-ekstraheerde metale het veranderinge in die partisie/verdeling van Cu en Zn uitgewys met verandering in klei-inhoud, maar die tendens was nie altyd konstant nie. Beide DTPA en $CaCl_2$ ekstraksie het toenemende beskikbaarheid van Zn in substrate uitgewys met toenemende soutinhoud. Soutinhoud het ‘n additiewe/toegevoegde tot sinergistiese

toksisiteitseffek saam met Zn vir *E. fetida*. In kombinasie met Cu het soutgehalte ook die geskatte biobeskikbaarheid van Cu verhoog soos uitgewys deur die $CaCl_{2,}$ ge-ekstraheerde fraksie, en het 'n additiewe effek gehad op die toksisitiet van Cu vir die erdwurm.

Die veldstudie kon nie die resultate van die laboratoriumstudie bevestig nie weens die belemmerende rol van vloede na swaar reënneerslae en daaropvolgende uitloging van soute en Cu. Die resultate van die eksperimentele ondersoek na die akute en chroniese effekte van NaCl op *E. fetida* het 'n LC50 van 5436 mg/kg NaCl en EC50 vir groei en kokonproduksie van 4985 en 2020 mg/kg NaCl opgelewer. Hierdie waardes het aangetoon dat erdwurms moontlik negatief beinvloed kan word in baie gronde wat 'n redelike gemiddelde konsentrasie van soute bevat. Soortgelyk kon *A. caliginosa* nie oorleef in natuurllike grond wat relatief lae soutkonsentrasies bevat het (EC=1.62 dS/m) nie terwyl voortplanting sterk ge-affekteer is by 'n lae EC waarde van 0.52 dS/m. *F. candida* en *E. doerjesi* kon oorleef in die grond met die hoogste soutgehalte (EC= 1.62 dS/m) maar hulle voortplanting is ernstig geknou vanaf 1.03 dS/m. In geheel blyk dit dat van allle taksa wat gebruik is, erdwurms die sensitiefste was vir die stres wat deur soutgehalte veroorsaak is. Die kennis kan nuttig wees in die bepaling van "veilige vlakke" van sout in gekontamineerde gronde. Die resultate van die vermydingstoetse het getoon dat *A. caliginosa* beide natuurlike en kunsmatig versoute gronde vermy het by konsentrasies wat heelwat laer was as dié wat deur *E. fetida* vermy is

Die gevolgtrekking is dat die invloed van klei en soutgehalte op die biobeskikbaarheid van Cu en Zn grootliks afhanklik is van die metale wat betrokke is en dat biobeskikbaarheid en toksisiteit normaalweg verminder het met verhoogde klei-inhoud, met die teenoorgestelde wat waar was in die geval van soutgehalte. Indien die spesies wat in die studie gebruik is beskou kan word as redelik verteenwoordigend van 'n wye reeks van grondorganismes, is die gevolgtrekking dat versouting van gronde nadelig sal wees vir meeste grondorganismes, selfs by relatief lae soutkonsentrasies. In die lig van die rol wat nuttige grondorganismes speel in verskeie grondprosesse wat bydraend is tot grondvrugbaarheid en volhoubare gebruik van gronde, word dit aanbeveel dat enige boerderypraktyk wat mag lei tot verhoging van die soutinhoud van landbougronde ontmoedig moet word.

DEDICATION

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 SOIL ECOSYSTEM

The soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fiber but also in the maintenance of local, regional and global environmental quality (Glanz, 1995). It is the habitat of plant roots and a diverse array of organisms - bacteria, fungi, protozoan and invertebrate animals, which contribute to the maintenance and productivity of agro ecosystem (Giller and Cadish, 1995).

Although, not apparent to the naked eye, soil is one of the most diverse habitats on earth and contains one of the most diverse assemblages of living organisms. A study has shown that a ton of soil in some parts of the world can contain around 10^{16} procaryotic cells (Curtis and Sloan, 2005). A single gram of soil has been estimated to contain several thousand species of bacteria (Torksvik et al., 1994). Other studies have indicated that globally about 1.5 million species of fungi (Hacksworth, 1991), 100,000 species of protozoan, 500,000 species of nematodes (Hawksworth and Mound, 1991), 3,000 species of earthworm (Lee, 1985), not to mention the other invertebrate groups of the mesofauna and macrofauna. The density of mites was put at 20,000-600,000 individuals per m^2 in temperate and tropical regions of the world (Lavelle and Spain, 2001), enchytraeids can be up to 200,000 individuals per m^2 but may be as low as hundreds or thousands in tropical forest and grassland (Athias, et al., 1974; Chiba et al., 1975), earthworms of 100-500 individuals per m^2 (Lee, 1985) and springtails with populations ranging from 100-670,000 individuals per m^2 (Petersen and Luxton, 1982).

Invertebrates are among the major components of soil biomass and play an important role in maintaining the structure and fertility of soil. Invertebrate-mediated processes such as drainage, aeration, and incorporation and degradation of organic matter are important in improving soil quality (Edwards and Lofty, 1977; Barber et al., 1998). These processes are enabled by their feeding behavior which enhances decomposition.

Decomposition is a biological process driven by decomposer organisms, which consists of a complex community of soil biota (Tian et al., 1992). For example, earthworms increase the decomposition of organic residues (Tian et al., 1995), microarthropods accelerate nutrient fluxes by influencing decomposition processes directly and indirectly (Moore et al., 1988). Termites and ants digest cellulose and lignified substances (Lee and Wood, 1971), millipedes break down plant litter and mix it with mineral soil which they ingest (Tian et al., 1995) while protozoan and nematodes increase N mineralisation by feeding on microflora (Bouwman et al., 1994).

Anthropogenic activities leading to pollution affect the beneficial soil fauna whose role in nutrient cycling processes has already been established (Lavelle et al., 1997, Tian et al., 1997). There are indications that pollution affects the structural and functional parts of their ecology. Such pollution affects the organisms to the extent that some of the organisms are even extinct before they are named or identified. Pollution of the soil environment also leads to accumulation of toxic substances in the soil organisms which cannot only be toxic to the organisms but transferred along the food chain (Abdul Rida and Bouche, 1994; Reinecke et al., 2000). This has been the concern of soil ecologists and ecotoxicologists over the last two decades since rice paddy fields irrigated with wastewaters from a zinc mine caused excessive cadmium (Cd) intake and adverse health effects in farmers who had consumed rice grown on this contaminated soil (Kobayashi, 1978).

1.2 POLLUTION OF THE SOIL ENVIRONMENT

Soil pollution is, strictly speaking, as old as soil itself. However, before human interference, the pollution used to be restricted to specific areas, such as locations with superficial metal ores, sites of volcanic activities, and tar works. Soil pollution can come from natural or anthropogenic sources. Although several substances causing pollution are naturally occurring, like Polycyclic Aromatic Hydrocarbon (PAH) (which originate from incomplete combustion e.g. as a result of forest fires) and metals, there is a dramatic increase in the level of soil contaminants which coincide with an extensive increase in the use of fossil fuel and industrialization (Jones et al., 1989). Rapid population and industrialization are two phenomena seen in the last century which have a great influence on global ecology. It is estimated that the world

population will reach 8,2 billion by the year 2025 (Depledge, 1992). This population growth would probably lead to a similar increase in the amount of pollutants released into the environment.

Soil pollution or contamination is the presence of manmade chemicals or other alterations to the natural soil environment. This type of contamination typically arises from rupture of underground, storage tanks, application of pesticides and herbicides, exhausts from automobile, contamination from smelter plants, and percolation of contaminated surface water to subsurface strata, leaching of wastes from landfills or direct discharge of industrial wastes to soil (Edwards, 2002). The most common chemicals involved are petroleum hydrocarbons, solvents, pesticides, and heavy metals (Forbes and Forbes, 1994). These chemical pollutants reach the soil through direct application, atmospheric fall-out, waste disposal and industrial effluents. These chemicals may be transient, have low toxicity and exert minor effects or, at the other extreme, be broad-spectrum biocides, persistent and even have the potential to bio-concentrate in organisms and food chains.

Pollutants can influence the ecological functioning of the soil system at virtually all trophic levels. At individual species level, they may kill soil organisms through direct acute toxicity, change or contaminate their food supply, or influence their reproduction by indirect effects on egg production and hatching. They may also affect their metabolism, growth and development, longetivity and sometimes genetic makeup. At the population level, they may affect the size, age, sex ratio, population structure and stability. At the community level, they affect the species diversity and may create an imbalance in the ecosystem functioning. They may also disrupt predator/prey relationships, relationships between soil-inhabiting organisms and plant, and above-ground plant species diversity (Edwards, 2002). In agricultural lands, perhaps some of the most important soil pollutants are the heavy metals.

1.3 METAL POLLUTION

The term heavy metal has been used to denote different meanings to different authors in the literature. Among ecotoxicologists, the term ‘Heavy metals (HM)’ is generally used to refer to metals that have shown to cause environmental problems. Those of major concern include: Cd, Cu, Ni, Cr, Pb, Co, V, Ti, Fe, Mn, Ag, Sn.

Forbes and Forbes (1994) listed mercury (Hg), cadmium (Cd) and lead (Pb) as the most hazardous heavy metals to humans and ecosystems and emphasized the significant dangers that copper (Cu), zinc (Zn), silver (Ag) and chromium (Cr) can also pose.

Heavy metals are byproducts of industrial activities and enter ecosystems through air, rivers and dumping (Forbes and Forbes, 1994). On a global scale there is now abundant evidence that humans have contaminated the environment with heavy metals (and other pollutants) from the poles to the tropics and from mountains to the abysmal depth (Samiullah, 1990). These metals are continuously released into the biosphere by volcanoes, natural weathering of rocks, and by human activities such as mining, combustion of fossil fuel, release of sewage and application of pesticides to agricultural lands.

At sites of historic and current mining and smelting operations, Cd, Pb, and zinc (Zn) have contaminated large areas of farmland (Asami, 1988; Dudka and Sajdak, 1992; Dudka et al., 1995a, b). In addition, the use of Pb in paints and of organic Pb compounds in gasoline has caused Pb to be widely dispersed in the general environment (Davies and Thornton, 1989; Nriagu, 1990). Locally, arable land can be strongly contaminated by pesticide and sewage sludge application in agriculture (Logan and Chaney, 1983; McBride, 1995). Widespread use of Zn in galvanized products has also contributed to the contamination of soil with Cd and Zn (Kabata-Pendias and Pendias, 1992). Elevated level of Cu and Ni may also be seen in areas of Cu-Ni mining and smelting (Li and Thornton, 1993; Dudka et al., 1995c; Mitchell and Barr, 1995).

Heavy metal pollution has become a great source of concern. Their contamination of soil and groundwater is not at all uncommon today. They interact with the soil matrix and may persist for a long period of time creating long-term hazards. Soil contamination with metals results in accumulation and subsequent toxicity to plants (Ernst, 1996; Zayed et al., 1998; Gimmler et al., 2002) microbes and invertebrates. In earthworms, heavy metals can reduce sperm count and induce spermatozoa damage (Cikutovic et al., 1993; Reinecke and Reinecke, 1997), reduce reproduction (Ma, 1983; 1984; Spurgeon et al., 1994).

The bioavailability of HM in soil is increasingly used as key indicator of potential risks that a contaminant poses to both the environment and human health. Hence the most exciting technical area in the risk assessment and remediation field today is contaminant bioavailability. Although risk assessment of metals is currently based on total metal concentration, it has been shown that this does not reflect the true uptake of metals by soil organisms. This is particularly important because estimates based on total metal concentration will lead to overprotection or forceful remediation of soil which is very expensive. Given that 'Risk Based Land Management' is increasingly being adopted as a cost effective management strategy for contaminated sites in terrestrial ecosystems, metal toxicity to soil animals and their uptake of contaminants are usually assumed a reflection of HM bioavailability.

1.4 BIOAVAILABILITY: A DYNAMIC CONCEPT

Bioavailability, as a concept has probably generated more discussion in ecological risk assessment of soil than any other concept. This is in one part due to different definitions given to it in the literature by different authors and its dynamic nature. This has led a few authors to believe that the dynamic approach of bioavailability in soil should comprise at least two distinct phases; the physico-chemically driven desorption process and a physiologically driven uptake and depuration process (McCarthy and Mackay, 1993; Peijenburg et al., 1997, 1999a).

For the purpose of this study, bioavailability will be considered in the sense suggested by Landrum et al., (1992). These authors defined bioavailability as comprising environmental availability, environmental bioavailability and toxicological bioavailability.

Environmental availability is that portion of the total environmental concentration of a chemical in the environmental matrix, be it soil, air or water, that is available for all fate and transport, uptake by the organism. Chemicals present in soil interact with specific soil constituents in a dynamic manner over time (desorption process), resulting in the sequestration of a portion of the chemical making it unavailable for interaction with biological receptors. Sequestration is a state in which a contaminant is segregated from and rendered unavailable to a receptor and arises from rate limiting processes involving contaminant interaction with the surrounding

matrix, such as phase transfer, complexation, and reversible chemical transformation. Sequestration is specific in relation to the combination of receptor, matrix, spatial and temporal scales, and route of exposure (Lanno, 2003).

Environmental bioavailability refers to that portion of the environmentally available portion that is eventually taken up by the organism. This requires identification of specific biotic species as endpoints. It is a well known fact that only an organism can measure bioavailability. Organisms however differ in their uptake of chemicals due to differences in behavior, feeding patterns and general physiology. The proportion of the total chemical in soil that is environmentally bioavailable therefore depends on the physiology and behavior of the soil animal.

Toxicological bioavailability is that portion of the chemical that is taken in by the organism that reaches the site of toxic action (STA). Thus the level in the organisms must reach some threshold value in the STA before effects, or toxicity start to occur. Once a chemical is taken up by an organism, it may be partitioned into biologically available, biologically unavailable, or storage fraction (Lanno et al., 1998). This is the depuration process. A biologically available metal can participate in essential metabolic functions or in the case of nonessential elements, or excess essential metal, contribute to toxicity. Biologically unavailable are those that are sequestered within the organisms. Sequestration in this sense means the partitioning of specific chemicals inside an organism into inert forms or pools that are biologically unavailable to the organisms (Lanno et al., 2004). Storage fractions are those stored in other tissues of the body. This process is called the depuration process. Toxicological bioavailability is thus the final determinant of toxicity (Hamelink et al., 1994). However, for a chemical to be toxic, it must first be environmentally bioavailable to the organism.

1.5 MEASURES AND ESTIMATES OF BIOAVAILABILITY

Measures of bioavailability may be either direct or indirect and biological while estimates can only be chemical and indirect. Direct biological measures of bioavailability are determinations of the actual amount of chemical taken up by the organism. Most of the time it provides the most accurate measure of bioavailability since it integrates all biotic and abiotic modifying factors of chemical bioavailability.

Two ways usually used to express biological bioavailability are Bioaccumulation (BA) or Critical Body Residue (CBR). Bioaccumulation is a direct measure of chemical concentration in an organism resulting from the net inward flux from uptake and depuration. It is usually regarded as that portion in the organism in the STA but below a toxic threshold. CBR, sometimes referred to as metal body burden (MBB) or lethal body concentration (LBC) are internal chemical concentrations that are associated with sublethal or lethal endpoints (McCarty and Mackay, 1993; Lanno et al., 1998, Wells and Lanno, 2001). Both approaches have been used to estimate bioavailability and toxicity of metals to plants, earthworm and springtails (Smit and Van Gestel., 1997; 1998; Posthuma et al., 1998). Both approaches and models however often only assume a steady state in tissue or whole organisms, limiting their applicability to metals that do not reach steady state over time.

Indirect biological measures of bioavailability include measuring of a response in organisms that can be linked with the bioavailability or toxicity of a certain chemical or stress. This could range from subcellular, biochemical markers up to whole organisms responses. Responses that have been observed in earthworms range from lethality to sublethal changes in biomarkers (e.g inhibition of certain enzymes, induction of metalothionein, Neutral Red Retention time (NRRT) by lysosomes (Weeks and Svendsen, 1996, Reinecke and Reinecke, 1998; Scott-Fordsmand and Weeks, 1998, 2000; Reinecke et al., 2004). Other indirect biological measures of bioavailability also include whole organism response such as weight change, cocoon production and viability and mortality. Although most indirect biological measurements of bioavailability are non specific (e.g NRRT) while others are specific for some groups of chemicals (e.g metallothioneins), they are constantly used in ecotoxicological or risk assessment of soil when used in laboratory or field study with appropriate or reference controls (Svendsen and Weeks, 1997, a, b; Spurgeon et al., 2000; Maboeta et al., 2002, 2003; Booth et al., 2003)

Indirect chemical estimates of bioavailability are estimates that determine chemical concentration in the exposure medium. This is either by determining the total metal concentration using vigorous extraction with acid or various liquid and solid-phase extraction techniques that sample some fraction of the chemical present in the test medium. This measurement only appears useful when used with or correlated

with the amount of the chemicals in organisms. Only an organism can determine whether a chemical is available or not. This measure is sometimes called a surrogate measure of biovailability by some authors (Lanno et al., 2004) or estimates (Reinecke and Reinecke, 2006) and proxy by others (Arnold et al., 2003). If and when it correlates with metal concentration in organisms, it is assumed by some authors to be a measure of bioavailability considering the cost and time of conducting bioassays (Lanno et al., 2004).

Direct chemical measurement of the bioavailability of a chemical in a soil sample is simply not possible since only an organism can indicate how much chemical is available to it and the amount depends on a plethora of biological factors such as the animal's feeding behaviour, mobility and interaction with other species.

1.6 FACTORS AFFECTING BIOAVAILABILITY OF METALS TO SOIL ANIMALS

Metal bioavailability is both metal and species dependent, and it is also dependent on the interaction between a chemical and an organism as a function of time (Rand, 1995). Hence, the accumulation, bioavailability and toxicity of metals to soil organisms not only depend on the characteristics of the organism itself but also on those of the chemicals and the environment (Beeby, 1993; Crommentuijn et al., 1997; Amorim et al., 2005a). This means that the relevant endpoints that relate to bioavailability should explicitly be taken into consideration, including the relevant exposure and uptake route, chemical fluxes and for specific biological species, their time dependency, and dynamic aspect as well as acclimation and redistribution processes within species. For the purpose of this study, factors affecting the bioavailability of metals in soil animals will be grouped under: soil type, metal kinetics, metal species and types and time.

1.6.1 Soil type

Theoretically, the most important soil characteristic influencing soil metal speciation and thus metal bioavailability are those that have to do with the amount of available sorption sites, pH, sorbed ions, and adsorption phases (Janssen et al., 1997a, Bradham, 2002). The amount of available sorption sites is related to the CEC.

Moreover, the adsorption process of metals in soil is dependent on various soil characteristics such as pH, clay, OM, and CEC (Harter, 1983; Elliot et al., 1986; Andersen and Christensen, 1988; King, 1988). Several studies have considered the effect of soil properties on the bioavailability of metals to soil organisms. Most of these studies identified those same factors influencing adsorption-pH, OM, CEC, clay content, as factors influencing the bioavailability of metals to soil organisms (Van Gestel, 1992, Amorim et al., 2005a, Van Gestel et al., 1995). Among the heavy metals, Cd, Cu, Pb and Zinc are the most studied.

Crommentuijn et al., (1997) found that increased pH and OM reduced the bioavailability of Cd to *F. candida*. Lock et al., (2000) also found that instead of clay and OM, pH and CEC were the most important factors affecting Zn and Cd ecotoxicity for *E. albidus* while using field and artificial soils. Lock and Janssen (2001a) found that the acute toxicity of Zn and Cd to the same oligochaete was determined mainly by pH and OM, followed by CEC using artificial soil. Amorim et al. (2005a) studied the effect of soil type and aging on the bioavailability and toxicity of Zn for *F. candida*. For freshly spiked soil, they found clay and OM are the most important factors. Spurgeon and Hopkin (1996a) also found pH and OM as the most important factors affecting Zn ecotoxicity to *E. fetida*. Peijenburg et al. (1999b) studied the effect of soil properties in 20 Dutch field soils on the bioavailability of Cd, Cu, Pb and Zn for *E. crypticus*. They found that pH and CEC were the most important factors, and that these differed with each metal.

However, a few other studies have shown that soil type may not play important roles in the bioavailability of metals to soil organisms and plants. Römbke et al. (2006) did not find any correlation between pH, CEC, OC and the toxicity of Zn to earthworms, springtails or plants while working on natural soils of European origin.

1.6.2 Metal speciation

Speciation and bioavailability are frequently interlinked because the speciation of the metal is often related to its bioavailability (Peakall and Burger, 2003). Although the total concentrations of metals are not always directly related to bioavailability and toxicity to organisms (Alexander, 2000), environmental quality criteria are usually based solely on the total metal concentration in the environmental matrix. The total

metal is usually present in a number of forms. Some forms or species are highly soluble, while others are so inert that their presence hardly influences the amount of the metal that is present in the soil solution phase.

In aqueous systems, bioavailability is often correlated with the free metal concentration, because the free ion is often the most bioavailable species of a dissolved metal. This is sometimes referred to as the Free Ion Activity Model (FIAM) (Campbell and Tessier, 1996). In view of a general lack of data on terrestrial systems, the quality objective of soil was derived from the aqueous quality objective by multiplying with an appropriate solid-liquid partition coefficient (Shea, 1988; Van Der Kooij et al., 1991; Van Leeuwen, et al., 1992). The partition coefficient is calculated as the ratio of the metal concentration in the particulate and liquid phases of the soil. This however rests on the assumption that toxic effects of compounds present in the particulate phase can be predicted on the basis of the chemical's concentration in the soil pore water, and that the pore water and the solid phase equilibrium are in chemical equilibrium. The pore water concept hinges mainly on the equilibrium partitioning (EP) concept.

EP is the theory that toxic effects and body residues in soil- and sediment-dwelling organisms can be predicted from the dissolved concentration in pore water. The theory assumes equilibrium partitioning between pore water and soil solids. According to the pore water hypothesis, the toxicity of chemicals is directly related to the concentration in the soil pore water, which is also influenced by soil characteristics (Van Gestel, 1997). With respect to the influence of soil type on bioavailability of metals, it has been proposed that the free metal ion activity in the pore water should be used instead of total metal (Sposito, 1984).

The actual environmentally available fraction of a pollutant for soil animals has been suggested to reside in this pore water (Belfroid et al., 1996) which can be measured as water extractable metal. This will of course depend on the soil type, feeding pattern and behavior of the organisms. There are studies confirming that this is true at least for organic chemicals in earthworms (Spurgeon and Hopkins, 1996a; Belfroid, 1994), Cd and Pb to *Eisenia andrei* (Peijnenburg et al., 1999a), *Enchytraeus crypticus* (Peijnenburg et al., 1999b), Zn for springtails (Smit and van Gestel, 1998). A study however reported that the uptake of Cd in *Folsomia candida* exposed to

artificial substrate (Crommentuijn et al., 1997) could not be correlated with the fraction of the metal in the pore water. This is probably an indication that metal bioavailability is not only (biological) species dependent but also dependent on the kinetics of the metal involved.

However, due to the àmount of time wasted analyzing the fraction in pore water, other less laborious sequential extraction methods have been described which can give an estimate of the chemically available fraction of the metal. This include the weak electrolyte extraction method using 0.01 M $CaCl_2$ or Ca $(N0_3)_2$ which estimates the mobile metal (exchangeable) and the diethyl triamine pentaacetic acid (DTPA) extraction method which estimates the mobilisable metal (complexed, adsorbed and carbonate forms) (Maiz et al., 1997).

Another concept, which has gained tremendous interest among ecotoxicologists, is the biotic ligand model (BLM), which was also first developed in the aquatic environment. In the BLM, both metal speciation and interactions of the metal at the site of toxic action are taken into account. The main assumption of the BLM is that metal toxicity occurs as the result of free metal ions (or other reactive metal species) reacting with binding sites at the organism–water interface (either physiologically active sites, leading to a direct biological response, or transport sites, leading to metal transport into the cell followed by an indirect biological response), which is represented as the formation of a metal–biotic ligand complexes. The concentration of these metal–biotic ligand complexes directly determines the magnitude of the toxic effect, independent of the chemical characteristics of the test medium. $Ca^{2+,}$ $Mg^{2+,}$ $Na^{+,}$ and $H^{+,}$ ions may compete for binding sites at the organism–water interface thus reducing the binding of toxic metal species to the BL and eventually reducing the toxicity of the reactive metal species (Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere and Janssen, 2002). Attempts to use the BLM concept for soil has been largely successful (Steenbergen et al., 2005; Lock et al., 2006), making some researchers to call it state-of-the-science concept to understand the bioavailability of metals in soil (Lock et al., 2006).

1.6.3 Metal kinetics

Metal kinetics is about the behaviour of each metal in the environmental matrix and in the test species. Metals differ in the way they are accumulated in, and assimilated and excreted by soil organisms. For elements such as Cd, and Pb, there is good evidence that the kinetics are slow, and accumulation of the elements, resulting in progressively higher tissue concentrations, may continue for the life span of the earthworm (Honda et al., 1984; Peramaki et al., 1992; Van Gestel et al., 1993; Neuhauser et al., 1995; Sheppard et al., 1997; Spurgeon and Hopkin, 1999, Lock and Janssen 2001a). This has necessitated the need for the metal's internal concentration in organisms to reach steady state before conclusions of ecotoxicological importance can be made in soil ecotoxicity testing. Steady state is a much preferred condition for standardisation of tests: prior to steady state, concentrations are changing rapidly and so do observable effects on the organisms. For other elements, such as Cr, Cu, Ni, and Zn, there are indications that the internal concentrations in earthworm, reach a steady state within a relatively short period of exposure (Peijnenburg et al., 1999a, Spurgeon and Hopkin, 1999; Lock and Janssen, 2001b). For *Eisenia andrei*, Cu and Zn reach their steady states within three days in some natural soils of European origin (Peijnenburg et al., 1999a).

1.6.4. Aging

The distribution of metal species and their ability to partition to the soil solution are not constant; they vary with the period of time that the metal has been present in the soil. This situation is referred to as ageing, and tends to immobilize the metals and render them less available for uptake than freshly added metals.

Part of the problem in extrapolating laboratory toxicity data to field realities is in the behaviour of the metals under field conditions that are different from the laboratory. La point et al. (1989) reported that this is mainly due to the effect of alterations of the chemical or the environment during this aging process. Since laboratory studies should mimic the most realistic situation and since freshly spiked soil do not allow the equilibration time that is required to resemble the common field situation, incorporating the effect of aging in the environmental risk assessment of

metal contaminated soil may contribute to a more realistic assessment regarding the impact of metals on terrestrial ecosystem (Lanno et al., 2004).

Effect of aging on ecotoxicity of Zn to springtail (Smit and van Gestel., 1998) and Cu to springtails and enchytraeids (Amorim et al., 2005a) has been reported. In both studies, aging was reported to affect the ecotoxicity of zinc and copper for springtails but no significant effect was observed for Cu to enchytraeids. These reports suggest that aging of metals does not always have a strong effect on the bioavailability and toxicity to soil organisms, with high emphasis on the biotic species involved. Thus, more research into aging of chemicals should be conducted with other metals and other species to ascertain the precise effect of aging of metals on soil fauna in ecotoxicological assessment of soil.

1.6.5. Mixed Metals

Ecological risk assessment of soil is often based on laboratory studies with single substance exposure (Van Straalen and Denneman, 1989: Wagner and Lokke, 1991; Van Straalen and Lokke, 1997). This introduces various uncertainties which are accounted for by the introduction of safety factors. However, organisms inhabiting polluted habitats are most times exposed to various toxicants simultaneously. Metals present in soil often come in mixtures of three to five, depending on the source of contamination (Spurgeon et al., 1994).

When performing toxicity tests with chemicals in soil, three types of interaction may be expected to occur:

1. chemical and physiochemical interactions with other constituents of the soil determining sorption and through that affecting bioavailability.
2. physiological interactions, affecting uptake from the soil solution and finally determining the quantity available at site of toxic action
3. interactions during the intoxication process, including combination with receptors, at the target site (Calamari and Alabaster, 1980).

During each of these three interactions, interactions of the chemical with other chemicals may occur.

Joint effect of these chemicals may be similar (Toxic Unit (TU) = 1), stronger (TU < 1) or weaker (TU > 1) than concentration addition. When TU = 1, it is said to be additive, when TU < 1, it is said to be synergistic and when TU > 1, it is said to be antagonistic. The TU model indicates that the concentration in the mixture is expressed as fractions of the EC_{50}'S of the individual toxicants. The data are tested against the null hypothesis of relative concentration additivity, which states that the effects of the mixture can be predicted from the sum of the TU of the individual compounds (Hermens and Leeuwangh, 1982; Kraak et al., 1994).

Few studies on mixed metal addition are available in the literature. Beyer et al. (1982) found antagonistic effects between uptake of Zn, Cd and Cu by earthworms. Khalil et al. (1996), on the contrary, found that the effect of mixtures of some metals such as Cu, Cd and Zn on the growth of juvenile *A. caliginosa* was only slightly less than additive. Posthuma et al. (1997) studied the effect of single and joint effect of Cu and Zn on *E. crypticus*. These authors found that the observed joint effect was similar to concentration addition when judged by external concentration and less than that for earthworm body concentrations. They also noted that copper reduced the sorption of Zn to soil, but Cu sorption was inert for Zn addition. Van Gestel and Hensbergen (1997) studied the detailed interaction of Cd and Zn to *F. candida,* making use of several endpoints. They found that the water solubility of Cd in the soil was significantly increased by the presence of Zn, whereas Cd did not affect the water solubility of Zn. They also reported that the effects of Cd and Zn are mainly antagonistic when growth is used as endpoint but mainly additive when reproduction is used. In their study, a less than concentration addition was also observed for body concentration. A less than concentration additive effect has been reported in the body metal concentration of polychaetes from several workers, for Cu and Zn (Posthuma et al., 1997), Cd and Zn (Posthuma et al., 1995) in *E. crypticus* and for Cd + Zn, Cd + Cu, and Cu + Zn in *E. andrei* (Weltje et al., 1995). From the foregoing, different effects are seen using different endpoints.

Despite these conflicting reports in the literature, concentration addition is an obvious choice when considering mixtures of chemicals that have similar modes of action and that do not interact. The TU approach was developed for chemicals having the same mode of action (Bliss, 1939) and only allows evaluation only if equitoxic

mixtures are antagonistic, additive or synergistic. As such, it does not allow evaluation of randomly chosen metal mixtures. However since metals do not all have the same mode of action, and that their mixtures may actually be random in the field, the Central Composite Design (CCD) was developed and compared with TU (Lock and Janssen, 2002). The CCD was developed to take advantage of predicting toxicity for random metal mixtures, by using multivariate test design for the development of surface response models.

Thus two models are presently available which describe the observed combined effects as a function of the toxicity of the individual components: Concentration addition (CA), based on TU approach and independent action (IA) which is based on response addition in CCD (Greco et al., 1992). Lock and Janssen (2002a) found that the differences between CA and IA predictions were largest for the mixture of Cu and Zn (essential elements) which has a steep dose-response relationship, while for the non-essential elements, it gave a similar prediction. They also reported that CA will predict a stronger effect for steep concentration-response curve (Cu and Zn) than IA and vice versa.

However, since most of these studies were conducted in a single soil type and since the ecotoxicity of metals does vary over several orders of magnitude, depending on soil characteristics (Lock et al., 2000), studies on toxicity of metal mixtures using different soil types will be informative in future research.

1.7 THE PROBLEM OF SALINITY

Saline soils often occur within irrigated land (Ayars and Tanji, 1999) in semi-arid or arid zones of the world. A report by an agency of the United Nations indicated that about 50% of irrigated areas of the world are either salinised or have the potential to be so in future (Tyagi, 1986). This is because current supplies of good quality surface ground water for crop production have not been able to keep pace with rapidly increasing water demands as a consequence of increased cropping intensities (Pimentel et al., 1999) and / or expansion in irrigated agriculture on marginal lands. Groundwater of different qualities is being used to make up the shortage of good quality waters for crop production. At certain places, this practice has lead to soil and drainage water contamination by a variety of pollutants. Salinity and sodicity are the

principal water quality concerns in irrigated areas receiving such waters (Ayars and Tanji, 1999).

The salinity of these soils occurs because salts accumulate in the surface and because rainfall is not sufficient to flush them from the upper part of the soil layer. The salts are primarily chlorides and sulphates of calcium, magnesium, sodium and potassium. The electrical conductivity of these soils is usually more than 200 or 400 mS/m depending on crop tolerance (Brady, 1990), while the exchangeable sodium percentage (ESP) is less than 15; the pH is usually less than 8.5. ESP = Exchangeable sodium (cmol/kg)/CEC (cmol/kg). Basically, soils having less than 0.15% of salt by dry weight of soil is considered not saline, those with 0.15-0.35%, 0.35-0.65%, and greater than 0.65% are considered as slightly saline, moderately saline and highly saline respectively (Fitzpatrick, 1974).

Salinity in irrigated lands has been reported in Australia (Ferdowsian et al., 1996), India (Rai and Singh, 1999), Iran (Jorenush and Sepaskhah, 2003), South Africa (Fey and deClercq, 2004) and most other countries of the world. Generally, salinity affects crop performance and beneficial soil biota leading to economic loss. In Australia, the cost of salinity to farmers has been estimated to be about $1 billion (SCLC, 1991) and that 450 of the endemic species of vascular plants are under threat of extinction from salinisation and hydrological change; approximately 60% of the > 4000 species of vascular plants in the south-western agricultural region are endemic, and 220 of the aquatic invertebrates of the wheat belt so far identified ‘will disappear’ (SSS, 2000). In Tanzania, salinity was reported to affect the dieback of *Acacia xanthophloea* (Benth) opening up the once densely forested Lerai area in Ngorongoro Caldera (Mills, 2006).

The role of salinity in influencing the bioavailability of metals to soil organisms has received little attention. The limited published data available however indicate that salinity affects the growth and reproduction of earthworms (Fischer and Molnar, 1997) and microorganisms (Lippi et al., 2000). Bryan and Langston (1992) have also indicated that salinity influences the toxicity of heavy metals to polychaetes. However, the influence of salinity on the bioavailability of metals to soil organisms under different soil textures remains to be tested.

1.8 EARTHWORM IN ECOTOXICITY TESTING

In the soil environment, invertebrates are very abundant and are expected to be good indicators of stress in their environment. However, because each and every soil invertebrate can not be tested, it became necessary to choose a species as a representative of other species in standard toxicity testing to evaluate the effect of toxic chemicals. Risk assessment of chemicals in soils is often based on single species toxicity tests. The purpose of ecotoxicological testing is not to protect the single species used in the test but because every single chemical can not be tested on every single species that need to be protected, single species tests are commonly used.

There are several reasons for selecting an earthworm as a representative species for the terrestrial environment. Earthworms are among the major components of soil biomass. They play an important role in maintaining the structure and fertility of soil. Earthworm-mediated processes like drainage, aeration, and incorporation and degradation of organic matter. They have beneficial effects on soil structure, disease control, and are of importance in improving soil quality (Edwards and Lofty, 1977; Barber et al., 1988; Edwards and Bohlen, 1992; Scott-Fordsmand and Weeks, 2000; Reinecke and Reinecke, 2004).

Earthworms are common in many soils, and are also extremely vulnerable to impacts on soil. They can assimilate and accumulate contaminants, making them ideal for assessing the effects of terrestrial pollutants and serving as biomonitors of soil quality (Samiullah, 1990; Bouche, 1992; Reinecke and Reinecke, 2004). They are practical to breed and use in both laboratory and field toxicity tests and are convenient to handle because of their relatively large size. Moreover, earthworms are an important part of the terrestrial food web and can constitute a significant component of the diet of birds, small mammals, reptiles, and other soil dwelling biota. Because of these characteristics, they have been adopted as important indicator organisms for assessing potential impacts of chemicals to soil organisms and to organisms in the terrestrial food web. Standardized tests have been developed to quantify effects of chemicals on worms (OECD, 1984).

The OECD test for earthworms is an ecotoxicological test performed in artificial soil; a mixture of sand (70%), clay (20%), peat (10%) and water in a

standard substrate and *Eisenia fetida*, is the preferred test organism. *E. fetida* was chosen because it is cosmopolitan and reproduces rapidly under laboratory breeding. The major advantage of this standardization is that it is practical, reproducible and allows comparability of results from different laboratories. Due to huge success with standardization of tests with earthworms, it has also been recommended for tests with collembolan (ISO, 1999) and enchytraeids (ISO, 2003).

Despite these advantages, it has been criticized on the basis of its high OM content, use of rare clay (Kaolin clay) and lack of aluminum, iron, and manganese oxides which are thought to influence bioavailability (McLaughin et al., 2002). Although this standard OECD soil has been criticised by some authors, it is still been used on the strength of these advantages in ecoxicological testing of soil.

Although, from an ecological point of view, the use of natural soil (Van Assche et al., 2002; Van Gestel and Weeks, 2004) and the most widespread local species in ecotoxicological testing have been recommended, the vast array of soil from different parts of the world that needs to be tested and standardized makes this suggestion a thing of the future.

1.9 LABORATORY MICROCOSMS, SEMI-FIELD AND FIELD STUDIES

Another setback for the OECD single species test is the problem of extrapolating from laboratory toxicity study to field realities. Such an extrapolation seems theoretically impossible, as the essential characteristics of an ecosystem are not incorporated in a single species laboratory test. In the field organisms are exposed under fluctuating environmental conditions to single, mixed contaminants in the presence of other species, of same or different ecological needs which may influence the bioavailability and toxicity of chemicals in the field as different from the lab. However, to undertake a comprehensive environmental risk assessment by way of a full-scale field study is impossible (Edwards, 1992); such studies are inherently expensive, the risk assessment would become an incredibly slow process, results would often be overshadowed by natural variation, and data would be influenced by widely fluctuating environmental conditions.

As an alternative to single species, it has been suggested that the laboratory microcosms or semi-field terrestrial model ecosystems can bridge the gap between single species laboratory tests and field studies. Such microcosms or terrestrial models contain organisms from several trophic levels. They allow us to perform experiments difficult to carry out in the field because of practical, technical and logistical reasons or legislation. In these models, several ecological factors can be controlled such as light, temperature, moisture, species diversity and abundance of the community introduced. This approach allows effects of various disturbances to be monitored on a large range of trophic and functional groups and to link them to ecosystem process changes such as nutrient dynamics or organic matter decomposition (Bogomolov et al., 1996; Filser and Krogh, 2002; Cortet et al., 2003).

However, the choice of methods to be used depends on the aim of the experiment. In instances where the effect of soil type is to be tested, OECD artificial soil is usually used as this allows for easy manipulation of the substrate. In such instances, a possible way of getting over the extrapolation problems is validating the laboratory findings in the field using a mesocosm. Experiments using a mesocosm to validate laboratory results show a promising way of solving the extrapolation problem (Svendsen and Weeks, 1997b).

1.10 AIMS

The aim of this study was to understand the roles of clay content and salinity in influencing the bioavailability and toxicity of Cu and Zn for earthworms. Another aim was to assess the tolerance of selected soil organisms to saline stress. The specific goals were:

1. to assess how clay content affects the partitioning, uptake and toxicity of Cu and Zn to earthworms
2. to assess how salinity affects the partitioning, uptake and toxicity of Cu and Zn to earthworms
3. to derive a quality criterion standard for toxicity of salt to earthworms
4. to assess how salinity affects life-cycle parameters of representatives of soil organisms
5. to determine the relevance of the earthworm avoidance test in predicting toxic effect of salt to this organism

6. to assess if a laboratory study on the influence of salinity on Cu to earthworms could be validated under field conditions.

1.11 THE CHOICE OF TEST SPECIES FOR THE PRESENT STUDY

An earthworm species (*E. fetida*) was chosen for most parts of this study because:

1. It is easy to culture and handle in the laboratory and are of relatively big size.
2. It is an important component of the soil biomass and enhances fertility of the soil through litter processing (Ecosystem engineers).
3. It is in direct contact with the soil matrix through its thin body and as such will be relevant in testing the pore water hypothesis.
4. There are also substantial data available on the bioavailability (of metals) in earthworms allowing comparison of results with those in the literature.

Aporrectodea caliginosa was initially considered as a test species for most of the laboratory trials because it is one of the most widespread soil-dwelling earthworms in many parts of the world including South Africa, for which a handful of ecotoxicity data are available. However, due to its slow breeding rate and the requirement of a large number of test animals for the present study, it was dropped in favour of *E. fetida*, the standard laboratory species. This species has been cultured in the ecotoxicology laboratory of the Department of Botany and Zoology, University of Stellenbosch since 1992. However, relevant results of the laboratory study were validated in the field with *A. caliginosa.*

1.11 THE CHOICE OF METALS FOR THE PRESENT STUDY

The present study is concerned with the influence of salinity and clay content on metal bioavailability which has direct bearing on agricultural lands in South Africa and other places in the world. Copper and zinc were chosen for the present study because Cu and Zn containing fungicides are used extensively on agricultural farms in South Africa and elsewhere. Copper oxychloride is widely used in vineyards in South Africa (London and Meyers, 1995) and Australia (Van-Zwieten et al., 2004). Although zinc contamination more often comes through smelting and mining activities, zinc fungicides are used in a few countries. For example, copper zinc

chromates is used in Australia (Van-Zwieten et al., 2004) while Mancozeb (containing 20% Mn and 2.5 % Zn) is used extensively by deciduous farmers in Western Cape region of South Africa (Reinecke et al., 2002). Since these fungicides are directly sprayed on crops to attack pests, they contribute significantly to soil contamination with copper (Malkomes, 1997) and zinc. Maboeta et al. (2003) reported that soil copper concentrations are elevated immediately after spraying with copper oxychloride in their experimental plots.

Agricultural lands are thus a major sink for these contaminants which may affect beneficial soil organisms (Van Gestel and Van Brummelen, 1996). Besides, some of these contaminants are washed down to nearby streams and river bodies leading to water pollution and detrimental effect to aquatic (Hodgkin and Hamilton, 1993) and intertidal organisms. Reinecke and Reinecke (2007) reported that pesticides could be transported to non target areas and water bodies by wind and run off. These pollutants can also accumulate in soil organisms (Helling et al., 2000; Snyman et al., 2000, 2002) causing stress. Once accumulated by soil invertebrates, they can be transferred in food chains, and so may end up in the diet of humans.

Despite the fact that Cu and Zn are essential metals, at high dosage, they become toxic to soil animals at elevated levels. Although Cu and Zn are not as toxic as Cd and Pb, at elevated level they have been found to be toxic (Spear, 1981) to soil organisms and humans. Prolonged consumption of large doses of Zn can result in some health complications such as fatigue, dizziness, and neutropenia (Hess and Schmid, 2002). Copper and zinc are essential because they are required for some metabolic activities in organisms and are most often found associated together in agricultural lands. However, they are likely to have different modes of action in organisms since they different roles in biochemical processes and so may not follow concentration addition model. Also, from previous studies, the kinetics of the two metals showed an early steady state response (Peijnenburg et al., 1999b), allowing for practicability of the experimental design. In this study, interest in these metals thus lies between essentiality and possible toxicity when present at elevated levels.

1.13 OUTLINE OF THE THESIS

This thesis aimed to assess how clay content and salinity influences the partitioning of Cu and Zn in soil as well as their uptake and toxicity to soil organism. The organisms used are described in Chapter 2, with a generalized discussion of the materials and methods used.

Chapter 3 and 4 describes how clay content influences the bioavailability of Zn and Cu to the earthworm Eisenia fetida using OECD soil. The results showed that despite both metals being essential metals, the influence of clay content was not essentially the same. The sections also highlighted the relevance of DTPA and CaCl2 extraction methods as surrogate measures of bioavailability for the two metals.

In Chapter 5 and 6, the influence of salinity on the bioavailability of Zn and Cu to the earthworm *E. fetida*, was studied using OECD soil. This was by done by using single and joint substance toxicity testing. NaCl as well as Cu and Zn were shown to individually affect life-cycle parameters in the earthworm. Joint effect of the metals and NaCl salt was either additive (for Cu) or synergistic (for Zn).

In Chapter 7, representatives of soil organism (*Folsomia candida*, *Enchytraeus doerjesi, Eisenia fetida* and *Aporrectodea caliginosa*) were exposed in laboratory studies to natural saline soils with the view to compare their sensitivity. The earthworm species were the most sensitive. Generally, the data showed that soil organisms are more sensitive than plants to saline stress.

In Chapter 8, since we found that the two earthworm species used in Chapter 7 were more sensitive than the Collembola and enchytraeid species, we studied their avoidance behaviour in natural and artificial saline soils. *A. caliginosa* was more sensitive than *E. fetida* in both types of soil, and probably would be useful as a species for possible screening of soils contaminated with salts.

In Chapter 9, an attempt was made to validate the results of Chapter 6, where we found additive interaction between Cu and salinity. The study was carried out with field microcosms using *A. caliginosa* and natural soil as opposed to *E. fetida* and OECD soil used in the laboratory study. Rainfall caused leaching of salts and Cu and

appeared to be the overriding factor. Although the study could not clearly assess the interaction of Cu and salinity in the field, it provided evidence of the effect of flooding on survival of *A. caliginosa*.

Chapter 10 provides a general conclusion of this thesis. An overview is given of the main findings of the laboratory and field studies. These were put in the context of environmental management and conservation of soil biodiversity. This information may assist decision makers and farmers on the likely dangers of agricultural practices that could increase salt contents of soils.

CHAPTER TWO

2.0 GENERAL MATERIALS AND METHODS

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

2.1 STUDY ANIMALS

E. fetida was used for most laboratory studies (Chapter 3-6, 9). Together with *E. fetida*, *A. caliginosa*, *Folsomia candida* and *Enchytraeus doerjesi* were used in Chapter 7. In chapter 8, *E. fetida*, and *A. caliginosa* were used.

2.1.1 *Eisenia fetida* Savigny 1826

2.1.1.1 Classification of *Eisenia fetida*

According to Storch and Welsch (1977), the classification of *E. fetida* is as follows:

Phylum: Annelida
Class: Clitellata
Subclass: Oligochaeta
Order: Opisthopora
Suborder: Lumbricida
Superfamily: Lumbricoidea
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: *Eisenia*
Species: *E. fetida*

2.1.1.2 Biology of *Eisenia fetida*

E. fetida is about 60-120 mm in length, with a diameter of 3-6 mm, and a segment of 80-120. The body is cylindrical but trapezoidal at the posterior end. It is hermaphroditic and reproduces sexually. Within four days of mating, cocoon

production begins (Venter and Reinecke, 1988). After reaching sexual maturity, each worm may produce 2-5 cocoons weekly (Edwards and Bohlen, 1992) which incubate for ± 23 days before hatching (Venter and Reinecke, 1988). Each cocoon produces ± 3 hatchlings. The species takes 7-8 weeks to complete its life cycle (Venter and Reinecke, 1988; Edwards and Bohlen, 1992) and individuals may live up to five years (Reynolds, 1977). It was found in the Palaeartic region, seldom above 1000 m but is now widespread in other temperate regions of the world, usually associated with cultivation, especially around major cities. It occurs only sporadically in the tropics, presumably from repeated re-introductions since populations seldom approach the density of those of other earthworm families and usually fail (Sims and Gerard, 1985).

2.1.2 *Aporrectodea caliginosa* **Savigny** 1826

2.1.2.1 Classification of *Aporrectodea caliginosa*

According to Sims and Gerard (1985), the classification of *A. caliginosa* is as follows:

Phylum: Annelida
Class: Clitellata
Subclass: Oligochaeta
Order: Opisthopora
Suborder: Lumbricida
Superfamily: Lumbricoidea
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: *Aporrectodea*
Species: *A. caliginosa*

2.1.2.2 Biology of *Aporrectodea caliginosa*

This is an endogeic earthworm species, which characteristically constructs burrows in highly mineralized soil horizons and mainly feeds on organic-rich soils. It is weakly pigmented and varies in size from 40-180 mm, in diameter from 3.5-7 mm and comprises between 120-146 segments (Sims and Gerard, 1985). This species can enter obligatory or facultative diapause under adverse environmental conditions, such

as drought or flooding (Sims and Gerard, 1985). Although it was initially known to occur in the western Palaearctic and eastern Nearctic, they were introduced into other temperate and tropical regions of the world mainly in cultivation, becoming extinct where native species have become locally extinct (Sims and Gerard, 1985). It is therefore widely distributed.

The life-cycle of this species is not as well studied as in *E. fetida*. *A. caliginosa* grows into a clitellate adult between 17-19 weeks (Graff, 1953 cited by Maleri, 2006). Clitellum development can however take as much as or more than 35 weeks. *A. caliginosa* produces between three and 27 cocoons per year (Von Wilcke, 1956; Satchell, 1967, cited by Maleri, 2006). The incubation time of the cocoon varies widely depending on the suitability of environmental factors like temperature and moisture. El Duweini and Ghabour (1965) reported an incubation period of 45-50 days at 20^0C, while Holmstrup et al. (1991) reported 62-84 days at 15^0C.

2.1.3 *Enchytraeus doerjesi* Westheide and Graefe, 1992

2.1.3.1 Classification of *Enchytraeus doerjesi*

According to NCBI (2008), the classification of *E. doerjesi* is as follows:

Phylum: Annelida
Class: Oligochaeta
Subclass: Tubificata
Order: Haplotaxida
Suborder: Tubificina
Superfamily: Enchytraeoidea
Family: Enchytraeidae
Genus: *Enchytraeus* (Henle, 1837)
Species: *E. doerjesi* (Westheide and Graefe 1992)

2.1.3.2 Biology of *Enchytraeus doerjesi*

The biology of this animal has not been studied extensively. The only information available at the moment is that of the authors who described this species. This animal ranges in length from 4-7 mm. Mean number of segments in hatched

juveniles is 18.4 while that in adults is 37. Length of time of embryological development at 21^0C is about 6.8 days while length of time from hatching to maturity is about 8.5 days. Total life span is between 63-95 days (Westheide and Graefe, 1992). Mean number of eggs in a cocoon at 21^0C is 5.1 with a range of one to 10 while up to a mean of 4.3 eggs could be produced per day (Westheide and Graefe, 1992)

2.1.5 *Folsomia candida* Willem 1902

2.1.5.1 Classification of *Folsomia candida*

According to Westheide and Graefe (1992), the classification of *F. candida* is as follows:

Phylum: Arthropoda
Class: Insecta
Subclass: Archaeognatha
Order: Collembola
Suborder: Arthropleona
Superfamily: Entomobryoidea
Family: Isotomidae
Subfamily: Proisotominae
Genus: *Folsomia*
Species: *F. candida*

2.1.5.2 Biology of *Folsomia candida*

This species is 1.5 to 3.0 mm in length at maturity. It is white or faintly yellowish in colour, and does not bear ocelli. There is a post-antennal organ behind the base of each antenna that probably detects airborne chemicals (Hopkin, 1997). Like all other collembolans, *F. candida* has a pair of thin-walled, closely apposed, eversible vesicles on the ventral side of the first abdominal segment. This structure is commonly known as the ventral tube, or collophore, and is involved in fluid exchange with the external environment. The ventral tube is an important exposure route for chemicals dissolved in soil pore water (Lock and Janssen, 2003a). The most distinguishing feature that separates *F. candida* from other members of the genus is

the presence of numerous (at least 16) stout setae on the ventral side of the manubrium of the furca.

Populations of *F. candida* consist exclusively of parthenogenetic females. At 20^0C, they take between 21 and 24 days to reach the sixth, or adult, instar when they are sexually mature. About 30 to 50 eggs are laid in each batch, which take seven to 10 days to hatch. The eggs are white, spherical, and 80 to 110 μm in diameter. Eggs maintained above 28^0C fail to hatch. The optimal temperature for hatching success is 21^0C (Fountain and Hopkin, 2005). At lower temperatures, the time span for each developmental stage is extended. An adult female may go through 45 molts in her lifetime with short reproductive instars (duration 1.5 days) alternating with longer non reproductive instars (duration 8.5 days) (Axelsen et al., 1997).

2.2 WORM PARAMETERS

In this study the following parameters were used as toxicological endpoints for the organisms used. They are weight change, mortality, cocoon production and internal metal concentration. The methods decribing how these were done are as included in the relevant chapters.

2.3 SOIL PARAMETERS

Soil parameters of interest in this study are pH, moisture, electrical conductivity, $CaCl_2$, DTPA and nitric acid extractable metal contents. These parameters were anlysed using standardized protccols which are further explained in the relevant chapters. The metal content of earthworm and soil was calculated by the following formulars:

Amount in worm (mg/kg) = {AAS reading * volume of sample (mL)}/mass of worm (g)

Amount in soil (mg/kg) = {AAS reading * volume of sample (mL)}/mass of soil (g)

CHAPTER THREE

3.0 ROLE OF CLAY CONTENT IN PARTITIONING, UPTAKE AND TOXICITY OF ZINC IN THE EARTHWORM *EISENIA FETIDA*

3.1 INTRODUCTION

Risk assessment and management of soils require accurate predictions of the bioavailability of contaminants in the soil matrix, because their toxicity is mainly a function of their bioavailability (Chapman and Wang, 2000). The bioavailability of toxicants in soil is increasingly used as key indicator of potential risks that contaminants pose to both the biological environment and to human health. Although risk assessment of metals is currently based on total metal concentration, this does not reflect the true uptake of metals by soil organisms (Alexander, 2000; Adriano, 2001). This is particularly important because estimates of risk based on total metal concentration will lead to overprotection or forceful remediation of soil which is very expensive.

A dynamic approach to understanding bioavailability in soil has been suggested which should comprise at least two distinct phases; the physicochemically driven desorption process, determining the environmental availability and a physiologically driven uptake and depuration process, determining the true bioavailability (McCarty and Mackay, 1993; Peijnenburg et al., 1997, 1999a). Bioavailability of metals in soil has been reported to be dependent on soil type, metal species, metal kinetics and age of the metal in the soil (Beeby, 1993; Van Gestel et al., 1995). The bioavailability and/or toxicity of metals in soil can vary over several orders of magnitude depending on soil modifying factors (van Gestel and van Dis, 1988; Lock et al., 2000). The effect of soil types on the bioavailability and toxicity of metals to soil organisms has been studied to a large extent. Most of these studies (Peijnenburg et al., 1999a, Lock et al., 2000 Lock and Janssen 2001a,c) reported that pH and cation exchange capacity (CEC) are the most important factors while others (Amorim et al., 2005a) reported a slightly different pattern. However, Van Gestel et al., (1995) noted that for each metal type, different combinations of soil parameters seemed to govern bioavailability.

Several soil properties were dealt with together in most of these studies. Many of these soil properties are auto-correlated to various degrees, and it is often difficult to distinguish the actual contribution of distinct factors (Basta et al., 1993) and to interpret results (Römbke et al., 2006). Although this approach may give relevant information on the combined effect of these properties, it would still mask individual roles of each soil parameter. Clay minerals are generally regarded as important natural ion exchange materials because they are generally coated with metal oxides (Jenne, 1988) and organic matter (Hart, 1982; Davis, 1984) providing surface characteristics that are important in the exchange of trace metals. Clay, because of its small particle size, has a large surface area per unit weight and exhibits surface charges that attract negatively and positively charged ions in water (Brady, 1990). Because of these properties, clay is believed to be a good adsorbent for heavy metal removal from water and waste water (Sajidu et al., 2006) and possibly from the liquid phase in soil. Soils high in clay minerals are believed to have a higher concentration of most trace metals than low-clayed soils (McBride, 1994). How this relates to the bioavailability of such metals needs to be quantified.

The normalization of toxicity data in some countries is still based on the clay and organic matter (OM) content. For example, the Dutch correction factor is based on normalizing toxicity data to 25% clay and 10% OM (Van Straalen and Denneman, 1989). Also, internationally agreed standardized soil is made up of artificial soil with a clay content of 20% (OECD 1984; 2004). Toxicity data for most pollutants are derived in laboratory tests with this soil constitution. However, in nature, clay content of soil may be less or more than 20% or 25% and hence the toxicity of the pollutants may vary depending on the clay content. If it can be shown that clay is a poor predictor of toxicity, then the inclusion of clay in the normalization of toxicity data, as it is presently done, should be questioned.

This study was therefore conducted with the aim of assessing the influence of clay content (keeping other factors as constant as possible) on the bioavailability of zinc to the earthworm *Eisenia fetida*. This species was chosen because it is easy to culture and there are relatively large data sets available in the literature on the biology and ecotoxicology of this species. Zinc was chosen because it has significant effects on earthworm growth, maturation rate and reproduction (Reinecke and Reinecke,

1996; Reinecke et al., 1997) and also because it is an important metal, causing toxicity when a cocktail of metals is present (Spurgeon and Hopkin, 1995; 1996b). The specific objectives were to assess the influence of clay content on the uptake of zinc in the body of the worms and also its effects on selected life-cycle parameters (mortality, growth, maturation, and reproduction). Another objective was to assess the influence of clay on zinc sorption by determining the fraction extractable by different chemical extraction methods and relating these to the observed biological responses.

3.2 MATERIALS AND METHODS

3.2.1 Test species

Specimens of *E. fetida* used for this study were age-synchronized from a culture kept in the laboratory of the Ecotoxicology Group, University of Stellenbosch, South Africa since 1992. Pre-clitellate worms between 6-8 weeks old were used in the experiments. The worms were acclimatized for at least 48 hours in Organisation for Economic Co-operation and Development (OECD) artificial soil prior to exposure (OECD, 2004)

3.2.2 Choice of clay type

At the beginning of this experiment, kaolin clay and bentonite, a type of montmorillonite clay, were obtained from Serina Trading Company, Cape Town, South Africa. These clay types were used to create an artificial soil system, as described in the OECD guidelines (1984; 2004). Two other treatment soils with each clay type were prepared by adjusting the clay contents to 5 and 40% clay, while the proportion of sand was adjusted based on the clay content. The pH, water holding capacity (WHC) and CEC of these soils were assessed. The pH-H_20 and WHC of these soils were determined according to OECD (2004) and ISO (1996) respectively. CEC was determined with the AgTu method in 0.4 M ammonium acetate at pH 7 (Chhabra et al., 1975). The pH and CEC of the substrates are shown in Table 1.

Table 1: The pH, water holding capacity (WHC) and cation exchange capacity (CEC) of artificial soils prepared with two clay types before pH adjustment. These parameters were measured after three days of soil preparation

Clay type	Clay content (%)	Organic matter (%)	pH	WHC (%)	CEC (mmol/kg)
Kaolin	5	10	3.61	57	128
	20	10	3.73	65	148
	40	10	3.94	84	157
Montmorillonite	5	10	5.45	59	121
	20	10	6.80	92	182
	40	10	8.41	135	233

Changes in the kaolin clay content increased the pH of the soil only by 0.33 units. Changes in the montmorillonite clay content by the same magnitude increased the pH of the soil to close to 3.0 pH units (Table 1). pH is the pre-eminent factor affecting the bioavailability of metals in soil (Impelliteri et al., 2003) and plays important roles in metal fraction distribution patterns (Lu et al., 2005). In order to adjust the pH of the montmorillonite clay group, it would be necessary to add $CaC0_3$ to one or two of the soil groups. This would however influence the bioavailability of the metal in the treatments receiving calcium supplement. Calcium can influence metal sorption to soil and uptake by soil animals (Kiekens, 1990; Kiewet and Ma, 1991). As clay content is the only variable of interest in this study, use of kaolin clay was therefore more appropriate.

3.2.3 Test soil, test procedures and experimental setup

The test soils consisted of OECD artificial soil. The first substrate type was prepared by mixing 10% finely ground (< 1 mm) sphagnum peat, 20% kaolin clay and 70% quartz sand (by dry weight), adjusted with $CaC0_3$ to pH 6.0 ± 0.5 (OECD 1984; 2004). The other two treatment soils were prepared as above except that the clay contents were adjusted to 5 and 40%. The test substance zinc chloride ($ZnCl_2$; purity,

98%; molecular weight =136.28) Merck, South Africa) was added as aqueous solution in the following concentrations (0, 250, 500, 750, 1000 mg/kg Zn) so as to make up 60% of the final water holding capacity of each treatment soil. Each treatment was replicated four times.

The treatment consisting of 500 g of soil was placed in a cylindrical plastic vessel of 2 l and allowed to equilibrate for two days before earthworms were introduced. Ten worms per container were used in each exposure regime and were introduced into the relevant test soil by placing them on the surface and allowing them to burrow in. The test containers were covered with a perforated lid to limit water loss due to evaporation and kept in 16 h light, 8 h dark at 20^0C (Reinecke and Kriel, 1981) in a climate chamber for 28 days. To maintain the worms during the exposure period in the test medium, the worm in each container was fed weekly with dried urine-free cattle manure ground and sieved (1mm) to provide 0.5 g per worm. Foods that were not eaten were removed on each sampling occasion to avoid build up of organic matter in the substrates. Sampling was done on day 7, 14 and 28 after worms were introduced into the substrates.

3.2.3.1 Soil parameters

Soil moisture content and pH were monitored on each sampling occasion. pH-H_20 was determined as described earlier. Soil moisture was determined by analyzing 5 g of sample with a Sartorius infrared moisture detector. Soil moisture content was kept constant by replacing the water loss with deionized water after each sampling. CEC was determined at day 1 and 28 as already described. For the determination of zinc contents of substrates, chemical extractions were carried out using three methods: $CaCl_2$, DTPA (di-ethylene-triamine-pentaacetic acid) and nitric acid extraction methods. For $CaCl_2$ and DTPA extractions, samples were taken at day 1 and 28 and a sequential procedure was followed according to those used by Maiz et al. (1997) and others. From each sample, 3 g of soil was shaken with 30 ml solution of 0.01 M $CaCl_2$ for 2 h prior to centrifugation at 4000 rpm for 15 min, the supernatant was removed for the determination of $CaCl_2$ extract. For the DTPA extraction, the residue was shaken with 6 ml solution of DTPA for 2 h prior to centrifugation at 3000 rpm for 10 minutes. The supernatant in both cases was filtered through Whatman 540 filter paper, acidified to 5% HNO_3 and stored at -4^0C until analysis by atomic absorption

spectrometry. Total zinc content was determined at day 1 and 28 by acid digestion as described by Maboeta et al. (2003).

3.2.3.2 Worm parameters

On each sampling occasion, growth, mortality, and internal zinc concentration (IZC) of worms were assessed while maturation rate and cocoon production were assessed only at day 28. Growth was determined by individually weighing each worm in each container, and comparing the mean weight with initial values. Mortality was assessed by stimulating the worm with a blunt probe and the earthworm was judged dead if no response could be observed. Worms not found during sampling was judged dead since earthworm tissue decompose easily in soil when dead. Cocoon production was determined by wet sieving the substrates (through a 2.0- and 1.0-mm sieve system). Cocoon number per worm was calculated by dividing the total number of cocoons by the number of surviving worms. It is assumed that a worm that died during the exposure couldn't have contributed to cocoon production since concentration of toxicants affecting cocoon production is often much lower than that affecting mortality. The net number of surviving worms in each container of the control soils (receiving 0 mg/ kg Zn) at the end of the test was eight, since two worms (one at day 7 and another at day 14) were removed for analysis of internal zinc concentration. This reduction in sample size (eight worms from the original 40) was accounted for in calculating cocoon production. Percentage of clitellate worms were determined by observing each worm for the absence or presence of clitellum.

To determine the IZC, whole worms (one per container except at day 28 when two were used) were randomly removed on each sampling occasion. They were placed in Petri dishes on moist filter paper for 24 hrs at 20^0C to allow depuration of their gut contents. Afterwards, they were frozen individually for metal analysis. The procedure for the metal extraction and analysis has been described by Maboeta et al. (2003). This was done by acid digestion with HNO_3 and spectrophotometric analysis.

3.2.4 Zinc analysis

The extracted soil and worm samples were analyzed for zinc by a Varian AA-1275 flame atomic absorption spectrophotometer (AAS). The AAS was calibrated by

5, 10 and 25 mg/L zinc standards. When samples were over-ranged, a serial dilution was made and samples were then analyzed. Previously spiked soil samples were analyzed and indicated a recovery above 90% with this procedure. For each batch of worm and soil digested, a blank was also prepared to detect possible contamination during the digestion process. Precision data were as follows: for 0.01 M $CaCl_2$ extract, average percent relative standard deviation (% RSD) = 2.0; for DTPA, average % RSD = 2.4 while % RSD for nitric acid extraction in worm and soil were 5.2 and 3.6 respectively.

3.2.5 Statistics

All data were checked for normality and homogeneity of variance with the Shapiro-Wilks W test and Levene's test respectively. For zinc concentrations in worm and in soil (nitric acid, DTPA and $CaCl_2$ extracts), one-way ANOVA was used to test for the effect of clay content along each nominal zinc concentration of substrates. When data were not normally distributed even after transformations, Kruskal-Wallis H-test was used. For the results of worm parameters (weight per sampling date, pooled mortality data, % clitellate and cocoon production), two-way factorial ANOVA was used when data were normally distributed and bootstrap statistics was used when data were not normally distributed even after transformations. Fischer's least significant difference (LSD) post-hoc test was used to determine the significance of differences in parametric cases while multiple comparison of 'z' and 'p' values was used in non parametric cases. Correlations between metals extracted by the three extraction methods and all worm parameters, and between worm IZCs concentrations and other endpoints in worm, and between clay and CEC were analyzed using Spearman-Rank correlation. Tests of similarity between DTPA zinc extracts of substrate at day 1 and 28 and $CaCl_2$ zinc extracts of substrates at day 1 and 28 were analyzed with Wilcoxon sign-paired test. All statistics were analyzed with STATISTICA 7.0.

3.3 RESULTS

3.3.1 Soil properties

The mean pH values of the unspiked treatments at the start of the experiment were 5.90, 6.04 and 6.24 in the 5%, 20% and 40% clay respectively. A decrease in pH with increasing Zn concentrations was observed for all soil groups. However, these pH decreases were in all instances below 0.5 units. pH increased during the 28-day exposure in all treatment groups. The mean increase was 0.42, 0.44 and 0.31 in the 5%, 20% and 40% clay respectively. At day 1, the CEC of the substrates increased with an increase in clay contents. The mean CEC values were 128 mmol/kg, 148 mmol/kg, and 157 mmol/kg in the 5%, 20% and 40% clay substrates respectively. At day 28, the CEC of the substrates increased slightly in all treatments. The mean CEC values were 140, 158 and 168 mmol/kg in the 5%, 20% and 40% clay respectively. Correlation between clay and CEC was significant at day 1 ($r = 0.949$, $P < 0.05$) and day 28 ($r = 0.956$, $P < 0.05$).

3.3.2 Total and available metals

The fractions of the total metal extracted at day 1 and 28 respectively by DTPA (6-93%) and (13-97%) were greater than those extracted by $CaCl_2$ (0-38%) and (0-27%), irrespective of the clay regimes. The correlation values computed between DTPA as well as $CaCl_2$ extracted zinc at day 1 and 28 and nitric acid extracted zinc were very strong ($r \geq 0.90$, $P < 0.05$). Generally, for nitric acid extracts at day 1 and 28, no significant effect ($P > 0.05$) of clay content was found for all zinc concentrations (Fig. 1).

For DTPA and $CaCl_2$ extracts at day 1, although there were a few significant effect of clay on Zn extractability, this did not follow a discernible pattern. At day 28, amounts of Zn extracted with DTPA in the 5% clay regimes were not significantly different ($P > 0.05$) at all nominal Zn concentrations. For $CaCl_2$ extraction at day 28, significantly higher amounts ($P < 0.05$) were extracted in the 5% clay than in 20 and 40% clay at 1000 mg/kg nominal Zn concentration while an opposite trend was seen at lower Zn concentrations.

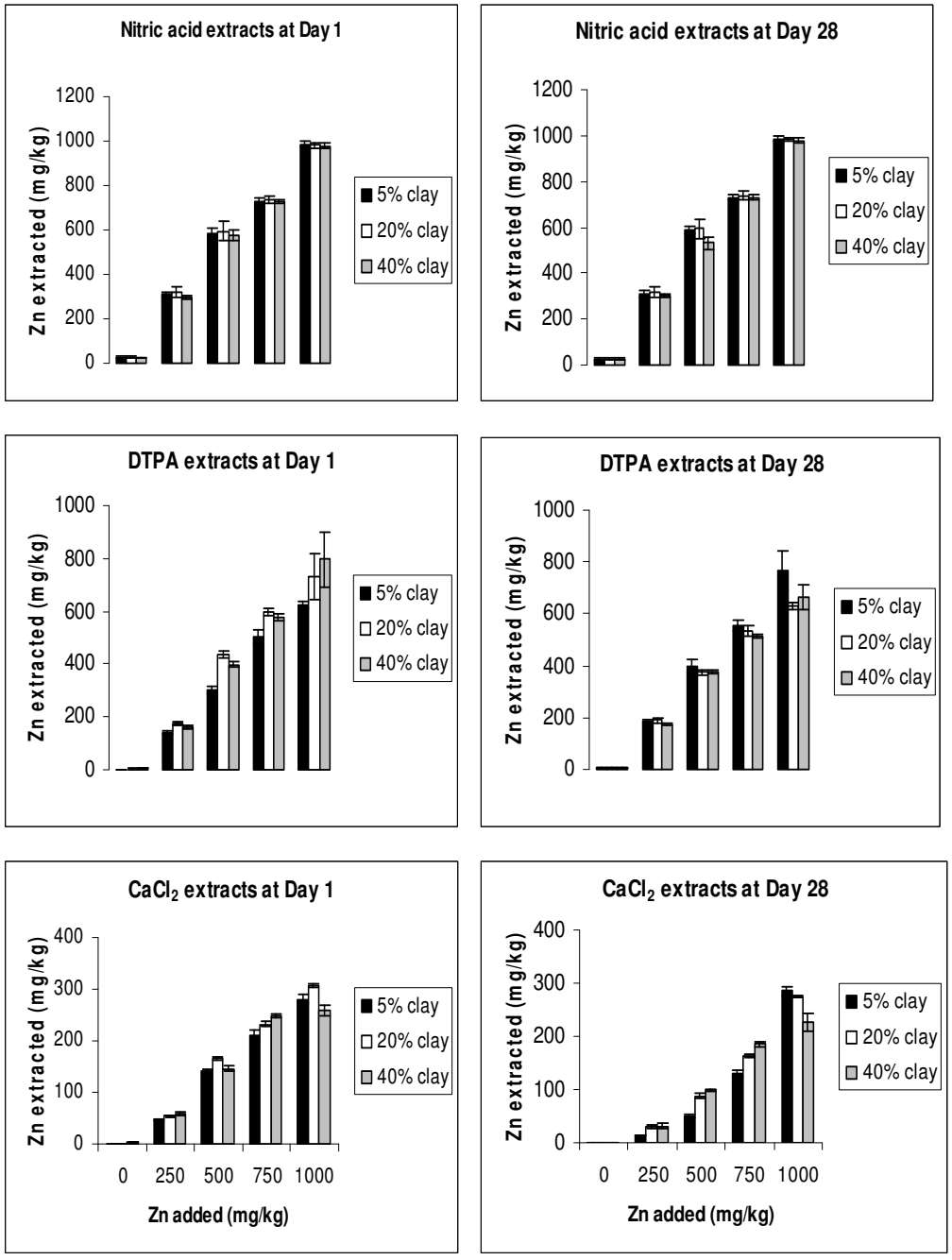

Fig. 1. The amounts of zinc extracted on day 1 and day 28 from differently spiked substrates using different extraction methods. Specimens of *Eisenia fetida* were exposed in these substrates for 28 days to the range of zinc concentrations under three clay regimes (error bars indicate standard error of mean).

3.3.3 Mortality, growth and reproduction

All mortalities occurred at days 7 or 14 and only at the higher Zn concentrations (750 and 1000 mg/kg Zn). Generally, mortality in these Zn concentrations increased as the clay level decreased (Fig. 2). Clay and Zn only had a significant effect ($P < 0.05$) on mortality at 1000 mg/kg Zn (Appendix 1). At this concentration, mortality at 5% clay was significantly ($P < 0.01$) higher than at other clay regimes, and that at the 20% clay was significantly higher ($P < 0.05$) than at the 40% clay regime.

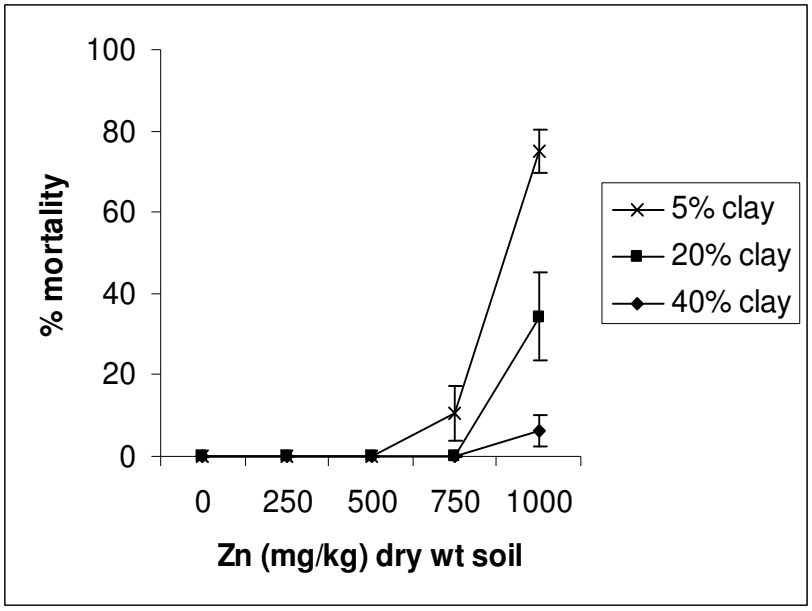

Fig. 2. Mean percentage mortality (pooled) during 28 days, of four groups, each consisting of ten pre-clitellate worms (*Eisenia fetida*), exposed to zinc under three different clay regimes at constant temperature (20^0C) and fairly constant soil moisture (error bars represent standard error)

Figure 3 shows the mean weight of worms exposed to zinc for 28 days under the three clay regimes. The mean weights of worms exposed in 250 mg/kg Zn substrates were not shown since they were essentially similar to those in unspiked substrates. There was an increase in mean weight of worms exposed to 0, 250 and 500 mg/kg Zn for all clay regimes.

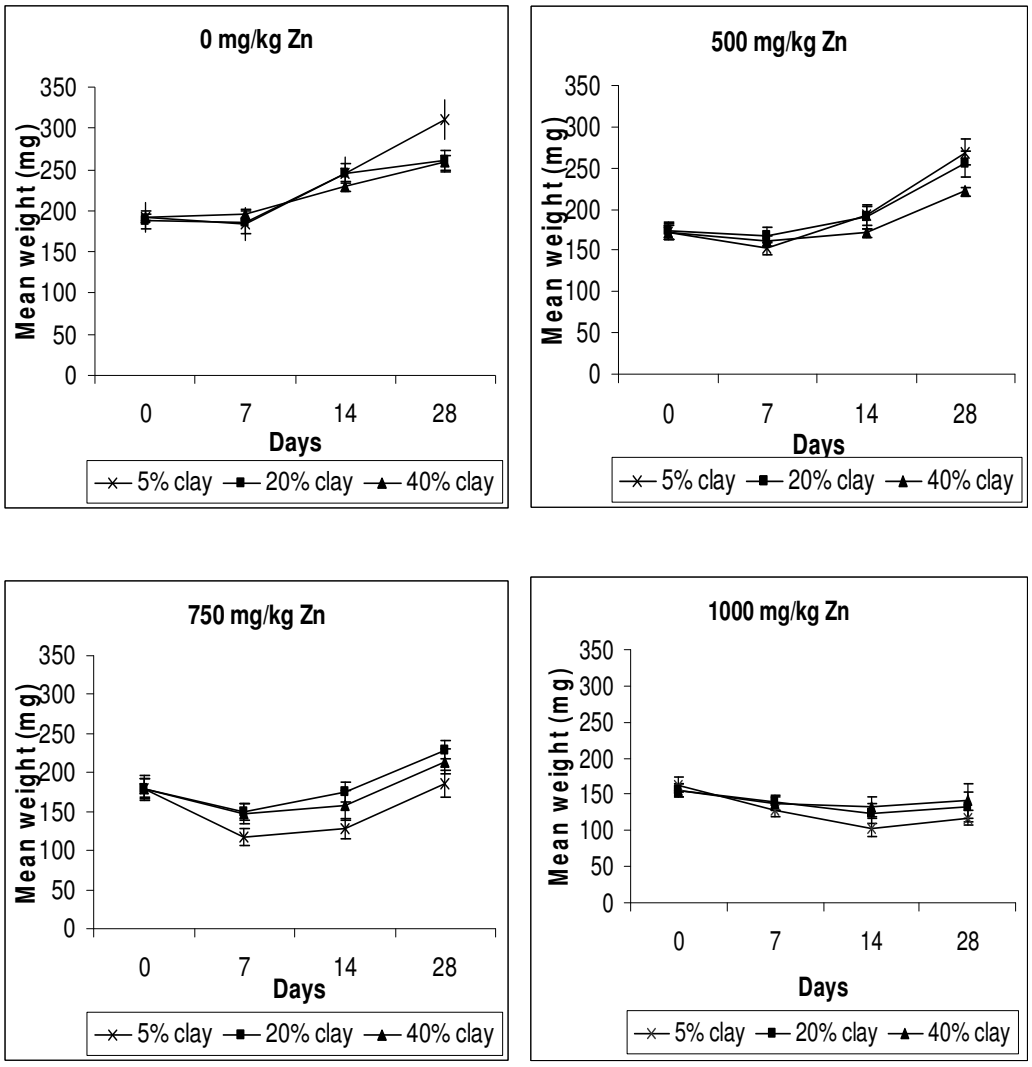

Fig. 3. Change in mean weight over time of four groups, each consisting of ten pre-clitellate worms (*Eisenia fetida*), exposed to a control and four zinc concentrations of OECD substrates adjusted to three clay regimes at constant temperature (20^0C) and fairly constant soil moisture (error bars represent standard error).

Those exposed to 750 mg/kg Zn had a fairly constant weight while those exposed to 1000 mg/kg Zn had a decrease in mean weight irrespective of clay content of substrates. Statistical analysis showed that interaction of clay and zinc on weight of worms was not significant (Appendix 2). Individually, clay had no significant influence on weight change while zinc showed significant influence ($P < 0.01$) on the weight of the worms. The significant differences (LSD) were demonstrated at day 14 and 28. At these days weight of worms in substrate concentrations of 750 and 1000 mg/kg Zn were significantly lower ($P < 0.01$) than those in the unspiked treatments (0 mg/kg Zn)

There was a clear dose-response relationship between zinc and cocoon production for all clay regimes (Fig. 4). No cocoons were produced at 1000 mg/kg Zn in all clay regimes and at 750mg/kg Zn in 5% and 20% clay regimes. The interaction of clay and zinc had no significant effect ($P > 0.05$) on cocoon numbers, but individually, clay and zinc had significant effects ($P < 0.05$) (Appendix 3). The mean number of cocoons produced was significantly higher in the 40% clay than the 5% clay, but cocoon production in both 40% and 20% on one hand and 20% and 5% on the other hand were not significantly different ($P > 0.05$). For zinc, the number of cocoons produced was significantly lower in worms exposed to either 500 or 750 mg/kg Zn when compared to unspiked treatments (0 mg/kg Zn) which was not significantly different from those exposed to 250 mg/kg Zn.

The results for clitella development also showed a dose-related response curve for zinc (Fig. 5). All the worms in all clay regimes became clitellate at 0 and 250 mg/kg Zn while none was clitellate at 1000 mg/kg Zn. Statistical analysis showed that interaction of clay and zinc had no significant effect ($P > 0.05$) on % of clitellate worms at all exposure concentrations (Appendix 4). Individually, the presence of clay had no significant effect on the % of worm becoming clitellate, but zinc had significant effect on % clitellate in the order: 0 = 250 > 500 = 750 > 1000 mg/kg ($P < 0.05$).

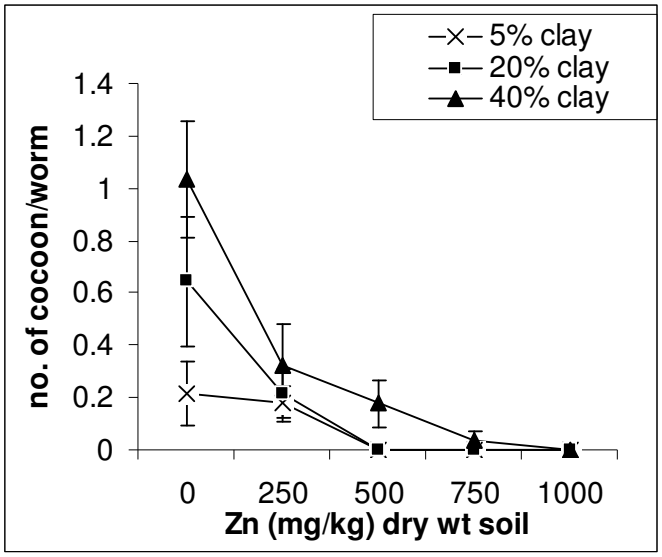

Fig 4. Mean number of cocoons/ worm (*Eisenia fetida*) produced after four weeks of exposure of four groups, each consisting of ten pre-clitellate worms, to zinc under three clay regimes at constant temperature (20^0C) and fairly constant soil moisture. (Error bars represent standard error)

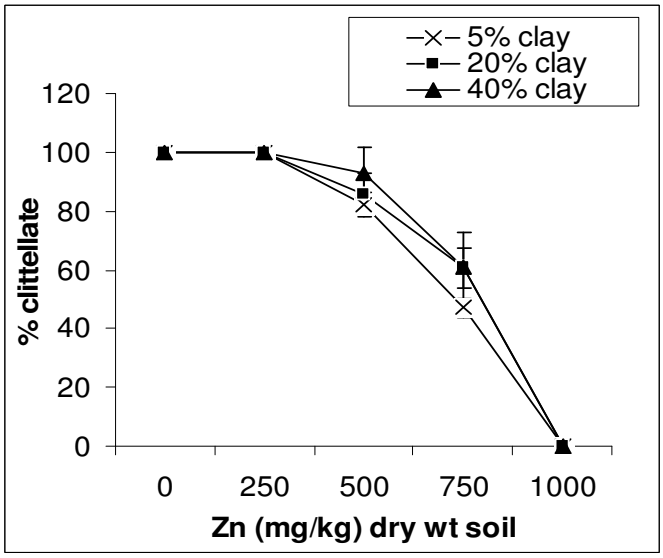

Fig 5. Mean percentage of four groups, each consisting of ten pre-clitellate worms (*Eisenia fetida*) reaching maturity after being exposed for four weeks to zinc under different clay regimes at constant temperature (20^0C) and fairly constant soil moisture (Error bars represent standard error).

3.3.4 Internal zinc concentrations in worms

There appeared to be fast uptake of zinc reaching the highest IZC level by day 7, after which values decreased until they were, by day 28, comparable with day 0 values (Fig. 6). The interaction of clay and zinc on IZC of the worms was not significant ($P > 0.05$) (Appendix 5). However, individually, the zinc levels of substrates showed highly significant differences (ANOVA, $P < 0.01$) in IZC at day 7 and 14. Post hoc analysis (LSD) showed that IZC of worms exposed to 750 mg/kg Zn (day 14, $P < 0.01$) and 1000 mg/kg Zn (day 7 and 14, $P < 0.01$) were higher than for worms in unspiked substrates (0 mg/kg Zn). Correlations between life cycle parameters and IZC of worms are presented in Table 2. Generally, weak correlations were found between all life cycle parameters and IZC. These correlations were weaker with increased time of exposure.

Table 2. Correlation coefficient (r) values between internal zinc concentration (IZC) and other worm parameters (weight, mortality, cocoon number and % clitellate worms) after exposure of *Eisenia fetida* to zinc under three clay regimes for 28 days (Numbers in brackets indicate the day data were taken).

Parameters	IZC (7)	IZC (14)	IZC (28)
weight (7)	- 0.429***		
Mortality (7)	0.364**		
weight (14)		- 0.412*	
Mortality (14)		0.307*	
weight (28)			- 0.325*
cocoon no (28)			- 0.416**
% clitellate (28)			- 0.497***
Mortality (28)			0.223

*significant at 0.05 confidence level **significant at 0.01 confidence level

***significant at 0.001 confidence level

3.3.5 Relationship between metal in worms, clay substrates and biological endpoints

Due to a large dataset obtained in this study, attempts were made to understand the relationship between Zn contents of substrates and worm parameters. For this reason, all soil and worm parameters in the different clay regimes were pooled. Values of nitric acid extractable zinc at day 28 were not included since these values were similar to day 1 values. Although the amount of zinc extracted with $CaCl_2$ correlated with most worm parameters slightly better than the amounts extracted in other ways, the correlation values computed were mostly within the same range (Table 3). The range was often below 0.1.

3.4. DISCUSSION

3.4.1 Zinc availability in substrates and its relationship with biological responses

The strong relationship between the amounts of zinc extracted respectively by $CaCl_2$, DTPA and nitric acid in our study, which was also reported by Dai et al. (2004), indicates that the available metals come from the same mineralogical sources. A decreasing availability of zinc in the liquid phase due to adsorption to clay particles was expected (Hobbelen et al., 2006), but this was not usually the case in this study. Although there appeared to be an increase in DTPA extractable zinc with reduced clay content by day 28, this pattern was not statistically significant at all zinc concentrations in the substrates. $CaCl_2$ extractable zinc in substrates showed significant differences among clay levels at most nominal zinc concentrations but the pattern was inconsistent. DTPA and $CaCl_2$ could not therefore explain the partitioning of zinc in this study. The lack of significant and consistent effect of clay content on partitioning of zinc observed in this study could be because zinc had a low adsorption capacity and a low affinity for kaolinite surface at pH 6.0 (Srivavastava et al., 2005). Moreover, Lock and Janssen (2003b) studied the influence of aging and soil properties on partitioning of zinc in 20 contaminated soils from Belgium. They found zinc partitioning, as shown by both pore water concentrations and $CaCl_2$ extracted

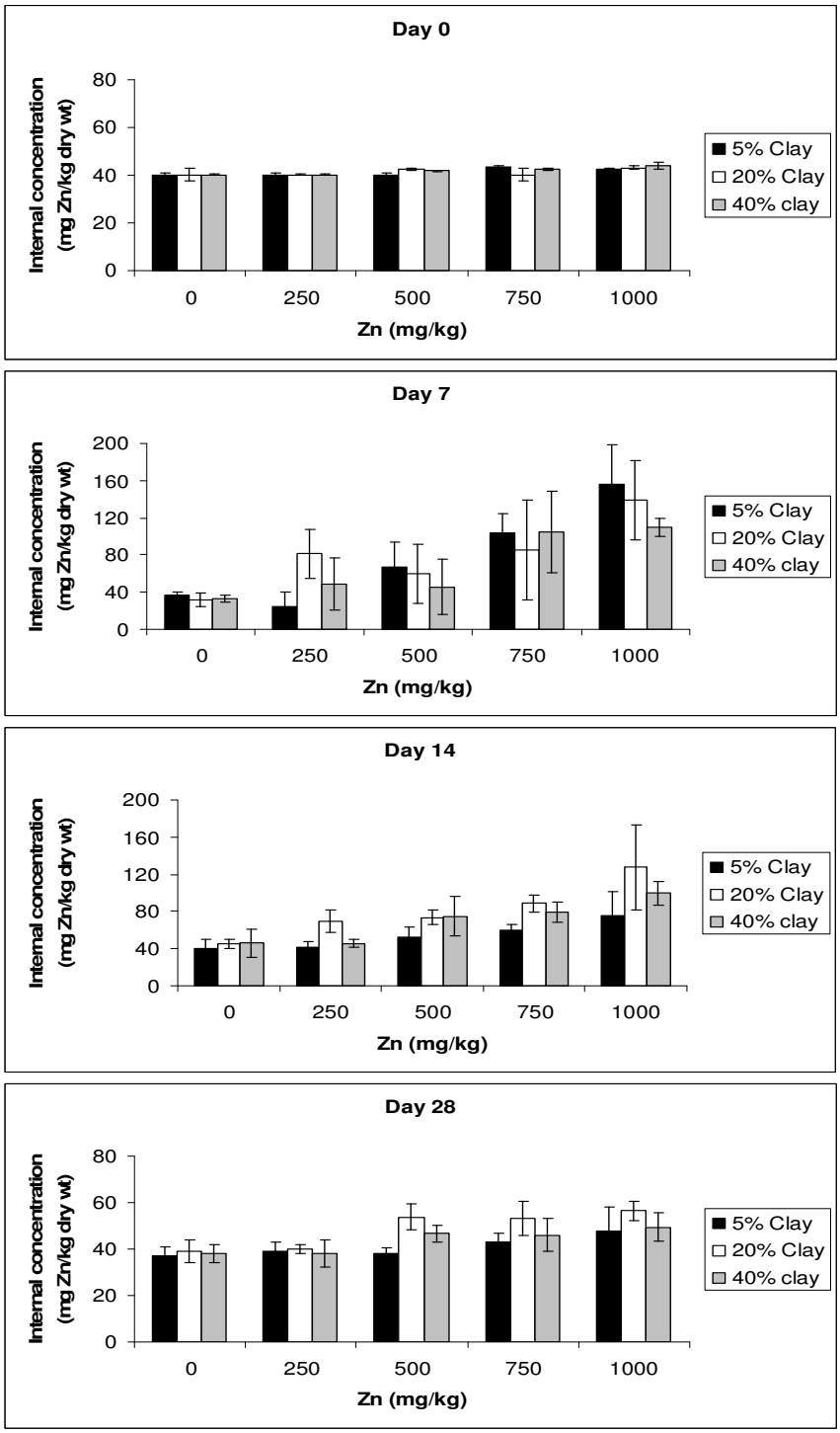

Fig 6. Mean zinc concentrations in specimens of *Eisenia fetida* after being exposed for 28 days in three clay regimes in an artificial soil system spiked with different concentrations of zinc (bars indicate mean of at least four worms and error bars indicate standard error of mean).

fractions, was significantly affected by pH whereas clay had no significant contribution.

Table 3. Correlation coefficient (r) values between amount of zinc extracted by three extraction methods ($CaCl_2$, DTPA and nitric acid extractions) and biological responses (weight, mortality, internal zinc concentration, cocoon number and % clitellate worms) for *Eisenia fetida* exposed for 28 days in artificial soil substrates adjusted to 5, 20 and 40% clay levels (numbers in brackets indicate the day data were taken).

Parameters	$CaCl_2$ (1)	$CaCl_2$ (28)	DTPA (1)	DTPA (28)	Nitric acid (1)
weight (7)	-0.555***		-0.506***		-0.384***
weight (14)	-0.814***		-0.777***		-0.723***
weight (28)	-0.717***	-0.767***	-0.690***	-0.720***	-0.708***
IZC (7)	0.484***		0.460***		0.345***
IZC (14)	0.574***		0.596**		0.632***
IZC (28)	0.493***	0.459**	0.490***	0.366*	0.477**
Cocoon no (28)	-0.698***	-0.645***	-0.662***	-0.704***	-0.717***
% clitellate (28)	-0.840***	-0.833***	-0.818***	-0.832***	-0.842***
Mortality (28)	0.635***	0.648***	0.569***	0.624***	0.617***

*significant at 0.01 confidence level **significant at 0.001 confidence level

***significant at 0.0001 confidence level

Conflicting results have been reported for the estimation of bioavailability based on chemical extraction. The results of Janssen et al. (1997a) suggested that the total metal concentration moderated by local soil characteristics could explain the bioavailability of zinc to *E. andrei* in 20 Dutch field soils. Boyd and Williams (2003) also reported that the total metal content could predict the toxicity of zinc to the nematode *Caenorhabditis elegans*. This is in contrast to the work of some authors who, despite using artificial substrate (Conder and Lanno, 2000) and remediated field soil (Conder et al., 2001) found that weak electrolytes e.g. $Ca(N0_3)_2$ is a better predictor of effect of zinc on *E. fetida*. In natural soils, Dai et al. (2004) found similar relations between the bioconcentration of zinc in *Lumbricus rubellus,* as well as *Aporrectodea caliginosa*, when correlated with DTPA and total metal. Hobbelen et al. (2006) did not find any significant relationship between accumulation of zinc by *Lumbricus rubellus* and *Aporrectodea caliginosa* when correlated with total, pore water or $CaCl_2$ zinc extracts in Dutch flood plain soils.

In our study the amount of $CaCl_2$ extracted zinc correlated slightly better with most of the biological responses, than total and DTPA extracted zinc contents and revealed a better understanding of time dependent availability of zinc over this short exposure period. It has been reported that the availability of zinc in soil decreases with time (Ford et al., 1997; Trivedi and Axe, 2000; Ma and Uren, 1997; Lock and Janssen, 2003b) and observations over a much longer time period are needed to confirm the findings of this study.

3.4.2 Influence of clay on zinc uptake and toxicity

The results presented here strongly suggest that while (kaolin) clay content had no significant influence on accumulation of zinc or its sub-lethal effects, it did at higher concentrations have a strong influence on lethality of zinc for the earthworm *E. fetida*. This could be explained by differences in bioavailability of zinc resulting from differences in clay content. Exposure concentrations of 750 mg/kg Zn and lower seemed to be below the acute toxicity threshold in most clay regimes. At 1000 mg/kg Zn however, the threshold was exceeded and differences in mortality were found between worm groups exposed to substrates with different clay levels. At this exposure concentration cocoon production and maturation would already have ceased and therefore not measurable as endpoints or effect parameters. The reason why there

was a significant effect of clay on mortality but not on growth at this highest zinc concentration is not clear. It may however be related to the change in sensitivity of earthworm upon disturbance at each sampling occasion. The contribution of worm disturbance at each sampling occasion to this observation could however not be quantified.

Most ecotoxicological models for predicting the effect of soil parameters on metal bioavailability focus on mortality as endpoint (Lock et al., 2000; Lock and Janssen, 2001b,c; Daoust et al., 2006) while sub-lethal endpoints are often neglected (Criel et al., 2008). Our results showed that it is not warranted to extrapolate directly from acute toxicity to chronic toxicity when explaining the effect of zinc in different soil types.

Kaolinite clay is the most abundant clay type in highly weathered tropical soils (Singh and Gilkes, 1992) and soils of African and Asian origin (Lim et al., 2002; Sei et al., 2002). Soils of European origin are mainly of Illite and Montmorillonite clay (Lock et al., 2000). However, kaolin clay has a lower CEC and WHC than montmorillonite clay (Table 1). Adsorption of metals to montmorillonite clay, and its binding capacity would therefore be higher than for kaolin clay. This would mean that the relative importance of clay content on bioavailability of zinc for this earthworm may increase if clay of higher CEC and WHC is present. It is however doubtful if this difference in adsorption capacity would have had a significant influence on bioavailability of zinc for this earthworm different from what is reported here. Lock and Janssen (2001d) reported that the LC_{50} of zinc to the potworm *Enchytraeus albidus* increased, only within an order of magnitude in OECD soil when kaolin clay was substituted for montmorillonite clay. An order of magnitude is an acceptable variation allowed in ecotoxicological tests (Edwards, 1984). Although attempts were made to limit variation in other soil variables so as to understand the role of clay content in this study, it was not possible to assess the separate roles of clay content and CEC. The strong positive correlation between clay content and CEC at the beginning and the end of this study indicates the strong interdependence of these variables.

Increase in clay content significantly increased cocoon production in unspiked substrates. This suggests that lack of clay could create a stressful environment for this

earthworm. Changes in clay content probably affect the behavior of the earthworm in the substrates. For example increase in clay content could limit earthworm movement, and therefore more energy may be saved which could be allocated for reproduction. Energy allocation mechanisms have been reported in soil animals (Crommentuijn et al., 1995). On the other hand, clay probably influences the availability of water, sorption of nutrients and other chemical reactions in the soil system. Moisture availability affects feeding activity directly or indirectly and influences the rate of clitella development (Reinecke and Venter, 1985). Reinecke and Viljoen (1990) reported that growth and reproduction in earthworms are strongly affected by the availability of food.

The effect of zinc on growth in the 20% clay treatment in our study was similar to those found in the literature (Spurgeon and Hopkin, 1996 a, b). However, cocoon production was lower in the control worms of our study and totally ceased at a lower zinc concentration than in these studies. This is because we started our exposures with younger (pre-clitellate) worms while fully clitellate worms were used in their study. Younger worms have been reported to be more sensitive to pollutants than older worms (Helling et al., 2000). We used pre-clitellate worms to evaluate maturation in our study. Our results further showed that maturation is a sensitive parameter for zinc toxicity. This is in agreement with previous studies that found worm maturity to be a sensitive and easily observable marker of sub-lethal zinc toxicity to *Eudrilus eugeniae* (Reinecke et al., 1997) and *Eisenia fetida* (Reinecke and Reinecke, 1996).

The fast uptake of zinc observed in this study was similar to those reported by previous researchers (Van Gestel et al., 1993; Peijnenburg et al., 1999a; Spurgeon and Hopkin, 1999; and Lock and Janssen, 2001a). Comparable IZC between worms in unspiked treatments and worms exposed to increasing zinc concentrations up to 1000 mg/kg Zn by day 28 suggests that regulation of zinc occurred in this organism as previously reported by other researchers (Lock et al., 2001a; Spurgeon and Hopkin, 1999). The weak correlation between IZC and other life cycle parameters showed that the internal concentration of zinc might not be a useful indicator for assessing toxicological effects (Lock et al., 2000). For example, no cocoons were produced at substrate concentrations of 750 and 1000 mg/kg Zn, although internal zinc

concentrations of these worms were comparable to control worms by day 28. Similar results were presented by Lock and Janssen (2001a) who worked with a maximum concentration of 560 mg/kg Zn and found reproduction of *E. fetida* ceased at the highest concentration, while the internal concentration of zinc was still at the same level as that of control worms.

3.5 CONCLUSION

Clay had no significant influence on the amount of zinc accumulated by the worms over the experimental exposure period and all of the concomitant biological endpoints except mortality. In unspiked treatments, clay had a significant positive effect on cocoon production which indicated that lack of clay could be a stressor. Zinc on the other hand, affected all the parameters tested significantly. Although, all chemical zinc extracts of substrate (acid digestion, DTPA and $CaCl_2$ extraction) used in this study correlated strongly with one another and in similar relations when correlated with the biological responses of worms, only $CaCl_2$ revealed the time dependent availability of this metal.

CHAPTER FOUR

4.0 INFLUENCE OF CLAY CONTENT ON PARTITIONING, UPTAKE AND TOXICITY OF COPPER IN THE EARTHWORM *EISENIA FETIDA*

4.1 INTRODUCTION

It is generally known from soil toxicity studies and ecotoxicological assessment that the bioavailability of a toxicant depends on various physicochemical and biological factors making it impossible to determine or deduce toxicity by simply measuring total toxicant concentrations in the soil (Peijnenburg et al., 1997; Lanno and McCarty, 1997; Marinussen et al., 1997). Due to these modifying factors, soil toxicants may never be 100% bioavailable.

The term bioavailability broadly describes the proclivity of a particular element or molecule for entering into or adhering onto living bodies (Impelliteri et al., 2003). Hence, only an organism can show how much of a toxicant is available to it. However, tissue concentrations of toxicants in organisms, especially essential metals that are regulated by most organisms, do not always correlate significantly with toxicological effects. Other (bio) markers and endpoints of toxicological effects are necessary in risk assessment of these metals if risk must be correctly predicted. These include other indirect biological responses like mortality, growth and reproduction (Reinecke and Reinecke, 1996; Reinecke et al, 1997; Helling et al., 2000). Some researchers relate bioavailability to some sort of extraction in substrates (Dai et al. 2004; Daoust et al., 2006) which can differentiate the components of toxicants in the soil chemical pool. These include nitric acid digestion of substrates which extracts the total metal in soil, as well as DTPA (di-ethylene-triamine-pentaacetic acid) or $CaCl_2$ extraction which extracts only the most labile forms in soil. The mobile (exchangeable) forms are extracted by $CaCl_2$ and mobilisable (complexed, adsorbed and carbonated forms) by DTPA (Maiz et al., 1997).

The bioavailability and or toxicity of metals in soil can vary over several orders of magnitude depending on soil modifying factors (Van Gestel and Van Dis, 1988; Lock et al., 2000). The effect of soil types on the bioavailability and toxicity of metals to soil organisms have been studied to a large extent. Most of these studies

(Spurgeon and Hopkin, 1996a; Peijnenburg et al., 1999b; Lock et al., 2000; Lock and Janssen 2001b,c Daoust et al., 2006) reported that pH and cation exchange capacity (CEC)/organic matter (OM) are the most important factors. In some of the studies, however, several soil properties were dealt with together. Many of these soil properties are auto-correlated to various degrees, and it is often difficult to distinguish the actual contribution of distinct factors and to interpret results (Basta et al., 1993; Amorin et al., 2005; Römbke et al., 2006). This approach, although it may give relevant information on the combined effect of these properties, would still mask individual roles of each soil parameter.

Clay minerals are generally regarded as important natural ion exchange materials because they are generally coated with metal oxides (Jenne 1988) and organic matter (Hart, 1982; Davis, 1984) providing surface characteristics that are important in the exchange of trace metals. Clay, because of its small particle size, has a large surface area per unit weight and exhibits surface charges that attract negatively and positively charged ions in water (Brady, 1990). Because of these properties, clay is believed to be a good adsorbent for heavy metal removal from water and waste water (Sajidu et al., 2006). Soils with high clay content are believed to have higher concentrations of most trace metals than those with low clay content (McBride, 1994). How this relates to the bioavailability of such metals needs to be quantified.

Internationally agreed standardized soil is made up of artificial soil with a clay content of 20% (OECD, 2004). Toxicity data for most pollutants are derived in laboratory tests with this soil constitution. However, in nature, clay content of soil may be less or more than 20% and hence the toxicity of the pollutants may vary depending on the clay content. This study was therefore conducted with the aim of assessing the influence of clay content on the bioavailability of copper to *Eisenia fetida*. The specific objectives were to assess the influence of clay content on the uptake of copper in the body of the worms and also its effects on selected life-cycle parameters (mortality, growth, and reproduction). Another objective was to assess the influence of clay on copper sorption by determining the fraction extractable by different chemical extraction methods and relating these to the observed biological responses.

4.2 MATERIALS AND METHODS

4.2.1 Test Species

E. fetida specimens used for this study were age-synchronized from a culture kept in the laboratory of the Ecotoxicology Group, University of Stellenbosch, South Africa since 1992. Clitellate worms between 17-20 weeks old (weighing 250-500 mg) were used in the experiments. The worms were acclimatized for 72 hours in untreated artificial soils and fed with the same type of food to be used in the experiment.

4.2.2 Choice of clay type

Montmorillonite clay has more adsorption properties than kaolin clay. However, a previous study had shown that changing the montmorillonite clay content of soil would result in substantial changes in other soil variables especially pH whereas little change was found for kaolin clay (Table 1). Addition of $CaCO_3$ to adjust pH to some of the treatments and not the others would however influence the bioavailability of metal in the treatments receiving calcium supplement. Calcium can influence metal sorption to soil (Kiekens, 1990) and also uptake of metals by animals (Kiewet and Ma, 1990). As clay content is the only variable of interest in this study, use of kaolin clay was therefore more appropriate

4.2.3 Test soil, test procedures and experimental setup

The test soils consisted of (OECD) artificial soil. The first substrate type was prepared by mixing 10% finely ground (<1mm) sphagnum peat, 20% kaolin clay and 70% quartz sand (by dry weight), adjusted with $CaCO_3$ to pH 6.0 ± 0.5 (OECD, 1984; 2004). The other two treatment soils were prepared as above except that the clay contents were adjusted to 5 and 40% clay, organic matter (OM) was held at 10% while sand was used to make up the remaining weight. The test substance copper oxychloride ($Cu_2Cl(OH)_3$) (trade name 'Virikop') was purchased from Agro-Serve (Pty) Ltd, South Africa. The substance which contains 850 g/kg active ingredient and 500 g/kg Cu was added as aqueous solution in the following concentrations (0, 80, 320 and 640 mg/kg Cu) based on a range finding test and information from the literature, as well as to include environmentally relevant concentrations. The

substance was then suspended in distilled water and mixed thoroughly with the corresponding soil so as to make up 60% of the final water holding capacity of each treatment soil.

The treatment consisting of 500 g of soil was placed in a cylindrical plastic vessel of 2 l and allowed to equilibrate for two days before earthworms were introduced. Ten worms per container were used in each exposure regime and were introduced into the relevant test soil by placing them on the surface and allowing them to burrow in. Four replicates were used for each exposure regime, bringing the total number of containers to four for each treatment. The test containers were covered with a perforated lid to limit water loss due to evaporation and kept in 16 h light, 8 h dark at 20^0C (Reinecke and Kriel, 1981) in a climate chamber for 28 days. To maintain the worms during the exposure period in the test medium, the worms in each container was fed weekly with dried urine-free cattle manure, ground and sieved (1mm) to provide 0.5 g per worm. The manure was rewetted with distilled water prior to use. Foods that were not eaten were removed on each sampling date to avoid build up of organic matter in the substrates.

4.2.3.1 Worm parameters

Sampling was done at day 1, 7, 14 and 28 after worms were introduced into the substrates. On these days, growth, mortality, and internal copper concentration (ICC) of worms were assessed, while cocoon production was assessed only at day 28. Growth was determined by individually weighing each worm in each container, and comparing the mean weight with initial values. Mortality was assessed by stimulating a worm with a blunt probe and the earthworm was judged dead if no response could be observed. Worms not found during sampling was judged dead since earthworm tissue decompose easily in soil when dead. Cocoon production was determined by wet sieving the substrates (through a 2.0- and 1.0-mm sieve system). Cocoon number per worm was calculated by dividing the total number of cocoons by the number of surviving worms. It is assumed that a worm that died during the exposure couldn't have contributed to cocoon production since concentration of toxicants affecting cocoon production is often much lower than that affecting mortality. The net number of surviving worms in each container of the control soils (receiving 0 mg/ kg Cu) at the end of the test was seven, since three worms were removed for analysis of internal

metal concentration. This reduction in sample size (12 worms from the original 40) was accounted for in calculating cocoon production.

To determine the internal metal concentration, whole worms (one per container except at day 28 when two were used) were removed on each sampling occasion. They were placed in Petri dishes on moist filter paper for 24 hrs at 20^0C to allow depuration of their gut contents. Arnold and Hodson (2007) have shown that 24 hrs is an adequate period for gut purging in *Eisenia spp*. Afterwards, they were weighed and frozen individually for metal analysis. The procedure for the metal extraction and analysis has been described by Maboeta et al. (2003). This was done by acid digestion with HNO_3 and spectrophotometric analysis.

4.2.3.2 Soil parameters

pH and soil moisture content were monitored on each sampling occasion. For pH-H_20, a suspension of (1:10, w/v) of the substrate was made in distilled water. The solution was stirred and the pH measurement was taken with a pH meter (Micro pH 2001, Crison). Soil moisture content was monitored on each sampling occasion by analyzing 5 g of sample with a Sartorius infrared moisture detector. Moisture loss was replenished by adding equivalent amount of de-ionised water. CEC was determined with the AgTu method in 0.4 M ammonium acetate at pH 7 (Chhabra et al., 1975). For the determination of copper contents of substrates, chemical extractions were carried out using three methods: $CaCl_2$, DTPA and nitric acid extraction methods. For $CaCl_2$ and DTPA extractions, samples were taken at day 1 and 28 and a sequential procedure was followed according to those used by Maiz et al. (1997) and others. From each sample, 3 g of soil was shaken with 30 ml solution of 0.01 M $CaCl_2$ for 2 h prior to centrifugation at 4000 rpm for 15 min, the supernatant was removed for the determination of $CaCl_2$ extract. For the DTPA extraction, the residue was shaken with 6 ml solution of DTPA for 2 h prior to centrifugation at 3000 rpm for 10 minutes. The supernatant was in both cases filtered through Whatman 540 filter paper, acidified to 5 % HNO_3 and stored at -4^0C until analysis by atomic absorption spectrophotometer (AAS). Total copper content was determined at day 1 and 28 by acid digestion of 1 g of each sample as described by Maboeta et al., (2003).

4.2.4 Copper analysis

The extracted soil and worm samples were analysed for copper by a Varian AA-1275 flame atomic absorption spectrophotometer. The spectrophotometer was calibrated by 5, 10 and 25 µg/ml copper standards. When samples were over-ranged, a serial dilution was made and samples were then analysed. Previously spiked soil samples were analysed and indicated a recovery above 75% with this procedure. For each batch of worm digested a blank was also prepared to detect possible contamination during the digestion process. Quality control was achieved by analysing reference materials independently prepared from the standards.

4.2.5 Statistics

All data were checked for normality and homogeneity of variance with Shapiro-Wilks W test and Levene's test respectively. When data were not normally distributed even after transformations, non parametric statistics were used. The result of all worm parameters where data were taken at more than one time (weight) was analysed using measures analysis of variance (ANOVA) per sampling time. For parameters where sampling was done once (cocoon production) or pooled (mortality), two-way factorial ANOVA was used when data were normally distributed and bootstrap non parametric statistics were used when data were not normal even after transformations. For copper concentrations in worms and in soil (nitric acid, DTPA and $CaCl_2$ copper extracts), one-way ANOVA was used to test for the effect of clay content along each copper level. When data were not normally distributed even after transformations, Kruskal-Wallis H-test was used. Fischer's least significant difference (LSD) post-hoc test was used to determine the significance of any differences between specific groups in parametric cases while multiple comparison of 'z' and 'p' values was used in non-parametric cases. Correlations between Cu extracted by the three extraction methods and all worm parameters, and between worm internal copper concentrations and other endpoints in worms were analysed using Spearman-Rank correlation. Tests of similarity between DTPA at day 1 and 28 and $CaCl_2$ at day 1 and 28 were analysed with Wilcoxon sign-paired test. All statistics were analysed with STATISTICA 7.0 software.

4.3 RESULTS

4.3.1 Soil properties

The mean pH values of the unspiked treatments at the start of the experiment were 5.61, 5.73 and 5.94 in the 5%, 20% and 40% clay respectively. A decrease in pH with increasing added copper concentrations was observed for all soil groups. However, these pH decreases were in all instances less than 0.5 unit. pH increased during the 28-day exposure in all treatment groups. The mean increase was 0.63, 0.45 and 0.30 in the 5%, 20% and 40% clay respectively. The CEC of the substrate, at day 1 increased with an increase in clay content. The mean CEC values were 128 mmol/kg, 148 mmol/kg, and 157 mmol/kg in the 5%, 20% and 40% clay respectively. At day 28, the CEC of the substrates increased slightly in all treatments. The mean CEC values were 140, 158 and 168 mmol/kg in the 5%, 20% and 40% clay respectively. Correlation between clay and CEC was significant at day 1 ($r = 0.949$, $P < 0.05$) and day 28 ($r = 0.956$, $P < 0.05$). The relationship between clay content and CEC could be described by the regression equation CEC = 26.87 log clay (%) +127.87 ($R^2 = 0.99$).

4.3.2 Mortality, growth and reproduction

Fig. 7 shows the % mortality in each of the soil groups pooled over the entire exposure period. All mortalities were found at days 7 or 14 and only at the higher Cu concentrations (320 and 640 mg/kg Cu). Generally, mortality in these Cu concentrations increased as the clay content decreased. There was a significant effect ($P < 0.05$) of the interaction of clay and copper on mortality (Appendix 6). The effect was however significant only at 640 mg/kg Cu. At this concentration, mortality at the 5% clay was significantly ($P < 0.05$) higher than at other clay regimes, and that at the 20% clay was significantly higher ($P < 0.05$) than at the 40% clay regime.

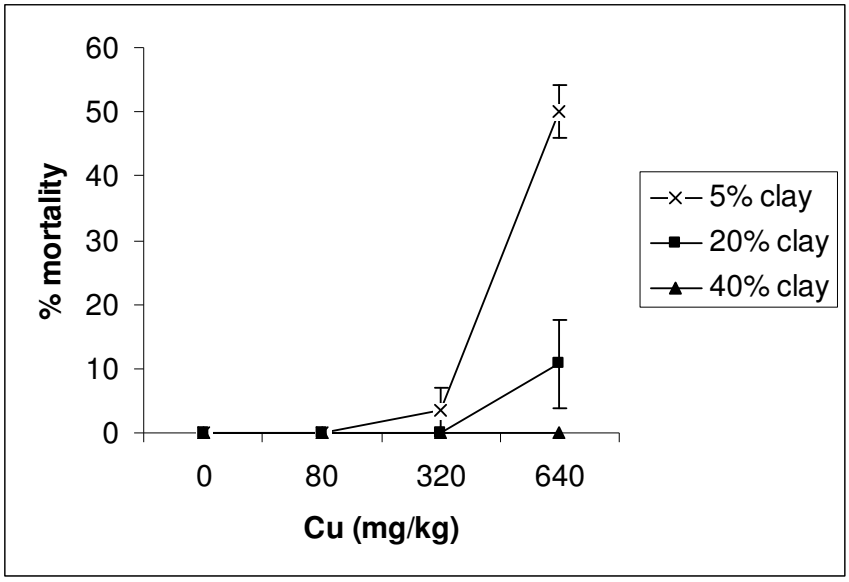

Fig. 7. Mean percentage mortality of four groups, each consisting of ten clitellate worms (*Eisenia fetida*), exposed to copper under different clay regimes at constant temperature (20^0C) and fairly constant soil moisture (error bars represent standard error).

Apart from a slight reduction in mean weight of worms at day 1, there was an increase in mean weight of worms in all exposure groups except for worms in the 5% clay soil group that were exposed to 640 mg/kg Cu (Fig. 8). The growth of the worms in response to copper in the three clay treatments showed different patterns. Generally, there was increased effect of copper toxicity on weight with reduction in clay content. Statistical analysis showed that the interaction of clay and copper was significant on weight at day 7, 14 and 28 (Appendix 7). At these days, exposed to either 320 or 640 mg/kg Cu, the mean weight of the worms in the 5% clay was significantly lower ($P < 0.01$) than those in the 20 and 40% clay groups. Also, significantly lower ($P < 0.01$) mean weight of worms in 20% clay group than in 40% clay groups were found at concentration of 640 mg/kg Cu at day 28.

The mean number of cocoons produced after 28 days at different exposure concentrations in the three soil groups is shown in Fig. 9. Generally, cocoon production was similar and not significantly different ($P > 0.05$) between the unexposed worms and worms exposed to 80 mg/kg Cu, irrespective of clay contents.

At concentrations higher than 80 mg/kg Cu, there was a general decline in cocoon production. The interaction of clay and copper had no significant effect ($P > 0.05$) on cocoon production, but individually, clay and copper had significant effects ($P < 0.05$) on cocoon production (Appendix 8). The mean number of cocoons produced in the substrate with 5% clay was significantly lower than in 20% ($P < 0.05$) and 40% clay ($P < 0.05$) clay treatments. The number of cocoons produced in the 20% clay group was almost significantly lower ($P = 0.06$) than in the 40% clay. For copper, the number of cocoons produced by worms was significantly lower ($P < 0.01$) in both the 320 and 640 mg/kg Cu exposures when compared to unspiked (0 mg/kg Cu) treatments.

4.3.3 Internal copper concentrations (ICC) of worms

Copper accumulation was below detection limit for all exposure groups at day 1 and 7. The ICC in both exposed and unexposed worms under the three clay regimes at day 14 and 28 is shown in Fig 10. Generally, there was increase in accumulation with increased copper concentrations of substrates, reduced clay content and increased time of exposure in all clay substrates. Statistical analysis showed that clay content had significant effect ($P < 0.05$) on ICC of worms. Generally, there were no significant differences in ICC between worms in 20 and 40% clay substrates for all Cu concentrations of substrates except at 320 mg/kg Cu at day 28 ($P < 0.05$). Differences in ICC between worms in 5 and 20% clay soils were only significant at 320 mg/kg Cu at day 14 ($P < 0.01$). However, differences in ICC between worms in 5 and 40% clay soils were significant at 80 mg/kg Cu at day 14 ($P < 0.05$), 320 mg/kg Cu at day 14 and 28 ($P < 0.01$), and 640 mg/kg Cu at day 28 ($P < 0.05$).

ICC was not correlated with the preceding life cycle effects because metal must be taken in before effects could be seen. Generally, a mild positive correlation was found between ICC and mortality, but there was a mild to fairly strong negative correlation between internal copper concentration and the duo of weight and cocoon production (Table 4). For weight the correlation was stronger with increased time of exposure, while for mortality it was weaker with increased time of exposure.

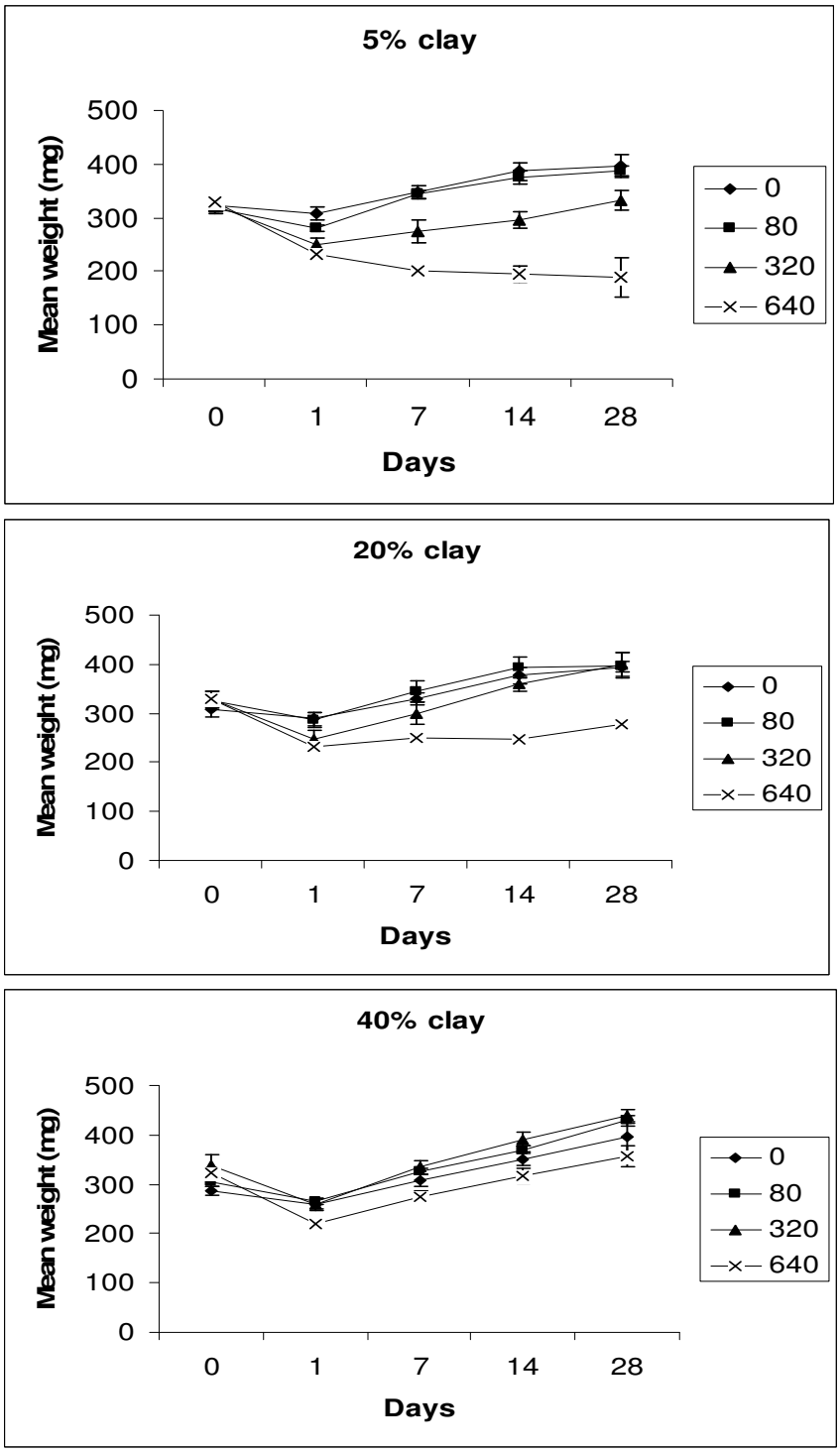

Fig. 8. Mean weight of four groups, each consisting of ten clitellate worms (*Eisenia fetida*), exposed to copper (mg/kg) under different clay regimes at constant temperature (20^0C) and fairly constant soil moisture (error bars represent standard error).

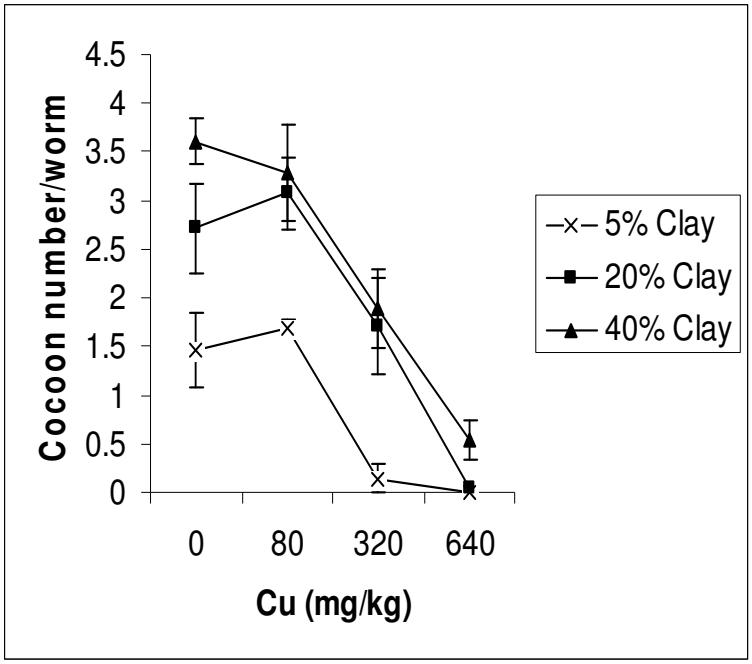

Fig. 9. Mean number of cocoons/ worm (*Eisenia fetida*) produced after four weeks of exposure of four groups, each of ten clitellate worms to copper under different clay regimes at constant temperature (20^0C) and fairly constant soil moisture. (Error bars represent standard error)

4.3.4 Total and available copper

The fraction of the total Cu extracted from soil at day 1 and 28 respectively by DTPA (0-88%) and (0-77%) were greater than those extracted by $CaCl_2$ (0-5%) and (0-6%), irrespective of the clay regimes. The correlation between DTPA and $CaCl_2$ extracted Cu at day 1 and 28 with nitric acid extracted copper was very strong ($r \geq 0.89$, $P < 0.05$). The amount of metals extracted from the three clay regimes by the three extraction methods at day 1 and 28 is presented in Fig. 11.

Generally, for nitric acid extracts, the amount extracted from all clay regimes were comparable for all Cu levels, and there were no significant differences ($P > 0.05$). For DTPA and $CaCl_2$ extracted Cu, there were only significant differences at 320 and 640 mg/kg Cu. For DTPA, the significant differences were at 320 mg/kg Cu at day 1 and 640 mg/kg Cu at day 28. At 320 mg/kg Cu at day 1, the amounts of Cu extracted from 5 and 20% clay were similar and significantly higher ($P < 0.01$) than

from 40%. At 640 mg/kg Cu at day 28, the amounts extracted at 5% clay group was significantly higher ($P < 0.05$) than at 40% clay group. For $CaCl_2$, significant differences were seen at day 1 and 28. At day 1, at the concentration of 320 mg/kg Cu, amounts extracted from 5% clay, was significantly lower ($P < 0.05$) than those

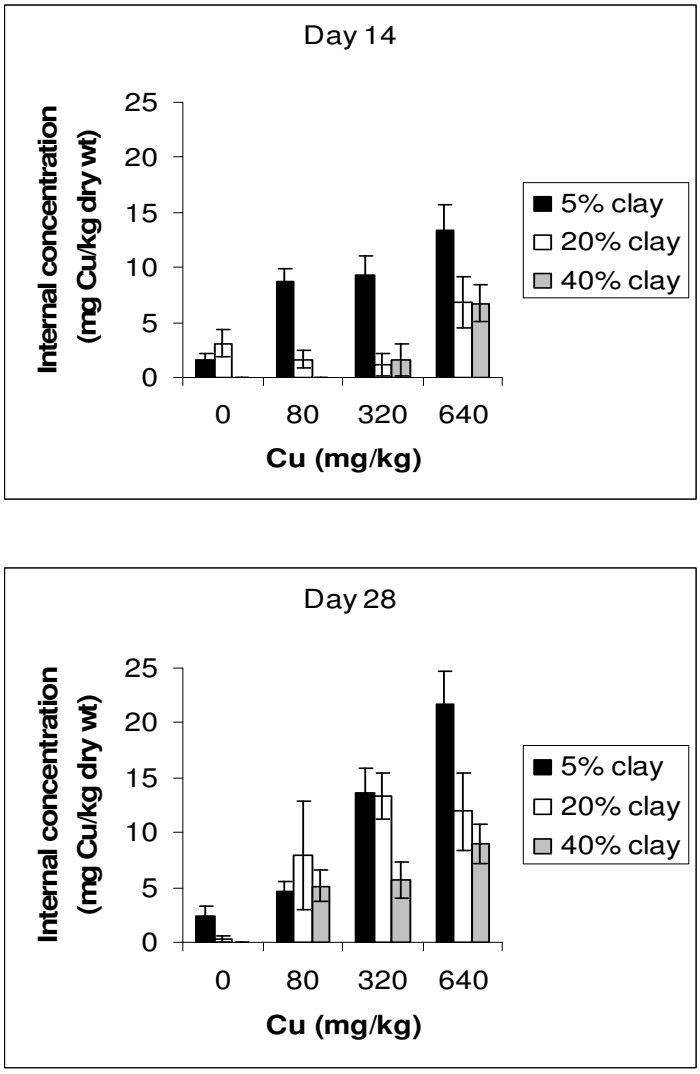

Fig.10. Mean copper concentrations in specimens of *Eisenia fetida* after being exposed for 28 days in three clay regimes in an artificial soil system spiked with different concentrations of copper (Bars indicate mean of at least four worms and error bars indicate standard error of mean)

from 20% and 40% clay, both of which could not be separated. The same pattern was seen at 640 mg/kg Cu but with a higher statistical significance ($P < 0.01$). The pattern

was fairly similar by day 28. At day 28, at the concentration of 320 mg/kg Cu, amounts extracted from 5% clay, was significantly lower ($P < 0.05$) than those from 20% and 40% clay, both of which could not be separated. At the concentration of 640 mg/kg Cu, the amounts extracted from 5% and 20% clay groups were significantly lower ($P < 0.05$) than from 40% clay group. Analyses of differences between Cu extracts at days 1 and 28 showed that the DTPA extracts at day 1 were significantly higher ($P < 0.01$) than at day 28 but there were no significant differences for $CaCl_2$ extracts between day 1 and 28.

4.3.5 Relationship between metal in worms, clay substrates and biological endpoints

Due to a large dataset obtained in this study, attempts were made to understand the relationship between Cu contents of substrates and worm parameters. For this reason, all soil and worm parameters in the different clay regimes were pooled. Table 5 presents the correlation between the amounts of metal extracted by the three extraction methods, in 5, 20 and 40% clay regimes and the endpoints in worms. Cu extracts were not correlated with the preceding worm parameter since worms must take up the metal before effects can be expected. The result showed that although all three Cu extracts correlated significantly with most worm parameters, DTPA extracted Cu correlated better with all the endpoints in worms, except for weight at day 1.

4.4 DISCUSSION

A recent study on the influence of illite clay content on Cu bioavailability and toxicity, which focused on survival of compost worms (Daoust et al., 2006), showed that clay significantly contributed to lowering the ecotoxicity of Cu, as measured by worm survival. The present study provides evidence that (kaolin) clay content is not only important in influencing the uptake and acute toxicity of copper, as shown by mortality, but also chronic effects as shown by its effect on growth of *E. fetida.*

The significant influence of kaolin clay content on uptake and toxicity of copper by *E. fetida* seen in this study could be explained by a decreasing availability

Table 4. Correlation coefficient (r) values between internal copper concentrations in worms (ICC) and other worm parameters (weight, cocoon number and mortality of worms) in exposure of *Eisenia fetida* to copper under three clay regimes for 28 days (days in bracket for all parameters).

Parameters	**ICC (14)**	ICC (28)
weight (14)	-0.399*	**NC**
weight (28)	NC	**-0.508***
cocoon no (28)	NC	**-0.625***
mortality	**0.561***	**0.366**

NC-parameters were not correlated

* significant at 0.05 level after Bonferroni adjustment

of metals in the liquid phase due to adsorption to clay particles (Hobbelen, et al., 2006). Kaolinite clay is the most abundant clay type in highly weathered tropical soils (Singh and Gilkes, 1992) and soils of African and Asian origin (Sei et al., 2002; Lim et al., 2002). Soils of European origin are mainly of Illite and Montmorillonite clay (Lock et al., 2000). However, Kaolin clay has a lower CEC and WHC than montmorillonite clay. Adsorption of metals to montmorillonite clay, and its binding capacity would therefore be higher than for kaolin clay. This would mean that the relative importance of clay content on bioavailability of copper for this earthworm may increase if clay of higher CEC and WHC is present. The use of kaolin clay in this study was necessary so as to avoid interference from pH, which is the pre-eminent factor affecting metal bioavailability in soil (Impelliteri et al., 2003) and CEC. The pH and CEC changes over time in this study were minimal and could not have significantly influenced our results.

The significantly higher cocoon production, with increased clay levels seen in this study after 28 days of exposure has not been reported elsewhere. This suggests that lack of clay could be a stressor on its own. This is similar to the effect of pH on

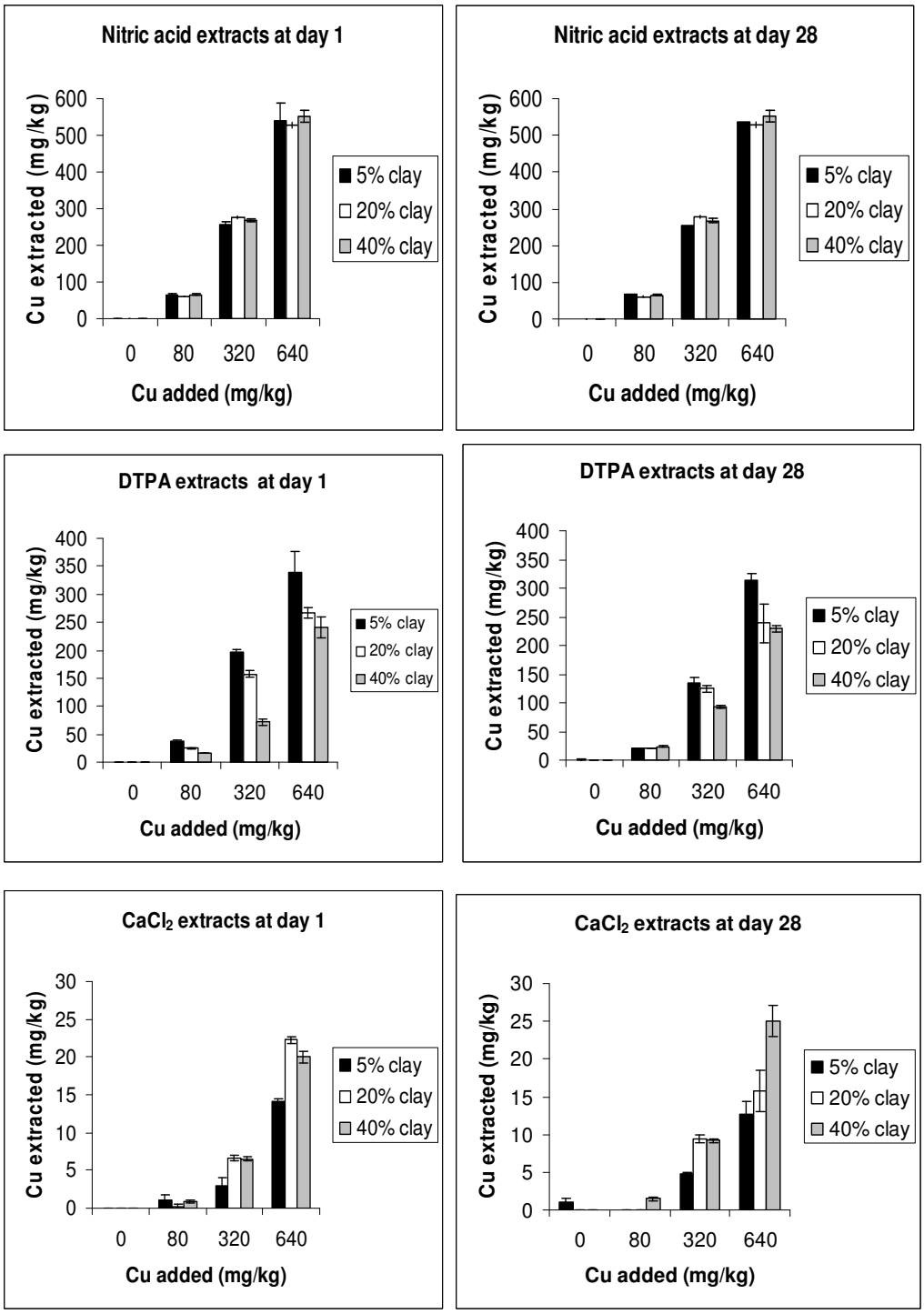

Fig. 11. The amounts of metal extracted on day 1 and day 28 from differently spiked substrates using different extraction methods. Specimens of *Eisenia fetida* were exposed for 28 days to the range of copper concentrations under different clay regimes in these substrates.

cocoon production already reported (Spurgeon et al., 2006). These soil properties probably affect the behaviour of the earthworm in the soil. For example increase in clay content could limit earthworm movement, and therefore more energy may be saved which could be allocated for reproduction. Energy allocation mechanisms have been reported in soil animals (Crommentuijn et al., 1995). On the other hand, clay could also influence the availability of water, sorption of nutrients and other chemical reactions in the soil system. Moisture availability affects feeding activity directly or indirectly and influences the rate of clitella development (Reinecke and Venter, 1985). Reinecke and Viljoen (1990) reported that growth and reproduction in earthworms are also strongly affected by the availability of food.

Table 5. Correlation coefficient (r) values between amount of copper extracted by three extraction methods ($CaCl_2$, DTPA and nitric acid extraction) and biological response (weight, internal copper concentrations, mortality, and cocoon number) in *Eisenia fetida* exposed for 28 days in artificial soil substrates adjusted to 5%, 20% and 40% clay levels. Numbers in brackets indicate the day data were taken.

Parameters	$CaCl_2$ (1)	$CaCl_2$ (28)	DTPA (1)	DTPA (28)	Nitric acid (1)	**Nitric acid (28)**
weight (1)	-0.643*	NC	-0.649*	NC	-0.694*	**NC**
weight (7)	-0.553*	NC	-0.633*	NC	-0.613*	**NC**
weight (14)	-0.493*	NC	-0.652*	NC	-0.586*	**NC**
weight (28)	-0.361	-0.338*	-0.584*	-0.500*	-0.460*	**-0.460***
ICC (14)	0.361	NC	0.581*	NC	0.444*	**NC**
ICC (28)	0.583*	0.606	0.760*	0.750*	0.743*	**0.743***
Mortality (28)	0.455*	0.393	0.559*	0.564*	0.491*	**0.491***
cocoon number (28)	**-0.633***	**-0.653***	**-0.800***	**-0.759***	**-0.703***	**-0.703***

NC-soil and worm parameters were not correlated

*-significant at 0.05 level after Bonferonni adjustment

The effects of copper on mortality, growth and reproduction seen in the 20% clay group in this study are similar to those found in the literature (Reinecke and Reinecke, 1996; Helling et al., 2000). The slow accumulation of copper in the present study was similar to those reported by previous researchers (Reinecke and Reinecke, 1996; Reinecke et al., 1997). It is however in contrast to reports by other researchers who found fast accumulation and elimination of copper, sometimes within the first three days (Peijnenburg et al., 1999a; Spurgeon and Hopkin, 1999). Despite increased accumulation of Cu with exposure time, a mild to fair relationship was found between internal copper concentration and life-cycle parameters. This may mean that the internal concentration of copper might not be a useful indicator for assessing toxicological effects as already reported by Lock et al., (2000). This may be as a result of copper regulation by the earthworm. Earlier researchers have demonstrated that copper could be regulated by earthworms (Spurgeon and Hopkin, 1999). These authors reported that regulation of copper in the earthworm *E. fetida* occurred primarily by excretion. They argued that the ability of earthworms to eliminate excess copper is probably dependent on the essential nature of the element.

For the estimation of bioavailability based on chemical extraction, conflicting results have been reported. Arnold et al. (2003) did not find any relationship between both $CaCl_2$ and DTPA extracted Cu and mortality and therefore suggested that they are not useful indicators of Cu bioavailability to *E. fetida*. Daoust et al. (2006) reported that the amount of $CaCl_2$ extracted Cu showed a good correlation with toxic response in *E. fetida* while the amounts of nitric acid extracted Cu (total metal) and DTPA extracted Cu did not. In natural soils, Dai et al., (2004) found the DTPA extracted Cu showed better relationship than total Cu extract when correlated with the accumulation in *Lumbricus rubellus* but no significant relationship was found for the two extracts when correlated with accumulation in *Aporrectodea caliginosa*.

In the present study, the amount of Cu extracted with DTPA showed better relationship with Cu accumulation in the worms as well as toxic response in *E. fetida* than total and $CaCl_2$ extracts. However, the nominal concentrations of Cu in worms were similar to those extractable by $CaCl_2$. For example, at an exposure concentration of 640 mg/kg Cu, the amounts extractable from the substrate by $CaCl_2$ and that extracted by acid digestion from the worm tissue were between 10 and 25 mg/kg Cu.

However, $CaCl_2$ extraction could not explain the partitioning of Cu as influenced by clay content. With more clay, there appeared to be more Cu extractable from the substrate, even after a repeat trial. This is in contrast to the general understanding of a reduced availability for uptake with increased clay content (Hobbelen et al., 2006) confirmed by the DTPA extract in this study. This apparent contrast could be because Cu is still partitioning between the liquid and solid phase in soil and has not yet reached equilibrium during this relatively short time in this laboratory study.

DTPA extracted Cu contents of soil correlated better with worm parameters than other extracts. It also gave a better understanding of time dependent availability of Cu in this study. It has been reported that the availability of Cu for uptake by organisms in soil decreases with time (Lock and Janssen, 2003c; Tom-Peterson et al. 2004). The 28-day exposure period in this study may be too short for equilibrium partitioning to be reached between solid and liquid phases and further study is needed over a longer period for this conclusion to be ascertained. Another study which extended over 16 weeks (Daoust et al., 2006) reported that the amount of $CaCl_2$ extracted Cu decreased significantly between days 0 and 16 in organic soil mixtures while a similar decrease was found for DTPA in soil substrates of low OM content. It further suggests a complex process of partitioning of metals in soil which is regulated by various soil factors. It is important to note that the OECD standard soil which contains a high amount of OM (10%) was used in this study. The effects of clay content may have been more pronounced in soils with lower OM as this high OM content could to a degree mask some of the influence of clay content on copper partitioning, uptake and toxicity.

4.5 CONCLUSION

Clay had a significant influence on the amount of copper taken up by the worms over the experimental exposure period and all of the concomitant biological endpoints except cocoon production. Clay, on its own, showed positive effect on cocoon production which indicated that lack of clay could be a stressor. Copper on the other hand, affected all the tested biological parameters significantly. There was also a significant effect of clay content on the estimated bioavailability of copper as determined by DTPA, suggesting that clay content influences the partitioning of this metal. The DTPA extract showed a better correlation than both total and $CaCl_2$

extracts when correlated with biological responses in worms. It also revealed a time dependent availability of Cu which makes it a suitable surrogate for showing the bioavailability of this metal.

CHAPTER FIVE

5.0 EFFECTS OF SALINITY ON PARTITIONING, UPTAKE AND TOXICITY OF ZINC IN THE EARTHWORM *EISENIA FETIDA*

5.1. INTRODUCTION

Salinisation of soil is a problem in many parts of the world. Primary salinisation is a natural phenomenon involving accumulation of salts through natural processes due to high salt contents in parent materials or groundwater. Secondary salinisation occurs frequently mainly as a consequence of over irrigation caused by improper management of irrigation facilities, poor soil internal drainage conditions or irrigation with water of poor quality (Yuan et al., 2007). Saline soils often occur in irrigated land (Ayars and Tanji, 1999) in semi-arid or arid zones of the world. About 50% of irrigated areas of the world are either salinised or have the potential to be so in future (Tyagi, 1986), underlining the extent of the problem.

Generally, salinity affects crop yield and beneficial soil biota, leading to economic losses. It affects the growth and survival of microorganisms (Lippi et al., 2000; Yuan et al., 2007), plants (Ramoliya et al., 2004; Kadukova and Kalogerakis, 2007) and soil animals (Hobel et al., 1992). It is also well known that the distribution and abundance of earthworms are influenced by soil salinity in various ecosystems (Lee, 1985).

Information on the effect of salinity on earthworms in different substrates is increasing gradually, but there are no existing soil quality guidelines for the protection of soil invertebrates against the relevant salt ions. Several recent researchers have taken the view that risk assessment of soils containing diffuse chemical mixtures should be performed in a site-specific manner due to the diversity and complexity of factors influencing toxicity (Posthuma et al., 2007). Information about the role of soil parameters and sensitivities of biota is therefore essential. It has been reported that NaCl in excess of 0.5% wet weight may be harmful to earthworms in activated sludge (Hartenstein et al., 1981). Fischer and Molnar (1997) found that mortality was significantly affected from 100 mM NaCl in organic manure and peat substrates, and cocoon production totally ceased at that same concentration while growth was

negatively affected at concentrations below or in excess of 60 mM NaCl. Bright and Addison (2002) used only a small number of worms in artificial soil substrate to obtain EC_{50}s of 4681 and 1884 mg/kg NaCl for growth and cocoon production respectively. These authors further derived an LC_{20} of 5534 mg/kg NaCl.

Moreover, these salt ions could be found together with other toxicants, especially metals in natural systems. In agricultural lands, repeated irrigation with water of poor quality and use of metal containing pesticides often lead to salinisation and accumulation of metals in the top layer of soil. For example, in Australia and South Africa where salinisation of agricultural soil is a concern metal containing pesticides are also used (Merry et al., 1983; Krause et al., 1996; Vermeulen et al., 2001). Calamari and Alabaster (1980) have highlighted three types of interaction that may occur among chemicals in soil, and their toxicity to soil organisms.

1. chemical and physiochemical interactions with other constituents of the soil determining sorption, affecting bioavailability.

2. physiological interactions, affecting uptake from the soil solution and finally determining the quantity available at the site of toxic action.
3. interactions during the intoxication process, including combination with receptors, at the target site.

During each of these three interactions, interactions of the chemical with other chemicals may also occur.

Few studies have been conducted on the influence of salinity on metal bioavailability. The limited published data available however indicate that salinity influences the toxicity of heavy metals to polychaetes in aquatic environments (Bryan and Langston, 1992). In soil, salinity tends to increase the bioavailability of metals. DTPA (di-ethylene-triamine-pentaacetic acid) extractable zinc were reported to increase with increasing levels of salinity in natural soil of Asian origin (Keshavarz et al., 2006) and sewage sludge (Pakpian et al., 2002). How the availability of these metals under saline conditions relates to their uptake and toxicity to soil biota is still largely unknown.

The aim of this part of the study was to provide data for the development of a quality criterion standard in Organisation for Economic Co-operation and Development (OECD) artificial soil substrate for the protection of earthworms against salinity. Also to investigate possible interactions between NaCl-salt and zinc ions in soil as well as how these interactions affect the uptake and toxicity of zinc to the earthworm *Eisenia fetida*. This species was chosen because it is easy to culture and there are relatively large data sets available in the literature on the biology and ecotoxicology of this species. Zinc was chosen because it has significant effects on earthworm growth, maturation rate and reproduction (Reinecke and Reinecke, 1996) and it is also a component of pesticides such as Mancozeb (Vermuelen et al., 2001) and copper zinc chromates (Ware, 1978) sprayed in agricultural lands. NaCl salt was chosen because it is the predominant salt in agriculturally induced salinisation in South Africa (Dallas and Day, 1993) and also in most inland saline water in Australia (Kefford et al., 2005), two arid regions where salinity is a concern.

5.2.0. Materials and methods

5.2.1. Test species

E. fetida specimens used for this study were age-synchronized from a culture kept in the laboratory of the Ecotoxicology Group, University of Stellenbosch, South Africa. Adult worms (18-20 weeks old) of between 250 and 500 mg were used in the experiments. The worms were acclimatized for 72 hours in OECD soil and were fed during this period with the same type of food to be used during the experiment consisting of dried cattle manure.

5.2.2 Test soils

A bulk sample of OECD test soils was prepared by mixing 10% finely ground sphagnum peat, 20% kaolin clay and 70% quartz sand, adjusted with $CaC0_3$ to pH 6.0 ± 0.5 (OECD, 2004). The maximum water holding capacity (WHC) of this soil was determined by using the procedure described by ISO (1996) after inundating the soil for 3 h in water and subsequently draining it for 2 h .The maximum WHC was 65%.

5.2.3 Test procedures and experimental setup

Two experiments were conducted. The first experiment was a single substance exposure with NaCl, while the second was a joint substance exposure with NaCl and Zn.

5.2.3.1 Experiment 1: single substance toxicity

Technical grade NaCl (artificial sea salt) purchased from Royal Salt Company Ltd, Parow East, South Africa, was added in solution form to 500 g sub-samples of soil to give concentrations of 0, 1000, 2000, 4000, 6000 and 8000 mg/kg NaCl by dry weight of soil. The salts were dissolved in the corresponding volume of de-ionised water so as to make up 60% of the final water holding capacity of each soil treatment. There were four replicates of each concentration.

The treated soils were placed in cylindrical plastic vessels of 2 l and allowed to equilibrate for two days before earthworms were introduced. Ten worms per container were used in each exposure regime and were introduced into the relevant test soil by placing them on the surface and allowing them to burrow in. The test containers were covered with perforated lids to limit water loss due to evaporation and kept in 16 h light, 8 hr dark at 20 ± 1^0C (Reinecke and Kriel, 1981) in a climate chamber for 28 days. To maintain the worms during the exposure period in the test medium, the worms in each container were fed weekly with ground sieved and dried urine-free cattle manure to provide 0.5 g per worm.

Mortality, growth and cocoon production were observed at day 28. LC_{50} (lethal concentration at which 50% of the population were killed) and EC_{50}s (Effect concentration at which a 50% reduction in a measured parameter) were calculated. Growth was determined by individually weighing each surviving worm from each container, and comparing the mean weight with initial values. Mortality was assessed by stimulating the worm with a blunt probe and an earthworm was judged dead if no response could be observed. Worms not found during sampling was judged dead since earthworm tissue decompose easily in soil when dead. The number of cocoons was determined at the end of the exposure by wet sieving the substrates (through a 2.0- and 1.0-mm sieve system).

5.2.3.2 Experiment 2: Joint substance toxicity

Three soil groups with differing salinity level were made by adding 0, 2000, or 4000 mg/kg NaCl by dry weight soil. These concentrations were chosen based on results of experiment 1, so as to assess sub-lethal effects in the joint substance exposures. Laboratory grade zinc chloride ($ZnCl_2$; purity, 98%; molecular weight = 136.28, Merck, South Africa) in several concentrations (0, 250, 500 and 750 mg/kg Zn dry weight of soil) were used in this experiment. The two salts (NaCl and $ZnCl_2$) were added in aqueous form and mixed thoroughly with the corresponding soil. The experimental design for exposure, pH and conductivity measurements are shown in Table 6. The treatment soils were prepared and worms were introduced as in Experiment 1 (single substance exposure). Exposure conditions were also as in experiment 1. Each treatment (of ten worms each) was replicated four times. Sampling was done at day 1, 7, 14 and 28 after worms were introduced to the substrates.

Mortality, growth, and internal metal concentrations were monitored on each sampling occasion while cocoon production was assessed at day 28 alone. Mortality, growth and cocoon production were determined as already explained in Experiment 1. Cocoon number per worm was calculated by dividing the total number of cocoons by the number of surviving worms. It was assumed that a worm that died during the exposure couldn't have contributed to cocoon production since concentrations of toxicants affecting cocoon production is often much lower than that affecting mortality. The net number of surviving worms in each container of the control soils (receiving 0 mg/kg Zn + 0 mg/kg NaCl) at the end of the test was seven, since three worms were removed from each container for analysis of internal metal concentration. This reduction in sample size (12 worms from the original 40) was accounted for in calculating cocoon production.

Table 6. The amounts of zinc and sodium chloride added in the experiments and the corresponding electrical conductivity and pH measured at day 1 after worms were introduced.

	Zinc concentrations		*Salt concentrations (NaCl)*		*Mean Electrical Conductivity (d S/m)*	
	mg/kg	*mM*	*mg/kg*	*mM*		*pH*
Experiment 1: NaCl alone	0	0	0	0	0.16	Nm
	0	0	1000	17	0.43	Nm
	0	0	2000	34	0.78	Nm
	0	0	4000	68	1.31	Nm
	0	0	6000	0	1.82	Nm
	0	0	8000	0	2.34	Nm
Experiment 2: NaCl+Zn	0	0	0	0	0.16	5.92
	250	3.82	0	0	0.24	5.61
	500	7.64	0	0	0.32	5.59
	750	11.46	0	0	0.41	5.44
	0	0	2000	34	0.78	5.82
	250	3.82	2000	34	0.86	5.63
	500	7.64	2000	34	0.95	5.54
	750	11.46	2000	34	1.01	5.32
	0	0	4000	68	1.31	5.78
	250	3.82	4000	68	1.4	5.63
	500	7.64	4000	68	1.48	5.49
	750	11.46	4000	68	1.56	5.34

Nm-not measured

To determine the internal metal concentration, one worm per container was removed on each sampling occasion, except at day 28 when all surviving worms were used. They were placed individually in Petri dishes on moist filter paper for 24 hrs at 20^0C to allow depuration of their gut contents. Afterwards, they were weighed and frozen individually for metal analysis. The procedure for the metal extraction by acid digestion has been described by Maboeta et al. (2003). Thereafter, samples were filtered using 0.45 µm cellulose nitrate filter paper into film boxes and stored at 4 ^{o}C until analysis.

For soil samples, electrical conductivity (EC) was measured at day 1 as described in SSS (1996). A soil-water extract (1:5; w/v) was made and measurements were taken with a conductivity meter (SM 802 pH/ EC/ TDS Meter, Spraytech). Soil moisture content and pH-(H_20) were monitored on each sampling occasion (Micro pH 2001, Crison for pH, Sartorius infrared moisture detector for moisture). Moisture loss was replenished by adding the equivalent amount of de-ionised water. For the determination of zinc contents of substrates, samples were taken at day 1 and 28 and chemical extractions were carried out using three methods: $CaCl_2$, DTPA and nitric acid extraction methods. For $CaCl_2$ and DTPA extractions, a sequential procedure was followed according to those used by Maiz et al. (1997). From each sample, 3 g of air dried sub-sample was shaken with 30 ml solution of 0.01 M $CaCl_2$ for 2 h prior to centrifugation at 4000 rev/min for 15 min. The supernatant was removed for analysis of metal contents. For the DTPA extraction, the residue of the $CaCl_2$ extraction was shaken with 6 ml solution of DTPA for 2 h prior to centrifugation at 3000 rev/min for 10 minutes. The supernatant was in both cases filtered through Whatman 540 filter paper, and stored at -4^0C until analysis. For nitric acid extraction, zinc contents were extracted by acid digestion as described by Maboeta et al., (2003).

For each batch of worm digested a blank was also prepared to detect possible contamination during the digestion process. The extracted soil and worm samples were analyzed for zinc by a Varian AA-1275 flame atomic absorption spectrophotometer (AAS). The Spectrometer was calibrated using 5-, 10- and 25 mg/l Zn standards. Previously spiked soil samples were analyzed and indicated a recovery above 90% with this procedure.

5.2.4 Chloride compensation

Since the EC of soil receiving Zn as $ZnCl_2$ increased slightly because of Cl^- increase, it was necessary to quantify the effect of Cl^- in Experiment 2. We therefore compared toxic effects on worms exposed to the highest concentration of NaCl (4000 mg/kg) with those exposed to 4000 mg/kg NaCl + highest concentration of Cl^- (813 mg/kg Cl) in the Zn treatments. Cl^- was added as KCl since it is known that KCl is not toxic at this concentration (Fischer and Molnar, 1996). Four replicates of the two groups of worms were used. Mortality and growth were assessed at day 1, 7, 14 and 28. Cocoon production was not assessed because worms did not produce cocoons at this concentration of NaCl.

5.2.5 Statistics

In experiment 1, the LC_{50} value for NaCl was calculated by using the Trimmed Spearman-Karber Program version 1.5. EC_{50} values of NaCl for growth and cocoon production were calculated by using the Linear Interpolation Method (USEPA, 1993). In experiment 2, all data were checked for normality and homogeneity of variance with the Shapiro-Wilks W test and Levene's test respectively. Growth data per sampling time, cocoon number, and pooled mortality data were analyzed using two-way analysis of variance (ANOVA) with salinity and zinc as variables while pH data by three-way ANOVA with salinity, zinc and time as variables. For zinc concentrations in worm and in soil (nitric acid, DTPA and $CaCl_2$ zinc extracts), one-way ANOVA was used to test for the effect of salinity along each zinc level. When data were not normally distributed even after transformations, Kruskal-Wallis H-test was used. Fischer's least significant difference (LSD) post-hoc test was used to determine the significance of any differences between specific groups in parametric cases while multiple comparison of 'z' and 'p' values was used in non-parametric cases. For the chloride compensation test, student's T-test was used to compare the means of growth data for each group at each sampling date. Tests of similarity between DTPA zinc extracts of substrate at day 1 and 28 and $CaCl_2$ zinc extracts of substrates at day 1 and 28 were analyzed with Wilcoxon sign-paired test. All data were analyzed with STATISTICA 7.0.

5.3.0. RESULTS

5.3.1. Experiment 1: Toxicity of NaCl as individual substance

There was no mortality in control soil (0 mg/kg NaCl) and soils exposed to 2000, and 4000 mg/kg NaCl. However, total mortality of worms occurred in all treatments exposed to 8000 mg/kg NaCl. The calculated LC_{50} for NaCl after 28 days was 5436 (confidence interval, CI, 5170-5716) mg/kg NaCl. There was no cocoon production as from 4000 mg mg/kg NaCl. The 28-day EC_{50} for cocoon production was 2020 (CI, 1467-2636) mg/kg. The 28-day EC_{50} for growth was 4985 (CI, 4605-5000) mg/kg NaCl.

5.3.2 Experiment 2: Joint substance toxicity

5.3.2.1 pH change

At day 1, spiking with Zn alone reduced the mean pH value of substrates by 0.48, whereas spiking with NaCl alone reduced the mean pH value of substrates by 0.14 units (Table 6). During the 28 day exposure period, pH increased slightly in all treatment groups, with the highest increase occurring between day 7 and 14. The range for pH changes in all treatments was 0.18 to 0.32. The increase was fairly similar in all treatments irrespective of NaCl or Zn concentrations. Statistical analysis showed that interaction of NaCl and Zn had no significant influence ($P > 0.05$) on pH. However, individually, NaCl, Zn as well as time had highly significant influence ($P < 0.01$) on pH in this study.

5.3.2.2 $CaCl_2$, DTPA and nitric acid extractable zinc in substrates

The amounts of $CaCl_2$, DTPA and nitric acid extractable zinc in the substrates at day 1 and 28 are shown in Fig 12. At day 1, no significant differences ($P > 0.05$) were found in the amount of zinc extracted at each zinc level in the three salt regimes by all three extraction methods. At day 28, for $CaCl_2$ and DTPA extractions, there were increases in extractable zinc content of substrates with increased salinity. These were however only statistically significant ($P < 0.05$) at the highest zinc level (750

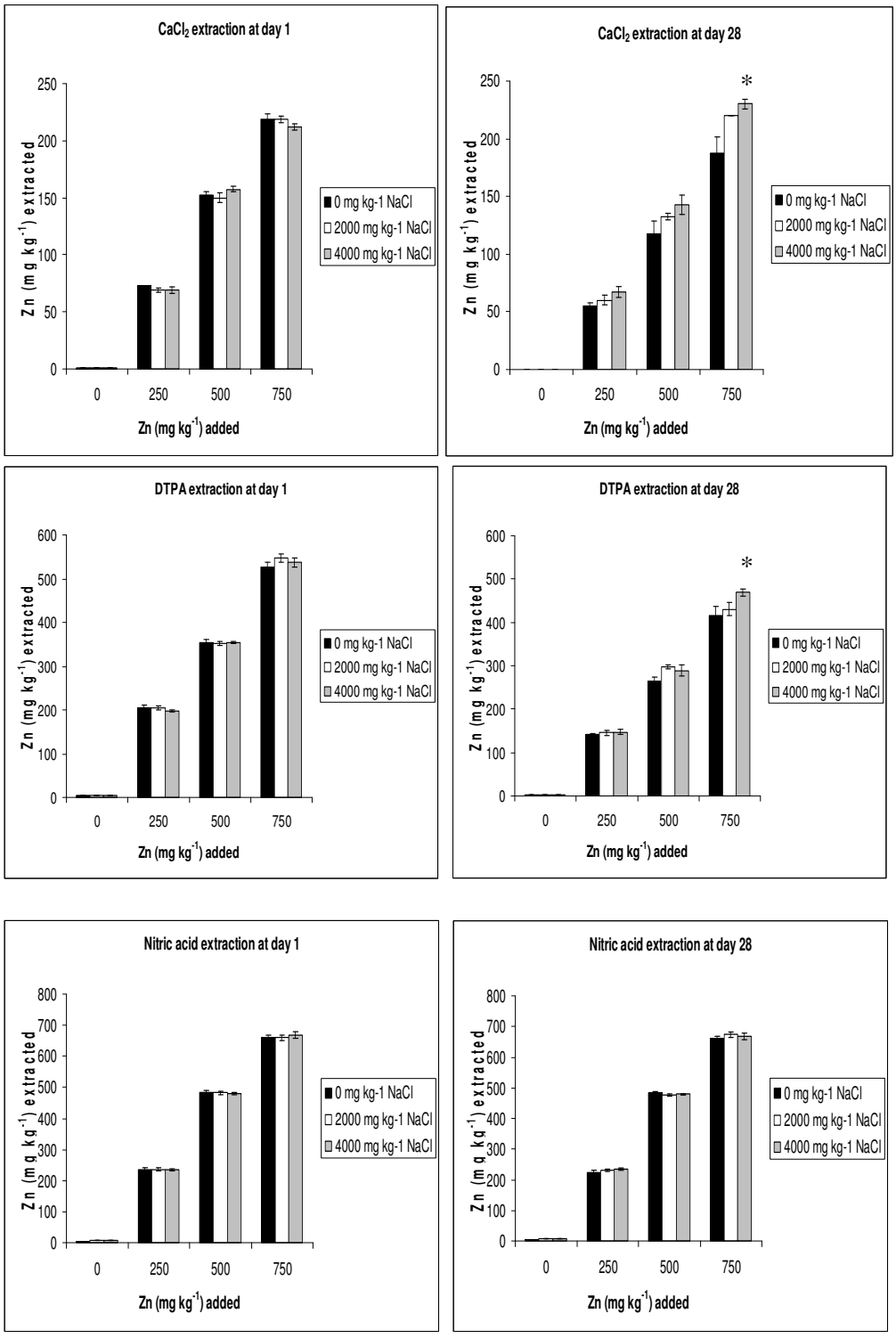

Fig 12 Mean (± SE) zinc concentrations extracted in saline and non saline substrates spiked with zinc as measured by Atomic absorption spectrophotometry after extraction with three extraction methods. * concentration significantly different from the non saline group ($P < 0.05$).

mg/kg Zn) for both extracts. For nitric acid extraction, no discernible pattern was seen. Analyses of differences between Zn extracts at days 1 and 28 showed that the $CaCl_2$ extracts at day 28 were significantly higher ($P < 0.01$) than at day 28 while DTPA extracts at day 28 were significantly lower ($P < 0.05$) than at day 1.

5.3.2.3 Mortality, growth and reproduction

Fig. 13 shows the % mortality in each of the substrate groups over the entire exposure period. Mortalities were found at all sampling dates with higher numbers of deaths occurring at days 7 and 14. There was no mortality in treatments with either NaCl or Zn alone. In treatments with a combination of NaCl and Zn, increased mortality was seen with increased concentration of both substances. This was only significant ($P < 0.05$) at 750 mg/kg Zn, in which case mortality in the 4000 mg/kg NaCl soil group was significantly ($P < 0.05$) higher than at 0 and 2000 mg/kg NaCl.

Fig. 14 shows the mean weight of worms exposed to zinc for 28 days under the three salinity levels. Generally, there was a decrease in worm weight in all treatments at day 1. Subsequently, from day 7, there was an increase in mean weight of worms in all exposure groups at all sampling occasions except for worms exposed to 750 mg/kg Zn and 4000 mg/kg NaCl substrates. The growth of the worms in NaCl and Zn treatments showed different patterns. In 0 mg/kg NaCl soil, there were no notable differences in mean weight of worms in all zinc concentrations. In 2000 mg/kg NaCl substrate, worms exposed to 750 mg/kg Zn had lower mean weight in comparison to worms in 0, 250 and 500 mg/kg Zn while in 4000 mg/kg NaCl exposu re group, a clear dose response curve of the effect of zinc was seen. Day 1 data were excluded from statistical analysis since the pattern of weight change could have been because of difference as a result of acclimatization of worms in non-saline substrates. Statistical analysis (ANOVA) showed that the interaction of salinity and zinc was significant so was their individual effects (Appendix 9).

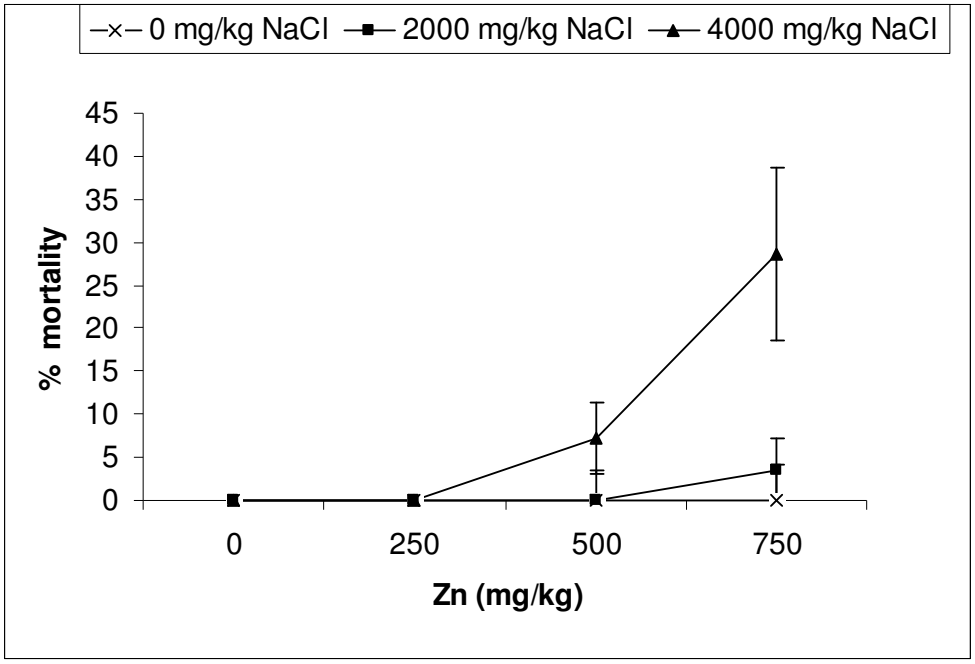

Fig 13. Mean (± SE) percentage mortality of four groups of ten worms (*Eisenia fetida*) each exposed to zinc for 28 days in saline and non saline OECD artificial soil substrates at constant temperature and moisture (n = 40 initially).

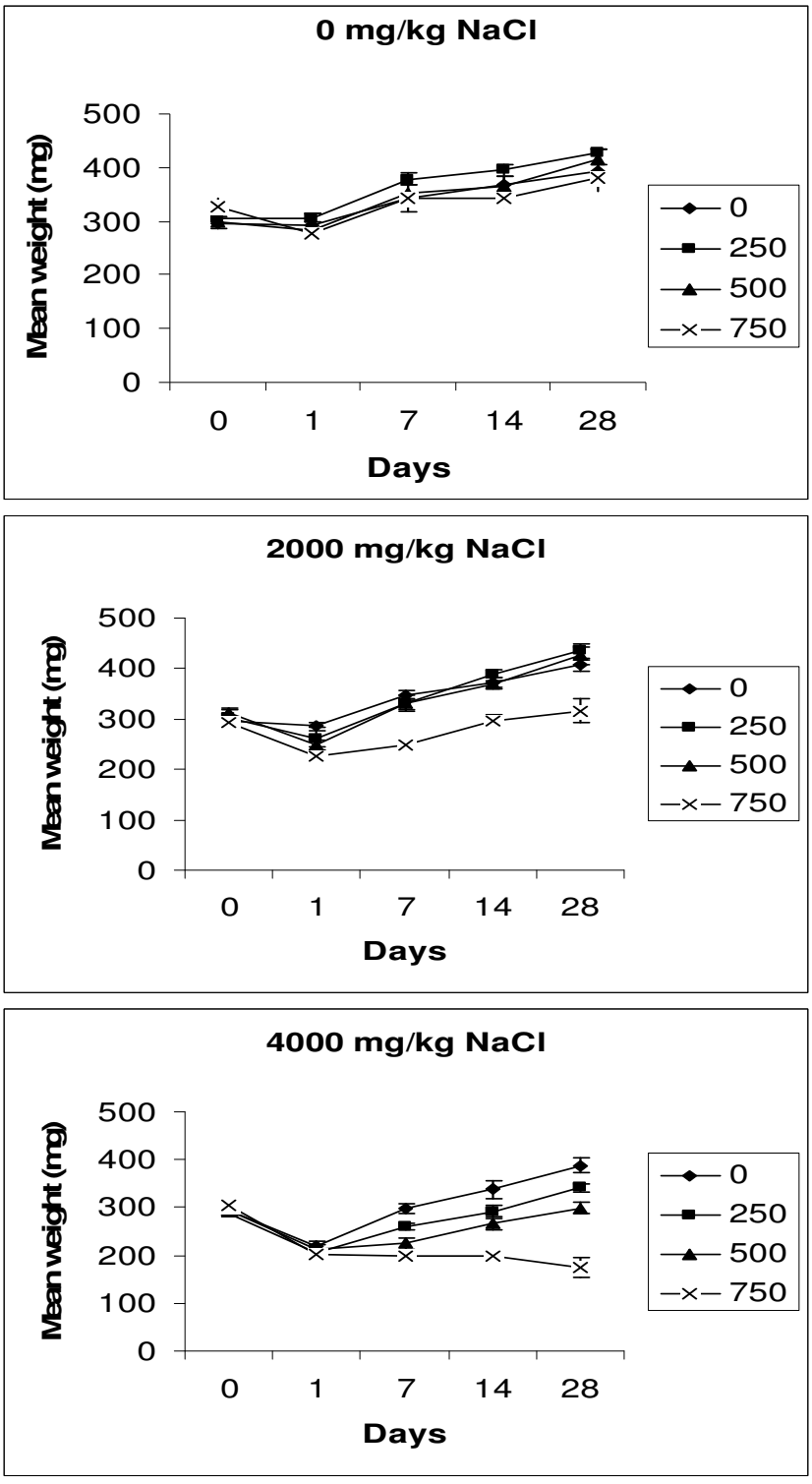

Fig 14. Mean (± SE) weight change of four groups of ten worms (*Eisenia fetida*) each exposed to different concentrations of zinc (mg/kg) in saline and non saline OECD artificial soil substrates at constant temperature and moisture.

Further post hoc analysis (Fischer's LSD) however showed that individually, zinc had no significant effect on the weight of the worms at all sampling times while salinity only had effect at day 7 and not the other days. At day 7, weight of worms in 4000 mg/kg substrate was significantly lower ($P < 0.01$) than for worms in 0 mg/kg NaCl and 2000 mg/kg NaCl. Post hoc analysis showed that the interaction (NaCl and Zn) effect was at day 7, 14 and 28. At day 7, the mean weight of worms in 2000 mg/kg NaCl was significantly ($P < 0.05$) lower than for worms in 0 mg/kg for Zn concentrations of 250 and 750 mg/kg. At day 14 and 28, mean weight of worms in 4000 mg/kg NaCl was significantly ($P < 0.01$) lower than for worms in 0 mg/kg NaCl and 2000 mg/kg NaCl for all zinc concentrations. For worms exposed in soil of 0 mg/kg and 2000 mg/kg NaCl, a significant difference in mean weight was only found at 750 mg/kg Zn in the order (0 mg/kg NaCl > 2000 mg/kg NaCl)

Fig. 15 shows the mean cocoon number produced at different exposure concentrations in the three salinity levels. Worms in 4000 mg/kg NaCl soil did not produce any cocoons, irrespective of zinc concentrations. In the 0 mg/kg NaCl soil group, cocoon production of worms exposed to 0 and 250 mg/kg Zn were similar and higher than at other Zn concentrations, while at 2000 mg/kg NaCl soil group, a clear dose response curve for the effect of Zn was seen. The interaction of salinity and zinc had a significant effect ($P < 0.05$) on cocoon numbers (Appendix 10). In all zinc concentrations from 250 mg/kg Zn onwards, the mean number of cocoons produced by worms in 0 mg/kg NaCl soil group was significantly higher than that produced in 2000 mg/kg NaCl, which was not significantly different from that produced in 4000 mg/kg NaCl soil group.

5.3.2.4 Internal concentration of zinc in worms

The internal concentration of zinc in both exposed and unexposed worms in the gradient of salinity is shown in Fig. 16. Generally, there were low zinc concentrations (mostly between 10 and 40 mg Zn/kg dry wt) in the body of the worms, irrespective of salinity levels. Statistical analysis showed that salinity only had

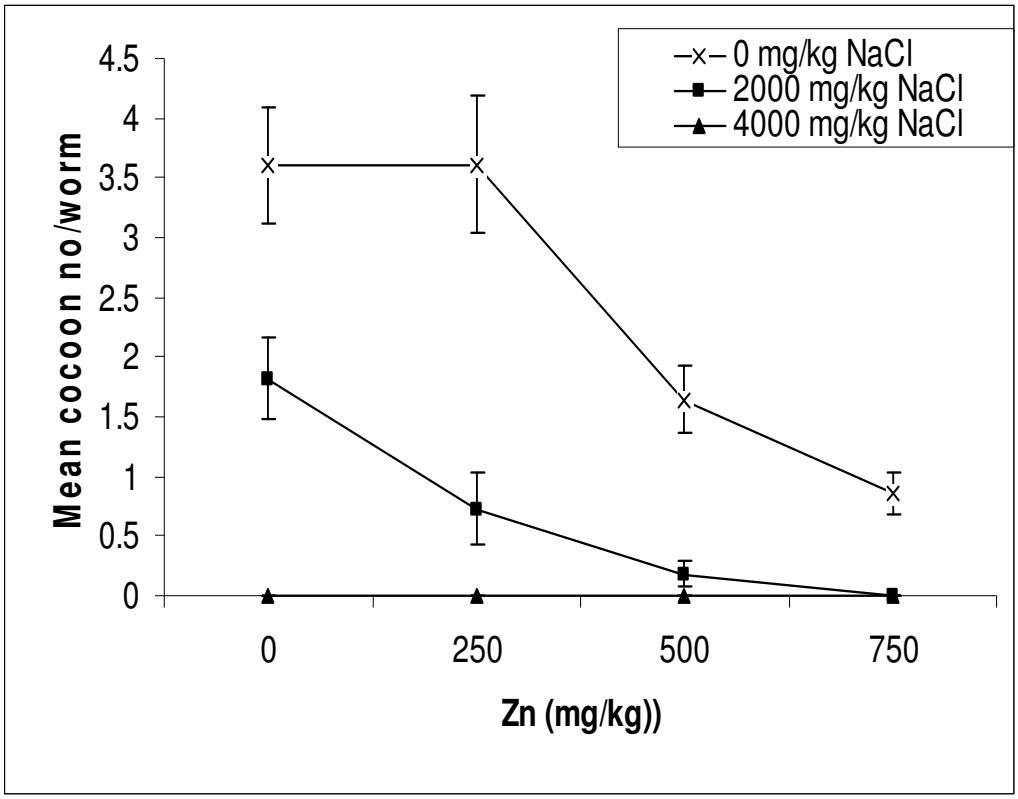

Fig 15. Mean (± SE) number of cocoons per worm produced after four-week exposure of four groups of ten worms (*Eisenia fetida*) each to zinc in saline and non saline artificial soil substrates at constant temperature and moisture.

significant influence ($P < 0.05$) on internal concentration of zinc in worms at day 7, and not any of the other sampling days (Fig. 16).

5.3.3 Chloride compensation

There was no mortality in all replicates of both groups (worms exposed to 4000 mg/kg NaCl and those exposed to 4000 mg/kg NaCl + 813 mg/kg Cl) during the 28-day exposure period. However, a highly significant effect ($P < 0.01$) on growth was found at days 7, 14 and 28. At these days weight of worms exposed to 4000 mg/kg NaCl were significantly higher than those exposed to 4000 mg/kg NaCl and 813 mg/kg Cl substrates.

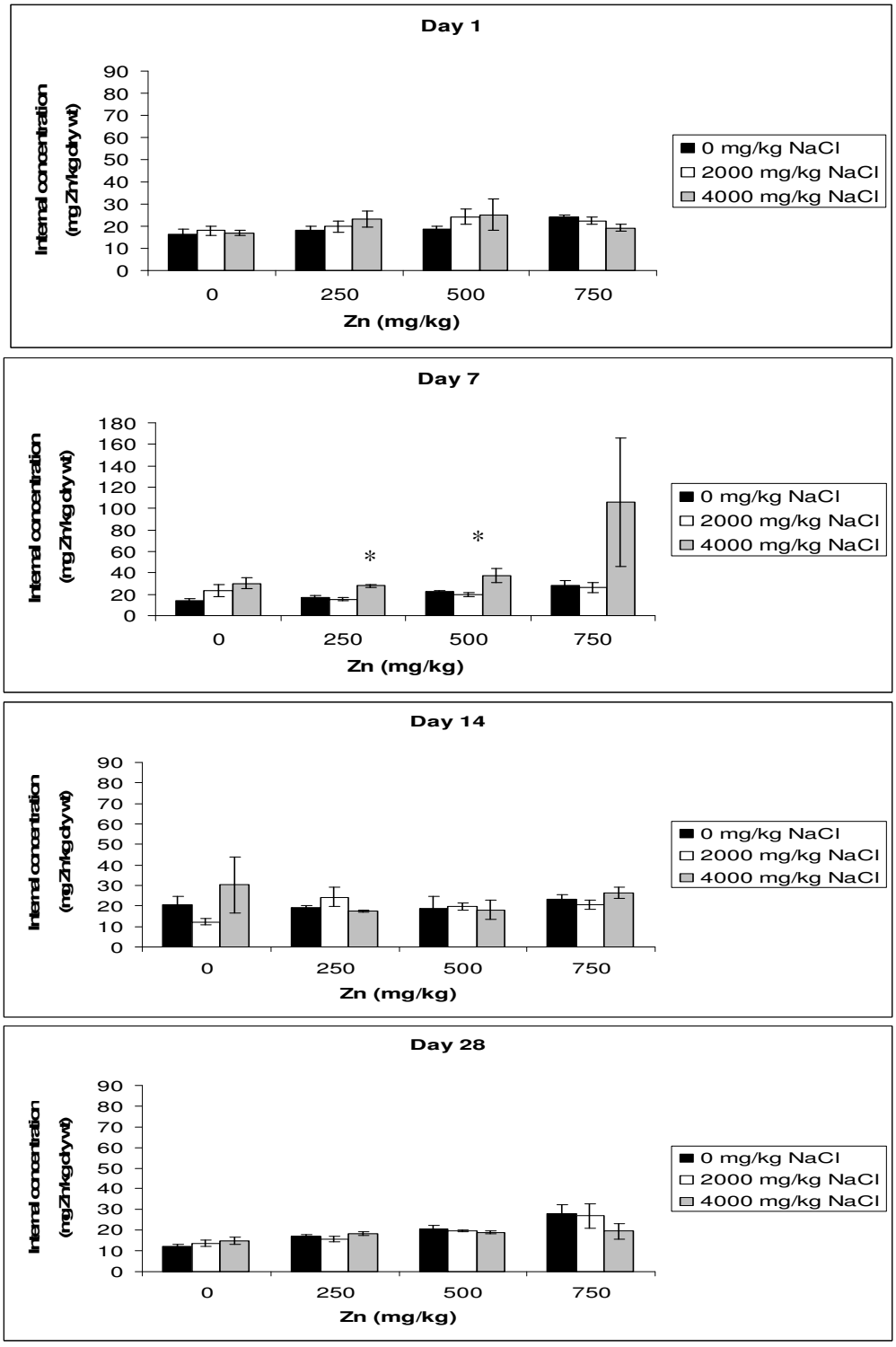

Fig 16. Mean (± SE) nitric acid extracted zinc concentrations in specimens of *Eisenia fetida* exposed to zinc in saline and non saline artificial soil substrates spiked with zinc for 28 days. * concentration significantly different from the non saline group ($P < 0.05$).

5.4.0 DISCUSSION

5.4.1 Toxicity of NaCl as individual substance

The results showed that increased salinity had harmful effects on growth, mortality and reproduction of this earthworm species. Of these parameters, cocoon production was the most sensitive parameter. Cocoon production was affected at concentrations far lower than those affecting growth and mortality. This is in agreement with another study (Bright and Addison, 2002) which showed cocoon production was a more sensitive parameter than mortality and growth in assessing the toxic effect of salinity. 28-day EC_{50} for cocoon production of 2020 mg/kg NaCl and for growth of 4985 mg/kg NaCl observed in this study were comparable to those available in the literature. Bright and Addison (2002) found 28-day EC_{50} for cocoon production of 1884 (CI, 1485-2284) mg/kg NaCl and growth of 4681 mg/kg NaCl. The LC_{50} observed in this study is lower than the LC_{20} of 5534 mg/kg NaCl observed by Bright and Addison (2002), although both studies used OECD soil substrate. The reasons for these differences in toxicity could be in the differences in sample size used. In our study, 40 worms (10 per unit, 4 replicates) were used in each exposure regimes while only 15 worms (5 per unit, 3 replicates) were used in their study. Although the robustness of a larger sample size would have increased the precision and accuracy of my data, other differences in conditions and worm age could also have played a role.

Soils are generally classified as saline when they have an EC of 4 dS/m or more (Sumner, 1995). Soils with a salinity of less than 2 dS/m are classified as non-saline for plants (Schoeneberger et al. 2002). This study showed that earthworm may be affected negatively at concentrations known to be safe for many plants. For the protection of soil organisms against salinity it is necessary to conduct more studies with organisms in different tiers, representing a greater diversity of soil biota.

5.4.2 Joint substance toxicity of NaCl and zinc

In the joint substance exposure experiment, salinity reduced the partitioning of zinc in the substrates as shown by increased values of labile zinc extracted by $CaCl_2$ and DTPA at day 28 of the test period. This is in agreement with results of other

researchers. Keshavarz et al., (2006) reported that bioavailable (DTPA extractable) zinc increased by 1 to 6.3% with increasing levels of salinity. In another study, DTPA extractable concentrations of zinc initially followed an increasing trend (from 0 to 2 dS m^{-1}) and reached a maximum concentration at EC value of 4 dS/m, after which a decreasing trend was observed with increasing EC value (Parkpian et al., 2002). Parkpian et al. (2002) reported that salinity could reduce the total metal extracted with nitric acid. No significant effect of salinity on nitric acid extraction was found in our study. The contrast between our study and theirs could be the result of the relatively lower salinity level we used. The highest salinity level in our study (EC = 1.31 dS/m) was lower than the lowest salinity group after control (EC = 2 dS/m) used by these authors. It was not necessary to adjust the EC of soil in our study to values of 2 dS m^{-1} or higher since earthworms would not survive in soils of these EC values. DTPA extractable Zn reduced significantly with time while the opposite was true for $CaCl_2$ extractable Zn fraction in our study. It is generally known that the availability of Zn reduces with time (Lu et al., 2005). However, increase in $CaCl_2$ extractable Zn in our study could have been contributed by salinity effect.

The toxic response observed in this study could however not be fully explained by the effect of salinity on partitioning or availability of zinc in the substrate alone. Although there was an increase in labile zinc concentrations, with increasing salinity, this was only significant for $CaCl_2$ and DTPA extraction (Fig. 12) when 750 mg/kg Zn was added. Calamari and Alabaster (1980) have suggested that the interaction of chemicals may occur in the substrate and at the point of entry into organisms among two or more chemicals. Our results further showed that there may have been interaction between the two substances at the point of entry into the organisms or at the site of toxic action. In the joint substance exposures, salinity and zinc had no significant negative effect on weight change and mortality as individual contaminants but their presence and possible interaction was detrimental for these parameters. The significant effect observed for salinity at day 7, which disappeared as the experiment progressed could be because the worms have not yet fully acclimatized to the substrate at day 7. This suggests the possibility of a synergistic mechanism between the two substances. However, the synergy between NaCl and Zn could be partly explained by Cl^- effect from the addition of zinc as zinc chloride. The results of the chloride compensation test showed that the contribution from Cl^-

compensation was significant for weight and not for mortality and could not fully explain the observed synergism between NaCl and Zn in this study. If the contribution of Cl^- is factored out, the joint effect of Zn and NaCl could be seen as additive rather than synergistic

Two possible explanations could be given for the observed interaction. Firstly, that salinity enhanced the availability of zinc in the substrates and hence increased uptake of zinc by the earthworm. However, because of its ability to regulate Zn, rapid elimination took place ensuring that the net worm body burden of zinc was comparable among worms in all salt regimes for all zinc concentrations. This could probably explain why the differences in toxicity of zinc between worms exposed in non-saline and saline substrates could not be explained by the differences in zinc accumulation which was only significant at day 7. Zinc is an essential metal which is regulated by earthworms (Lock and Janssen, 2001). Fast accumulation and elimination of zinc has been reported by many researchers e.g. (Spurgeon and Hopkin, 1999). The process of elimination was not investigated in this study. This prevents us from making conclusions about the mechanism involved.

Apart from increasing the availability of zinc in the substrates, NaCl itself joined with zinc to evoke additive to synergistic toxicity at the point of entry into the earthworm. NaCl was toxic to these organisms, as shown by all life-cycle parameters tested in this study, especially cocoon production. This suggests the possibility of an additive to synergistic mechanism between the two substances. The results presented are strongly supportive of these explanations. Further studies focusing on both zinc and Na ions in the tissue of the worm could unravel the exact mechanism of this interaction.

A simple way to express the interaction of NaCl and Zn, in our study would be by joint action/toxicity. This is in contrast to results of other studies where only the cationic constituent of the salt was considered. Recently, Lock et al. (2006) while developing the Biotic Ligand Model (BLM) reported that an increase in Na^+ up to 15 mM would reduce cobalt toxicity to *Enchytraeus albidus* by a factor close to 3. Steenbergen et al. (2005) also showed that Na^+ did protect *Aporrectodea caliginosa* against Cu^{2+} toxicity. Also, an increase in Na^+ up to 13 mM reduced zinc toxicity to *Daphnia magna* by a factor of 3.1 (Heijerick et al., 2002). All these authors suggested

that the protection of these organisms by Na^+ against metal ions was due to competition at the biotic ligand. The BLM has gained increased interest among ecotoxicologists in the last few years, and proved useful in explaining the effect of competing ions on metal toxicity to various organisms (Steenbergen et al., 2005). However, the current data, in view of the aforementioned data, showed that above a certain threshold, these competing ions may not be protective but may rather become toxic to the organism. For example, in 4000 mg/kg NaCl (or 27 mM Na), Na^+ was not protective against zinc toxicity as reflected by all parameters tested while in 2000 mg/kg NaCl (13.5 mM Na) in comparison to the 0 mg/kg NaCl soil group, there were higher although non significant toxic effects as shown by the figures for mortality and weight change. It is difficult to set a narrow range for this threshold since this study did not include a salt level between 0 and 13 mM Na in the joint substance exposures, the range in which the threshold could lie.

5.5 CONCLUSION

This part of the study showed that salinity can have detrimental effects on earthworms at concentrations considered safe for many plant species. However, direct comparisons cannot be made and conclusions should be made with caution because of differences in bioavailability of chemicals between the artificial soil and other soils. We determined 28-day LC_{50} of 5436 mg/kg NaCl. The EC_{50}'S for growth and cocoon production were 4985 and 2020 mg/kg NaCl respectively. In the joint substance exposures, salinity in the range used in this study slightly increased the availability ($CaCl_2$ and DTPA extractable fractions) of zinc in the substrates. The combined effect of salinity and zinc was more severe than when worms were exposed to either contaminant. An additive to synergistic interaction between salinity and zinc seems to affect their toxicity to *Eisenia fetida*. The apparent synergy could only be partly explained by Cl^- effect from addition of zinc as $ZnCl_2$. This study indicated the detrimental effects salinisation of soils could have on earthworms. When combined with sub-lethal concentrations of zinc, it could especially affect reproduction. However, because of differences in the effect of Na, Ca, K, and Mg ions, more studies need to be done incorporating all of these ions before these preliminary conclusions could be firmly established. Field studies are needed to confirm the observed biological effects under environmentally relevant conditions.

CHAPTER SIX

6.0 THE COMBINED STRESS EFFECTS OF SALINITY AND COPPER ON THE EARTHWORM *EISENIA FETIDA*

6.1 INTRODUCTION

Irrigation and use of agrochemicals have been features of conventional agricultural practice for long and their use improved crop yield and food production (Lee, 1985). It is however doubtful if these practices enhance sustainability of environmental resources. Repeated irrigation (with water of poor quality) and use of pesticides could lead to salinisation of soil and metal contamination respectively, especially in the top layers of soil.

Salinisation of soil is a problem in many parts of the world. Saline soils often occur in irrigated land (Ayars and Tanji, 1999) in semi-arid or arid zones of the world. About 50% of irrigated areas of the world are either salinised or have the potential to be so in future (Tyagi, 1986), underlining the extent of the problem. Generally, salinity affects crop yield and beneficial soil biota, leading to economic losses. It affects the growth and survival of microorganisms (Lippi et al., 2000; Yuan et al., 2007), plants (Ramoliya et al., 2004; Kadukova and Kalogerakis, 2007) and soil animals (Hobel et al., 1992). It is also well known that the distribution and abundance of earthworms are influenced by soil salinity in various ecosystems (Lee, 1985).

Other than salinisation, another soil contamination problem is that of metal contamination. Metal contamination of the soil environment, apart from natural occurrence, could be caused by industrial activities and the use of metal containing agrochemicals (Vorobeichik, 1998). One such agrochemical is copper oxychloride which is a widely used fungicide in agricultural land (Krause et al., 1996). Although it is an essential mineral, the toxic effects of excessive amounts of Cu are well documented: it significantly affects survival, growth and reproduction of earthworms (Svendsen and Weeks, 1997; Helling et al., 2000; Maboeta et al., 2002, 2003), damages internal organs and compromise lysosomal membrane integrity in snails (Snyman et al., 2000, 2002) and several other soil organisms.

Most studies on the effect of salinity or copper on soil organisms have hitherto focused on individual substance toxicity. However, in natural systems, especially in agricultural lands, environmental stressors like salinity and copper could be present simultaneously. Existing studies mostly neglect to address the fact that in soil, the overall joint effect is the result of interaction at various levels, inside and outside the body of the test organisms (Posthuma et al., 1997). As a result, data generated from individual substance exposures could not be used to predict the toxicity to soil organisms exposed to myriads of contaminants simultaneously in the field. Mixture toxicity experiments may reflect the actual pollution of ecosystem in a more realistic way than experiments in which toxicants are tested individually (Spurgeon et al., 1994).

Mixture toxicity of contaminants may follow an additive or non-additive (antagonistic or synergistic) model. According to the terminology generally accepted for describing the toxicity of a mixture, when the toxicity of a mixture corresponds exactly to the sum of the fractions of the individual component the effect is referred to as "additive". When toxicity is greater or lower than the sum of the fractions of the individual components, the effect is referred to as "non additive": synergistic in the former case or antagonistic in the latter ((Mohapatra and Rehanganjra, 1996; Tao et al., 1999). However, recent data showed that more complex response patterns, such as dose level/ratio-dependent synergism/antagonism do occur (Jonker et al., 2005). Dose level-dependent deviation indicates a situation where deviation from reference model at low dose levels is different from the deviation at high dose levels. For instance, antagonism may be observed at low dose levels and synergism at high dose levels. Dose ratio-dependent deviation indicates a situation where deviation from reference model depends on the composition of the mixture (Jonker et al., 2005).

Few studies have been conducted on the joint influence of salinity and metals on sorption processes and toxicity to soil organisms. Available literature data suggest that salinity tends to increase the bioavailability of metals in substrates. DTPA (di-ethylene-triamine-penta acetic acid) extractable copper, zinc, manganese and lead were reported to increase with increasing levels of salinity in sewage sludge (Keshavarz et al., 2006). Pakpian et al. (2002) found DTPA extractable zinc increased by 1 to 6.3% with increasing levels of salinity in natural soil of Asian origin. In

Chapter 5, it was demonstrated that salinity significantly increased the DTPA and $CaCl_2$ extractable Zn in Organisation for Economic Co-operation and Development (OECD) artificial soil. The latter authors further suggested an additive to synergistic interaction between salinity and Zn in their combined toxicity to the earthworm *Eisenia fetida*. How salinity may influence the bioavailability and toxicity of Cu, another essential metal, remains speculative.

The aim of this paper was to investigate possible interaction between NaCl-salt and copper ions in soil as well as how these interactions affect the uptake and toxicity of copper to the earthworm *Eisenia fetida*. This species was chosen because it is easy to culture and there are relatively large data sets available in the literature on the biology and ecotoxicology of this species. Copper as copper oxychloride was used because it is one of the major fungicides used in irrigated agricultural land (Krause et al., 1996) such as vineyards which could also be under threat of salinisation. NaCl salt was chosen because it is the predominant salt in agriculturally induced salinisation in South Africa (Dallas and Day, 1993) and also in most inland saline water in Australia (Kefford et al., 2005), two arid regions where salinity of soils is a concern.

6.2 MATERIALS AND METHODS

6. 2.1 Test species

E. fetida specimens used for this study were age-synchronized from a culture kept in the laboratory of the Ecotoxicology Group, University of Stellenbosch, South Africa. Adult worms (9-12 weeks old) of between 250 and 400 mg were used in the experiments. The worms were acclimatized for 72 hours in OECD soil and were fed during this period with the same type of food to be used during the experiment consisting of urine free, dried and grinded cattle manure.

6.2.2 Test procedures and experimental setup

OECD soil was prepared by mixing 10% finely ground sphagnum peat (<1 mm), 20% kaolin clay and 70% quartz sand, adjusted with $CaC0_3$ to pH 6.0 ± 0.5 (OECD, 2004). Technical grade NaCl (artificial sea salt) purchased from Royal Salt Company Ltd, Parow East, South Africa was added in the following concentrations: 0,

1000, 2000 and 4000 mg/kg NaCl/soil dry weight (DW) to achieve a gradient of electrical conductivity values. Copper was added as copper oxychloride ($Cu_2Cl(OH)_3$) (trade name 'Virikop') which was purchased from Agro-Serve (Pty) Ltd, South Africa. The test substance (850 g/kg a.i. and 500 g/kg Cu) was added in the following concentrations: 0, 20, 80, 320 and 640 mg Cu/kg DW soil. The concentrations were chosen in this way to include nominal concentrations of Cu normally found in agricultural soils in South Africa and elsewhere. The substances NaCl and Cu were added singly and jointly, in aqueous solution and mixed thoroughly with the artificial soil (each consisting of 500 g sub-sample) in an amount that leads to 60% of the final water holding capacity of the soil. The experimental design for exposure, pH and conductivity measurements are shown in Table 7.

The spiked soils were placed in cylindrical plastic vessels of 2 l and allowed to equilibrate for three days before earthworms were introduced. Ten worms per container and four containers per treatment were used. The worms were introduced into each container by placing them on the surface and allowing them to burrow in. The test containers were covered with perforated lids to limit water loss due to evaporation and kept in 16 h light, 8 hr dark at 20 ± 1^0C (Reinecke and Kriel, 1981) in a climate chamber for 28 days. Worms were fed weekly with ground, sieved and dried, urine-free cattle manure (0.5 g per worm).

6.2.2.1 Worm parameters

Sampling was done at day 7, 14 and 28 after worms were introduced into the soil. Measured parameters were growth, mortality, and internal copper concentration of worms. Growth was determined by weighing each worm individually from each container, and comparing the mean weight with initial values. Mortality was assessed by stimulating the worm with a blunt probe and an earthworm was judged dead if no response could be observed. The number of cocoons produced was determined at the end of the exposure by wet sieving the soil (through a 2.0- and 1.0-mm sieve system), counting the number of cocoons and dividing by the number of surviving worms.

Table 7. The amounts of Cu and NaCl added in the experiments and the corresponding electrical conductivity and pH measured at the beginning (day 0) of the experiment. (EC = electrical conductivity).

Treatment Groups	*NaCl (mg/kg)*	*Cu (mg/kg)*	*Mean EC (dS/m)*	*Mean pH*
Control	0	0	0.13	5.94
NaCl alone	1000	0	0.43	5.88
	2000	0	0.74	5.76
	4000	0	1.29	5.74
Cu alone	0	20	0.13	5.85
	0	80	0.15	5.81
	0	320	0.21	5.78
	0	640	0.25	5.65
NaCl+ Cu	1000	20	0.44	5.85
	1000	80	0.44	5.84
	1000	320	0.53	5.73
	1000	640	0.58	5.63
	2000	20	0.74	5.74
	2000	80	0.76	5.71
	2000	320	0.79	5.67
	2000	640	0.85	5.65
	4000	20	1.30	5.73
	4000	80	1.31	5.70
	4000	320	1.35	5.64
	4000	640	1.41	5.59

To determine the internal copper concentration, one worm per replicate was sampled at each sampling occasion, except at day 28 when all remaining worms were used. Worms were placed singly in Petri dishes on moist filter paper for 24 hrs at 20^0C to allow depuration of their gut contents. Afterwards, each worm was frozen at -4^0C for metal analysis. The procedure for the metal extraction by acid digestion has been described by Maboeta et al. (2003). Thereafter, samples were filtered (using 0.45 µm cellulose nitrate filter paper) into film boxes and stored at 4^oC until spectrometric analysis.

6.2.2.2 Soil parameters

For soil samples, electrical conductivity (EC) was measured at day 1 as described in SSS (1996). A soil-water extract (1:5; w/v) was made by making a suspension of 5g soil in 25ml distilled water and measurements were taken with a conductivity meter (SM 802 pH/ EC/ TDS Meter, Spraytech). Soil moisture content and pH-(H_20) were monitored on each sampling occasion (Sartorius infrared moisture detector for moisture). Moisture loss was replenished by adding equivalent amounts of de-ionised water. For the determination of copper contents of soil, samples were taken at day 28 and chemical extractions were carried out using three methods: $CaCl_2$, DTPA and nitric acid extraction methods. For $CaCl_2$ and DTPA extractions, a sequential procedure was followed according to those used by Maiz et al. (1997). From each sample, 3 g of air dried sub-sample was shaken with 30 ml solution of 0.01 M $CaCl_2$ for 2-h, prior to centrifugation at 4000-rev/min for 15 min. The supernatant was transferred for analysis of metal contents. For the DTPA extraction, the residue of the $CaCl_2$ extraction was shaken with 6 ml solution of DTPA for 2 h and then centrifuged at 3000-rev/min for 10 minutes. The supernatant was in both cases filtered through Whatman 540 filter paper, and stored at -4^0C until analysis. For nitric acid extraction, copper contents were extracted by acid digestion as described by Maboeta et al. (2003).

6.2.3 Copper analysis

The extracted soil and worm samples were analyzed for copper by flame atomic absorption spectrophotometer (AAS) (Varian AA-1275). The AAS was calibrated by 1, 5, and 10 mg/l Cu standards. When samples were over-ranged, a

serial dilution was made and samples were then analyzed. Previously spiked soil samples were analyzed and indicated a recovery above 90% with this procedure. Further, quality control was achieved by analysing reference materials prepared independently of the standards. This also indicated a recovery above 90%.

6.2.4 Statistics

All data were checked for normality and homogeneity of variance with the Shapiro-Wilks W test and Levene's test respectively. Growth data per sampling time and cocoon number were analyzed using two-way analysis of variance (ANOVA) with salinity and Cu as variables. When interaction was significant, effects were interpreted as more or less than additive, when non-significant they were interpreted as additive (Boone, 2008). For Cu concentrations in worm and in soil (nitric acid, DTPA and $CaCl_2$ Cu extracts), one-way ANOVA was used to test for the effect of salinity along each Cu level. When data were not normally distributed even after transformations, Kruskal-Wallis H-test was used. Fischer's least significant difference (LSD) post-hoc test was used to determine the significance of any differences between specific groups in parametric cases while multiple comparison of 'z' and 'p' values was used in non-parametric cases. The EC_{50} values of NaCl and Cu for growth and cocoon production were calculated by using the Linear Interpolation Method (USEPA, 1993). ANOVA data were analysed using STATISTICA 7.0 software.

6.3 RESULTS

6.3.1 pH and Electrical Conductivity (EC) changes

Spiking with either Cu or NaCl reduced the pH in all treatments slightly, but usually below a mean of 0.3 units. Spiking with combination of both substances caused further reductions in pH values, but not below a mean of 0.5 units from the control soil pH (Table 7). pH values increased slightly in all treatment groups after 28 days exposure period. The overall range for pH increase in all treatments was 0.29 to 0.51. Spiking with Cu increased the EC values between 0.02 and 0.13 dS/m depending on the amounts of Cu added.

6.3.2 $CaCl_2$, DTPA and nitric acid extractable copper in soil

There were no significant differences ($P > 0.05$) among salinity groups for all nominal Cu concentrations for DTPA and nitric acid extractable Cu contents. In the case of $CaCl_2$ extractable Cu, fractions in 320 and 640 mg/kg were significantly higher with increasing salinity levels (fig. 17).

6.3.3 Individual toxicity: NaCl and Cu

No mortality was found in both the NaCl and Cu single substance tests. Mean weight of worms over the exposure period increased in all treatments receiving either Cu or NaCl relative to substance concentrations (fig. 18). Statistical analysis (ANOVA) showed that individual NaCl and Cu had significant effects on weight of the worms. For NaCl, significant differences were found at 4000 mg/kg NaCl at days 7, 14 and 28. On these days the weight of worms exposed to 4000 mg/kg NaCl was significantly lower ($P < 0.01$) than those exposed to 0 mg/kg NaCl. The EC_{50} for growth was 3586 mg/kg NaCl. For Cu, significant differences were found at 320 mg/kg ($P < 0.01$) and at 640 mg/kg ($P < 0.01$) at day 7, 14 and 28. On these days, the weights of these worms were significantly lower than for worms exposed in 0 mg/kg Cu spiked soil. The EC_{50} for growth was 530 (Confidence Interval, 497-563) mg/kg Cu.

The mean cumulative cocoon number produced over four-week exposure period at different NaCl and Cu concentrations is shown in figure 19. Worms exposed to either 640 mg/kg Cu or 4000 mg/kg NaCl soil did not produce any cocoons. The EC_{50} for cocoon production was 309 mg/kg Cu (Confidence Interval, 224-400) and 2000 mg/kg NaCl (Confidence Interval, 1711-2421).

6.3.4 Mixture toxicity: NaCl and Cu

The different combinations of NaCl and Cu did not lead to mortality in any of the treatment groups during the 28-day exposure period. Mean weight of worms over

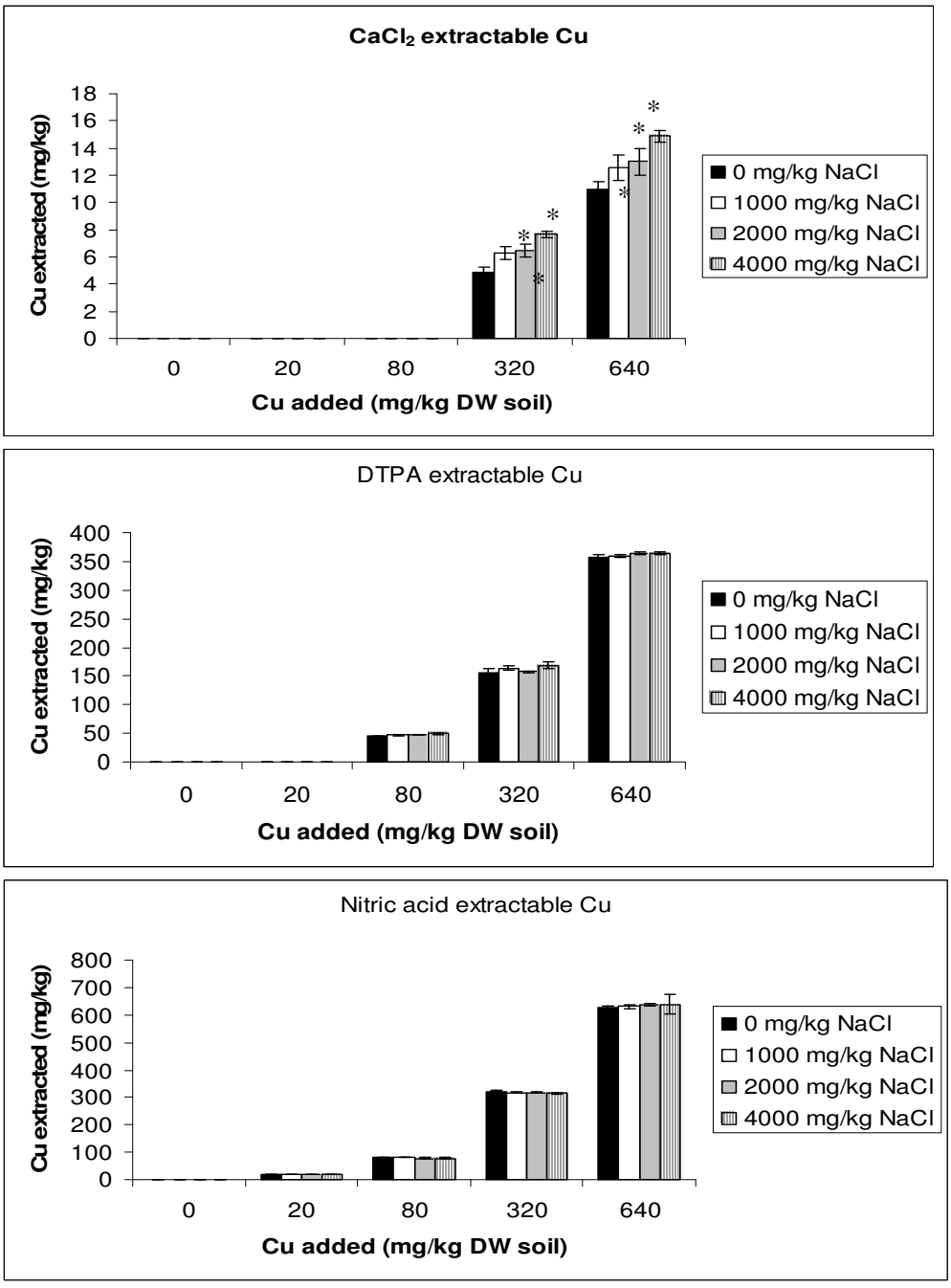

Fig. 17. Mean (± SE) $CaCl_2$ DTPA, and nitric acid copper concentrations extracted in saline and non-saline artificial soil substrates spiked with copper. * Concentration significantly different from the non-saline group (ANOVA, $P < 0.05$).

the 28-day exposure period increased in all treatments except for worms exposed to combination of 640 mg/kg Cu and either 2000 or 4000 mg/kg NaCl as well as worms exposed to a combination of 320 mg/kg Cu and 4000 mg/kg NaCl. To simplify results of multiple combinations of both substances, a case by case basis of interaction was computed. This was by adjusting the percentage weight change of the control worms (in unspiked soil) to 100%, and other data were benchmarked against the control worm data. Significance of the differences was confirmed with ANOVA, and Fischer's LSD post-hoc test. Only weight change by day 28 was used since effects were similar but more pronounced at day 28 than at other days. Table 8 shows the mean % weight change (gain or loss) of worms exposed to single and mixed NaCl and Cu, with reference to worms in unspiked soil and the type of interaction between them. Generally, more than additive effects were found at higher concentrations while

less than additive effects were found at lower concentrations of both substances. However, this pattern was not significant for most combinations, indicating a general pattern towards additive interaction, except for combination of 2000 mg/kg NaCl and 640 mg/kg Cu where more than additive effects found was significant. In all NaCl soil groups, cocoon production of worms exposed to 20 and 80 mg/kg Cu were similar and higher than at other Cu concentrations (Fig. 19). The interaction of NaCl and Cu had no statistically significant influence (ANOVA, $P = 0.14$) on cocoon numbers (Appendix 11). The interaction between NaCl and Cu could be expressed as additive for all Cu-NaCl combinations.

6.3.5 Internal copper concentration in worms

The internal copper concentration at days 7, 14 and 28 in both exposed and unexposed worms in the gradient of salinity is shown in Fig. 20. Generally, there was increased internal copper concentration with increased substrate Cu concentration and exposure times. For most nominal concentrations of Cu in soil, there was no significant difference ($P > 0.05$) in internal copper concentration in worms exposed to Cu without NaCl and those with 1000, 2000, or 4000 mg/kg NaCl. The only exception being at 320 mg/kg Cu at day 28 where internal copper concentration was higher for worms exposed in 1000 and 2000 mg/kg NaCl than those in non-saline soil (Fig. 20).

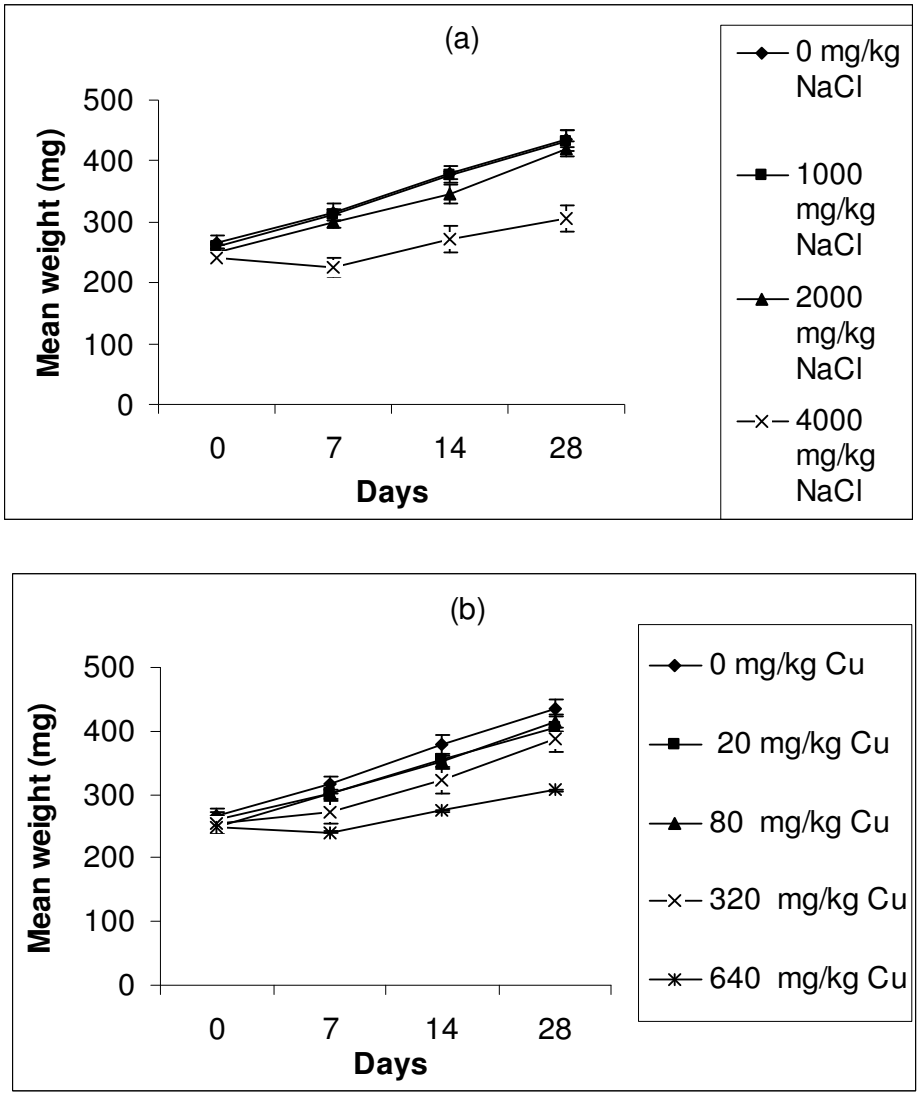

Fig 18. Mean (± SE) weight change of worms (*Eisenia fetida*) exposed to (a) copper or (b) sodium chloride in OECD substrates during a 28-day period.

Table 8. Mean percentage weight change of worms (± SE) exposed to Cu and NaCl singly and as mixtures as a function of the response of unexposed worms after 28 days in OECD artificial soils

	Effects (as % deviation from response of control worms)			
Cu + NaCl concentrations (mg/kg)	Cu alone	NaCl alone	(Cu+NaCl)	Interaction type (significance)
20+1000	0.5 ±12.9	6.0 ± 7.2	1.1 ± 6.0	more than additive (ns)
20+2000	0.5 ±12.9	7.7 ± 5.8	15.3 ± 8.9	less than additive (ns)
20+4000	0.5 ±12.9	-54.8 ± 13.6	-42.8 ± 20.8	less than additive (ns)
80+1000	-4.6 ±13.9	6.0 ± 7.2	15 ± 14.3	less than additive (ns)
80+2000	-4.6 ±13.9	7.7 ± 5.8	5.9 ± 7.9	less than additive (ns)
80+4000	-4.6 ±13.9	-54.8 ± 13.6	-27.7 ± 6.7	less than additive (ns)
320+1000	-16.2 ± 3.8	6.0 ± 7.2	3.9 ± 9.4	less than additive (ns)
320+2000	-16.2 ± 3.8	7.7 ± 5.8	-0.9 ± 6.8	less than additive (ns)
320+4000	-16.2 ± 3.8	-54.8 ± 13.6	-106.3 ± 10.7	more than additive (ns)
640+1000	-66.8 ± 3.4	6.0 ± 7.2	-80.2 ± 3.4	more than additive (ns)
640+2000	-66.8 ± 3.4	7.7 ± 5.8	-108.7 ± 0.8	more than additive (s)
640+4000	-66.8 ± 3.4	-54.8 ± 13.6	-167. 7 ± 3.5	more than additive (ns)

ns- non-significant

s- significant at 95% confidence level by ANOVA and Fischer's LSD tests.

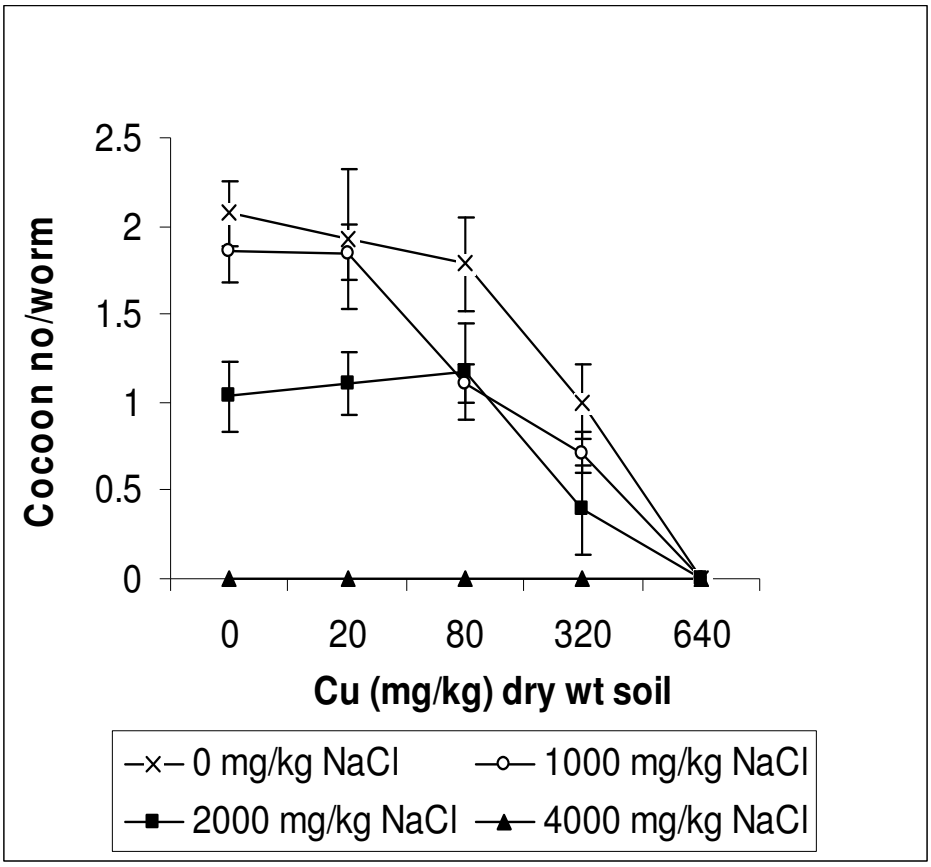

Fig 19. Mean (± SE) number of cocoons per worm produced after four-week exposure of *Eisenia fetida* to copper saline and non-saline artificial soil at constant temperature and moisture.

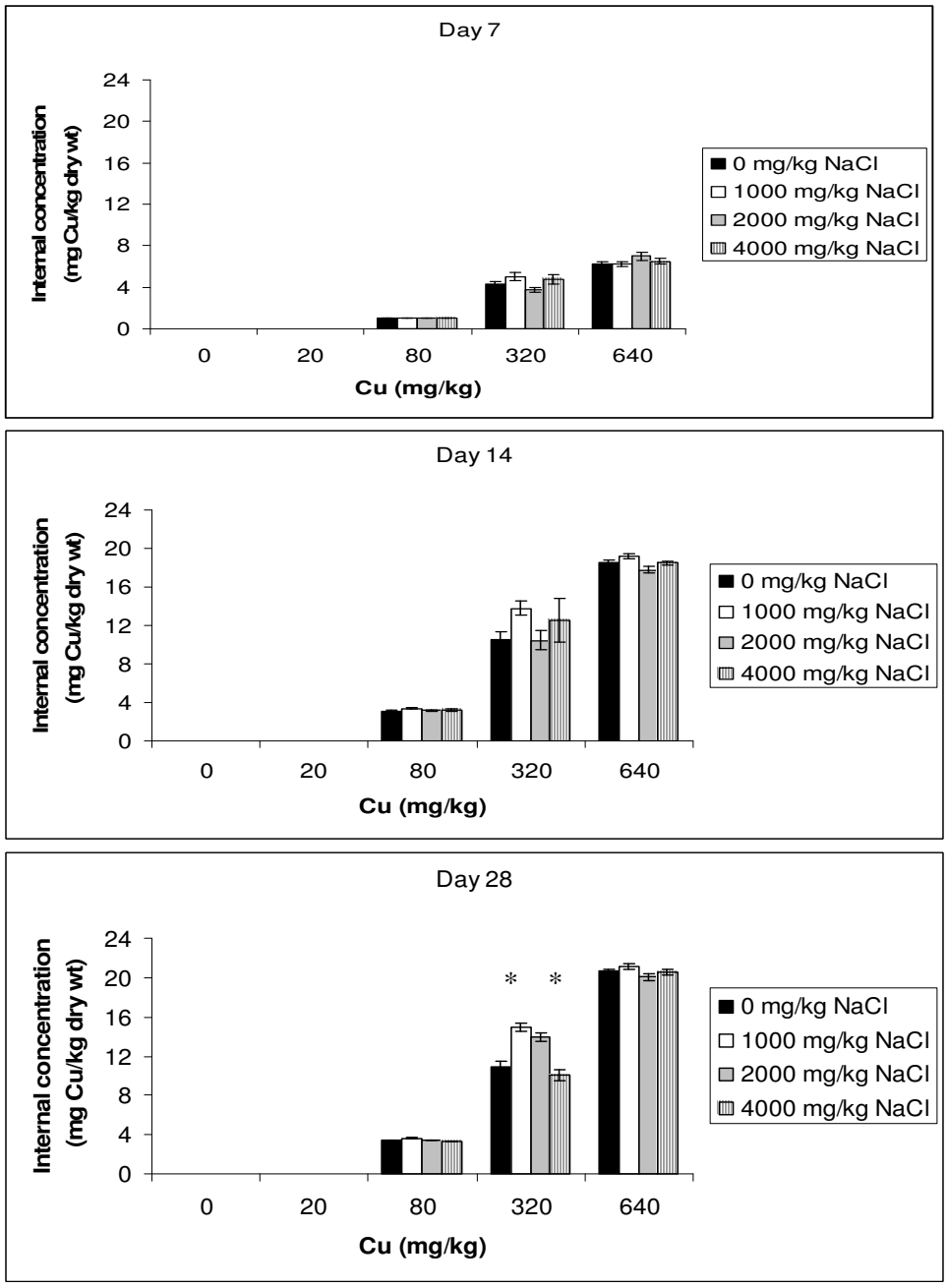

Fig 20. Mean (± SE) nitric acid extracted tissue copper concentrations in *Eisenia fetida* exposed to copper for 28 days in saline and non-saline OECD artificial soil .*Concentration significantly different from the non-saline group (ANOVA, P< 0.05).

6.4 DISCUSSION

Spiking of soil with either NaCl or Cu did not change the pH of soil significantly. This excluded interference of pH differences on the results of this study. pH is the pre-eminent factor influencing the bioavailability of contaminants in soil (Impelliterri et al., 2003). Moreover, addition of Cu as copper oxychloride did not affect considerably the electrical conductivity of the already adjusted saline soil, and so the effects of interaction on worms could be attributed mainly to the addition of NaCl and Cu. This study showed that with increased salinity, there was increased labile Cu in the soil as shown by our data for $CaCl_2$ extracted Cu (Fig. 17). This suggests that salinity reduced the partitioning and increased the potential bioavailability of Cu in soil. This is similar to the observation of Pakpian et al. (2002) who found bioavailable (DTPA extractable) Cu in sewage sludge to increase with increasing salinity up to 8 dS/m. These findings indicate that the potential bioavailability of Cu in organic substrates may increase with increasing salinity. A similar pattern was also found for the influence of salinity on Zn bioavailability ($CaCl_2$ and DTPA extracted) in a prior study using OECD soil (Chapter 5). Although Parkpian, et al. (2002) reported that salinity could reduce the nitric acid extracted and increase the DTPA extracted Cu, no significant effect of salinity on these Cu concentrations was however found in our study. The difference between our findings and theirs could be the result of the relatively low salinity used in the present study. The highest salinity level in our study (EC = 1.29 dS /m) was lower than the lowest salinity (EC = 2 dS/m) used by these authors. We could not use soil of EC values higher than 2 dS/m since earthworms would not survive (Chapter 5).

Calamari and Alabaster (1980) have highlighted three possible types of interaction that may occur among chemicals in soil, and their toxicity to soil organisms. These include chemical and physiochemical interactions of chemicals in the substrates which could affect the partitioning of one or both chemicals. It could also be physiological interaction of chemicals at point of entry into the organism affecting uptake from the soil solution and finally determining the quantity available at the site of toxic action. Lastly, it could be interaction at the site of toxic action, including combination with receptors, at the target site. Since salinity reduced the partitioning of Cu in soil, rendering more Cu available in solution, the mainly additive

effects found for growth and cocoon production suggested that Na^+ probably also interfered with Cu at the point of entry into the earthworm. The interaction of NaCl and Cu at point of entry might be because Na^+ occupies the biotic ligand with Cu. However, it is important to note that NaCl also occupies the ligand to such an extent as to exert a toxic effect of its own. This was obvious since NaCl presented as an individual substance, had a toxic effect on growth and cocoon production. In doing so, it might also compete with Cu, and the surplus Cu^{2+} in the substrate as a result of increased salinity might not bind on the biotic ligand bringing the net interaction to additivity. This could probably explain why the interaction of NaCl and Cu was mainly additive for most combinations.

The mostly additive interaction of NaCl and Cu on life cycle parameters observed is in contrast to results of other studies where only the cationic components of the salts were considered. Recently, Lock et al. (2006), while using the Biotic Ligand Model (BLM), reported that an increase in Na^+ up to 15 mM reduced cobalt toxicity to *Enchytraeus albidus* by a factor close to 3. Steenbergen et al. (2005) also showed that Na^+ did play a role in protecting *Aporrectodea caliginosa* against Cu^{2+} toxicity. These authors suggested that the protection of these organisms by Na^+ against metal ions was due to competition at the biotic ligand. The BLM has gained increased interest among ecotoxicologists (Steenbergen et al., 2005; Lock et al., 2006) in the last few years, and proved to be useful in explaining the effect of competing ions on metal toxicity to various organisms. However, our data showed that Na^+ when added as NaCl may not always be protective because it increases the labile Cu in soil and on its own may evoke toxicity to the organism. Present BLM data are mostly based on acute toxicity. It is not clear whether the differences between my results and those of other authors were because I used sub-lethal endpoints while they used mortality. Responses to sub-lethal concentrations of chemicals may be of various kinds, including behavioral, which may not necessarily amount to a direct toxic effect as in acute toxicity.

The effect of NaCl as individual contaminant on cocoon production was similar to those presented in the literature. For example, the 28-day EC_{50} found in this study, of 2000 mg/kg NaCl, was similar to the 1884 mg/kg and 2020 mg/kg NaCl found in OECD soil by Bright and Addison (2002) and Chapter 5 respectively.

However, the EC_{50} of 3586 mg/kg NaCl for growth observed here was lower than 4681 and 4985 mg/kg NaCl found by Bright and Addison (2002) and Chapter 5 respectively. Since younger worms have been found to be more sensitive to pollutants (Helling et al., 2000), these differences could be due to differences in worm age. For example, in chapter 5, worms aged between 20 and 24 weeks were used whereas worms between 9 and 12 weeks old were used in the present study.

My results on growth and cocoon production of worms in test soil with Cu alone could not be compared directly with those found in the literature since test durations differed. Spurgeon and Hopkin (1995) found a 21-day EC_{50} for cocoon production of 716 mg/kg Cu while we observed a 28-day EC_{50} of 309 mg/kg Cu. For growth an EC_{50} of 601 mg/kg Cu was calculated by Spurgeon and Hopkin (1995) whereas our study determined a value of 530 mg/kg Cu. The 28-day LC_{50} was > 640 mg/kg Cu in our study while a value of 519 mg/kg Cu was obtained in OECD soil (pH 7.0) by Maboeta et al. (2004). A 14-day LC_{50} of 643 mg/kg Cu (Neuhauser et al., 1985) and 683 mg/kg Cu (Spurgeon et al., 1994) and a 56-day LC_{50} of 555 mg/kg Cu (Spurgeon et al., 1994) have previously been reported for this earthworm. Despite difficulties in direct comparison, growth and mortality data that I got were within the broad range reported in the literature.

One would expect an increase in uptake and accumulation of Cu in worms with an increase in estimated bioavailability of Cu in soil, as a result of increased salinity. This was not verified in most cases. The only exception was at Cu concentration of 320 mg/kg, at day 28, where significantly higher internal Cu concentrations was found in worms exposed to 1000 and 2000 mg/kg NaCl soil than in non saline soil (Fig. 19). Even here at this Cu concentration, no increase was evident in worms exposed to a concentration of 4000 mg/kg NaCl. This apparent contrast and inconsistent pattern makes it difficult to use internal Cu concentration of worms to explain the interaction of NaCl and Cu on toxicity to this earthworm. It also probably showed that interaction of NaCl and Cu took place at the point of entry as earlier discussed. However, an explanation for the weakness of internal Cu concentration as a reliable marker/endpoint could be the result of elimination or sequestration of Cu in the body of the worms since Cu is an essential metal known to be regulated by the earthworm *Eisenia fetida* (Spurgeon and Hopkin, 1999). The more

than additive effect of NaCl and Cu at higher concentrations could then be because more energy is spent in regulation of Cu at higher concentrations than at lower concentrations. Changes in this direct energy budget could render the worm more vulnerable.

At lower concentrations of both contaminants, there is a tendency towards additive interaction, whereas at higher concentration especially of Cu, there seems to be a tendency towards non-additivity (synergistic) interaction. However, in most field agricultural soils, Cu content of soil is usually below 640 mg/kg Cu. The soil copper content determined in 19 vineyards in the Western Cape, South Africa amounted to a mean of 9 mg/kg with a maximum of 27 mg/kg Cu (I. van Huyssteen, personal communication). In German vineyards, Wittassek (1987) measured a copper content between 190 and 350 mg/kg Cu in old vineyards and copper contents of 45-50 mg/kg Cu in vineyards established after 1960. Vavoulidou et al. (2005) reported a Cu content of < 200 mg/kg Cu in Greek soils of various agricultural uses. In agricultural soils of European Mediterranean area, Micó et al. (2006) found mean Cu content of 23 mg/kg Cu. It is therefore not evident that increasing salinity of soils (when induced by NaCl) will, apart from other possible detrimental effects of salinity, have a synergistic effect to increase Cu toxicity, at field relevant concentrations. This is in contrast to information available for Zn, another essential element, in the previous experiment (Chapter 5). I described a possible synergistic effect of NaCl and field relevant Zn concentrations on the earthworm *E. fetida* in a laboratory study using OECD soil as in the present study. This highlights a possible difference in the way salinity may affect Cu and Zn toxicity to earthworms in spite of the fact that both are essential metals and therefore regulated.

6.5 CONCLUSION

This study examined the combined stress effects of salinity and Cu on the earthworm *Eisenia fetida* by monitoring interactions in soil and in the organism. With increased salinity, $CaCl_2$ extractable Cu increased. At high concentrations, both NaCl and Cu, as individual contaminants had severe effects on growth and cocoon production of this earthworm. Their combined effects on these parameters were mainly additive. The only exception was at high concentrations of NaCl and Cu, where more than additive effects were found for growth. Despite an increase in

estimated bioavailability of Cu in soil (extracted with $CaCl_2$) with increased salinity, no consistent significant increase was found in Cu contents in worm tissue. Based on the current findings it is therefore unlikely that an additive interactive effect will occur in field situations, where both salinity and Cu may jointly present a problem.

CHAPTER SEVEN

7.0 COMPARATIVE STUDY OF THE EFFECTS OF SALINITY ON LIFE-CYCLE PARAMETERS OF FOUR SOIL DWELLING SPECIES (*FOLSOMIA CANDIDA, ENCHYTRAEUS DOERJESI, EISENIA FETIDA AND APORRECTODEA CALIGINOSA*)

7.1 INTRODUCTION

Salinisation of soil is one of the common problems under irrigated agriculture especially in areas of low rainfall and high evaporative demand (Sumner, 1995) including South Africa and Australia. Salinisation of soil occurs either as a result of natural processes due to high salt contents in parent materials or irrigation with water containing high soluble salts. About 50% of irrigated areas of the world are either salinised or have the potential to be so in future (Tyagi, 1986), underlining the extent of the problem.

In South Africa, saline soils are found in irrigated agricultural lands (ARC-ISCW Staff, 2004) in semi arid and arid regions which make up 90% of total land (UNESCO, 1977). The Western Cape region is known for its elaborate agricultural activities where wine and fruits are produced in large quantities. To keep pace with the demands of agriculture in this area, irrigation is undertaken from rivers like the Berg and Breede rivers. Dryland and irrigation salinity is extensive in these river catchments, where effects on crop production have been reported (Biesenbach and Inc, 1989; Moolman and deClercq, 1993; Fey and deClercq, 2004). Salinity affects crop performance and beneficial soil biota, and could lead to economic losses. It affects the growth and survival of microorganisms (Lippi et al., 2000; Yuan et al., 2007), plants (Ramoliya et al., 2004; Kadukova and Kalogerakis, 2007) and soil organisms (Hobel et al., 1992).

Data on environmental effects of salinity on soil organisms are mostly collected from laboratory experiments with artificial soils and synthetic chemicals (Fischer and Molnar, 1997; Chapter 5). In most cases this makes extrapolation from laboratory data to field realities difficult. La Point et al. (1989) had noted that one of the major causes for the lack of predictive value of laboratory tests can be found in the effect of

alterations of the chemical being tested or in the environment. These arguments suggest that toxicity data derived from artificial soil substrates using chemicals may not be predictive of effects in field situations. The use in most studies of technical or laboratory grade NaCl is not environmentally realistic, as NaCl does not enter the environment in its pure form. Also differences in soil properties between artificial and field soil would further compound the problem. For instance, the toxicity of NaCl to the earthworm, *Eisenia fetida* is lower in natural composted soil than in artificial OECD soil (Robidoux and Delisle, 2001) suggesting the relevance of using natural soil in assessing the effects of salinity on organisms. Laboratory experiments can thus be in good agreement with responses of organisms at contaminated (salinised) sites, if the experiments are performed in representative soil from the site (Marinussen et al., 1997).

It is well known that soil invertebrates vary in their sensitivity to pollutants (Lock and Janssen, 2001c,e; Amorim et al., 2005a). The effect of salinity on an organism with a particular feeding behavior and ecological role can not be directly extrapolated for another organism with a different behavior and role in soil. We therefore studied the effect of salinity on different organisms at different levels in the food chain, with different feeding regimes, life-style modes and taxonomic positions. This study was designed as part of a larger study aimed at assessing the influence of salinity on different tiers of soil organisms. In this contribution, we studied the effect of salinity on life-cycle parameters of the collembolan *Folsomia candida*, enchytraeid *Enchytraeus doerjesi* and two earthworm species *E. fetida* and *Aporrectodea caliginosa* using natural saline soils.

These four genera are important in ecotoxicological studies because they are frequently used in toxicity tests, and standardized tests are available for the representatives of the first three genera. Collembola are insects occurring abundantly in the surface layers of most soils. They are an integral part of soil ecosystems and are vulnerable to the effects of soil contamination. The abundance and diversity of Collembola have been used widely to assess the environmental impact of a range of pollutants on soils (Fountain and Hopkin, 2005). They feed on fungi or algae and thus indirectly contribute to the decomposition of leaf litter. The species *F. candida* Willem 1912 is most abundant in organic soils and has been used in several laboratory

studies (e.g Amorim et al., 2005a). Enchytraeids, just like earthworms, interact with the mineral layer of the soil, and because of their soft body, are often sensitive to contamination of the soil environment. *E. albidus* is the "standard "enchytraeid species (ISO, 2001). However, this species performed poorly in some natural soils (Kuperman et al., 1999), the reason why other species are often used in studies with natural soils. *E. doerjesi* is a recently discovered species (Westheide and Graefe, 1992), easy to culture and fast growing, which makes it an ideal object for population studies. It was discovered in laboratory cultures of terrestrial enchytraeids originating from France, Holland and the Philippines. This species seems to reproduce better at 25^0C than at 20^0C, which could be an indication of its tropical origin (P. Voua-Otomo, unpublished data). Initial trials showed that this species reproduced well in the natural soil that we used in this study. Two eco-physiologically different earthworm species were used. *E. fetida* Savigny, 1826 is an epigeic (litter-dwelling) earthworm species. It is recommended as a reference test species for soil toxicity testing (OECD, 2004). *A. caliginosa* Savigny, 1826 is an endogeic species, and interacts with the mineral layer of soil. It is a very common earthworm species in many natural soils, and therefore could show direct possible effect of salinity on earthworms in the field.

7.2 MATERIALS AND METHODS

7.2.1 Test organisms

A culture of the *F. candida* has been maintained in the Ecotoxicology laboratory of the department of Botany and Zoology, Stellenbosch University for about 5 years on a layer of Plaster of Paris mixed with activated charcoal and were kept in a climate chamber at 20^0C at a light/dark regime of 16/8 h. Granulated dry yeast was added weekly as food. For the experiments, juvenile specimens aged 10-12 days were used. The culture of *E. doerjesi* has been maintained in the laboratory for about 3 years in moist soils. The substrate is made up of 70% fine sand and 30% coarse sand which was mixed together and moistened. The worms were kept at 20^0C and fed once a week with flakes of oatmeal. Adult (clitellate) worms were hand-picked from the stock culture, with the aid of a dissecting microscope, and randomly assigned to a waiting dish. Specimens of the earthworm *E. fetida* were age-synchronized from a culture kept in the laboratory for several years. Adult worms (18-20 weeks old) of between 250 and 500 mg were used in the experiments. *A.*

caliginosa specimens were collected at grassland close to the Eerste River in Stellenbosch, Western Cape, South Africa by digging and hand-sorting. This species occurs abundantly in this area which had no known history of pesticide use or abnormal salinity. Only adult worms of between 400 and 800 mg with fully developed clitella were selected for the experiment.

7.2.2 Test soil

Soil used in the experiments was collected from the same plot of land on Robertson Experimental Farm, Robertson, Western Cape, South Africa (33^{o} 50'.028'' S, 19^{o} 53'.492'') where soil salinity varied over relatively short distances. By using soil from the same plot, near homogenous soil samples, in terms of other soil characteristics than salinity, were ensured as far as possible. This plot was bordered by an olive plantation to the left, and a cattle ranch to the right. On the two other sides were buildings and a gravel road. The olive plantation was established in 1995 and copper oxychloride has been used for a number of years to control pests. Two areas on the plot were identified initially, based on differences in EC values. A bulk soil sample was collected from each of these two areas, by digging and collecting the top 10 cm layer. Plant cover in the two plots was similar. These plants included the grass *Cynodon dactylon* and herb *Polygonum aviculare*. No macrofauna was found in either soil at the time of collection. These two bulk soil samples were brought to the laboratory, air dried, sieved (2 mm) and thoroughly mixed individually, after carefully removing the surface organic materials and fine roots. Since copper oxychloride was sprayed in the plot next to the site where the soil was collected, we therefore analyzed the soil for metal contents (Cu, Zn, Cd and Pb) using nitric acid digestion as described by Maboeta et al. (2003). The EC of the soil samples was determined as described in SSS (1996). A soil-water extract (1:5; w/v) was made and measurements were taken with a conductivity meter (SM 802 pH/ EC/ TDS Meter, Spraytech). Soil samples representing a range of EC values were prepared by serial dilution. We succeeded in preparing five soil samples with varying EC values. The main properties of these soils are presented in Table 1. The soil sample with a relatively low EC of 0.08 dS/m was regarded as control soil. pH-H_20 was determined with a pH meter (Micro pH 2001, Crison) in a 1:10 w/v suspension of 5g of each sample. The maximum water holding capacity (WHC) of this soil was determined by using the procedure described by ISO

(1996) after inundating the soil for 3 h in water and subsequently draining it for 2 h. The soil moisture content was determined with the aid of a Sartorius infrared moisture detector. The clay fraction was determined by sedimentation velocity and organic matter content was estimated from the determination of carbon which was done by combustion method using elemental analyser (Euro EA). Exchangeable cations and anions were extracted with 1 M ammonium acetate (1:10, soil: extractant ratio), shaken for 2 h and Ca, Mg, K and Na were analyzed by atomic absorption spectrophotometry (Pharmacia LKB-Ultrospec III). Exchangeable anions were extracted with distilled water (1:5, soil: H_20) shaken for 2 h and Cl, NO_3, PO_4 and SO_4 ions in extracts were analyzed by ion chromatography (Dionex DX 120).

7.2.3 Test procedures

For all tests, the soils were moistened with de-ionised water to achieve 55% of the maximum water holding capacity of each soil. Each treatment had four replicates and the exposures were run for 28 days in a climate chamber at 20^0C and light/ dark cycle of 16/8 h. This temperature was selected since it is the recommended temperature for *E. fetida*, *F. candida* and *E. doerjesi* (OECD, 2004; ISO, 1998; Römbke and Moser, 2002), although a lower temperature (15^0C) is considered optimal (Daugbjerg, 1988) for *A. caliginosa*. However 20^0C was used in this study to maintain consistency with the temperature used for the three other species.

The enchytraeid test was performed as described by OECD (2000). Ten adult worms with well developed clitella were introduced into plastic containers (50 mL), each containing 20 g of soil which had been mixed with required de-ionised water. Food (oat meal – 10 mg) was added weekly to each container. At the end of the test, the organisms were immobilized with alcohol and fixed with Bengal red as reported by Römbke and Moser, (2002). After 12 hours, the animals were coloured red and the solution was spread in a box and observed under the binocular microscope and counted. Adult survival and number of juveniles were noted. Adults and juveniles were easily distinguished by size and presence or absence of clitella.

Table 9. The physicochemical properties of five soils prepared by mixing soils collected form Robertson Experimental farm, Western Cape, South Africa

	Soil Groups				
	1	**2**	**3**	**4**	**5**
Parameters					
WHC (%)	36.7	36.4	36	36.4	36.4
Clay (%)	5.5	5.5	5.5	6.1	6.2
pH (H20)	9.4	9.4	9.3	9.3	9.2
OM (%)	< 1	< 1	< 1	< 1	< 1
EC (dS/m)	0.08	0.52	1.03	1.31	1.62
Exchangeable cations (mmol/kg)					
Ca	56.2	58.1	61.9	66	72.4
Mg	12.9	14.6	17.2	18.5	20
Na	56.9	79	100.6	105	111.1
K	3.1	3.5	4	4.3	4.5
Exchangeable anions (mmol/kg)					
Cl	3.4	27.2	57.5	76.4	105.4
NO_3	0.8	1.6	2.6	4.9	7.9
PO_4	4.2	4.8	5.5	5.3	5.1
SO_4	1.8	12.4	25.8	34.3	50.7
Cu (mg/kg)	8.2	7.9	7.9	7.8	7.6
Zn (mg/kg)	26.5	26.2	25.9	25.7	25.3
Pb (mg/kg)	ND	ND	ND	ND	ND
Cd (mg/kg)	ND	ND	ND	ND	ND

ND - not detected

The Collembola test was performed as described by ISO (1998). Ten juvenile organisms (10-12 days) were introduced into plastic containers (50 ml), each containing 32 g of dry soil which had been mixed thoroughly with de-ionised water. Food (dry yeast – 5 mg) was added weekly to each container. At the end of the test, the organisms were extracted to alcohol with a modified Berlese-Tullgren extraction chamber after 6 hrs (Jackson and Raw, 1966). To enhance complete recovery of organisms from the substrates, each extracted test vessel was emptied into a container filled with distilled water, and gently mixed with the aid of a spatula to allow the organisms trapped in the soil to float on the surface. Digital photographs of the water surface and the organisms were taken using a specialized microscope (Leica MZ16 A) fitted with a digital camera. Adult and juvenile organisms on the images were later counted using the computer software Image Tool. Total number of adults and

juveniles were estimated from the numbers recovered from the extraction and the photographs.

The earthworm tests were performed as described by OECD (2004). Worms were sorted from the soil, washed, weighed individually, and added to the relevant test soil containing 500 g of dry soil, which had been mixed thoroughly with de-ionised water. In the test with *E. fetida*, 10 specimens which had a low mean weight (293 mg) were added to each treatment replicate. For *A. caliginosa*, which had a higher mean weight (601 mg), only six worms were used per container. To maintain the worms during the exposure period, the test containers were covered with a plastic sheet to limit water loss. To increase rates of growth and cocoon production, finely ground fresh horse manure (dried and rewetted to 75% water content) was added as a source of food in both tests. To ensure sufficient food, 5 g dry weight of food was added weekly to each container. In the test with *E. fetida*, manure was added on the soil surface as food, while for *A. caliginosa*, it was mixed with surface soil (Spurgeon et al., 2000). Apart from survival and reproduction, growth was also assessed in the earthworms, since initial pilot trials showed low earthworm reproduction in this natural soil. Survival and growth were monitored at day 7, 14 and 28, while cocoon production was assessed at day 28. Survival was assessed by stimulating the worm with a blunt probe and an earthworm was judged dead if no response could be observed. Worms not found during sampling were judged to be dead since earthworm tissue decomposes easily in soil. Growth was determined by individually weighing each surviving worm from each container, and comparing the mean weight with initial values to calculate weight change. The number of cocoons was determined at day 28 by wet sieving the substrates (through a 2.0- and 1.0- mm sieve system). Cocoon number per worm was calculated by dividing the total number of cocoons by the number of surviving worms. It was assumed that worms that died during the exposure period could not have contributed to cocoon production since concentrations of toxicants affecting cocoon production is often much lower than those affecting mortality.

7.2.4 Statistics

Data were presented as mean ± SE. All data generated were tested for normality and homogeneity of variance by Lillifor's and Levene's tests respectively. In cases

where data did not pass either test, log-transformation was used. One-way analysis of variance (ANOVA) was used to test for differences in survival and reproduction rates among EC levels for all organisms as well as growth per sampling time for the earthworm species. In cases where assumption of normality was violated, even after data transformation, Kruskal-Wallis H-test was used. Fischer's least significant difference (LSD) post-hoc test was used to determine the significance of any differences between specific groups in parametric cases while multiple comparison of 'z' and 'p' values was used in non-parametric cases.

7.3 RESULTS

7.3.1 Survival of soil organisms

There were no mortalities in the control soils of the enchytraeid and earthworm tests. In the collembolan test, mean survival rate in the control soil was 78%. The effect of salinity on survival of the four different species is presented in Fig. 21. Survival of *F. candida* and *E. doerjesi* was not significantly affected in the EC range used in this study. However, for the two earthworm species, survival was affected significantly. Survival was affected more in *E. fetida*, where a significantly lower survival was found at an EC of 1.03 dS/m, and all worms died at an EC of 1.31 dS/m. For *A. caliginosa*, survival was significantly affected at an EC of 1.31 dS/m, while total mortality occurred in soil with the highest EC (1.62 dS/m).

7.3.2 Reproduction of soil organisms

The effects of salinity on reproduction of all four soil dwelling species after 28 days of exposure to saline soils are presented in Figure 22. Reproduction was affected more in the earthworm species than in the enchytraeid and collembolan species. For juvenile production in *F. candida* and *E. doerjesi*, a clear dose response relationship was seen with salinity which was fairly similar for both species.

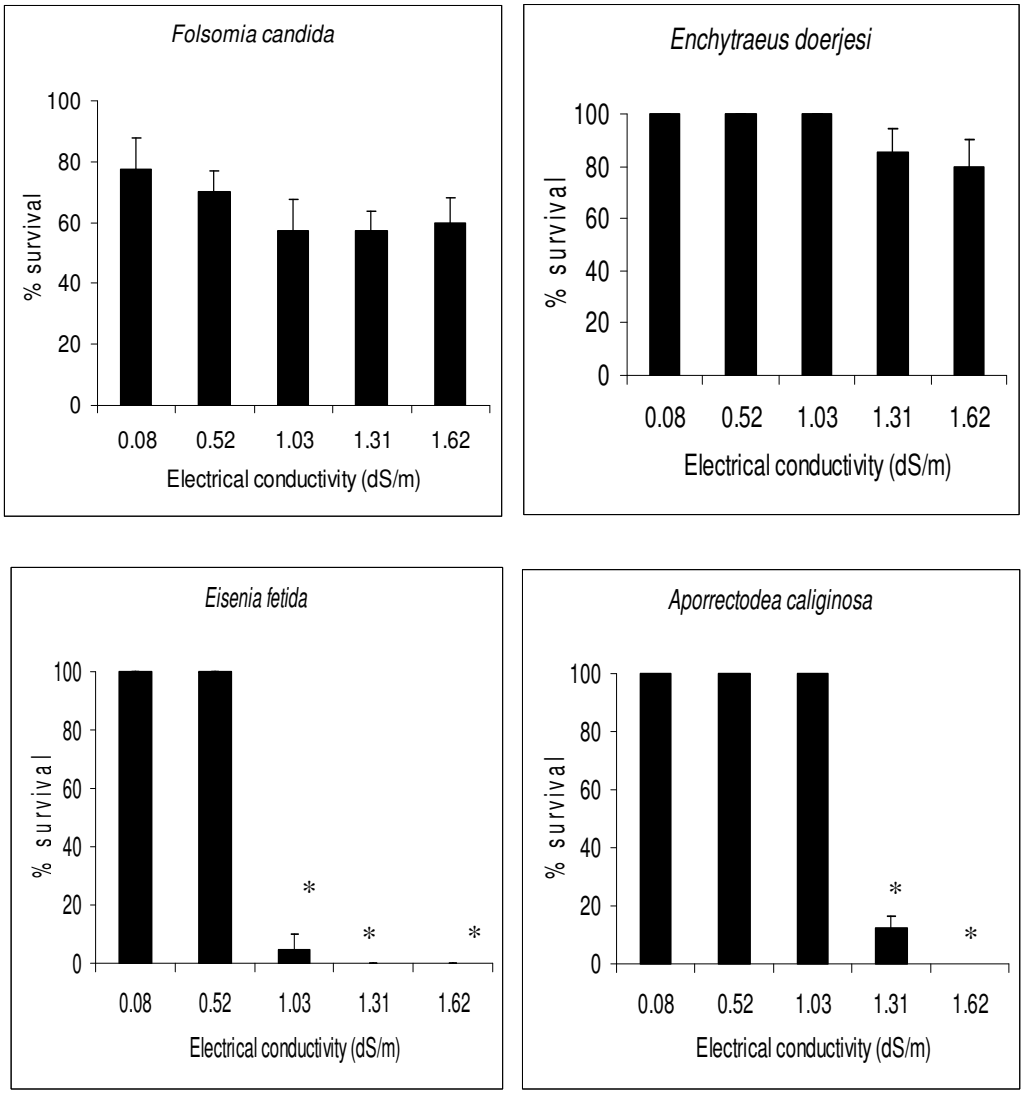

Fig 21. Survival of four soil organisms exposed for four weeks in natural soils of varying salinities under controlled laboratory conditions (error bars represent standard error). * significantly different from control groups.

However, a significantly lower juvenile production ($P < 0.05$) for both species was only found at an EC of 1.03 dS/m . Total cessation of reproduction occurred earlier for *E. doerjesi* (at EC of 1.31 dS/m) than for *F. candida* (at EC of 1.62 dS/m). The earthworm species only produced cocoons in the control soil (0.08 dS/m) during the 28-day test (Fig. 22).

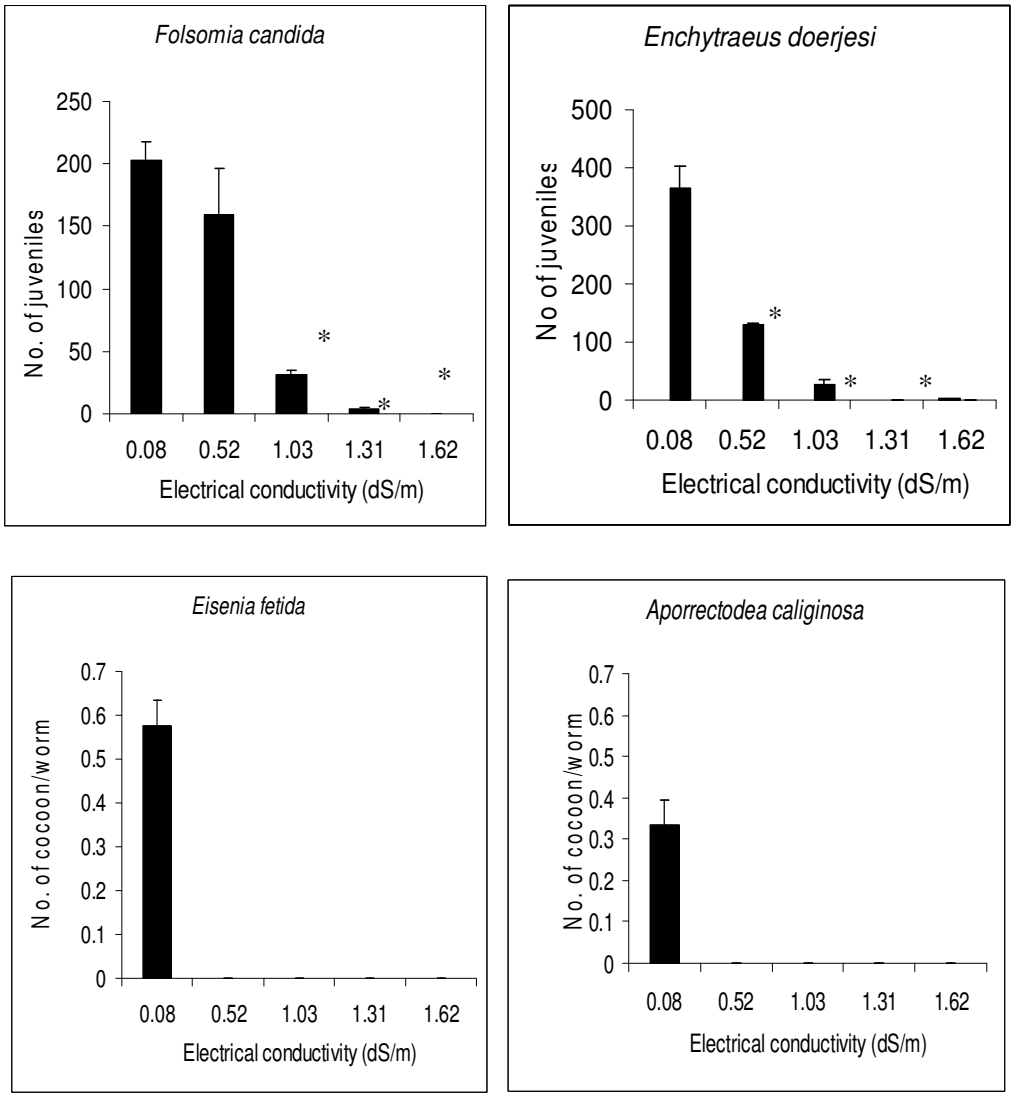

Fig 22. Reproduction of four soil organisms exposed for four weeks in natural soils of varying salinities under controlled laboratory conditions (error bars represent standard error). * significantly different from control groups.

7.3.3 Growth of earthworms

Growth of earthworm species over a period of 28 days is shown in Fig. 23. Generally, growth decreased with an increase in the EC of substrates. For *E. fetida*, the mean weight change of worms exposed to soil with 0.52 dS/m was not

significantly different (P > 0.05) at all sampling dates from those exposed to the control soil. However, significantly lower mean weights (P < 0.01) were found for worms exposed to soils of 1.03 dS/m and higher, from day 7 to day 28.

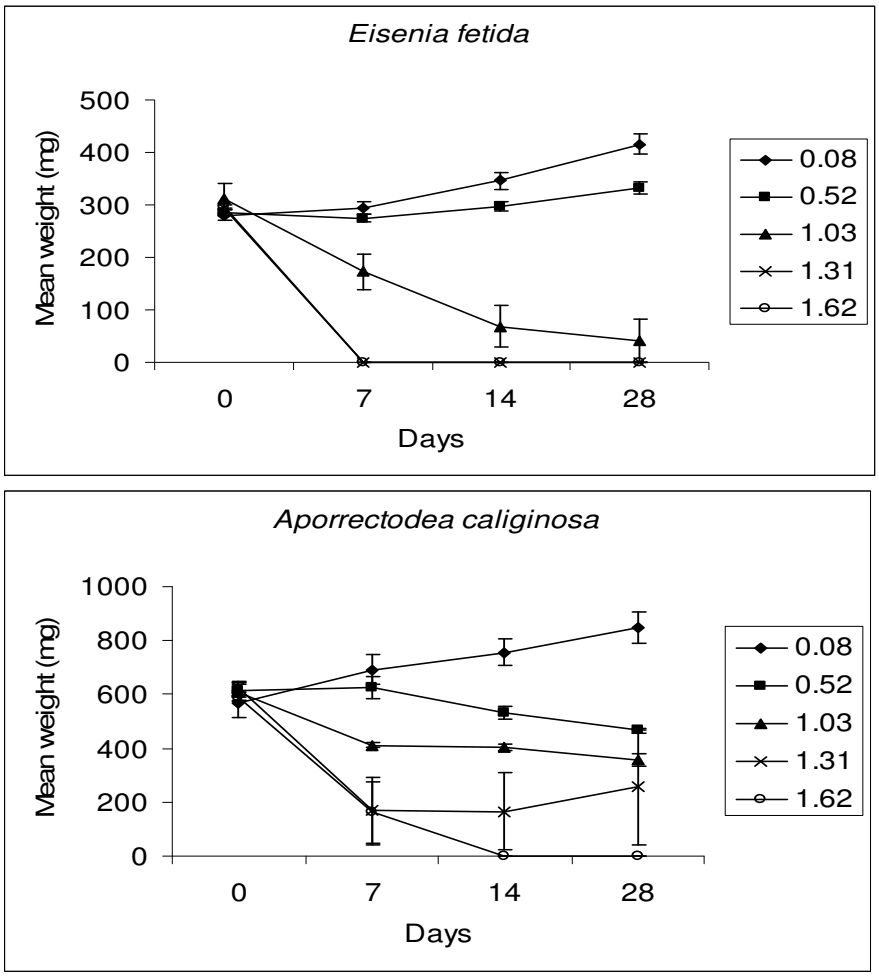

Fig. 23. Growth of two earthworm species (*Eisenia fetida* and *Aporrectodea caliginosa*) exposed for four weeks in natural soils of varying electrical conductivities (dS/m) under controlled laboratory conditions (error bars represent standard error).

Specimens of *A. caliginosa*, exposed to soil with EC of 0.52 dS/mand higher at day 14 and 28 had a significantly lower (P < 0.01) mean weight in comparison to worms in control soil At day 7, only worms exposed to soil of EC of 1.03 dS/m and higher had a significantly lower (P < 0.01) mean weight than the control worms.

7.4 DISCUSION

Salinity of soils collected from the Robertson area, in the Western Cape South Africa is dominated mainly by NaCl and less so by Ca salts (Table 9). This is in agreement with the findings of Dallas and Day (1993) who reported that NaCl is the predominant salt in agriculturally induced salinisation in South Africa. Apart from changes in salinity (and concomitant ionic concentrations), variation in other physico-chemical properties of soil was minimal (Table 9). These variations were small enough to allow the effects observed in this study to be based mainly on changes in salinity alone. Validity criteria were achieved for survival of all species tested except for *F. candida* where only about 78% of adult specimen could be recovered. It is not clear if this reduction in survival is due to low recovery of animals due to sampling methodology or less than optimum soil conditions for this species. However, in other studies, less than 80% survival had similarly been reported in natural and OECD soils (Smit and Van Gestel, 1998).

Reproduction was detrimentally affected in soil with an EC of 1.03 dS/m and higher in all four soil-dwelling species used in this study. Survival of the earthworms, *E. fetida* and *A. caliginosa* (Figs. 21 and 22) was also affected. This showed the detrimental effects that salinisation of soil could have on soil fauna, as also reported by other researchers (Hobel et al., 1992). Soils are generally classified as saline when they have an EC of 4 dS/m or more (Sumner, 1995). Soils with a salinity of less than 2 dS m^{-1} are classified as non-saline for plants (Schoeneberger et al., 2002). Our study showed that these four soil-dwelling species, representing a range of different groups, would be affected negatively at concentrations considered to be "safe" for many plants. In a survey of occurrence of salinity in the Western Cape, South Africa, Fey and Declerq (2004) reported that salinity of soils ranged from as low as 0.2 to as high as 9 dS m^{-1}. In some sugarcane fields in Zimbabwe, Rietz and Haynes (2003) reported EC's ranging from 0.1 to 24.8 dS/m . It can therefore be expected that many generally beneficial soil organisms would be totally absent in most saline soils in southern Africa and elsewhere, which falls into the range considered as non-saline for plants.

Salinity in the range used in this study had no significant effect on survival of *F. candida* and *E. doerjesi*, whereas it had harmful effects on reproduction at intermediate salinity levels, and caused total cessation of reproduction at higher

salinities. This indicates that reproduction was a more sensitive parameter than survival in the assessment of toxic effect of salinity to these organisms. This observation was also true for the earthworm species investigated in this study. This pattern has been reported previously in a study on the sensitivity of reproduction and survival parameters to cadmium for a range of soil arthropods (Crommentuijn et al., 1995). For some species, such as the oribatid mite *Platynothrus peltifer*, these authors found that reproduction was inhibited at concentrations far below those affecting survival.

Effects on survival and reproduction occurred at lower EC values in the earthworms than in *F. candida* and *E. doerjesi*. This indicates that the two earthworm species were more sensitive to saline stress than the collembolan and enchytraeid species. Between the two earthworm species, *E. fetida* appeared to be the more sensitive species since survival was seriously affected at 1.03 dS/m whereas no significant effect on survival was found for *A. caliginosa* at this salinity level. This observation is in contrast to other studies where other chemicals were used. In a study aimed at comparing the sensitivity of four earthworm species to zinc, Spurgeon et al. (2000) found that survival and reproduction of *A. caliginosa* were more sensitive endpoints for zinc than they were in *E. fetida* although both were within the same order of magnitude. In another study, Kula and Kokta (1992) found that *A. caliginosa* was more sensitive to insecticides parathion and propoxur than *E. fetida* in OECD soil substrates. It therefore appears that the relative sensitivity of these two species of earthworm depends on the chemical to which the worms were exposed (Heimbach, 1985). The relatively small differences in the sensitivities of the two earthworm species to salinity in our study suggest that for this pollutant, which has a number of diverse effects, differences in species sensitivity may be lower than for chemicals such as pesticides or other xenobiotic compounds with a single mode of action (Spurgeon et al., 2000).

Although the responses of *F. candida* and *E. doerjesi* were similar for survival and reproduction in the saline soils, *E. doerjesi* appeared to be slightly more sensitive since juvenile production ceased at a lower EC than for *F. candida*. Overall, for the four species, sensitivity to salinity increased in the order: *F. candida* < *E. doerjesi* < *A. caliginosa* < *E. fetida.* There were no comparable data available in the literature on

the sensitivities of these organisms to salinity. Most other studies were done on the metal sensitivity of *F. candida*, *E. fetida* and *E. crypticus* or *E. albidus*. Amorim et al., (2005a) reported that *F. candida* was less sensitive to Cu than the enchytraeids *E. albidus* and *E. luxuriosus*. Similarly, *F. candida* was less sensitive to Mn and Cd than the earthworm *E. fetida* which was also less sensitive than the enchytraeid species *E. albidus/crypticus* (Lock and Janssen, 2001c; Kuperman et al., 2004). However, for Hg and Cr, *E. fetida* appeared to be the least sensitive (Lock and Janssen, 2001e, 2002b) of these three species. It appears therefore that the differences in sensitivity are as a result of differences in the nature of contaminants. Differences in sensitivity between the worm species and *F. candida* might be explained by the different routes of uptake and exposure conditions. It has been argued that earthworms are more susceptible to metal pollution than many other groups of soil invertebrates (Bengtsson et al., 1992) and this appears to be more valid for salinity than for metals. Thus, in comparison to the enchytraeid and collembolan species, toxicity data for earthworms may be more useful in setting ''safe levels'' for soil salinity in a species sensitivity distribution model (Posthuma et al., 2007).

In this study, *E. fetida* could not survive in soils with an EC of 1.31 dS/m, whereas there was no mortality in OECD soil with a similar EC (Chapter 5). It is difficult to compare the two studies since artificial OECD and NaCl-salt were used in Chapter 5 while natural soil with different ionic constitution and soil properties was used in the present chapter. It is well known that the bioavailability of chemicals in soil varies depending on the soil properties and binding capacity of each soil (Spurgeon and Hopkin, 1996a). The combination of lower clay and organic matter contents and higher pH in soil used in the present study would mean that the binding capacity of this soil is lower than the OECD soil, and could thus partly explain the increase in toxicity of salt to the earthworm. Due to the diversity and complexity of factors influencing soil toxicity it would therefore also be important to perform risk assessment of salinity in soils in a site-specific manner (Posthuma et al., 2007).

7.5 CONCLUSION

This study showed that salinity up to an EC of 1.62 dS/m did not have a significant effect on survival of *F. candida* and *E. doerjesi* whereas significant effects were found on the earthworms *E. fetida* and *A. caliginosa*. Reproduction of all four

soil organisms was significantly impaired at 1.03 dS/m while absolute cessation of reproduction occurred at an EC of 0.52 dS/m for the earthworms and 1.31, 1.62 dS/m for the enchytraeid and collembolan species respectively. *E. fetida* was the most sensitive species followed by *A. caliginosa*, *E. doerjesi* and *F. candida*. This study showed the detrimental effects salinisation of soils could have on soil organisms, especially in the way it could affect reproduction. Site-specific field studies are needed to confirm the observed biological effects under environmentally relevant conditions.

CHAPTER EIGHT

8.0 AVOIDANCE BEHAVIOUR OF TWO ECO-PHYSIOLOGICALLY DIFFERENT EARTHWORMS (*EISENIA FETIDA* AND *APORRECTODEA CALIGINOSA*) IN NATURAL AND ARTIFICIAL SALINE SOILS

8.1 INTRODUCTION

Salinisation of soil is a rising problem in most arid and semi arid areas. Poor irrigation and drainage management are normally the main causes of salinisation and as the water table rises, salts dissolved in the groundwater, reach and accumulate at the soil surface through capillary movement (Rietz and Heinz, 2003) Much information is available on the effects of salinity on physico- chemical properties of soil (Sumner, 1995), and on plants (Ramoliya et al., 2004; Kadukova and Kalogerakis, 2007), and knowledge of its effects on beneficial soil organisms is building up gradually.

Salinity affects the growth and survival of microorganisms (Lippi et al., 2000; Rietz and Heinz, 2003; Yuan et al., 2007), and soil enchytraeids (Hobel et al., 1992). It has been reported that NaCl in excess of 0.5% wet weight may be harmful to earthworms in activated sludge (Hartenstein et al., 1981). Elduweini and Ghabbour (1965) reported that high salinity resulting from excessive irrigation can limit earthworm populations in some situations. Fischer and Molnar (1997) found that mortality was significant at 100 mM NaCl in organic manure and peat substrates, and cocoon production ceased totally while growth was negatively affected at concentrations below or in excess of 60 mM NaCl. In chapter 5, a 28-day LC50 of 5436 mg/kg NaCl and 28-day EC50 for growth and cocoon production of 4985 and 2020 mg/kg NaCl respectively were found for the earthworm *Eisenia fetida* in an OECD substrate. It was concluded that earthworms would be severely affected at salt concentrations considered to be safe for many plants.

Since most agricultural lands are either under the threat of salinisation or likely to be so in the future (Tyagi, 1989), there is a need for rapid soil screening methods for salinity in affected areas. Avoidance tests are increasingly regarded as a quick method for determining the potential harmfulness of contaminated soil (Lukkari

et al., 2005). It has been suggested that avoidance behaviour could be used as a meaningful indicator since the endpoints measured can sometimes be related to life-cycle effects (Amorim et al., 2008a). In the case of earthworms, avoidance of contaminated soils would have serious ecological impact since they are a major component in many soils and help to enhance nutrient turnover and soil aeration (Edwards and Bohlen, 1996).

Earthworms have chemical receptors in their prostomium, and possess high locomotory capacities; they can therefore sense pollutants and avoid polluted soils (Stephenson et al., 1998). Avoidance behavior of earthworms to various chemicals has been reported by many authors (e.g Garcia et al., 2008) and appears very promising as a quick assessment method of risk posed by a contaminated soil. When avoidance behaviour is shown by earthworms, it is often at concentrations lower than those affecting life-cycle parameters, indicating its relevance as predictive marker of impending effect at individual and population levels (Garcia 2004; Lukkari et al., 2005). As of now, the test has been shown to be suitable for many pollutants and mixture of pollutants (ISO, 2006). In a few cases, non avoidance of earthworms to certain chemicals (organophosphate pesticides and lead nitrate) has also been reported (Reinecke et al., 2002, Hodge et al., 2000). For salinity, only tangential evidence exist that they show avoidance in sandy soil soaked with several dilutions of sea water (Piearce and Piearce, 1979).These authors had performed their test before the first draft of the OECD guideline (1984) was proposed. Since they did not use a standard OECD soil or natural soil, direct comparison of data collected in the avoidance test with those collected in acute and chronic tests (for which substantial information is already available) is not possible.

It is well known that different earthworm species vary in their avoidance response to contaminated soils (Piearce and Piearce, 1979; Lukkari and Haimi, 2005). This study therefore aimed to compare the avoidance behaviour of two eco-physiologically different earthworms, *Eisenia fetida*, a suitable laboratory species also known for its composting abilities, and *Aporrectodea caliginosa*, a field relevant species. The specific aims were to compare the avoidance behaviour of these two earthworms in substrates of different soil properties and ionic constitutions as well as

to ascertain if the avoidance response is as sensitive as the most sensitive life-cycle parameter identified in parallel studies (Chapters 5 and 7).

8.2. MATERIALS AND METHODS

8.2.1 Test organisms

E. fetida specimens used for this study were taken from a culture kept in the laboratory of the Ecotoxicology Group, University of Stellenbosch, South Africa. Adult worms of between 300 and 600 mg were used in the experiments. *A. caliginosa* specimens were collected at grassland close to Eerste River in Stellenbosch, Western Cape, South Africa by digging and hand-sorting. The species occurs abundantly in this area which had no known history of pesticide use. These soils are not known to be saline. Only adult worms of between 400 and 800 mg with fully developed clitella were selected for use in the experiment.

8.2.2 Test soil

Two test soils were used in this experiment: OECD soil (OECD) and Robertson soil (ROBS). The OECD soil was prepared as described by OECD Guideline (2004). It consisted of 70% sand, 20% kaolin clay and 10% sphagnum peat by dry weight. The pH was adjusted to 6.0 ± 0.5 by $CaCO_3$. The maximum water holding capacity (WHC) was 65%. ROBS soil was collected from a farmland at Robertson Experimental Farm, Robertson, Western Cape, South Africa (33^o 50'.028'' S, 19^o 53'.492'') E ($33^0$50'S, $19^0$50'E). The collection, preparation and treatment of this soil have previously been described (Chapter 7). Two bulk soil samples, initially identified based on differences in electrical conductivity (EC) were collected from the top 10 cm layer. These were brought to the laboratory, air dried, sieved (2 mm) and thoroughly mixed individually, after carefully removing the surface organic materials and fine roots. The EC of these soil samples was determined as described in SSS (1996). A soil-water extract (1:5; w/v) was made and measurements were taken with a conductivity meter (SM 802 pH/ EC/ TDS Meter, Spraytech). Soils with intermediate EC were prepared by serial dilution. This soil had a pH > 9, organic matter (OM) <1%, clay 6%, maximum WHC of 36%.

8.2.3 Test procedures

The two earthworm species were each tested separately in both OECD and ROBS soils using similar salt concentrations. The worms were acclimatized for 24 hrs in the respective soils using either unspiked soil (OECD) or lowest salinity soil (ROBS). For the OECD soil, samples were spiked with technical grade NaCl (artificial sea salt) purchased from Royal Salt Company Ltd, Parow East, South Africa in the following concentrations: 0, 500, 1000, 2000, 4000 mg/kg. The concentrations were chosen to include those used in previous tests (chapter 5 and 7). The total amount of salt required for each concentration was added at once into deionised water and mixed with the total volume of soil for that concentration to achieve 55% of the WHC for each soil. For ROBS soil, that is a natural saline soil, the EC of the soil was adjusted to 0.08, 0.30, 0.52, 1.03, 1.33 dS/m to include EC values used in Chapter 5. The soil was moistened with deionised water to achieve 55% of the maximum WHC of the soil. The inclusion in this study of these salt concentrations (for OECD soil) and these EC values (for ROBS soil) was done to allow comparisons with the results of previously mentioned authors. After the soils were prepared, they were allowed to equilibrate for two days before being used in the experiments.

The avoidance test was performed as described by ISO (2006). Treated soils were introduced into the corresponding plastic containers. The cylindrical plastic containers (115 cm area, 10 cm height) were divided into two sections by drawing a line on the outside and labelling it with the name of the corresponding treated soil. Using a piece of plastic fitted transversally as a divider in the vessel, one half of the vessel was filled with saline soil, the other filled with control soil. For control purposes, control soils were used on both sides of the divided containers. The volumes of soil used were 300 g and 250 g (dry wt) for the ROBS and OECD soils respectively on each half of the container. Five replicates were used for each treatment. After the soils were introduced, the plastic divides were removed and 10 adult earthworms were placed on surface at the dividing line between the two halves of each test container (Fig. 24).

1 2 3 4 5

Fig. 24. Experimental steps in earthworm avoidance test: (1) introduction of the movable wall in the centre of the test vessel; (2) introduction of one test soil; (3) introduction of the other test soil; (4) movable wall is removed; (5) placement of the earthworms in the centre of the soils; At test end, reintroduction of the wall to separate the soils and counting of the organisms present in each side is done.

The vessels were then closed with transparent, perforated lids. The tests were carried out in a climate chamber at 20 ± 1^0C with a light/dark cycle of 16/8 hr for all treatments. The animals were not fed during the test. At the end of the test period of 48 h, the control and the contaminated soil sections were carefully separated by inserting the plastic divide and the number of earthworms was counted in each section of the vessels. Individuals found between the sections (on the separating line) were counted as 0.5 for each side, irrespective of the space occupied by the body in each side.

8.2.4 Statistics

Results are presented in graphs in terms of average of net response (NR) expressed as percentage and calculated as follows:

NR = {(C-T)/N}*100

where

C = worms observed in the control soil;

T = worms observed in test soil; N = total number of worms per replicate.

A positive (+) net response indicates avoidance and a negative net response (-) indicates a non-response (or attraction) to the saline soil. In accordance with the Draft Guideline for the Earthworm Avoidance Text (ISO, 2006), the habitat function of soils is considered to be limited if on average > 80% of worms are found in the control soil. In addition, one-way analysis of variance (ANOVA) was used on the raw data and data derived from NR to assess the concentration at which significant differences were found in the number of worms choosing the control soil. Tukey and Fischer's least significant difference (LSD) post-hoc tests were used to determine the significance of any differences between specific groups in all cases Also, a pairwise T-test was used to determine if there were differences between the number of worms choosing the control and the treated soil for each concentration. Calculations were performed using the statistical software package Statistica 7.0. Further, the avoidance effect expressed as the number of affected worms (i.e. those which avoided the treated part of the test vessel), was used as endpoint. From this, EC50's were calculated by

using the Linear Interpolation Method (USEPA, 1993) after confirmation that about 50 ± 10% of worms had chosen either side of the dual control chamber.

8.3 RESULTS

For both species, in both soils, no worm died or escaped during the exposure period. The proportions of worms in the two untreated sides were within 60%: 40% as specified in the ISO guideline (ISO, 2006). Therefore validity criteria were achieved in the tests. The Net Response (NR) showing avoidance of *E. fetida* and *A. caliginosa* in saline OECD and ROBS soils are shown in Figs. 25 and 26. Based on the 80% effect criterion (ISO, 2006), significant avoidance was achieved earlier at concentrations of 1000 mg/kg NaCl and higher for *A. caliginosa* than at 2000 mg/kg NaCl and higher for *E. fetida* in OECD soil. In ROBS soil, there was significant avoidance of soils with EC of 0.52 dS/m and higher for *A. caliginosa* whereas significant avoidance was only achieved in soils with EC of 1.03 dS/m and higher for *E. fetida.*

Using one-way ANOVA and the Tukey test in post-hoc comparison, results were consistent with earlier results obtained using NR and 80% trigger value. The only exception being in ROBS soil for *A. caliginosa* where significant avoidance was found at 0.30 dS/m (Tukey, $P < 0.01$). However, when the LSD post hoc test was used the results were different, with more significant differences being found in addition to those identified in the 80% trigger threshold. These all involved *E. fetida* in both OECD and ROBS soils. Significant avoidance was found in OECD soil with 500 mg/kg NaCl (LSD, $P < 0.05$) and ROBS soil with EC of 0.52 dS/m (LSD, $P < 0.01$).

Using the T-test for the results in the OECD soil, significant avoidance was found for all test concentrations for both earthworms. *E. fetida* showed significant avoidance (T-test, $P < 0.05$), of soils with a concentration of 500 mg/kg NaCl and higher (T-test, $P < 0.05$). Results were similar for *A. caliginosa,* except that the confidence level increased (T-test, $P < 0.01$) as from 1000 mg/kg NaCl. In ROBS soil, significant avoidance was found from 0.52 dS/m onwards for *E. fetida* (T-test, $P < 0.05$), and the confidence level increased (T-test, $P < 0.01$) as from 1.03 dS/m. *A. caliginosa* showed significant avoidance from 0.30 dS/m (T-test, $P < 0.01$) onwards.

The avoidance EC50 values obtained here were compared to results from acute and chronic tests with the same soils in chapters 5 and 7 (Table 1). In order to allow for a direct comparison of data, only concentrations used for the acute and chronic tests were used in calculating the avoidance EC50 values. The resulting EC50 values of *E. fetida* for avoidance were lower than for reproduction, growth and survival in OECD soil. A similar pattern was seen for *A. caliginosa* in ROBS soil.

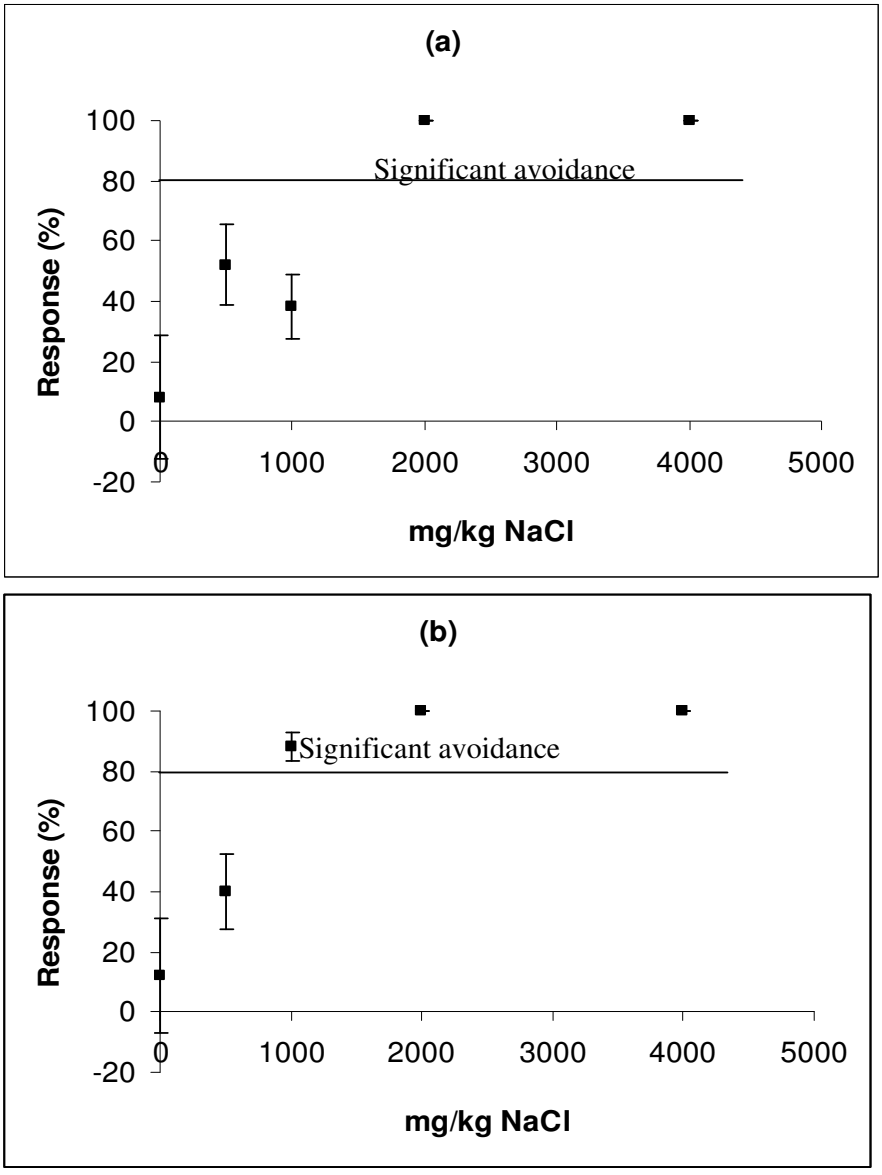

Fig . 25. Mean (± SE) net response of five groups, each consisting of ten worms (a) *Eisenia fetida* (b) *Aporrectodea caliginosa* after 48 hour exposure in an avoidance chamber with OECD soil adjusted with varying amounts of NaCl under controlled

laboratory conditions. Lines indicate the trigger value of 80% for the effect on the individuals.

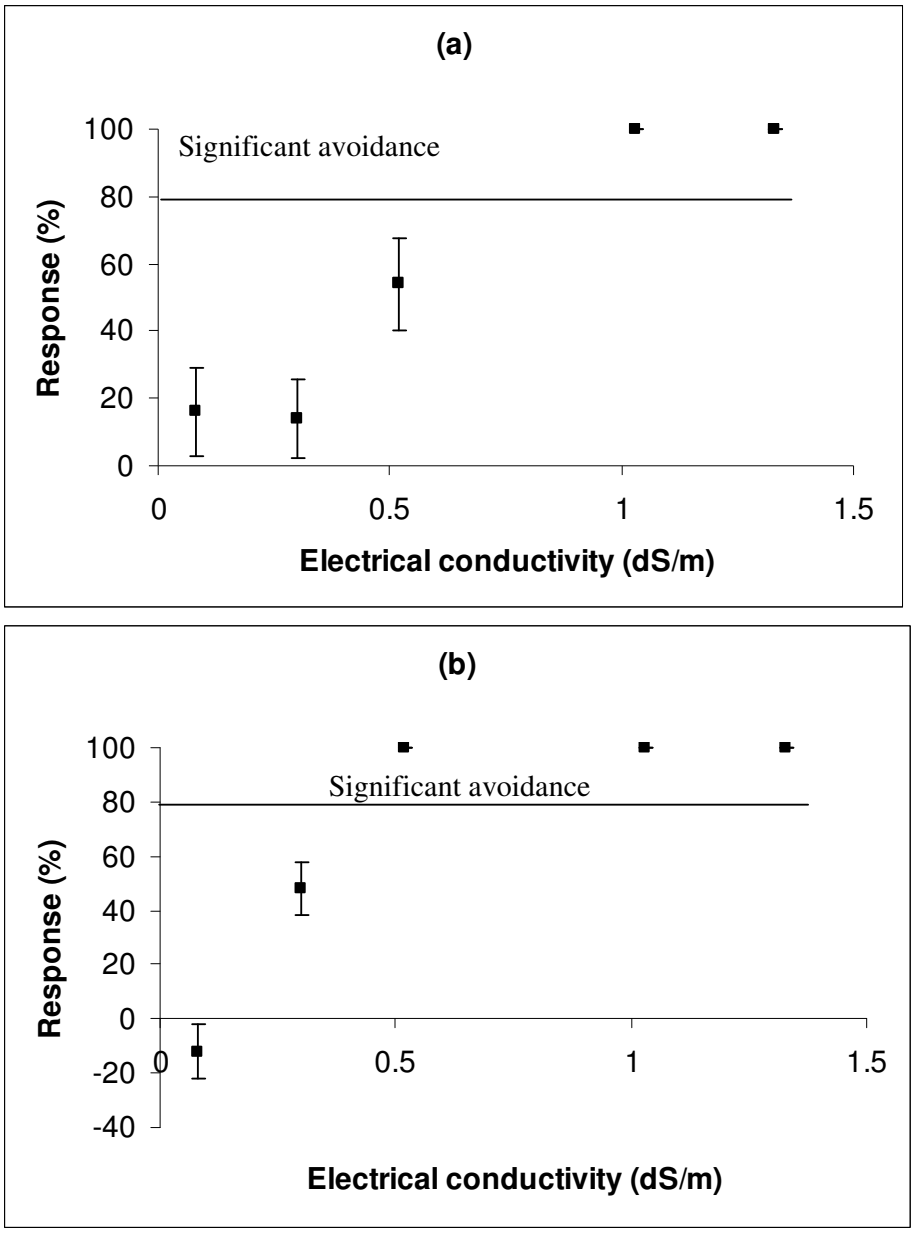

Fig 26. Mean (±SE) net response of five groups, each consisting of ten worms (a) *Eisenia fetida* (b) *Aporrectodea caliginosa* after 48 hour exposure in an avoidance chamber with natural soils of varying salinity collected from Robertson Experimental Farm in South Africa and kept under controlled laboratory conditions. Lines indicate the trigger value of 80% for the effect on the individuals.

Table 10. Comparison of LC50 and EC50 values for life cycle parameters and avoidance response of *Eisenia fetida and Aporrectodea caliginosa* exposed to salts using OECD and natural soil (collected from Robertson Experimental Farm, South Africa) (result from present chapter and Chapters 5 and 7)

	LC50(Confidence Interval)	EC50 (Confidence Interval)		
Substrates/pollutants	Survival	Growth	Reproduction	Avoidance
OECD/NaCl (mg/kg)				
E. fetida	**5436 (5170-5716)***	**4985 (4605-5000)***	**2020 (1467-2636)#**	**1164 (397-1490)**
A. caliginosa	**ND**	**ND**	**ND**	**667(391-833)**
ROBS/salts (dS/m)				
E. fetida	**0.75(0.73-0.77)***	**0.40 (0.37-0.47)***	**0.29(0.29-0.29)***	**0.56 (0.44-0.71)**
A. caliginosa	**1.15(1.08-1.22)***	**0.29(0.29-0.29)***	**0.29(0.29-0.29)***	**0.26**

estimated based on the results of Chapter 5

- * estimated based on the results of Chapter 7
- ND=not determined

However, for *E. fetida* in OECD soil, the EC50 value for avoidance was higher than for reproduction, lower than for survival but in the same range as for the effect on growth.

8.4 DISCUSSION

The response of *A. caliginosa* in the avoidance test was by an order of two more sensitive than *E. fetida* in both substrates (Table 10). The data for growth in ROBS soil partly followed the same pattern. This suggests that irrespective of soil properties, and ionic constitution of salt, *A. caliginosa* appears to be the more sensitive of the two species to saline stress as shown by its avoidance response. Since the reason for performing the avoidance tests in natural saline soils containing a mixture of salt and also in OECD soil containing only NaCl, was to obtain ecologically relevant data for both earthworm species as well as to see to what extent one could extrapolate results obtained in artificial soil to those obtained in field soil, it therefore appears that data collected in OECD soil could be relevant in field situations. Although ROBS salt used here is primarily Na and Ca dominated, trace

quantities of Mg and K salts are present (Chapter 7). These findings further suggest that earthworms could avoid salinised soils with different contamination histories.

The difference in avoidance response of these two earthworm species could be due to differences in chemoreceptor characteristics (Stephenson et al., 1998), physiological and morphological characteristics (Edwards and Bohlen, 1996) as well as behavioural and ecological characteristics (Lukkari and Haimi, 2005). It is difficult to use the ecological roles of these organisms alone to explain their sensitivity in the avoidance test since the contribution of each of these characteristics is not yet known. For example, in a study of the avoidance behaviour of three ecologically different earthworms to soil spiked with a mixture of Cu/Zn, the endogeic earthworm *A. caliginosa* was compared to two epigeic earthworm species *Dendrobeana octahedra* (which does not usually mix surface soil) and *Lumbricus rubellus* (which mixes surface soil) (Lukkari and Haimi, 2005). The result showed that *D. octahedra* was the most sensitive followed by *A. caliginosa*. These results as well as our own provide further confirmation that the ecological roles of earthworms may not, on their own, be an accurate predictor of their avoidance behaviour towards chemicals.

The higher sensitivity of the avoidance behaviour of *A. caliginosa* in OECD and ROBS soils compared to that of *E. fetida* contradicts the results of Piearce and Piearce (1979) who found *E. fetida* to be more sensitive than *A. caliginosa* in an avoidance test using sand as substrate. The difference in substrates could have been responsible for differences in response since it is well known that soil properties on their own can affect the avoidance behaviour of soil organisms (Amorim et al., 2005b, 2008b). Also it contradicts what I have obtained (Chapter 7 and Table 10) for survival of these organisms in ROBS soil, where *E. fetida* showed a more sensitive response to salinity than *A. caliginosa*. This could indicate that the tendency to avoid saline soil may not in all cases be a reliable predictor of lethality (acute effects). However, it must be noted that *E. fetida* specimens used in chapter 7 in the acute toxicity test were 3 months old and age synchronised while those used in the avoidance tests in the present chapter were not age synchronised since it is not requirement of the ISO guideline for avoidance tests. It was not possible to determine the precise age of *A. caliginosa* specimens that were used in the tests since they were collected from the field. The differences in sensitivities obtained between the avoidance and acute tests

could be age related. Also, as pointed out by Lukkari et al. (2005), in acute and chronic tests, organisms are forced to live in homogeneously contaminated test soil for the duration of the test and therefore have no possibility to exercise a choice, in contrast to avoidance tests where they do have a choice.

The comparison of the EC50 values in the avoidance tests with those obtained for growth and LC50 values for survival (Table 10) indicates that avoidance response is clearly more sensitive than growth and survival. When compared with reproduction, it is either more sensitive or within the same order of magnitude. This was obvious for *E. fetida* in OECD soil and for *A. caliginosa* in ROBS soil. In earlier studies reproduction was by far the most sensitive life-cycle parameter for the effect of salinity on earthworms in OECD (Chapter 5) and natural soil (Chapter 7). This study has thus shown that avoidance behavior could be as sensitive an endpoint as reproduction in assessing the effect of salinity on earthworms. This is similar to the results obtained by Garcia (2004) for avoidance behaviour of earthworms to metals. This author reported EC50 values for avoidance that were lower than the mortality values but in the same order of magnitude as those for reproduction. Lukkari et al. (2005) reported that *A. tuberculata* avoided soil metal concentrations that were lower than those that induced responses in acute toxicity and reproduction tests.

However, this lower or quite similar response for both the avoidance test and the chronic reproduction test were not always obtained. In this study, reproduction and growth for *E. fetida* in ROBS soil were more sensitive endpoints than avoidance. This suggests that the predictive value of the avoidance tests for *E. fetida* might be low in natural soils. This is similar to the findings of Amorim et al. (2005b) who exposed the potworm *Enchytraeus albidus* to three different fungicides in avoidance tests. For benomyl, carbendazim and phenmedipham tested in OECD and standard European soil (LUFA) the avoidance EC50 values were in the same order of magnitude as the mortality LC50 values, but clearly higher than the respective EC50 values for reproduction. A larger data base is therefore required before a reliable relation between avoidance response and endpoints such as mortality/reproduction could be established.

8.5 CONCLUSION

Avoidance tests with *E. fetida* and *A. caliginosa* showed clear responses of earthworms to low salinity in both OECD artificial and ROBS soils. Of the two earthworm species, *A. caliginosa* was the more sensitive in the avoidance tests irrespective of soil properties or ionic constitution of substrates. The results of the reproduction and avoidance tests with *A. caliginosa* were mostly in good accordance. The avoidance tests could be used to determine whether a saline field soil would be potentially harmful to this earthworm species or as a useful rapid screening tool for the assessment of potentially harmful saline soil in cases where reproduction is not possible or below the required validity criteria due to natural, ambient soil properties (Amorim et al., 2005b; Chapter 7). Apart from the need for a larger data base concerning the relation between avoidance and mortality/reproduction responses in soils of different properties, information is also required on the role of contamination histories before the avoidance test can be generally accepted as a time-saving alternative to long-term tests. However, these results suggest that the avoidance test for salinity could be used as a suitable screening method showing first tendencies of saline stress on the habitat function of soils, especially in large saline areas.

CHAPTER NINE

9.0 EFFECTS OF FLOODING, SALINITY AND COPPER ON THE EARTHWORM *APORRECTODEA CALIGINOSA*: A MICROCOSM FIELD STUDY

9.1 INTRODUCTION

Copper contamination of the soil environment, apart from natural occurrence, could be caused by industrial activities and the use of metal containing agrochemicals (Vorobeichik, 1998). One such agrochemical is copper oxychloride which is a widely used fungicide in agricultural land (Krause et al., 1996). Through repeated spraying, Cu can accumulate in the top layer of soil. Although it is an essential mineral, the toxic effects of excessive amounts of Cu are well documented. It, for example, significantly affects survival, growth and reproduction of earthworms (Svendsen and Weeks, 1997a; Helling et al., 2000; Maboeta et al., 2002, 2003), damages internal organs and compromise lysosomal membrane integrity in snails (Snyman et al., 2000, 2002, 2005) and several other soil organisms.

In agricultural land, not only pesticide use could present problems for beneficial, non-target soil organisms, salinisation of soil may present another problem, especially in most arid and semi arid areas (Tyagi, 1989). Salinity affects growth and reproduction of plants (Kadukova and Kalogerakis, 2007), and beneficial soil organisms (Yuan et al., 2007; Fischer and Molnar, 1997). In South Africa, irrigation induced salinity is a problem especially in the Western Cape region (Fey and Declerq, 2004). The Western Cape region is known for wine production and copper oxychloride is often used by deciduous fruit farmers for fungal control (London and Meyers, 1995). It is therefore likely that both salinity and copper contamination would be a problem in some instances.

Few studies have reported the joint effect of salinity and copper contamination on sorption processes in substrates and their toxicity to soil organisms. Available literature data suggest that salinity tends to increase the bioavailability of Cu in substrates. DTPA (di-ethylene-triamine-penta acetic acid) extractable copper was reported to increase with increasing levels of salinity in sewage sludge

(Keshavarz et al., 2006). Salinity significantly increased the $CaCl_2$ extractable Cu in Organisation for Economic Co-operation and Development (OECD) artificial soil and had additive interaction with Cu in toxicity to the earthworm *Eisenia fetida* (Chapter 6). Since most of these studies were either conducted in semi-soil (artificial soil/sewage sludge) or as laboratory studies, how these results relate to the combined effect of these contaminants in the field remains to be tested.

Laboratory toxicity tests are usually conducted under stable ambient conditions, while exposures in ecosystems occur in a fluctuating climate. As pointed out by Jones and Hart (1998), a number of factors could contribute to the observed differences between laboratory and field effects, of which differences in susceptibility between species are the most important. These authors also identified environmental factors (e.g. rainfall) as another factor that could create uncertainties when extrapolating from the laboratory to the field. The method of contaminant application is also important. For example granular application of the pesticide carbofuran can cause greater toxicity than when sprayed as a solution (Jones and Hart, 1998). Since *E. fetida*, the species often used in laboratory tests, is not a field relevant species (because it is mainly found in compost heaps and not in soil) it is necessary to study the combined effect of salinity and copper with a field relevant species, and under field relevant conditions.

Although a fully fledged field study is often laborious, time consuming and difficult to interpret, the use of simulated field systems or microcosms could reduce such uncertainties (Burrows and Edwards, 2002). We therefore decided to use this methodology to study the combined effects of salinity and copper on earthworms under field conditions. During the study period heavy rainfall and unexpected flooding of field plots occurred which influenced the experimental outcome but also allowed for an assessment of the effects of flooding.

The species selected, *Aporrectodea caliginosa,* is an endogeic earthworm species common in many agricultural soils in South Africa. It is sensitive to both salinity (Piearce and Piearce, 1979) and copper (Maboeta et al., 2003). This study was therefore designed to investigate the joint effect of increasing salinity and copper, on this earthworm species under field relevant conditions. The specific aims were to determine how salinity influenced the sorption processes of Cu in the soil and its

uptake by the earthworm, as well as how the combination of both salinity and Cu may affect survival, growth and reproduction.

9.2. MATERIALS AND METHODS

9. 2.1 Test species

A. caliginosa specimens were collected from a grassland close to the Eerste River in Stellenbosch, Western Cape, South Africa by digging and hand-sorting. This species occurs abundantly in this area which had no known history of pesticide use. Conductivity testing of the habitat soil did not show elevated levels of salinity. Only adult worms of between 400 and 800 mg with fully developed clitella were selected for the experiment. They were acclimatized for one week in the test soil prior to use in the experiment.

9.2.2 Test soil

The test soils were collected from a farmland at Robertson Experimental Farm, Robertson, Western Cape, South Africa (33^{o} 50'.028'' S, 19^{o} 53'.492'') E (33^{0}50'S, 19^{0}50'E). The collection, preparation and treatment of these soils are described in chapter 7. These soils had a pH: 9-9.5, organic matter (OM): <1%, clay: 6%, maximum WHC: 36% and electrical conductivity (EC) of between 0.08 and 1.62 dS/m Since there were significant mortality at higher salinity (chapter 7), only soils with EC of 1.05 dS/m or lower were used in the present study. The soils were moistened to 55% of the maximum WHC.

9.2.3 Test procedures and experimental setup/semi field study

Specimens of *A. caliginosa* were exposed to soil of increasing salinity (EC = 0.08, 0.62 and 1.05 dS/m) in purpose built microcosms placed under field conditions. The microcosms were made from polyethylene plastic pipes of diameter 10 cm and height 15 cm. The bottom of each pipe was sealed using nylon and medium density polyethylene (mesh size 1 mm). This was then screwed to the base of the microcosm and secured with plastic holders ensuring that earthworms could not enter or escape. Each microcosm was loaded with 2 kg of the corresponding soil up to the 13 cm mark and allowed to equilibrate for two days before earthworms were introduced. After the

earthworms were introduced, 10 g of grinded manure, rewetted with 10 ml of water, was mixed were about 160 g of the corresponding soil and added until each microcosm was filled to the brim. The food was added on the top layer to maintain the worms during the exposure period, so as to mimic what happens in soil where organic matter content of top soil is higher than in the sub-surface. Earthworm and predator entry into the microcosms from above was prevented by placing a net (mesh size of 1mm), which was secured with a plastic holder, over the top of the microcosms. There were 12 microcosms for each salinity group, with 15 worms in each microcosm, giving a total of 36 microcosms containing 540 worms.

For the field experiment, a flat grassland area next to the Eerste River in Stellenbosch, was prepared. A plot of 3 x 3- m plot was marked out, and separated with the aid of pegs and rope (Fig. 27). This plot was demarcated into three strips: two sampling strips of 1 x 3 m^2 with a boundary strip of the same area (1x3m^2) in between them.

Fig. 27. The researcher spraying the fungicide copper oxychloride in plots where microcosms were inserted, on grassland close to the Eerste River in Stellenbosch, South Africa. Each subplot received microcosms loaded with natural soil of different salinities.

Each sampling strip was divided into three sub-plots: one for each salinity level. The 36 soil filled cosms containing the worms were sunk 15 cm into the corresponding sub-plot such that for each sampling strip, there were 18 microcosms and 6 for each salinity level. One sampling strip was sprayed with water (untreated) while the other was sprayed with copper oxychloride (treated). In the treated plot, Effekto Virikop copper oxychloride (Active ingredient: 850 g/kg) was applied at a concentration of 5 g/L of distilled water, as recommended. This gave an effective copper oxychloride concentration of 4.25 g/L active ingredient. The solution was applied with a Matabe compress spraypack. The volume sprayed was 1.5 L/m^2. The control plot was water-sprayed with the same volume as in the treated plot. The spraying event took place on the day the microcosms were inserted and subsequently at two-week intervals. Sampling was done at day 14 and again at day 28 after the microcosms had been inserted in the field. Although the field test was originally designed to run for 56 days, extensive flooding of the experimental plots occurred, causing high worm mortality as witnessed by the presence of large numbers of dead worms on the surface of the microcosms after the flood subsided. The test therefore had to be terminated on day 28. Two microcosms from each treatment were sampled at day 14 while the remaining four were sampled at day 28. However, at this stage soil of an intermediate salinity level (EC= 0.30 dS/m) was introduced after two weeks by inserting additional microcosms with lower salinity levels in order not to miss out on possible trends at lower salinity levels. These microcosms were harvested two weeks after the earlier ones, and were treated as previously described.

9.2.3.1. Worm parameters

On each sampling day, growth, mortality, and internal copper concentration (ICC) of worms were assessed. Mortality was assessed by stimulating the worm with a blunt probe and an earthworm was judged dead if no response could be observed. Worms not found were assumed to be dead since dead earthworms in soil disintegrate within a few days. Growth was determined by weighing each worm individually from each microcosm, and comparing the mean weight with initial values. The number of cocoons produced was determined at the end of the exposure by wet sieving the substrates (through a 2.0- and 1.0-mm sieve system), and counting the number of cocoons. To determine the ICC, four worms per treatment were removed and placed

singly in Petri dishes on moist filter paper for 24 hrs at 20^0C to allow depuration of their gut contents. Afterwards, they were frozen singly for metal analysis. The procedure for the metal extraction by acid digestion is described in Chapter 6. Thereafter, samples were filtered, using 0.45 µL cellulose nitrate filter paper, into film boxes and stored at 4 ^{o}C until spectrometric analysis.

9.2.3.2 Soil parameters

Electrical conductivity (EC) of soil samples was measured at each sampling date by making a saturated paste extract in distilled water (1:5, w: v) and measuring with a conductivity meter. Soil moisture content and pH-(H_20) were monitored on each sampling occasion (Micro pH 2001, Crison for pH, Sartorius infrared moisture detector for moisture). For the determination of copper contents of substrates, samples were taken at days 14 and 28 and chemical extractions were carried out using three methods: $CaCl_2$, DTPA and nitric acid extraction methods as described in chapter 6.

9.2.4 Copper analysis

The extracted soil and worm samples were analyzed for copper by a Varian AA-1275 flame atomic absorption spectrophotometer (AAS). For each batch of worms and soil digested and analysed, a blank was also prepared to detect possible contamination during the digestion process. Previously spiked soil samples were analyzed and indicated a recovery above 90% with this procedure. Quality control was achieved by analysing reference materials independently prepared from the standards.

9.2.5 Statistics

All data were checked for normality and homogeneity of variance with the Shapiro-Wilks test and Levene's tests respectively. Data on EC, pH, survival and % weight change per sampling time and cocoon number were analyzed using two-way analysis of variance (ANOVA) with salinity and Cu as variables. For Cu concentrations in worm and in soil (nitric acid one-way ANOVA was used to test for the effect of salinity along each Cu level. When data were not normally distributed even after transformations, Kruskal-Wallis H-test was used. Fischer's least significant

difference (LSD) post-hoc test was used to determine the significance of any differences between specific groups in parametric cases while multiple comparison of 'z' and 'p' values was used in non-parametric cases. ANOVA data were analysed using STATISTICA 7.0 software.

9.3. RESULTS

9.3.1 Rainfall/Changes in physicochemical properties of soil

High precipitation occurred for most days of the experimental period with flooding of microcosms occurring at the time of the second sampling (after 28 days) (Fig. 28).

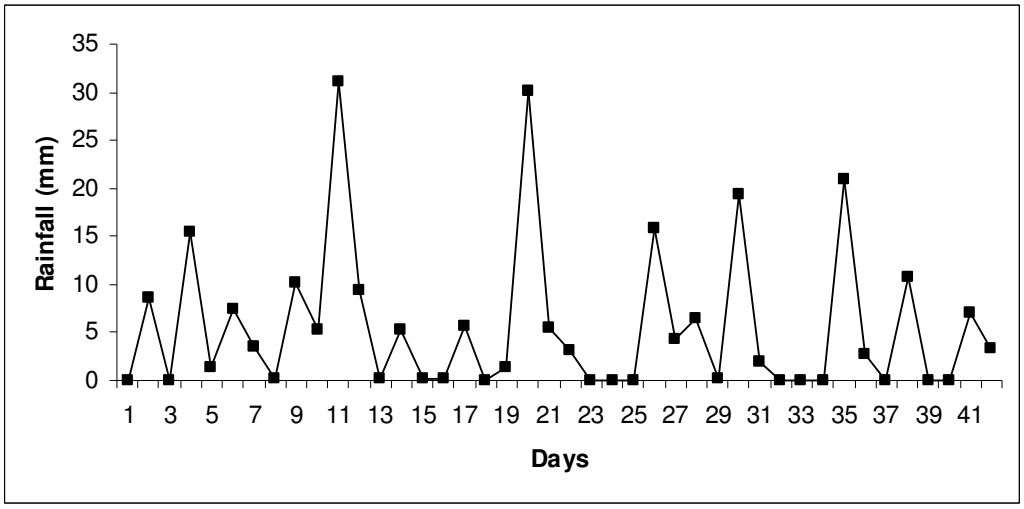

Fig. 28. Mean daily rainfall in the Stellenbosch area during the field microcosm experiment with *Aporrectodea caliginosa* (Day 0 was 20 August, day 14 was 3 September, day 28 was 18 September, 2008, while day 42 was 1 October, 2008).

The mean moisture content of the soil in the microcosms was 10.9% at day 1 when the microcosms were taken to the field. At day 14, the moisture content rose to 20.1% (above the maximum WHC) and further to 21.9% when measured at day 28. There was a general increase in pH of all soils irrespective of treatments with copper oxychloride or water as the experimental exposure period progressed (Table 11).

Table 11. Changes in soil pH and electrical conductivity (EC) over a 28 day period in the field exposure microcosm experiment with earthworms (*Aporrectodea caliginosa)* in a salinity gradient with or without copper treatments.

Parameter		*Copper treated*		*Untreated*	
pH	Day 0	Day 14	Day 28	Day 14	Day 28
	8.72	8.82	9.16	8.71	9.05
	8.99	9.02	9.20	9.1	9.29
	8.78	8.83	8.74	9.05	8.99
	8.55	8.83	8.96	8.95	8.96
EC (dS/m)					
	0.08	0.09	0.09	0.06	0.06
	0.3	0.11	0.10	0.09	0.08
	0.62	0.09	0.09	0.11	0.11
	1.05	0.09	0.09	0.16	0.16

However, the pH changes were usually below 0.5 units. There were no significant individual or joint effects of salinity and Cu (ANOVA, $P > 0.05$) in the pattern of pH increase. There was a substantial reduction in the EC values of all soil salinity treatments by day 14 and this remained so until day 28 (Table 11). There were no significant differences (ANOVA, $P > 0.05$) in the pattern of EC reduction between copper treated and untreated microcosms.

9.3.2. $CaCl_2$, DTPA and nitric acid extractable copper in substrates

The amounts of Cu extractable by both $CaCl_2$ and DTPA were below the detection limit for all treatment groups (salinity and copper treatments). The amounts of Cu extracted by the nitric acid method along the salinity gradient in microcosms from both treated and untreated plots are shown in Fig. 29. Generally the Cu content of soil was generally low and within a range of 3-5 mg/kg Cu. There was no significant ($P > 0.05$) increase in Cu content of soil in the treated microcosms, and along the salinity gradient.

9.3.3 Survival, growth and reproduction

The survival of worms along the salinity gradient in the treated and untreated microcosms is shown in Fig. 30. At day 14, irrespective of the spraying regime, survival of worms was mostly above 70% for all treatments, except for treatments having an initial EC value of 1.05 dS/m. There was no significant effect of interaction between salinity level and copper (ANOVA, $P > 0.05$) on survival. The individual effect of copper treatment was also not significantly (ANOVA, $P > 0.05$) different from the control. However, salinity had a significant effect on survival of the worms (ANOVA, $P < 0.01$).The LSD test revealed significant differences ($P < 0.01$) in survival between worms exposed to soil with an EC of 1.05 dS/m and other salinity treatments. At day 28, no worms survived in the microcosms with an initial EC of 0.3 dS/m, which were introduced two weeks later than others (See methods). Although the survival in all other treatments fell below 40% at this day, the basic pattern was similar to the data obtained at day 14. However, the individual and joint effect of salinity and copper were not significant (ANOVA, $P > 0.05$) at this day.

There was however a significant effect (ANOVA, $P < 0.05$) of salinity as an individual parameter on percentage weight change of worms. The LSD test revealed that worms exposed in soils with an initial EC of 0.62 and 1.05 dS/m gained significantly less weight ($P < 0.05$, and $P < 0.01$ respectively) than those in 0.08 and 0.30 dS/m soils. By day 28, there was a general reduction in weight already gained at day 14 to values close to their initial weight at day 0. In the copper sprayed treatments, all worm weight changes were negative whereas in the water sprayed treatments, they were all positive except in the soil with the highest salinity. However, interaction between, as well as individual effects, of salinity and copper had no significant effect (ANOVA, $P > 0.05$) on weight change by this day between the control and experimental treatments.

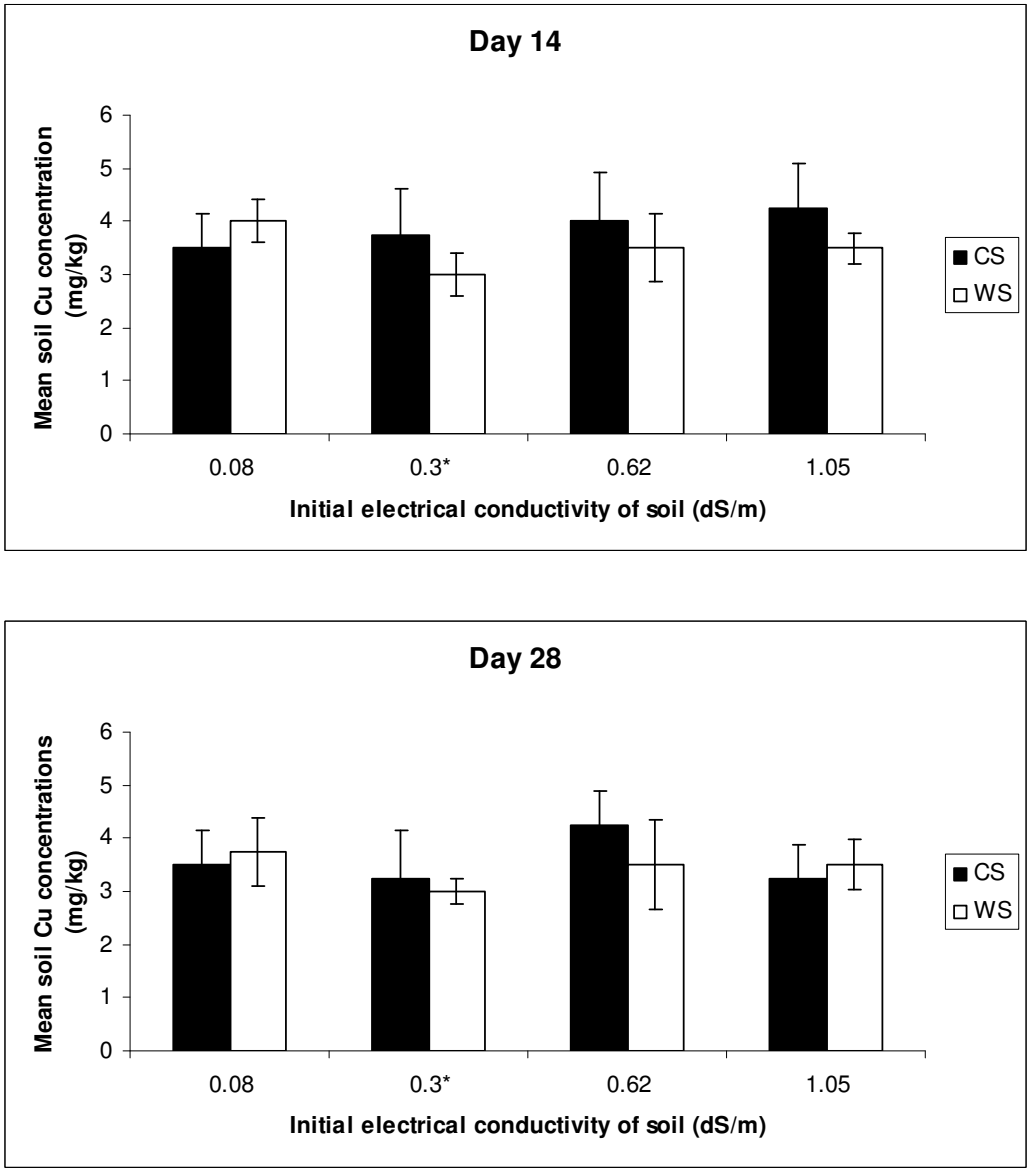

Fig. 29. Mean (± SE) nitric acid extracted Cu content in soils of increasing salinity after spraying with copper oxychloride (CS) or water (WS) in a field microcosm experiment. * Microcosms were introduced two weeks later than for others. See also Table 1 for changes in salinity over time.

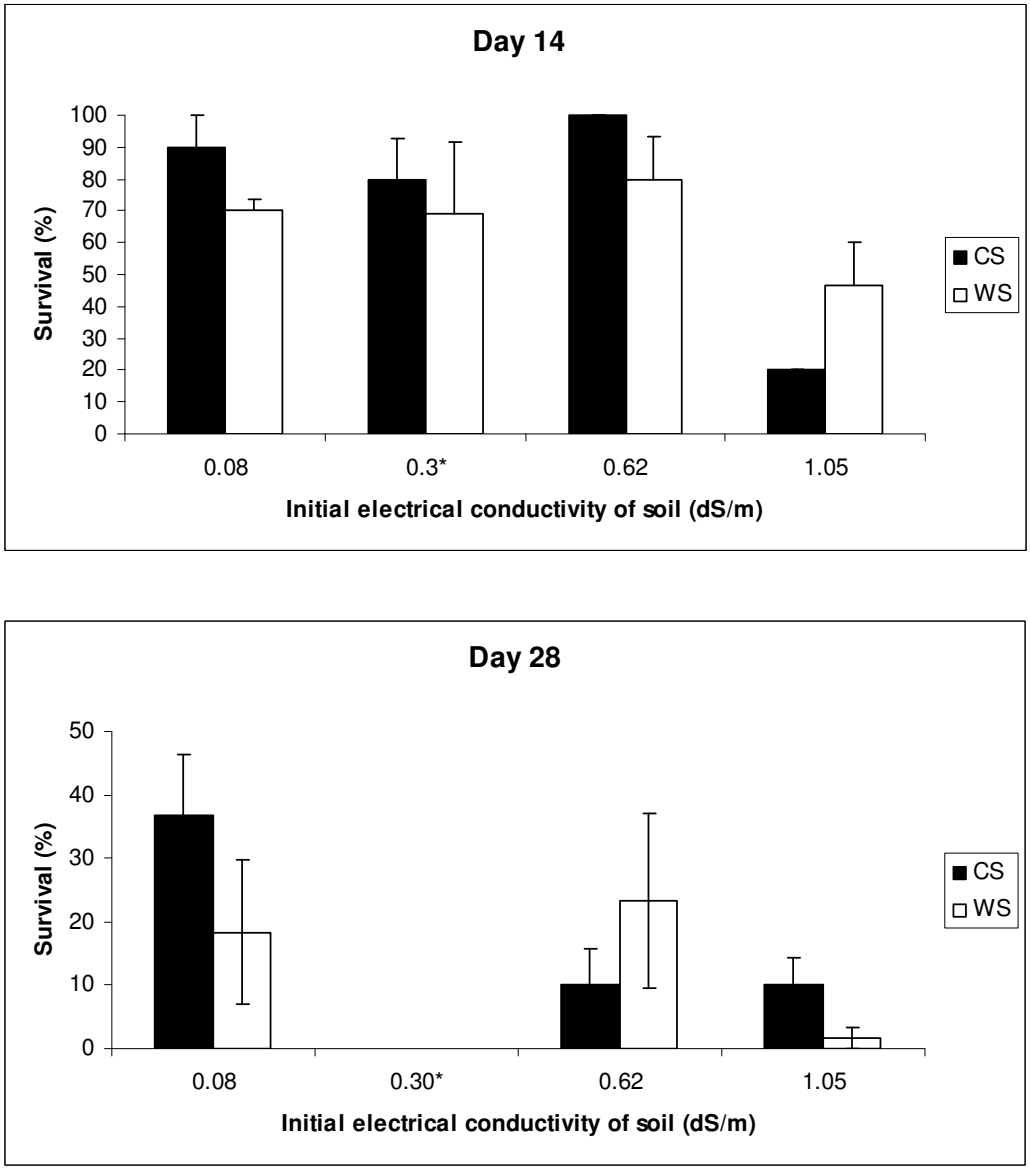

Fig. 30. Mean (± SE) survival of earthworms (*Aporrectodea caliginosa*) in soils of increasing salinity after spraying with copper oxychloride (CS) or water (WS) in a field microcosm experiment. * Microcosms were introduced two weeks later than for others. See also Table 1 for changes in conductivity over time.

Mean percentage weight change of worms over the 28-day exposure period is shown in Fig 31. At day 14, there was a general weight gain in all treatments. Interaction of salinity and copper treatments, as well as copper as an individual parameter, had no significant effects (ANOVA, $P > 0.05$) on weight change of worms.

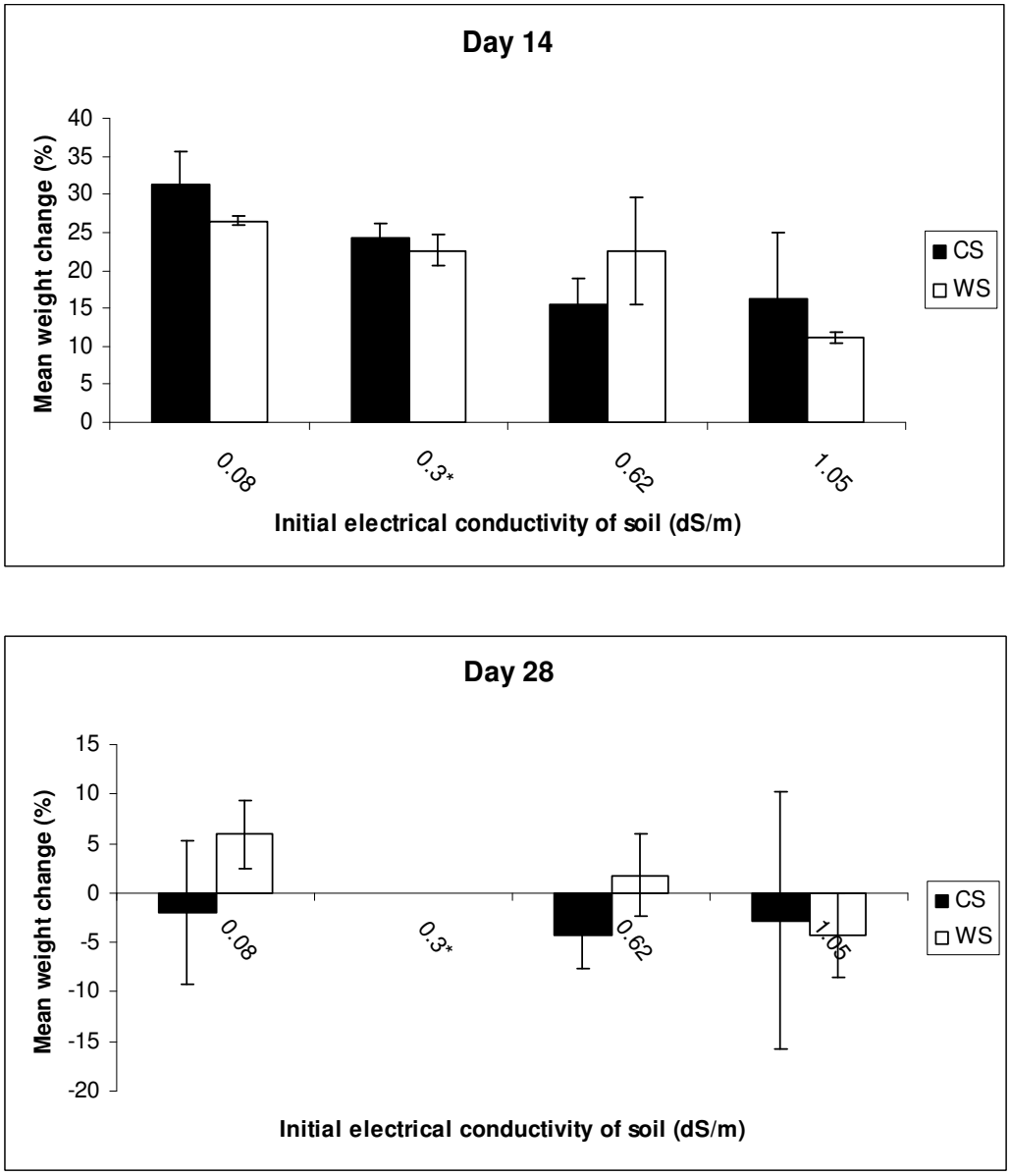

Fig. 31. Mean (± SE) percentage weight change over 28 days of earthworms (*Aporrectodea caliginosa*) exposed in soils of increasing salinity after spraying with copper oxychloride (CS) or water (WS) in a field microcosm experiment. * Microcosms were introduced two weeks later than for others. See also Table 1 for changes in conductivity over time

No cocoons were produced by day 14 in any of the treatments. By day 28 only a few cocoons were produced in soils of the lowest initial EC values (Figure 32). Although cocoon production was less in copper treated microcosms in comparison with untreated microcosms, this was without statistical significance (T-test, $P > 0.05$). In two non-saline microcosms that were not Cu treated, 2 and 1 cocoons were found but no worms survived in these microcosms.

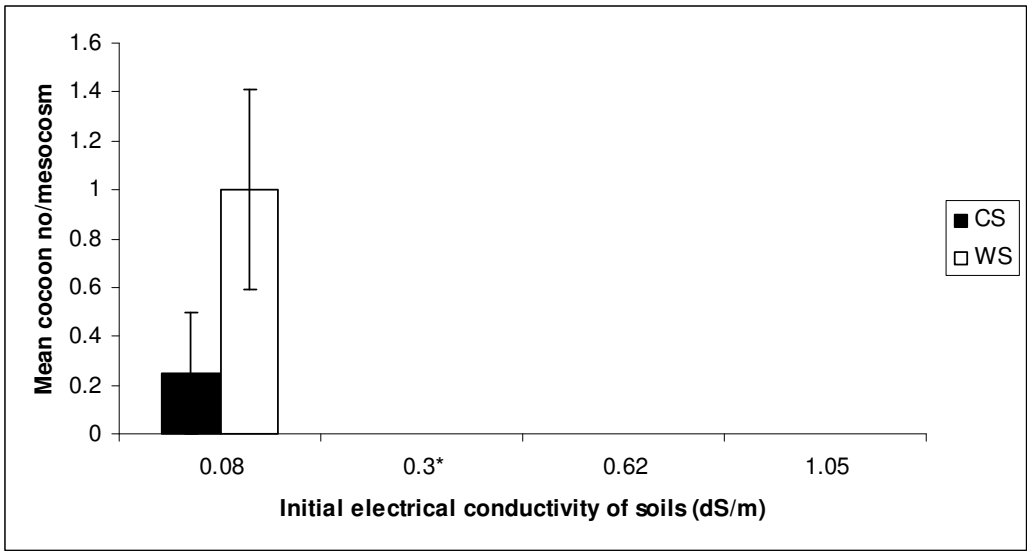

Figure 32. Mean (± SE) cocoon production per microcosm of earthworms (*Aporrectodea caliginosa*) exposed in soils of increasing salinity after spraying with copper oxychloride (CS) or water (WS) in a field microcosm experiment.

* Microcosms were introduced 2 weeks later than for others. See also Table 11 for changes in conductivity over time

9.3.4 Internal copper concentration in worms

The internal copper concentration (ICC) at days 14 and 28 in worms of both treated and untreated microcosms along the gradient of salinity revealed low Cu concentrations in worms (Fig. 33). Generally, ICCs of worms were between 6-8 mg/kg Cu and were not dependent on salinity, Cu treatment or their interaction (ANOVA, $P > 0.05$).

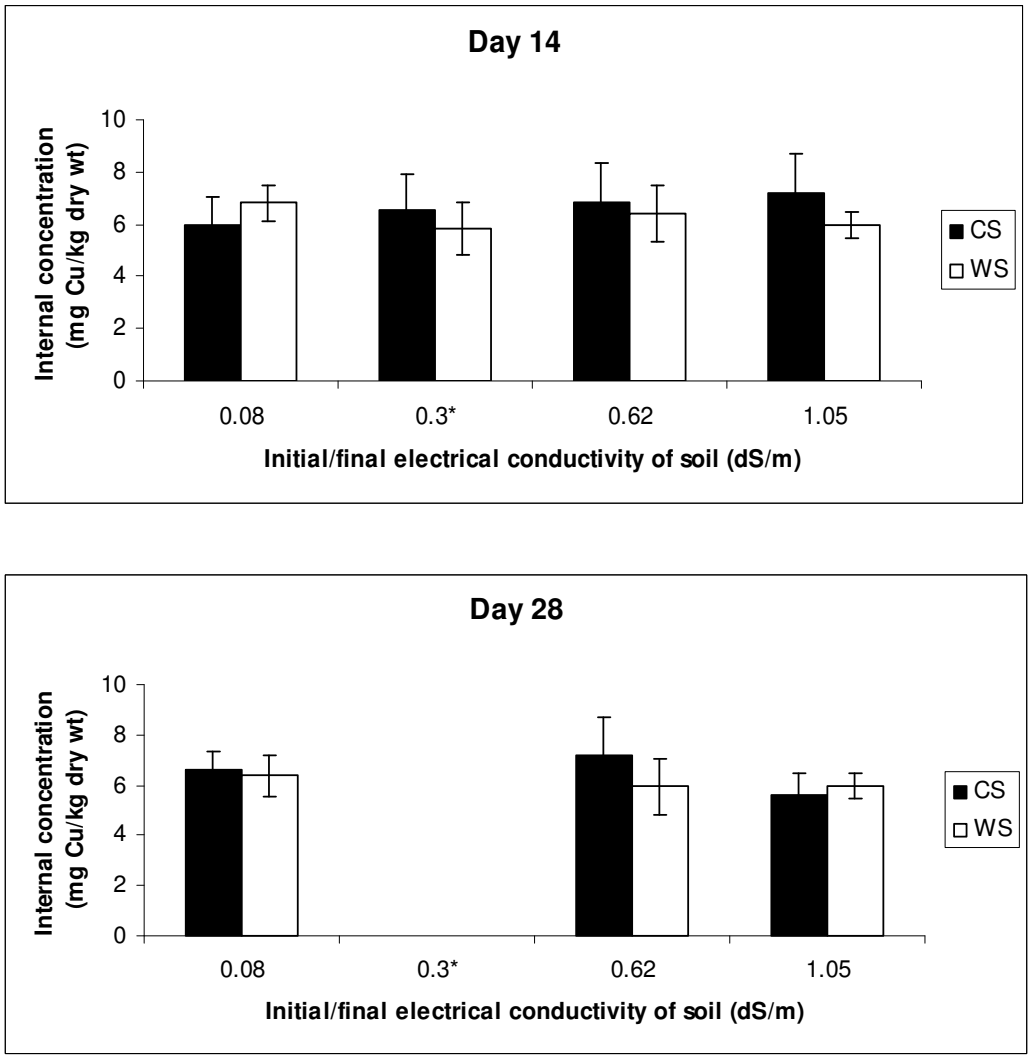

Fig. 33. Mean (± SE) copper concentrations in specimens of *Aporrectodea caliginosa* exposed to soils of increasing salinity after spraying with copper oxychloride (CS) or water (WS) in a field microcosm experiment. * Microcosms were introduced two weeks later than for others.

9.4. DISCUSSION

Salinity had a significant effect on earthworm survival and weight change at day 14 but this was not observed at day 28. One would expect that this toxic effect would have been more pronounced with increasing exposure time. Since this was not so at day 28, it may indicate that other factors could have played an over-riding role in the toxicity pattern as the experimental exposure period progressed. Analysis of the salinity of soil treatments over the exposure period indicated a substantial reduction in the EC values of soil, such that values were fairly similar in all treatments by days 14 and 28 (Table 11). Despite this reduction in EC, toxicity found at day 14 suggested that the effect of salinity found here could be seen as a worst-case scenario. The effect of salinity on *A. caliginosa* found in this study could not be compared directly with that found for this species in the laboratory (Chapter 7). This is mainly because EC changes found in the field microcosms were not observed in the laboratory study. The laboratory study was conducted in a closed system and at fairly constant temperature and moisture conditions, excluding the role and influence of varying environmental factors on EC.

Application of copper oxychloride at the recommended dosage at two-week intervals for a month period had no significant effect on earthworms in this study. This was not strange since Maboeta et al., (2003) applied copper oxychloride once a month for three months at concentrations simulating a nine times spraying frequency and found no immediate response on biomass and worm density of *A. caliginosa* over the short term and only a delayed effect at a much later stage. In vineyards, the effect of copper oxychloride application on soil organisms is often due to many years of spraying and accumulation of Cu (Van Zwieten et al., 2004). In the study of Maboeta et al. (2003), Cu content of soil and worms increased with increased Cu exposure whereas no accumulation in soil and worms were found in the present study, which was possibly due to the lower relative concentration of copper oxychloride used and the shorter exposure period. Also the influence of environmental factors can not be ignored. EC reduction and lack of Cu accumulation could have been caused by extreme environmental factors (mainly rainfall) which caused flooding/waterlogging of the microcosms especially in the last two weeks of the experiment. When flooding occurs two effects may be possible. Firstly, there could be increased availability of

chemicals for uptake with increased moisture content of soil (Van Gestel and van Diepen, 1997). Secondly, there could be percolation of chemicals through leaching from the soil to underground water (Smit et al., 1997). In this study it appeared that the latter seemed to be the case for most part of this study. Leaching of salts and Cu from the microcosms could be deduced from the reduced EC values measured in all treated soils after 28 days in the field (Table 11) as was also evident from the lack of Cu accumulation in both the soil (Fig. 29) and worms (Fig. 33). It is likely that this took place gradually until a large proportion of the unbound salts and Cu were leached out. It was not possible to determine the leaching rate since data were only collected at two-week intervals, but it appears that the contaminants (at least for salt) were not fully leached out in the initial exposure period since effects were found at day 14. However, it is necessary to note that collection, handling and processing of soil would have led to serious disturbance of the soil structure, which could cause increased infiltration and porosity. These factors could have pre-disposed the soil to excessive leaching. It is possible that such a large amount of rainfall as witnessed in this observation period might not have led to leaching of contaminants from the soil column if the soil was in its natural, undisturbed state. This possibility should be considered when interpreting results obtained with microcosms in which soil structure was mechanically disturbed.

Apart from the effect of flooding on chemicals in soils, direct effect of flooding on the organisms was evident in this study. By day 28 of the experiment, survival of worms in all treatments irrespective of salinity or Cu levels was lower than 50% (Fig. 29). Some of the worms were found dead floating in water, suggesting that they died because of a lack of oxygen, a situation caused by flooding. Also, recovery of a few cocoons in soil with the lowest salinity (EC = 0.08 dS/m) where no worms survived, showed that conditions may have been suitable for the worms until the flooding occurred. Contrasting results of the influence of flooding on survival and abundance of *A. caliginosa* have been reported in the literature. Zorn et al. (2005) reported that flooding effects did not show a clear pattern on abundance and biomass of *A. caliginosa* in a Dutch flood plain. Ausden et al., (2001) found hardly any *A. caliginosa* in flooded grassland in Western Europe while Keplin and Broll (2002) found a reduced number of representatives of this species in flooded grasslands in Germany. In contrast, Pizl (1999) found an increase in relative abundance of *A.*

caliginosa after summer flooding in the former Czechoslovakia. A direct comparison between these studies and the current one is hardly possible unless the exact conditions and timing of observations could be compared. Earthworms can be resilient and populations may recover soon after flooding (Zorn et al., 2005) in spite of the damaging effects observed during flooding.

The apparent effect of leaching of salts and Cu in this study did not allow a proper assessment of the interactive effect of salinity and Cu and the observed non interaction could therefore not be considered to be reliable. In the earlier laboratory study (chapter 6) with *E. fetida*, it was concluded that an additive interaction between salinity and Cu should be expected at Cu concentration ranges likely to be found in agricultural soils. Increased salinity had an additive effect with Cu up to a concentration of 320 mg/kg Cu. Cu content of most agricultural soils often fall below 100 mg/kg Cu.

In a review comparing laboratory tests with field trials of pesticides, Jones and Hart (1998) suggested that there can be significant limitations on the usefulness of field trials in earthworm risk assessment because of a lack of control of earthworm species present and the prevalent environmental conditions. They suggested the use of microcosms to overcome some of the limitations. In this study, the use of microcosms allowed me to have some control over earthworm species present but not over variable environmental conditions.

9.5. CONCLUSION

The roles of individual and interactive effects of salinity and copper could not be demonstrated clearly in this field experiment, most probably because salts were leached from the microcosms as a result of extensive flooding. Rainfall was high during this study period despite conducting these tests close to spring time. This caused leaching of contaminants and could have been the reason for the low toxicity. The study did however provide evidence that flooding could also have a severely negative effect on survival and growth of *A. caliginosa*.

CHAPTER TEN

10. GENERAL CONCLUSION

With the increasing global demand for food and looming economic and climatic uncertainties, there is more likelihood that higher input agriculture will be required to address these challenges. This inevitably involves the increased use of irrigation practices and pesticides, both of which could lead to increased salinisation and metal contamination of agricultural lands.

Metal pollution is currently of higher concern in developed, industrialised countries than in developing countries. In developed countries like for example The Netherlands, USA, and Germany, industrial activities have over many decades left a backlog of metals in industrial and even in some urban areas. In many cases, levels of metals are way above the background concentrations. It can be expected that in developing countries, like South Africa, mining activities and use of metal containing pesticides in agriculture are gradually increasing the metal concentrations of soils. Although, this concentration might still be considerd to be within "acceptable" levels, it is likely that with increased activities, this would become a serious problem in the future.

These issues raise the need to understand the fate and behaviour of metals in soils, in order to gain knowledge of their bioavailability for beneficial soil organisms in order to implement adequate protective measures of the soil environment when necessary. A plethora of factors are known to affect the bioavailability of metals in soils. These include soil properties, age of metals in soil, ionic strength of the soil among others. While attention before now has been on the role of organic matter content, pH and cation exchange capacity, little has been done on the role of clay content and salinity. South Africa houses a large variety of soil types, ranging from those that are very sandy to those with very high clay content. In order to utilize and protect these soils fully, an understanding of the role of clay and salinisation on toxicity of metals was studied. This work therefore investigated the role clay content and salinity plays in influencing the toxicity of Cu and Zn to soil organisms mainly earthworms, and in some cases collembolans and enchytraeids.

This study revealed that (kaolin) clay content had little effect on the bioavailability of sub-lethal concentrations of Zn to earthworms (Chapter 3) whereas it had a significant influence on both sub-lethal and lethal concentrations of Cu (Chapter 4). The basic effects were such that with an increase in clay content, (bio) availability of metals was reduced. The differences in the effect of clay content on the bioavailability of these two essential metals could be explained by differences in their sorption properties. Other workers have reported that Zn was a mobile metal while Cu tended to adsorb strongly to the surface of clay particles and organic matter. Although, montmorillonite clay was not used in this study due to the inherent variation it caused in other soil properties, its stronger adsorption properties indicate that clay content might become a more important factor in soils that consist predominantly of montmorillonite clay. It is therefore necessary to study the effect of different clay types on the metal bioavailability to earthworms. Clay type varies from region to region. Kaolin clay is more common in tropical soils while montmorillonite clay is more common in temperate soils. These differences suggest that simply adopting standardized soil ecotoxicity testing guidelines that were developed in one region (for example, for European soils), in other parts of the world without recourse to local soil properties, might bring some levels of uncertainty into toxicity evaluation (for example, in tropical areas).

This study revealed that soils with a relative low salt content that would normally have them categorized as non-saline, were detrimental to a wide range of soil-dwelling species (*Folsomia candida, Enchytraeus doerjesi, Eisenia fetida and Aporrectodea caliginosa*) tested in this study. All four taxa did not survive and/or had significantly lower reproduction in natural/artificial soils with an EC value lower than 2 dS/m (Chapter 7). In view of the fact that soils are generally classified as non-saline for plants when they have EC values less than 2 dS/m, it therefore appears that soil organisms may generally be more sensitive to salinity than many plants. Of the four taxa, the earthworm species showed higher sensitivity to salinity than the collembole and enchytraeid. If this finding can be confirmed by more studies by including additional taxa, earthworms could serve as a useful representative organism for assessing “safe” salt levels in soil for purposes of protecting soil biodiversity.

Sensitive avoidance behaviour of earthworms when exposed to saline soil at relatively low salt concentrations (Chapter 8) further showed their relevance as good bio-indicators of saline conditions. These findings (on their sensitivity and avoidance of salts) suggest that the absence of soil organisms, especially earthworms, in partially saline soil may therefore be a good indicator of poor soil quality since in their absence, the productivity of such soils in terms on nitrogen mineralization, organic matter decomposition, and other functions would be impaired.

The combined effect of salinity and Zn on earthworms revealed that their interaction was either additive or synergistic (Chapter 5). The combined effect of salinity and Cu was also additive (Chapter 6). These findings indicate that in agricultural soils that could be affected by both salinity and metal contamination, a joint toxic effect could be expected. Although the Cl^- effect could have been responsible for the synergistic interaction found for salinity and Zn, the increase in availability of metals (extracted by $CaCl_2$ and DTPA) with increased salinity, showed that an interaction leading to a more than additive effect can not be ruled out completely. Caution should therefore be taken when employing conventional agricultural practices that may encourage extensive irrigation and pesticide usage in cases where it could be avoided, since these are the main sources of salinisation and metal contamination in agricultural lands.

Although the attempt to understand the joint effect of salinity and Cu under field conditions by using field microcosms was largely complicated by the influence of heavy rainfall and eventual leaching of contaminants, the prominent role played by environmental conditions was brought to the fore (Chapter 9). This finding showed the limiting influence that environmental factors can have on field data collection and stresses the challenges involved in field validation of laboratory data. It underlines the statements of other researchers who have also suggested that field studies of this kind are often laborious, time consuming and that the results are sometimes overshadowed by the interference of varying environmental factors. Since one of the main goals of ecotoxicological assessment in soil is to predict effects of chemicals under field conditions, more and better ways of conducting and analysing field data needs to be explored.

Leaching of salts as observed in the field study highlights the possibility of using irrigation with water of good quality to flush out excessive salts beyond the reach of beneficial organisms and plants. The implications of this in the management of underground water however need to be quantified. In highly salinised soils, other known methods of salinity management might also be helpful. These include:

- better management of annual crops and pastures
- planting of perennial, deep-rooted crops and grasses
- installing systems that drain excess surface and sub-surface water and pump out groundwater
- planting salt-tolerant crops and grasses and
- developing new industries that use the saline resources (eg, saline aquaculture and harvesting salt on a commercial basis).

On the whole, this study provided baseline data that could be helpful in assessing the toxicity of two essential metals (Cu and Zn) with reference to clay and salt contents of soil. The study also highlighted likely differences between the behaviour of these two essential metals, and as such care must be taken when using models described to predict the toxicity of one to predict the other. The study further provided data on salinity tolerance of four taxa, and highlighted the detrimental effects that salinisation of soils have on these taxa. This data would be primarily helpful for farmers and policymakers who have to decide on the need to discourage the use of poor irrigation schemes which can increase salt contents of soils because it can affect soil biodiversity and thereby hamper soil organisms in their roles of enhancing or contributing to soil fertility.

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12. 0 APPENDIX A

Appendix 1: Analysis of Variance table showing the individual and interactive effect of clay content and zinc on mortality of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Variables	*SS*	*DF*	*MS*	*F*	*p*
CLAY	12.23333	2	6.11667	23.42553	0.000001
ZINC	91.26667	4	22.81667	87.38298	0.000001
CLAY*ZINC	48.93333	8	6.11667	23.42553	0.000001

Apendix 2: Analysis of Variance table showing the individual and interactive effect of clay content and zinc on weight change of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period

Time	*Variables*	*SS*	*DF*	*MS*	*F*	*P*
Day 0	CLAY	837	2	419	0.742	0.483250
	ZINC	1113	3	371	0.658	0.583474
	CLAY*ZINC	2511	6	419	0.742	0.619496
Day 7	CLAY	957	2	479	0.816	0.448541
	ZINC	18805	4	4701	8.018	0.000057
	CLAY*ZINC	5646	8	706	1.204	0.318525
Day 14	CLAY	396	2	198	0.244	0.784290
	ZINC	95131	4	23783	29.351	0.000001
	CLAY*ZINC	10746	8	1343	1.658	0.135563
Day 28	CLAY	3563	2	1781	1.601	0.212901
	ZINC	168028	4	42007	37.761	0.000001
	CLAY*ZINC	21163	8	2645	2.378	0.13439

Appendix 3 Analysis of Variance table showing the individual and interactive effect of clay content and zinc on cocoon production of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Variables	*SS*	*DF*	*MS*	*F*	*p*
CLAY	0.880952	2	0.440476	5.71324	0.008529
ZINC	2.051020	2	1.025510	13.30147	0.000095
CLAY*ZINC	0.598639	4	0.149660	1.94118	0.132358

Appendix 4. Analysis of Variance table showing the individual and interactive effect of clay content and zinc on maturation of worms exposed in OECD artificial soil substrates

Variables	*SS*	*DF*	*MS*	*F*	*p*
CLAY	5.2	2	2.6	0.027	0.972997
ZINC	84955.1	4	21238.8	223.023	0.000001
CLAY*ZINC	885.2	8	110.6	1.162	0.342789

Appendix 5. Analysis of Variance table showing the individual and interactive effect of clay content and zinc on zinc accumulation of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Time	*Variables*	*SS*	*DF*	*MS*	*F*	*p*
Day 7	CLAY	1483.5	2	741.8	0.20791	0.813060
	ZINC	79773.6	4	19943.4	5.58977	0.000969
	CLAY*ZINC	11735.6	8	1466.9	0.41116	0.908203
Day 14	CLAY	7145.3	2	3572.7	3.1384	0.052973
	ZINC	24236.5	4	6059.1	5.3227	0.001348
	CLAY*ZINC	2938.7	8	367.3	0.3227	0.953130
Day 28	CLAY	641.43	2	320.72	3.6537	0.033853
	ZINC	1483.27	4	370.82	4.2245	0.005475
	CLAY*ZINC	353.23	8	44.15	0.5030	0.847532

Appendix 6. Analysis of Variance table showing the individual and interactive effect of clay content and copper on mortality of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Variables	*SS*	*DF*	*MS*	*F*	*p*
CLAY	7.87500	2	3.937500	31.50000	0.0000001
COPPER	17.41667	3	5.805556	46.44444	0.0000001
CLAY*COPPER	19.45833	6	3.243056	25.94444	0.0000001

Apendix 7:Analysis of Variance table showing the individual and interactive effect of clay content and copper on weight change of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Time	*Variables*	*SS*	*DF*	*MS*	*F*	*p*
Day 0	CLAY	951	2	476	0.700	0.503028
	COPPER	4085	3	1362	2.005	0.130599
	CLAY*COPPER	3199	6	533	0.785	0.587387
Day 7	CLAY	2795	2	1398	1.725	0.192591
	COPPER	67257	3	22419	27.670	0.000001
	CLAY*COPPER	19349	6	3225	3.980	0.003724
Day 14	CLAY	16480	2	8240	9.710	0.000424
	COPPER	121696	3	40565	47.801	0.000001
	CLAY*COPPER	36438	6	6073	7.156	0.000045
Day 28	CLAY	50152	2	25076	14.428	0.000025
	COPPER	136045	3	45348	26.092	0.000001
	CLAY*COPPER	34336	6	5723	3.293	0.010974

Apendix 8: Analysis of Variance table showing the individual and interactive effect of clay content and copper on cocoon production of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Variables	*SS*	*DF*	*MS*	*F*	*p*
CLAY	942.125	2	471.063	22.5060	0.0000001
COPPER	2492.167	3	830.722	39.6894	0.0000001
CLAY*COPPER	209.208	6	34.868	1.6659	0.1577990

Appendix 9. Analysis of Variance (ANOVA) table for the individual and interactive effect of salinity and zinc on weight change of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period

Time	*Variables*	*SS*	*DF*	*MS*	*F*	*P*
Day 0	SALINITY	837	2	419	0.742	0.483250
	ZINC	1113	3	371	0.658	0.583474
	SALINITY*ZINC	2511	6	419	0.742	0.619496
Day 7	SALINITY	97552	2	48776	66.550	0.000001
	ZINC	32496	3	10832	14.779	0.000002
	SALINITY*ZINC	17193	6	2866	3.910	0.004151
Day 14	SALINITY	85760	2	42880	55.588	0.000001
	ZINC	51221	3	17074	22.134	0.000001
	SALINITY*ZINC	16222	6	2704	3.505	0.007824
Day 28	SALINITY	104485	2	52242	43.369	0.000001
	ZINC	95100	3	31700	26.316	0.000001
	SALINITY*ZINC	45930	6	7655	6.355	0.000125

Appendix 10. Analysis of Variance (ANOVA) table for the individual and interactive effect of salinity and zinc on cocoon production of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Varaiable	*SS*	*DF*	*MS*	*F*	*p*
SALINITY	50.21098	2	25.10549	83.4406	0.000001
ZINC	18.14413	3	6.04804	20.1013	0.000001
SALINITY*ZINC	13.39031	6	2.23172	7.4173	0.000032

Appendix 11. Analysis of Variance (ANOVA) table for the individual and interactive effect of salinity and copper on cocoon production of worms (*Eisenia fetida*) exposed in OECD artificial soil substrate over a 28-day period.

Variable	*SS*	*DF*	*MS*	*F*	*p*
SALINITY	3.81701	2	1.90850	11.9979	0.000067
COPPER	24.22925	4	6.05731	38.0795	0.000001
SALINITY*COPPER	2.08095	8	0.26012	1.6352	0.141668

SS-sum of square, DF-degree of freedom, MS-mean square, F-F values, P- probability level

13.0 APPENDIX B

Papers published, in press or accepted from the present study:

1. **Owojori,** O.J., Reinecke, A.J., Rozanov, A.B., 2008. Effects of salinity on partitioning, uptake and toxicity of zinc in the earthworm *Eisenia fetida*. Soil Biology and Biochemistry. 40, 2385-2393

2. **Owojori**, O.J., Reinecke, A.J., Rozanov, A.B., 2009. Role of clay content in partitioning, uptake and toxicity of zinc in the earthworm *Eisenia fetida*. Ecotoxicology and Environmental Safety. 72, 99-107.

3. **Owojori**, O.J., Reinecke, A.J., Rozanov, A.B., 2009. The combined stress effects of salinity and copper on the earthworm *Eisenia fetida.* Applied Soil Ecology. 41 (3), 277-285.

4. **Owojori**, O.J., Reinecke, A.J., 2009. Avoidance behaviour of two eco-physiologically different earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*) in natural and artificial saline soils. Chemosphere. 72 (3), 279-283

5. **Owojori**, O.J., Reinecke, A.J., Voua-Otomo P., Reinecke, S.A., in press. Comparative study of the effects of salinity on life-cycle parameters of four soil dwelling species (*Folsomia candida*, *Enchytraeus doerjesi, Eisenia fetida* and *Aporrectodea caliginosa)*. Pedobiologia. 10.1016/j.pedobi.2008.12.002

6. **Owojori**, O.J., Reinecke, A.J., Rozanov, A.B., in press. Influence of clay content on bioavailablility of zinc in the earthworm *Eisenia fetida.* Ecotoxicology and Environmental Safety.