A comparison of biomarkers in assessing the combined effects of pesticide mixtures on non-target soil invertebrates

By

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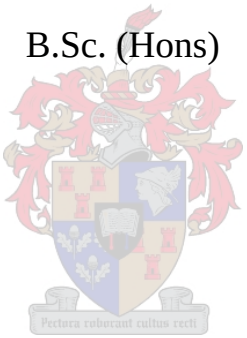

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DECLARATION

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

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ABSTRACT

Agricultural environments are usually contaminated with mixtures of antropogenically introduced chemicals as a result of pesticide spraying, which can affect beneficial, non-target soil invertebrates, such as earthworms negatively. Most studies on mixture toxicity have focused on interactions of chemicals with similar structures and mechanisms. However, chemical mixtures may occur as conglomerates of diverse structures and toxicological mechanisms in the environment.

This study was aimed at assessing the effects of pesticides singly, and in a mixture, on earthworms, using lifecycle parameters (growth and reproduction) and biomarkers (neutral red retention (NRR) assay and acetylcholinesterase (AChE) inhibition) as endpoints. Thus, to determine whether any interactions occurred between the pesticides as shown by the measured endpoints. Another aim was to validate the use of the chosen biomarkers for assessing mixture toxicity.

The pesticides used were from three groups: organophosphates, heavy metal-containing pesticides and pyrethroids. From these three groups, four of the most commonly used pesticides in the orchards and vineyards of the Western Cape, South Africa, were chosen, namely chlorpyrifos (organophosphate), azinphos-methyl (organophosphate), copper oxychloride (heavy metal-containing fungicide) and cypermethrin (pyrethroid). Earthworms were exposed in the laboratory to a range of concentrations of chlorpyrifos and copper oxychloride singly, and in 1:1 mixtures of these pesticides in artificial soil, for four weeks. After the exposure period, the biomass change was determined as measure of growth, and cocoon production, hatching success and number of hatchlings per cocoon were determined as measures of reproduction.

Growth (biomass change) and reproduction (cocoon production) were affected by the highest concentration treatment (20mg/kg) of chlorpyrifos, but copper oxychloride and the mixture of the two pesticides showed no observable effects on lifecycle parameters. Dose related effects on NRR times were however determined for both pesticides and the mixture. Dose related effects on AChE activity were found for chlopyrifos and the mixture of the two pesticides, but not for copper oxychloride. Short-term exposures (48 hours) of earthworms to the following pesticides in artificial groundwater: chlorpyrifos,

copper oxychloride, azinphos-methyl, cypermethrin, chlorpyrifos-copper oxychloride, chlorpyrifos -azinphos-methyl and chlorpyrifos-cypermethrin, were done followed by the determination of AChE inhibition. Dose related effects were exhibited on the AChE activity of earthworms exposed to chlorpyrifos, a mixture of chlorpyrifos and copper oxychloride, azinphos-methyl, and a mixture of azinphos-methyl and chlorpyrifos. Copper oxychloride, cypermethrin and the mixture of chlorpyrifos and cypermethrin had no effect on AChE activity. Earthworms died at the highest exposure concentration of the mixture of chlopyrifos and cypermethrin.

Results have shown that although the pesticides did not cause observable effects on lifecycle parameters, there were effects at subcellular and biochemical level, as shown by the biomarkers. Mixtures of pesticides, in some instances, affected earthworms differently from their single components, indicating interactions between the pesticides in mixtures, as shown by the measured endpoints. The NRR assay proved to be a good general biomarker of soil contamination, and the AChE activity could also be a valuable tool in assessing the effects of organophosphate mixtures and mixtures of organophosphates and pesticides from other groups.

OPSOMMING

Nie-teiken organismes, soos erdwurms, word negatief beïnvloed deur mengsels van antropogeniese chemikalieë in landbou-omgewings. Die meeste studies wat handel oor die toksisiteit van chemiese mengsels het tot dusver gefokus op chemikalieë van dieselfde aard en met dieselfde meganismes van werking. Mengsels van chemiese stowwe kan egter as konglomerate van 'n verskeidenheid strukturele eienskappe en met verskillende toksiese meganismes in die omgewing aangetref word.

Tydens die studie is gepoog om die effekte van enkel pestisiede sowel as mengsels daarvan op erdwurms te bestudeer, deur van lewensloop kenmerke (groei en voortplanting) en biomerkers (neutraalrooi retensietyd - NNR en inhibisie van asetielcholienesterase -AChE) as eindpunte gebruik te maak. 'n Verdere doel van die studie was om vas te stel of daar enige wisselwerkings tussen die verskillende pestisiede plaasvind, soos aangetoon deur die gemete eindpunte, en verder ook om die gebruik van die gekose biomerkers as maatstawwe van mengseltoksisiteit te evalueer.

Die pestisiede wat gebruik is, is van drie verskillende groepe afkomstig: organofosfate, swaarmetale en piretroiede. Van hierdie drie groepe is vier van die pestisiede wat vry algemeen in boorde en wingerde in die Weskaap, Suid-Afrika, gebruik word, geïdentifiseer. Hierdie stowwe is chlorpyrifos (organofosfaat), azinphos-metiel (organofosfaat), koperoksichloried (swaarmetaalbevattende fungisied) en sipermetrien (piretroied).

Erdwurms is in die laboratorium aan 'n reeks konsentrasies van chlorpyrifos en koperoksichloried as enkel toksikante en as 1:1 mengsels in kunsmatige grond, vir vier weke blootgestel. Voor en na die blootstellingsperiode is die biomassa van die wurms, as maatstaf van groei, bepaal en kokonproduksie, uitbroeisukses en getal nakomelinge per kokon bepaal as maatstawwe van voortplantingsvaardigheid. Groei (biomassaverandering) en voortplanting (kokonproduksie) is beinvloed deur behandeling met die hoogste konsentrasie (20 mg/kg) chlorpyrifos, terwyl geen effek van koperoksichloried of die mengsel van hierdie twee pestisiede gevind is nie. Daar is

gevind dat beide die pestisiede, enkel en in die mengsel, die NRR tye beinvloed het. Die AChE aktiwiteit is beinvloed deur chlorpyrifos en die mengsel, maar nie deur die koperoksichloried nie.

Korttermyn blootstellings van erdwurms (48 uur), in kunsmatige grondwater, van erdwurms aan chlorpyrifos, koperoksichloried, azinphos-metiel en sipermetrien as enkel toksikante en mengsels van chlorpyrifos-koperoksichloried, chlorpyrifos-azinphos-metiel en chlorpyrifos-sipermetrien, is gedoen en gevolg deur die bepaling van AChE inhibisie. Koperoksichloried, cypermetrien en die chlorpyrifos-sipermetrien mengsel het geen waarneembare effek op die AChE aktiwiteit gehad nie ?????. Die erdwurms wat blootgestel is aan die hoogste konsentrasie in die mengsel van chlorpyrifos-sipermetrien het doodgegaan.

Die resultate het getoon dat die pestisiede nie in die korttermyn die lewensloopkenmerke in enige waarneembare mate geaffekteer het nie maar daar was effekte op sellulêre en biochemiese vlakke soos aangetoon deur die biomerkers. Sommige mengsels van die pestisiede het die erdwurms verskillend van die enkelstowwe geaffekteer. Daar het dus wisselwerking tussen sommige van die pestisiede wat in mengsels aangewend is, plaasgevind, soos aangetoon deur die gemete eindpunte. Die NRR toets, as breë-spektrum biomerker was 'n goeie maatstaf van kontaminasie in grond en daar is aanduidings dat die AChE aktiwiteit, as 'n spesifieke biomerker, 'n nuttige maatstaf kan wees om die effekte van organofosfaatmengsels en mengsels van hierdie chemiese groep en die van ander chemikalieë aan te toon.

DEDICATION

I would like to thank God Almighty without whom I would not have come this far. I would also like to thank my parents and siblings who have supported me throughout my pre- and post-graduate studies. The sacrifices that they have made are greatly appreciated. I would also like to thank my friends, who have given me the strength throughout my post-graduate studies.

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CHAPTER 1

INTRODUCTION

Agricultural environments are often contaminated with anthropogenically introduced chemicals. These chemicals rarely occur in isolation in agricultural soils due to various products that are sprayed. A large quantity of pesticides is sprayed to combat pests, and fertilizers used to nourish plants. These chemicals entering the environment produce unwanted residues, which pose a great threat to non-target organisms. When a toxic substance is introduced into the environment, it interacts with other constituents of the environment and becomes more or less available to organisms. The bioavailability and toxicity of chemicals depend on the species of the target organism, behaviour of the chemical and the conditions of the ambient environment. Chemicals need to be taken up by the organism in order to be toxic. If there is no uptake, there is no toxicity, regardless of the concentration of the chemical in the environment (Sheppard *et al* 1997). When uptake by the organisms takes place, it is followed by interaction with receptors, and toxicity manifests (Tao *et al* 1999).

The response of organisms exposed to several chemicals simultaneously requires consideration of the interactions between the chemicals inside and outside the organism. Effects of mixtures of toxic chemicals can be additive, synergistic (greater than additive), or antagonistic (smaller than additive). It is also possible for chemicals to act independently of each other, affecting different target sites in an organism. Most toxicological data available to date are however related to single chemicals. While this information might be sufficient for gaining knowledge of the characteristics of chemicals, it lacks the detail necessary for evaluating toxic effects of chemical mixtures (Malich *et al* 1998). Predicting the toxicity of mixtures based upon the knowledge of individual chemicals only can lead to wrong conclusions. Mixture toxicity experiments reflect the actual hazard of contaminated environments better that experiments in which effects of single toxicants alone are tested. Quantifying mixture effects, contributes to the improved extrapolation of laboratory data to the field, as the presence of toxicant

mixtures in the field is one of the factors determining differences between laboratory and field toxicity (Weltje 1998).

Most studies available on chemical mixtures focus on the toxicological interactions of chemicals having similar structures and mechanisms. For example, there is a number of studies done on mixtures of chemicals with fairly similar structures and mechanisms, such as heavy metals. Marino *et al* (1998) did a study on Cu-Cd interactions in earthworms and found that exposure of earthworms to Cd before exposure to copper increased the amount of copper taken up, while Tao *et al* (1999) determined the synergistic effect of copper and lead uptake by fish, and found lead to facilitate the uptake of copper. Korthals *et al* (2000) determined the joint toxicity of Cu and Zn to a terrestrial nematode community in an acid sandy soil and found the combined effects of combined exposure to be additive or less than additive. Kraak *et al* (1999) did a study on short-term ecotoxicity of a mixture of five metals (Cu, Zn, Ni, Cd and Pb) to the zebra mussel *Dreissena polymorpha* and found that the accumulation of each metal by the zebra mussel was not influenced by the presence of the other four metals. In the study of Weltje (1998), of mixture toxicity and tissue interaction of Cu, Zn, Cd and Pb in earthworms in laboratory and field soils, it was found that toxic effects were mainly antagonistic for total soil concentrations.

In the agricultural industry, it is common practice to apply more than one pesticide simultaneously or in sequence, to treat different pest species. This tendency to use a mixture of pesticides is also supposed to be a means of avoiding the development of pest resistance to a single chemical (Scharf *et al* 1997). Some of these chemicals leach into the soil and may affect non-target organisms as single substances, but often also as mixtures (Lytle and Lytle 2002). A few studies have been done on organic pesticide mixtures and their effects on non-target organisms. Springett and Gray (1992) studied the effects of repeated low doses of the herbicide, glyphosphate, the fungicide Captan and the insecticide azinphos-methyl on the earthworm *Aporrectodea caliginosa* and found that there were interactions between pesticides in combination. Glyphosphate and Captan had a lesser effect on growth and mortality than glyphosphate alone. Azinphos-methyl and

Captan had an effect less than that of azinphos-methyl alone on growth and mortality. Marinovich *et al* (1996) also did a study of the effect of pesticide mixtures of dimethoate, azinphos-methyl, diazinon, primiphos methyl and benomyl, and found mixtures to be more toxic to protein synthesis of *in vitro* human nervous cells than single compounds. Steevens and Benson (2000) determined the interactions of chlorpyrifos and methyl mercury using the amphipod, *Hyalella azteca,* and found methyl mercury antagonized the effects of chlorpyrifos on acetylcholinesterase inhibition. Richardson *et al* (2001) also did a study analyzing the additivity of *in vitro* inhibition of cholinesterase by mixtures of chlorpyrifos-oxon and azinphos methyl-oxon on brain and serum of rats, and found that the compounds resulted in greater than additive effects at higher concentrations. Lytle and Lytle (2002) did a study on the uptake and loss of chlorpyrifos and atrazine by *Juncus effuses* in a mesocosm study with a mixture of the pesticides and found that the mixture affected the uptake of chlorpyrifos more than that of atrazine. Jin-Clark *et al* (2002) evaluated the effects of atrazine and cyanazine on chlorpyrifos toxicity in *Chironomas tentans*, and found that these herbicides conferred synegystic effects on chlorpyrifos.

Because of their numerous functions in terrestrial ecosystems, earthworms have often been chosen as experimental organisms for toxicity testing, representing the primary decomposers of the soil fauna (Lokke and Van Gestel 1998). These organisms were therefore also used in the present study. Through their action, earthworms have a major impact on the fragmentation of organic material. They mix organic and inorganic fractions of the soil, which is of great importance for the soil fertility and stability. While contributing to the process of decomposition, earthworms also affect soil aeration, water transport and soil structure (Reinecke and Reinecke 1998). Often earthworms are referred to as the predominant component of the soil fauna, in terms of biomass, which makes them an important food source for many predatory soil organisms (Lokke and Van Gestel 1998). These organisms have been used extensively in environmental monitoring, especially as biological monitors of heavy metal and organophosphate pollution (Sheppard *et al* 1997). Impacts of pollutants in the soil environment can be evaluated either by measuring direct toxic effects or long-term effects on earthworm populations.

Earthworms are known to accumulate heavy metals when exposed to them (Marinussen *et al* 1997). They are sensitive to many chemicals and tend to concentrate some chemicals inside their bodies (Reinecke and Reinecke 1998).

Pesticides and other chemicals introduced into the soil may alter the behavior of earthworms. Behavioral changes may have the effect that earthworms migrate to non-contaminated areas in order to minimize contact with chemicals. This can cause reduction in surface casting and an increase in leaf litter in the contaminated areas. The changes in the environment caused by man's industrial and agricultural activities have influenced earthworm populations in many parts of Southern Africa. As a result there is a general absence of indigenous species and a dominance of introduced species in cultivated areas (Reinecke 1983).

Although there is no single species of earthworm that is sensitive to all chemicals, the European species *Eisenia fetida* Savigny, 1826 is widely considered a model species and is prescribed as a test organism by the Organization for Economic Co-operation and Development (OECD) in Europe, and the Environmental Protection Agency (EPA) in the USA (Lokke and Van Gestel 1998). This species is commonly found in places where large concentrations of organic matter are decaying in the Northern Hemisphere and is frequently collected from compost heaps and manure piles. Individuals of this species have been studied as potential waste decomposers as well as a protein source in animal feed (Reinecke and Kriel 1980).

The greatest advantage of using this species as a test organism is that it can easily be cultured in large quantities in the laboratory and because of its relatively short lifecycle and high reproductive rate, synchronized cultures can be obtained. This allows for long-term studies of successive generations (Reinecke and Reinecke 1998). Various studies have been conducted on the lifecycle parameters of *E. fetida* (Venter and Reinecke 1988, Viljoen and Reinecke 1988, Reinecke and Viljoen 1990, Reinecke and Viljoen 1991). This species has a great reproductive ability. Reproductive potential is influenced by environmental conditions, soil conditions, as well as the availability of food (Reinecke

and Viljoen 1990). Cocoon production probably occurs for the largest part of the life span of the individuals. The mean period of cocoon incubation at optimum temperature is 23 days, with an average of 2.7 offspring per cocoon (Venter and Reinecke 1988). The cocoons are quite resistant to unfavorable temperature and moisture. The offspring will attain sexual maturity within 40 to 60 days under favorable conditions (Venter and Reinecke 1988).

Although pesticides are extensively used by the agricultural sector, little information is available about their sub-lethal effects on beneficial non-target organisms such as earthworms. Many studies on the effects of pesticides to earthworms have focused on acute lethal effects (Cathey 1982, Robidoux *et al* 1999, Miyazaki *et al* 2002). Mortality as a measure of a population's sensitivity to a chemical is regarded as neither a sensitive nor a relevant ecological parameter (Vermeulen *et al* 2001). Sublethal stress caused by the presence of a contaminant may not kill the organism, but may divert energy from growth and reproduction to ensure the survival of the organism. Growth and reproduction may therefore be affected by exposure to contaminants before mortality occurs. These parameters are therefore more relevant to measure as effects of contaminants on populations, as they can show detrimental effects long before mortality occurs. An effect on the growth and reproduction may affect the population at a later stage (Maboeta *et al* 2003). Other sublethal effects at the below individual level, such as effects at the sub-cellular and enzymatic levels, will show effects even earlier than lifecycle parameters and are also important tools for determining effects before they are manifested at organismal or population level. The sustainable use of agrochemicals therefore requires that extensively used chemicals should be assayed for their effects on beneficial non-target organisms such as earthworms, using tests at different levels of organization.

The fate and effects of pesticides in the environment are determined by a number of physical and chemical properties, such as temperature, pH and whether the environment is terrestrial or aquatic. However, how these properties affect the interactions among

mixtures of pesticides and how that can influence uptake and toxicity are not clear (Lytle and Lytle 2002). The bioavailability of chemicals to earthworms in soil is largely determined by the soil pore water concentration (Lokke and Van Gestel 1998). Belford *et al* (1995) determined the importance of different routes of uptake for earthworms, based on studies in which earthworms were exposed to a number of chlorobenzenes in soil, water, and via contaminated food. They concluded that the relative importance of oral uptake compared to uptake from pore water increased with increasing lipophility of the chemical and increasing organic content of the soil. The stronger a chemical is adsorbed to the soil, the more oral uptake contributes to the body burden of the chemical in earthworms (Lokke and Van Gestel 1998). Because earthworms are semi-aquatic, living in the soil water layers, uptake experiments can be done in aqueous media to exclude the influence of adsorption processes associated with the solid phase of the soil (Kiewiet and Ma 1991). In this study earthworms were exposed in both media (soil and water).

Toxicological testing of pesticide mixtures becomes difficult because of the great number of potential pesticide mixtures in the environment. New chemicals for which no data is available are produced every day. There is therefore need for more data on toxicity of pesticides to non-target organisms, in order to select chemicals that can do the least harm. There is also need for more general information about the mode of action of different pesticide types on organisms.

Among the most commonly used pesticides, also in South Africa, are organophosphates, pyrethroids, carbamates, chlorinated phenols, and heavy metal pesticides. Organophosphates and carbamates are known to disrupt the central and peripheral nervous systems in vertebrates and invertebrates by inhibiting the activity acetylcholinesterase, an enzyme that is involved in the chemical transmission of impulses between neurons (Dembele *et al 2000*). Organophosphorous pesticides are used throughout the world to control a large variety of insects and other invertebrates, fungi, birds, mammals, and herbaceous plants. These pesticides are usually short-lived under

most environmental conditions. They are widely variable in toxicity to aquatic and terrestrial organisms (Hoffman *et al* 1995).

Chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$), also known, in South Africa, by the trade names Dursban® and Lorsban®, is a broad-spectrum organophosphate insecticide widely used for agricultural pest control, to combat pests such as ants, scale insects and cutworms. It is also used for house hold and garden use to control pests such as mosquitos, flies and bedbugs. It is highly volatile with a high vapour pressure (Lytle and Lytle 2002). It is one of the most commonly used insecticides in orchards and vineyards of the Western Cape, (Schulz 2001) and its effects were therefore examined during this study. Chlorpyrifos is toxic to freshwater fish, aquatic invertebrates, and estuarine and marine organisms. It is reported to have an LC_{50} of 1077mg/kg in adult *E. fetida* (Eason *et al* 1999). Although this LC_{50} is high, chlorpyrifos might have adverse sub-lethal effects on these animals at lower concentrations

As chlorpyrifos has been extensively used worldwide for nearly four decades and a considerable database on its toxicity exists. It is known to inhibit acetylcholinesterase activity, along with many other organophosphates. Richards and Kendall (2002) suggested that chlorpyrifos also inhibits DNA and protein synthesis. It is also shown in some studies that uptake of chlorpyrifos by plants is influenced by the presence of other chemicals such as herbicides (e.g. atrazine) (Lytle and Lytle 2002). Some studies have demonstrated that chlorpyrifos interacts additively with the organometal methyl mercury with survival as the endpoint, although *in vivo*, methyl mercury antagonizes the effects of chlorpyrifos on acetylcholinesterase activity of the amphipod *Hyalella azteca* (Steevens and Benson 2000).

Azinphos-methyl ($C_{10}H_{12}N_3O_3PS_2$), is another commonly used organophosphorous pesticide in the orchards and vineyards of the Western Cape. It is a persistent broad spectrum insecticide, and its persistence in soil is quite variable (Schulz 2001). It is fairly immobile in soil because it adsorbs to soil particles and has low water solubility. It has a low leaching potential and therefore is unlikely to contaminate groundwater. It is used

primarily for foliar application against leaf feeding insects. It is toxic by inhalation, dermal absorption and ingestion. Springett and Gray (1992) studied the effects of azinphos-methyl on the earthworm *Aporrectodea caliginosa* in laboratory cultures, and found that it reduced the growth rate of the earthworms. Marinovich et.al (1996) compared the effects of pesticide mixtures on nerve cells *in vitro* to single pesticides, with azinphos-methyl as one of the pesticides and found that it inhibited acetylcholinesterase activity and protein synthesis. During the present study the short-term effects of azinphos-methyl singly as well as in a mixture with chlorpyrifos on earthworms were investigated.

Pyrethroids are pesticides that also represents an increasing proportion of the world's pesticide sales. Their lack of persistence in the terrestrial environment, coupled with the slow development of pest resistance, has made them popular for both agricultural and public health application (Ray and Forshaw 2000). Pyrethroids are also neurotoxicants but they act on a target different to that of organophosphates. Their major site of action has been shown to be the voltage-dependent sodium channels (Costa 1988). While some neurotoxic substances have a specific action on a specific biochemical process, others such as pyrethroids, are likely to exert their effects by interacting with more than one biological site (Costa 1988). Another target for pyrethroids is the voltage-dependent chloride channels, which are found in nerve, muscles, and salivary glands. These channels are modulated by protein kinase C, and their function is to control cell excitability. The decrease in chloride open channel state serves to increase excitability and therefore to synergize pyrethroid action on the sodium channels (Ray and Forshaw 2000). Some organophosphates can enhance pyrethroid toxicity and some organophosphates have a greater potential to synergize pyrethoids than others (Ray and Forshaw 2000). Cypermethrin, a commonly used pesticide in South Africa, is highly toxic to non-target invertebrates, such as spiders (Araneae), true bugs (Heteroptera), and sawfly larvae (Moreby 2001). Short-term effects of cypermethrin singly as well as in mixture with chlorpyrifos on earthworms were determined in this study.

Most studies on mixtures of organic pesticides have concentrated on mixtures of organophosphorus pesticides or chlorinated phenols (Marinovich *et al* 1996, Richardson *et al* 2001, Jin-Clark *et al* 2002). Less information is available on mixtures of heavy metal containing pesticides and those of organophosphates and heavy metal containing pesticides (Steevens and Benson 2000). Copper oxychloride is one of the most commonly used heavy metal containing fungicides in orchards and vineyards of the Western Cape region (Helling *et al* 2000), and its effects were examined during the present study singly and in combination with chlorpyrifos. Environmental contamination of soils by copper, apart from natural occurrence, is caused by the use of agrochemicals, such as copper oxychloride. Although copper is an essential metal, it is toxic to earthworms in high concentrations. Earthworms do not accumulate very high body concentrations at high exposure levels of copper, but are still negatively affected by the metal (Helling *et al* 2000). Some authors have shown that copper causes mortality and sublethal injury to earthworms at lower concentrations than that of lead and zinc (Reinecke *et al* 2002).

Copper oxychloride ($ClCu_2H_3O_3$) is applied under the commercial name Virikop® at a rate of 1.25 to 7.5 kg/ha in South African vineyards, with several applications per season. Copper concentrations of as much as 50 µg/g, have been found in soil immediately after the spraying season (Reinecke *et al* 2002). The mean soil copper content determined in 19 vineyards in the Western Cape amounted to 9 mg Cu per kg in soil on average, with a maximum of 27 mg Cu per kg in soil (I. Van Huyssteen, personal communication, in Helling *et al* (2000)). The monitoring of earthworm communities in orchards and vineyards revealed a very low earthworm abundance, which could probably be attributed partly to the intensive usage of the copper-based fungicide (copper oxychloride).

The LC_{50} of Cu for *E. fetida* varies between 100 and 1000 mg Cu per kg of soil. Copper oxychloride affects growth and reproduction of *E. fetida*, with considerable impact shown on reproduction at an exposure concentration of 15.92 mg Cu per kg substrate and higher (Helling et. al. 2000). This fungicide is also known to affect earthworms at the subcellular level by affecting the lysosomal stability of the coelomocytes of the

organisms (Reinecke *et al* 2002). If beneficial organisms such as earthworms are to be protected from high Cu levels in soils, it is important to determine effects and toxic stress caused by this metal, before they manifest at the population level.

The use of sensitive sub-organismal tests or biomarkers, which show effects early, is therefore important. According to Van Gestel and Van Brummelen (1995), a biomarker is defined as any biological response to an environmental chemical at the below individual level, measured inside an organism or in its products, indicating a departure from the normal status, which cannot be detected from an intact organism. Biomarkers have been used extensively in the laboratory to document and quantify exposure to, and the effects of, environmental contaminants on organisms (Svendsen and Weeks 1997). Because they measure effects at the sub-organismal levels which are targeted first by toxicants, they have the advantage of reacting rapidly to exposure and are able to show integrated effects of multiple stressors (Svendsen and Weeks 1997)

A broad spectrum of xenobiotics can alter the normal functioning of the organism's body. Xenobiotically induced sublethal cellular pathology reflects perturbations of function and structure at molecular level. Many toxic substances or their metabolites result in cell injury by reacting primarily with biological membranes (Moore 1985). Examples of membrane damage include changes in cellular compartmentalization, such as injury to lysosomes or mitochondria. Many xenobiotics induce alterations in the bounding membrane of the lysosome, leading to destabilization (Moore 1980).

Injury resulting in destabilization of the lysosomal membrane bears a quantitative relationship to the magnitude of stress response. Release of degradative hydrolytic enzymes from the lysosomal compartment into the cystol may result from destabilization of the lysosomal membrane, which may result in lysosomal fusion with other intracellular vacuoles leading to the formation of pathologically enlarged lysosomes (Moore 1988). Lysosomes are an ideal starting point for investigations of generalized cellular injury in organisms. This role of lysosomes may be important as a detoxification system. As with other detoxification systems, this process is effective until the storage capacity of the

lysosomes is overloaded, or lysosomes are damaged directly by the accumulated contaminant (Lowe *et al* 1995).

Lysosomal responses have been used as a biomarker of stress, due to exposure of cells to metals, utilizing a method using the vital dye, neutral red. This biomarker has also been employed for hemocytes of the common garden snail *Helix aspersa* (Snyman *et al* 2000), as well as the lysosomes of the coelomocytes of earthworms (Svendsen *et al* 1996, Svendsen and Weeks 1997, Reinecke *et al* 2002). The neutral red retention assay is an assay based on the ability of viable cells to incorporate and bind neutral red and is used to determine lysosomal damage.

Neutral red is a weak cationic, vital dye that penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in the lysosomes. When exposed to toxic stress, such as exposure to toxic heavy metals, the integrity of the lysosomal membrane is affected and, depending on the degree of damage to the membrane, the dye accumulated in the lysosomal vacuole diffuses out to the cystol, staining it light red. The assay is based on the rate at which the leaking of neutral red takes place. The neutral red retention time is calculated by determining the time needed for the dye to leak into the cystol of 50% of the cells observed (Reinecke and Reinecke 1999). It has proven to be reliable and practical in assessment of the adverse effects of anthropogenic heavy metal contamination at subcellular level for different earthworm species (Svendsen *et al* 1996, Svendsen and Weeks 1997, Reinecke *et al* 2002). Therefore this biomarker was selected in the present study to measure the stress response of earthworm coelomocytes, especially to metal exposure.

Earthworm coelomocytes, the cells used in this study, are contained primarily within the fluid in the coelomic cavity. They play an essential role in cell-mediated immunity by reacting to invading pathogens or foreign material by phagocytosis. They consist of five major types: basophils, neutrophils, acidophils, granulocytes and chloragogen cells. Neutrophils are highly adherent cells and tend to be flattened with a thinly spread and

nearly transparent cytoplasm, with irregular and indistinct cell margins. Their sizes vary from 12-50 µm, depending on the degree of flattening. The nucleus is large (8-10 µm). The cytoplasm of neutrophils reacts weakly with most cytoplasmic stains (Stein and Cooper 1978).

Acidophils are highly granular cells comprised of two groups, Type I (20-30 µm) in diameter and Type II (10-15µm) in diameter. The cytochemical reactions of the cytoplasm are often obscured by the granules. Granulocytes are amoeboid in appearance with an irregular outline. They contain numerous prominent granules which are more widely dispersed than those of acidophils. The nucleus (5-9 µm) is randomly located within the cell. Chloragogen cells are also highly granular, but markedly different from either acidophils or granulocytes. They usually occur in clusters and pseudopodia are inconspicuous or absent. Nuclei are frequently obscured by the granules (Stein and Cooper 1978).

Basophils are the most numerous of the coelomocytes, comprising approximately 60-70% of the cell population. The majority of the basophilic cells is 8-15 µm in diameter, but may vary between 5 µm and 30 µm. The cytoplasm is not heavily granular and the nucleus is spherical to ovoid, (4-8 µm in diameter) and located centrally or peripherally. They have large petaloid pseudopodia, extending from the cell surface (Stein and Cooper 1978). These cells were selected for counting in this study, because they are abundant, and less granular. They can also adhere to the glass of the microscope slides due to the amoeboidal characteristics, and therefore be observed easily.

In the context of contaminant biomonitoring in earthworms, the neutral red assay cannot be used to measure effects of toxicants targeting the functioning of the systems, such as the nervous system, in exposed organisms. Cholinesterase inhibition is the primary mode of organophosphate toxicity and the measure of this inhibition has become a standard for determining organophosphate exposure (Richards and Kendall 2002). Organophosphorous pesticides (OP's) therefore affect neurotransmission if

acetylcholinesterase is inhibited. As the role of neurotoxins such as OP's were investigated during the present study, this biochemical biomarker was also selected.

Signal transmission in the nervous system involves electric transmission along the surface of the axon and chemical transmission of impulses between neurons. Acetylcholine is the major chemical transmitter between neurons. It is discharged at a nerve synapse, moves across the synapse and binds to the acetylcholine receptor in the postsynaptic membrane. The binding initiates an electric impulse in the next neuron, and the message is passed on (Moriarty 1999). Termination of the signal transmission occurs when acetylcholine is rapidly hydrolyzed into acetate and cholin by acetylcholinesterase released from the post-synaptic membrane, immediately after signal transmission. Acetylcholinesterase activity can be inhibited by toxicants, such as organophosphates, when they bind irreversibly to the enzyme. The binding (Moriarty 1999) removes functional acetycholinesterase molecules, thereby causing an accumulation of acetylcholine at the nerve synapses, and a continuous stimulation of the nerves and their target muscles (Peakall 1992).

The method most commonly used to measure acetylcholinesterase activity is that of Ellman (1961). This method consists of providing the enzyme, acetylcholinesterase, with a substrate, acetylthiocholine, which, if hydrolyzed, releases the thiocholine and acetic acid. The quantity of thiocholine obtained is proportional to the enzyme activity of acetylcholinesterase (Ellman *et al* 1961) and is measured spectrophotometrically. This method is therefore based on the coupling of following reactions:

Acetylthiocholine $\xrightarrow{\text{(enzyme)}}$ thiocholine + acetate

Thiocholine + dithiobisnitrobenzoate = yellow colour (Ellman 1961)

Acetylcholinesterase inhibition has been used extensively as a biomarker in studies of effects of chlorpyrifos and mixtures of other insecticides with chlorpyrifos *in vivo* and *in vitro* on a number of different organisms. This biomarker has also been used to test a number of pesticides on different species of earthworms (Stenersen 1979). Steveens and

Benson (2000) used it in assessing the interactions of chlorpyrifos and methyl mercury on the amphipod *Hyalella azteca,* and it was also used by (Richardson *et al* 2001) in analyzing the additivity of *in vitro* inhibition by mixtures of chlorpyirifos-oxon and azinphos-methyl-oxon on rat brain and serum. Jin-Clark *et al* (2002) also used the method in a study to determine the effects of atrazine and cyanazine on chlorpyrifos toxicity in *Chironomus tentans*, as also did Richards and Kendall (2002) in a study of biochemical effects of chlopyrifos on *Xenopus laevis*. According to Scaps *et al* (1997), it is not a good biomarker for measuring the effects of heavy metals on *E. fetida* because unlike organophosphates, heavy metals do not seem to affect AChE activity.

It is important to establish the relationship between these biomarkers and lifecycle parameters such as growth and reproduction of organisms, in order to provide some degree of ecological relevance (Reinecke *et al* 2002). It is also important to use different biomarkers in mixed toxicity because an organism may be affected differently by different contaminants on different target sites. For instance, acetylcholinesterase activity may be a good biomarker for measuring the effects of organophosphates, but not in measurements of heavy metal toxicity (Scaps *et al* 1997). The question then becomes: what happens when an organism is exposed to a mixture of an organophosphate and a heavy metal and can biomarkers be used to determine interactions in a mixture?

Because the chosen pesticides are commonly used in the orchards and vineyards of the Western Cape, South Africa, they are likely to occur as mixtures in the field. These mixtures can have devastating effects on the beneficial soil organism such as earthworms if synergistic interactions occur. The null hypothesis in this study was that single pesticides and mixtures of pesticides from different groups would not have different effects on the lifecycle parameters of non-target soil invertebrates (earthworms), and that biomarker results would not be affected by the pesticide mixtures of different groups and their single components. The bioavailability and uptake of the pesticides studied would also not be affected by the presence of other pesticides in a mixture. The simple additive model is therefore expected to apply.

The aim of this study was to determine whether binary mixtures of toxicants would affect earthworms differently than single substances. The substances used in this study were from three different groups of pesticides, the organophosphate group, the pyrethroids and the heavy metal containing group. The endpoints measured were lifecycle parameters and biomarkers. The lifecycle parameters were biomass change, as a measure of growth, and cocoon production and hatching success, as measures of reproduction. The biomarkers used were the neutral red retention assay (measuring lysosomal integrity) and the acetylcholinesterase assay (measuring inhibition of acetylcholinesterase).

The specific aims of this study therefore were:

1. To assess the effects the pesticides singly and in a mixture on lifecycle parameters of non-target organisms (earthworms)

2. To assess the effects of the pesticides at sub-organismal level on earthworms exposed singly and in a mixture using biomarkers

3. To determine if there are any differences in results as a result of the different exposure media

4. To determine if there are any interactions between the pesticides as shown by the measured endpoints

5. To compare and validate the use of the chosen biomarkers in assessing mixture toxicity

CHAPTER 2

MATERIALS AND METHODS

2.1 Study species

Eisenia fetida Savigny, 1826, was chosen for this study. This species occurs naturally in northern Europe in places rich in organic matter (Lokke and Van Gestel (1998).

The classification according to Simms and Gerard (1985) is as follows:

Phylum:	Annelida
Subphylum:	Clitellata
Class:	Oligochaeta
Order:	Haplotaxida
Suborder:	Lumbricina
Superfamily:	Lumbricodea
Family:	Lumbricidae
Subfamily:	Lumbricinae
Genus:	*Eisenia*
Species:	*Eisenia fetida* (Savigny, 1826)

It is usually found in damp rotting vegetation, wet decaying leaf litter and under sodden logs where pH ranges from 4.3-7.5. It is also found, on standing manure heaps and sewage filter beds where it can tolerate low concentrations of ammonia (Sims and Gerard 1985). It is widely considered a model species and is prescribed as a test organism for toxicity testing by the Organization for Economic Co-operation and Development (OECD) in Europe and the Environmental Protection Agency (EPA) in the USA (Lokke and Van Gestel 1998). *E. fetida* is cultured in our laboratory under controlled conditions (20^{o}C and 60% RH) and originated from individuals brought from Europe. All worms for the present study came originally from this stock culture. Cocoons were collected from the stock culture, they were then hatched in distilled water in Petri dishes and hatchlings were reared in cow manure to obtain synchronized cultures of the same age.

Homogenic groups (with regard to weight) of adult (clitellate) worms of the same age, were selected for each treatment, and used for all exposure concentrations and replicates.

2.2 *Preliminary Experiments*

Preliminary experiments were done in soil to determine the range of concentrations to be tested in the final experiments. Ten clitellate worms were subjected to four concentrations (0.02 mg/kg, 0.2 mg/kg, 2 mg/kg and 20 mg/kg) of chlorpyrifos in 400g of artificial soil (OECD 1984), with a control. The exposure period was four weeks, and worms were weighed at the beginning and the end of the exposure period, to determine biomass change as a measure of growth. Cocoons were sorted from the substrate after four weeks and kept in multiwell plates in distilled water for four weeks. The cocoons were checked daily for hatchlings. The number of cocoons, hatching success and number of hatchlings were determined for each treatment. Four worms were removed from the substrate after the exposure period, and used for the neutral red retention assay. Three worms were removed from each treatment and prepared for the determination of the acetylcholinesterase activity utilizing Ellman's Method (Ellman 1961).

Adult worms were also exposed to 4 different concentrations of chlorpyrifos (0.02 mg/kg, 0.2 mg/kg, 2 mg/kg and 20 mg/kg) in artificial groundwater (Kiewiet and Ma 1991), and a control. Worms were starved prior to exposure by putting them in Petri dishes on moist filter paper for 48hours. Four worms were subjected to 400ml of the groundwater for 48hours. After the exposure period, acetylcholinesterase activity of whole worm homogenate was measured using Ellman's Method.

No preliminary experiments were done on the copper oxychloride exposures because lethal and sublethal concentrations of copper oxychloride are known for *E. fetida* from previous studies in our laboratory (Helling *et al* 2000, Reinecke *et al* 2002).

2.3 *Exposures in soil*

Artificial soil was used as a medium of exposure in this part of the study. This was prepared according to the method described by the OECD (1984). It consisted of 70% washed silica sand, 20% kaolin clay and 10% peat moss thoroughly mixed by hand, and $CaCO_3$ was added to give a pH of 6.0±0.5. The pH was measured with a Crison micro pH meter 2001 (KCL electrode), by shaking the substrate sample in distilled water of known pH and measuring it directly. The sand was obtained from the region of Kraaifontein (Cape Town, South Africa) in an open field at a depth of ±1.5m. Before use the sand was rinsed thoroughly with water until the water that came out was clear. The sand was then dried at 70°C and sieved to a particulate size of 850 ≤ 500 µm. The koaline clay was obtained from T. REINDERS™ Potters supplies (Kraaifontein), and the peat moss used was SHAMROCK™ Irish peat moss, obtained from the Stodels nursery in Durbanville.

Test pesticides used were dissolved in ± 240ml of distilled water, and thoroughly hand mixed with the substrate to give the desired concentrations per dry weight and a moisture content of 60-65%, determined by analyzing 1g of substrate with a Sartørius infrared moisture detector. Ten clitellate worms were put in a cylindrical glass container (±3.6cm radius and ±16.5cm height) with 400g of this substrate. Four replicates (done sequentially) were used for each treatment (two replicates for the copper oxychloride treatments). A piece of black plastic was put on top of the substrate in the containers to avoid drying out of the substrate. Each container was then covered with a piece of gauze to keep the worms from escaping. Containers were kept in a climate room of 20°C and 60% relative humidity for the exposure period of 4 weeks. The containers were kept in the dark by covering them with black plastic. Worms in each container were fed weekly with 2.5g of urine-free cattle manure that had been previously dried, ground and sieved to a particle size of between 100 and 500µm. Concentrations used were, control (no pesticide), 0.02, 0.2, 2.0, and 20 mg kg^{-1} pesticide in the substrate. Pesticides used were the organophosphate chlorpyrifos (480g/l active ingredient) and copper oxychloride (Virikop C®: copper oxychloride 850g/kg = 500g/kg Cu) singly. Binary mixtures of

chlorpyrifos and copper oxychloride in 1:1 ratios of the same concentrations were also used.

2.4. *Experiment using artificial groundwater*

Experiments were also carried out using artificial groundwater, which consisted of 100mg $NaHCO_3$, 20mg $KHCO_3$, 200mg $CaCl_2.2H_2O$, and 180mg $MgSO_4$ per liter of distilled water, as a medium of exposure (Kiewiet and Ma 1991). The artificial groundwater had a pH of ±8.2. Pesticides were dissolved in the groundwater to give the desired concentrations of, control (no pesticide), 0.002, 0.02, 0.2, and 2 mg/l of groundwater. These concentrations were of a lower range than those used in the soil because the preliminary experiments showed the pesticides were more bioavailable in water than in soil and the exposed organisms could not tolerate higher concentrations. Four replicates of each treatment were used. Worms were exposed singly to chlorpyrifos, copper oxychloride, azinphos-methyl and cypermethrin. Exposures were also done in binary mixtures of 1:1 ratio of the same concentrations. Mixtures of chlorpyrifos-copper oxychloride (organophosphate/heavy metal), chlorpyrifos-azinphos-methyl (organophosphate/organophosphate) and chlorpyrifos-cypermethrin (organophosphate/pyrethroid) were used.

Clitellate *E. fetida* of the same age of were used. Prior to exposure they were kept on moist filter paper in Petri dishes at a temperature of 20^{o}C for 48 hours so they could empty their gut contents. This was done to avoid polluting the groundwater with fecal matter during the exposure period. The Petri dishes were kept under black plastic to avoid light from negatively affecting the worms. Earthworms were exposed in 400ml of aerated artificial groundwater in 500ml beakers in a climate room of 20^{o}C for 48 hours under black plastic to avoid light. The water was acclimated by putting it in the climate room of 20°C for 24hours before exposing the worms. Four worms were put in each beaker. After exposure, two worms were taken from each replicate and prepared for acetylcholinesterase activity measurement.

2.5 *Lifecycle parameters*

Each of the ten worms from each replicate of each treatment of the artificial soil exposures was washed, dried with a paper towel, put onto a weighing boat with water to avoid the drying out of the animal and weighed on a Sartørius balance. The mean mass of the ten worms from each jar was determined at the beginning and the end of the experiment. Percentage biomass change was calculated and used as a measure of growth.

At the end of the four week exposure period, cocoons were hand-sorted from the substrate by emptying the substrate from each jar and spreading it onto a tray. A magnifying lamp was used to enhance the visibility of the cocoons. The cocoons were then counted and put separately in wells of multiwell plates with distilled water. The multiwell plates were incubated in the climate room at 20°C for four weeks, in the dark under black plastic. Hatchlings were recorded daily and removed from the water during this period. The total number of cocoons and number of hatchlings per cocoon were determined, and the hatching success calculated. These were used to determine the effects of pesticides and the pesticide mixtures on the reproduction of the worms.

2.6. *Biomarkers*

2.6.1 **Neutral-red retention assay**

This biomarker was only measured in worms exposed in the artificial soil medium as the NRR assay measures stress response of the animals, and the artificial groundwater experiments also affected the worms with other stress factors (see discussion). After the exposure period of four weeks, three worms were removed from the substrate of each replicate, thus 12 worms from each exposure concentration. Each worm was washed in distilled water and blotted dry on filter paper. 20 µl of coelomic fluid containing coelomocytes was collected from each worm in a syringe containing 20 µl of earthworm Ringer solution (Appendix1A (1)). This was done by inserting the needle into the coelomic cavity posterior to the clitellum, with the worm bent double to increase

pressure. A stock solution (Appendix1A (2)) of the neutral red dye was prepared by mixing 20 mg of neutral red (Toluyne Red) with 1 ml of dimithylsulfoxide (DMSO) in an Eppendorf tube. To make up a working solution, 10 µl of the stock solution was mixed with 2.5 ml of the earthworm Ringer solution. 20 µl of the cell suspension in ringer was placed onto a microscope slide and left for about 20 seconds for the cells to adhere to the surface. 20 µl of the working solution (Appendix1A (3)) was then added to the cell solution on the slide. The slide was then covered with a cover slip and transferred to a microscope with 400X magnification, where observation was started immediately and divided into two minute intervals. During these intervals the slide was scanned randomly and the number of basophilic cells with fully stained cytosols and the number with unstained cytosols counted. After each 2 minute observation period the slide was put into a moisture chamber for 2 minutes to prevent it from drying out. Observations were ended when the ratio of cells with stained cystols was over 50% of the total number of cells counted. This interval was noted as the neutral red retention time.

2.6.2 **Acetylcholinesterase activity**

This biomarker was measured in earthworms from artificial soil and groundwater exposures. Because worms exposed in soil water had their gut contents eliminated before the exposure period, they could be used directly for the analysis. However, worms exposed in artificial soil had to eliminate their gut contents after removal from the substrate and before the analyses. This was done in order to get a clear sample of the homogenate without the unnecessary gut content probably also containing microorganisms, that could interfere with the analysis. At the end of the exposure period in soil, three worms were removed from the substrate of each container and put on moist filter paper in Petri dishes for 48 hours to allow them to empty their gut contents before analyzing them. All worms collected and treated in this way were frozen in 2 ml plastic tubes with closed lids at -80°C until they could be homogenized.

To obtain a suspension of worm material to be analyzed, each worm was defrosted and homogenized as follows: A constant ratio of worm mass versus buffer volume was used

during the homogenization. Each worm was weighed and pH8 phosphate buffer (Appendix1B (1)) was added (4× w/v). The worm was then put into a small Petri dish on ice and cut into small pieces using a scalpel and fine forceps. The worm pieces were then transferred to a cold glass Kimble tube with the amount of buffer determined (4× w/v). The worm was homogenized for 1 minute at setting five with a Polytron homogenizer. The homogenate was divided into two equal quantities and transferred to two 1.5 ml Eppendorf tubes using a plastic Pasteur pipette. The homogenate was then centrifuged in a microcentrifuge (Haraeus Biofuge *fresco* 1998) at 13.0G per minute at 4°C for 30 minutes. The supernatant was removed with a 100µl mircropippete and two aliquots of ±300 µl put in two 0.6 ml microcentrifuge tubes and frozen at -80^{o}C until acetylcholinesterase activity could be determined.

Acetylcholinesterase activity was measured using a modified method of the Ellman assay (Ellman *et al* 1961). Acetylthiocholine iodide (Appendix1B (2)) was used as the substrate with dithionitrobenzene (DTNB) as reagent (Appendix1B (3)). These, as well as the buffer were prepared and kept in ice to maintain a constant cold temperature. The homogenate was unfrozen and held on ice to keep it cold. 10 µl of the homogenate, 90 µl of pH 7 phosphate buffer (Appendix1B (1)), 50 µl of DTNB and 50 µl of acetylthiocholine iodide were mixed in each well of a 96 multiwell plate. The absorbance was read on a Multiscan spectrophotometric plate reader at 405 nm. Readings were taken kinetically at two minute intervals over a period of 10 minutes. A blank consisting of buffer, substrate and DTNB solutions was also read. (For the experiment chlorpyrifos, copper oxychloride and the mixture thereof in artificial groundwater, 25 µl of homogenate, 75 µl of buffer, 50 µl of DTNB and 50 µl of acetylthiocholine iodide were used; and readings were taken kinetically at four intervals over a period of 5 minutes). This was done because this experiment was treated as a preliminary experiment. The absorbance over time was determined and referred to as relative acetylcholinesterase activity. A standard was prepared by mixing a cocktail of different worm homogenates of the same species and this standard was used as a reference for the reading of each plate. An average of three readings was taken for each worm, as well as the standard. A standard curve was plotted to ensure the stability of the enzyme substrate

and a linear curve meant that the enzyme assay was working. Protein analysis was done on some of the samples as well as the standard using a single cell module Life Science UV/Vis Spectrophotometer (Beckman DU®530) to ensure that all samples were homogeneous (the ratio of buffer to homogenate was the same in all samples). The homogenate (75 µl) was mixed with pH7 phosphate buffer (325 µl) and 100 µl of the solution was transferred into a 1 ml cuvette in the spectrophotometer. A blank, consisting of pH 7 phosphate buffer, was read before each run. An average of the three readings was taken for each sample.

2.7. **Statistical analysis**

The data in this study were analyzed by using version 6 of STATISTICA data analysis software system, (StatSoft Inc. 2003). Values were presented as the mean ± SD (standard deviation). The probability levels used for statistical significance were $p<0.05$ for all tests. Differences between treatment groups were checked for significance by means of a one-way analysis of variance (ANOVA). Differences between the different pesticide treatments were checked for significance by means of a factorial ANOVA, followed by an all pair-wise multiple comparison test (Student Newman-Keuls) (see Appendix 3).

CHAPTER 3

RESULTS

3.1 **Preliminary exposures of *E. fetida* to chlorpyrifos**

3.1.1 **In soil**

Worms from all the pesticide treatments and control survived but lost weight. There were no statistically significant differences in biomass change or reproduction between the different treatments. All the exposure treatments showed shorter mean neutral red retention times than the control, but there was no dose-response relationship. The highest concentration treatment (20 mg/kg) had the lowest AChE activity of all the treatments.

The chosen concentrations of chlorpyrifos (0.02 mg kg^{-1}, 0.2 mg kg^{-1}, 2.0 mg kg^{-1}, and 20 mg kg^{-1} in substrate) thus proved to be sublethal for *E. fetida*, as shown by these preliminary experiments, and it was decided that these concentrations would be used for the final experiments in soil. Although there were no statistically significant differences observed between the different treatments with regard to lifecycle parameters, a number of repetitions were done during the final experiment to substantiate that.

3.1.2. **In artificial groundwater**

Some of the earthworms (50%) exposed to 20mg/l of chlorpyrifos in artificial groundwater died before the end of the exposure period of 48hours. It was then decided that a lower range of exposure concentrations (0.002 mg/l, 0.02 mg/l, 0.2 mg/l and 2 mg/l) would be used for the final experiment. The neutral red retention times for all treatments, including the control were very short (see chapter 4.3.1), it was therefore decided that the neutral red retention assay would not be used as a biomarker in the artificial groundwater experiments. Chlorpyrifos showed a dose related effect on AChE activity.

3.2 **Final exposures of *E. fetida* to chlorpyrifos and copper oxychloride in soil**

3.2.1 **Lifecycle parameters**

3.2.1.1 *Growth* (*biomass change*)

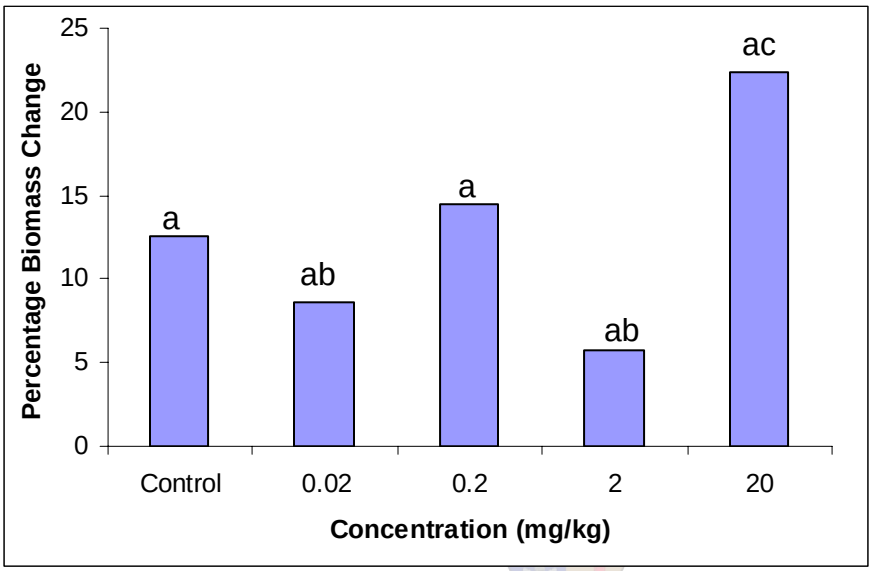

Figure 3.1 Mean percentage biomass change (weight loss) of *E. fetida* after 4 weeks of exposure to different concentrations of chlorpyrifos in soil (n=40). Different letters indicate that the means are significantly different among treatments (ANOVA; $F=34.95$, $df=1,4$; $p<0.05$).

All the earthworms from all the concentration treatment groups survived, and all earthworms lost weight, including those of the control. As illustrated in Figure 3.1, earthworms exposed to 20 mg/kg of chlorpyrifos lost more (22.4%) weight than the rest of the concentration treatment groups. The percentage weight loss of these earthworms was significantly different ($p<0.05$) to the 0.02 mg/kg and the 2mg/kg concentration treatments, and not significantly different to the control (Figure 3.1). Earthworms exposed to 2 mg/kg had the least percentage weight loss (5.7%), but were not significantly different ($p>0.05$) to other treatment groups except the 20 mg/kg treatment.

Earthworms exposed to different concentrations of copper oxychloride also lost weight and showed no significant differences ($p>0.05$) in biomass change among all the treatments (Figure 3.2).

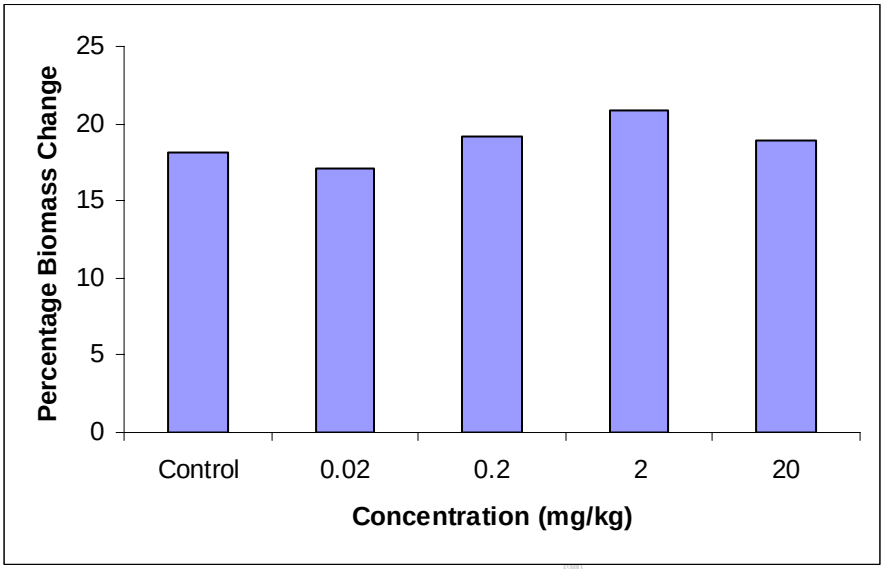

Figure 3.2 Mean percentage biomass change (weight loss) of *E. fetida* after 4 weeks of exposure to different concentrations of copper oxychloride in soil (n=20).

Earthworms exposed to a binary mixture of chlorpyrifos and copper oxychloride also showed no significant differences ($p>0.05$) among all the treatment groups (Figure 3.3).
In Figure 3.4 the biomass changes found in the three pesticide treatments are compared. There were no statistically significant differences ($p>0.05$) exhibited by biomass change as a measure of growth at any of the concentrations between the three pesticide treatments (see Tables 1(a.1 and a.2)).

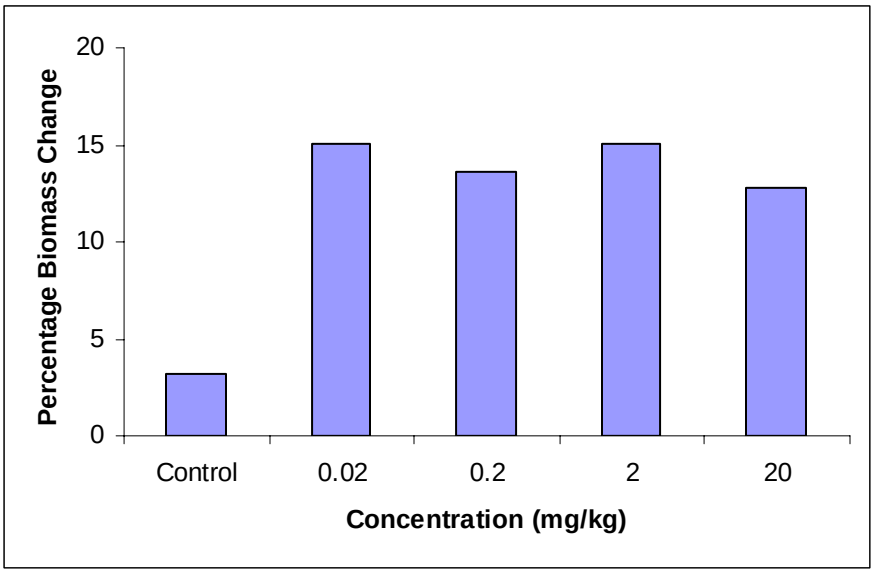

Figure 3.3 Mean percentage biomass change (weight loss) of *E. fetida* after 4 weeks of exposure to different concentrations of a 1:1 mixture of chlorpyrifos and copper oxychloride (n=40).

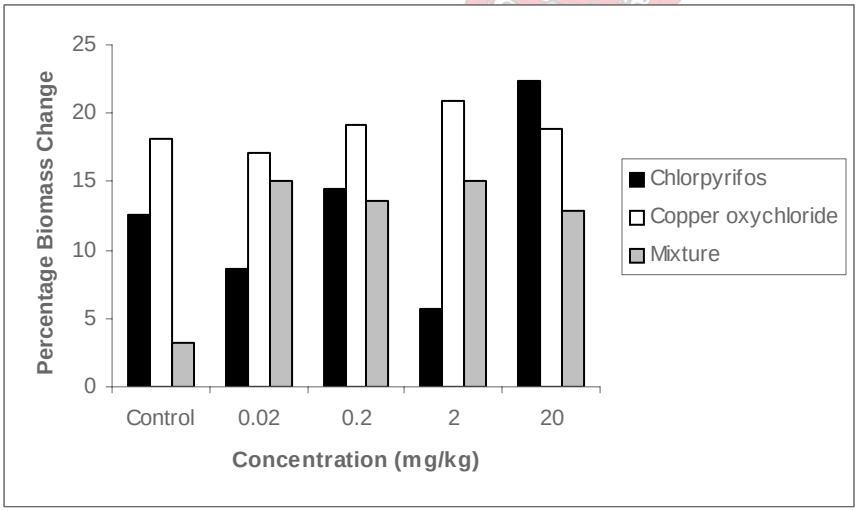

Figure 3.4 Percentage biomass change (weight loss) of *E. fetida* exposed to different concentrations of chlorpyrifos (n=40), copper oxychloride (n=20) and 1:1 mixture of chlorpyrifos and copper oxychloride (n=40) (see Tables 1(a.1and a.2)).

3.2.1.2 *Reproduction*

Figure 3.5 represents the mean number of cocoons produced by earthworms exposed to different concentrations of chlorpyrifos, copper oxychloride and a binary mixture of the two pesticides. Earthworms exposed to the highest concentration treatment (20 mg/kg) of chlorpyrifos produced a significantly (ANOVA; $F=676.67$; $df=1,4$; $p<0.05$) lower mean number of cocoons, compared to the rest of the chlorpyrifos exposure treatments. Earthworms exposed to copper oxychloride showed no significant differences ($p>0.05$), in the number of cocoons produced, in the different concentration treatments. The earthworms exposed to the mixture of chlorpyrifos and copper oxychloride also did not exhibit any significant differences ($p>0.05$) among the different concentration treatments in terms of the number of cocoons produced. There were no significant differences ($p>0.05$) in the number of cocoons produced at any of the concentrations between the three pesticide treatments (see Tables 1(b.1 and b.2)).

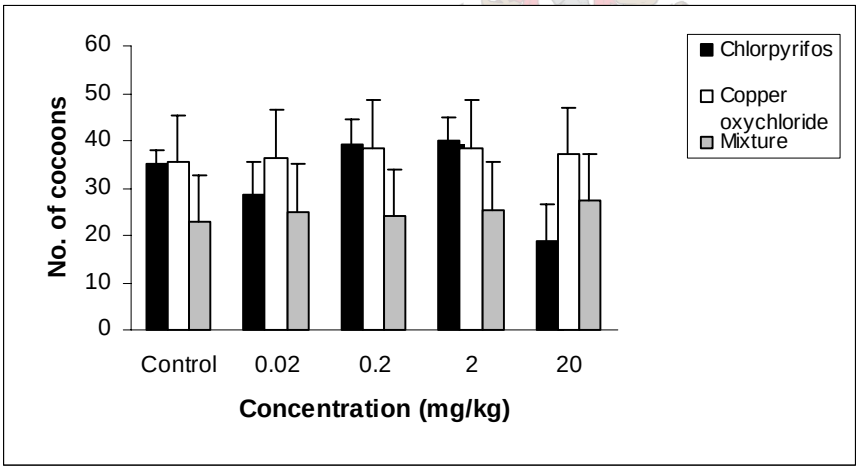

Figure 3.5 Mean number of cocoons ±SD produced by *E. fetida* after 4 weeks of exposure to different concentrations of chlorpyrifos (n=40), copper oxychloride (n=20) and a 1:1 mixture of chlorpyrifos and copper oxychloride (n=40) (see Tables 1(b.1and b.2)).

There were no significant differences in the hatching success of earthworms exposed to different concentrations of chlorpyrifos, copper oxychloride or the mixture of chlorpyrifos and copper oxychloride ($p>0.05$) (Figure 3.6 and Tables 1(c.1and c.2)).

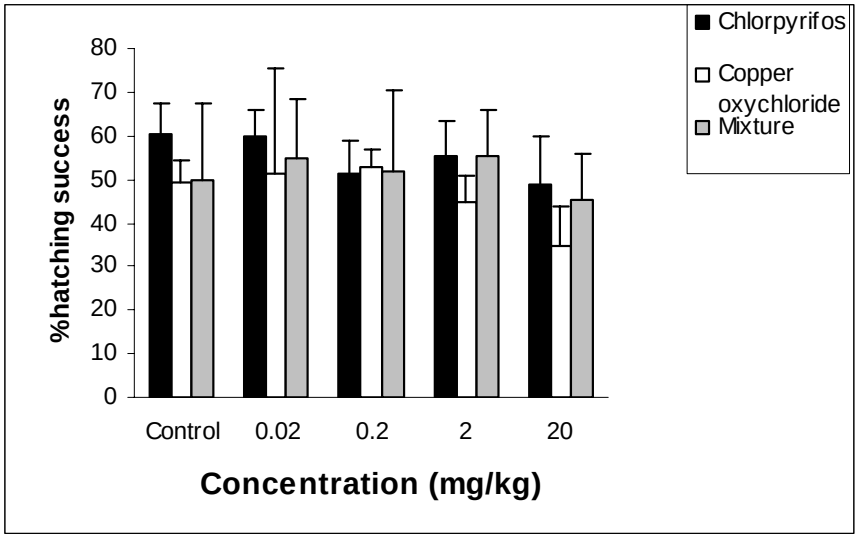

Figure 3.6 Percentage hatching success (Mean ±SD) of cocoons produced by *E. fetida* after 4 weeks of exposure to different concentrations of chlorpyrifos (n=40), copper oxychloride (n=20) and a 1:1 mixture of chlorpyrifos and copper oxychloride (n=40) (see Tables 1(c.1 and c.2)).

Figure 3.7 represents the mean number of hatchlings per cocoon produced by earthworms exposed to different concentrations of chlorpyrifos, copper oxychloride and a binary mixture of the two pesticides. The number of hatchlings per cocoon showed no significant differences ($p>0.05$) among the concentrations of chlorpyrifos, copper oxychloride and the mixture, or between the different pesticide treatments.

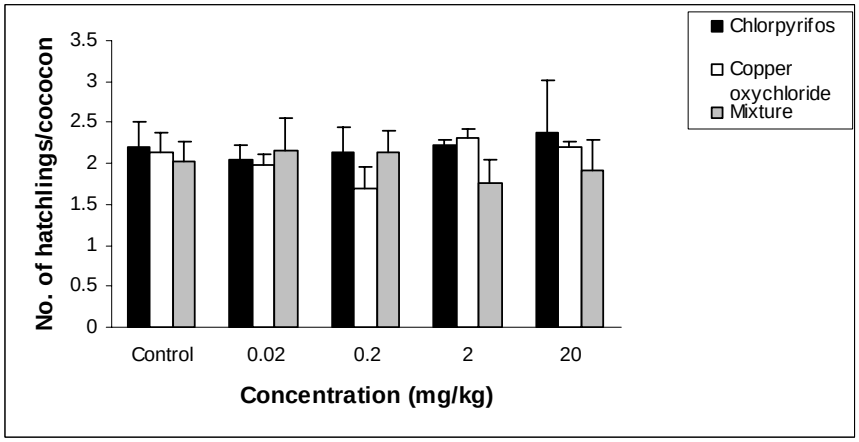

Figure 3.7 Mean number of hatchlings per cocoon (±SD) produced by *E. fetida* after 4 weeks of exposure to different concentrations of chlorpyrifos (n=40), copper oxychloride (n=20) and a 1:1 mixture of chlorpyrifos and copper oxychloride (n=40) (see Tables 1(d.1 and d.2).

3.2.2 **Biomarkers**

3.2.2.1 *Neutral Red Retention Assay*

Figure 3.8 illustrates the neutral red retention times of earthworms exposed to different concentrations of chlorpyrifos. Control worms had a significantly ($p<0.05$) high NRR time compared to the rest of the concentration treatments (see Table1(e.1)). A slight dose-related effect was exhibited by the NRR times of cells from worms treated with different concentrations of the pesticide. The decrease in retention time between the control and the 0.02 mg/kg treatment was significant ($p<0.05$). The NRR times of the lowest concentration treatment (0.02 mg/kg) and the highest concentration treatment (20 mg/kg) differed significantly ($p<0.05$) from each other. The two middle concentration treatments (0.2 mg/kg and 2mg.kg) did not differ significantly from each other ($p>0.05$). These concentration treatments had significantly ($p<0.05$) lower NRR times than the control, but not significantly ($p>0.05$) lower times than the 0.02 mg/kg treatment, and also not significantly ($p>0.05$) higher times than the 20 mg/kg treatment.

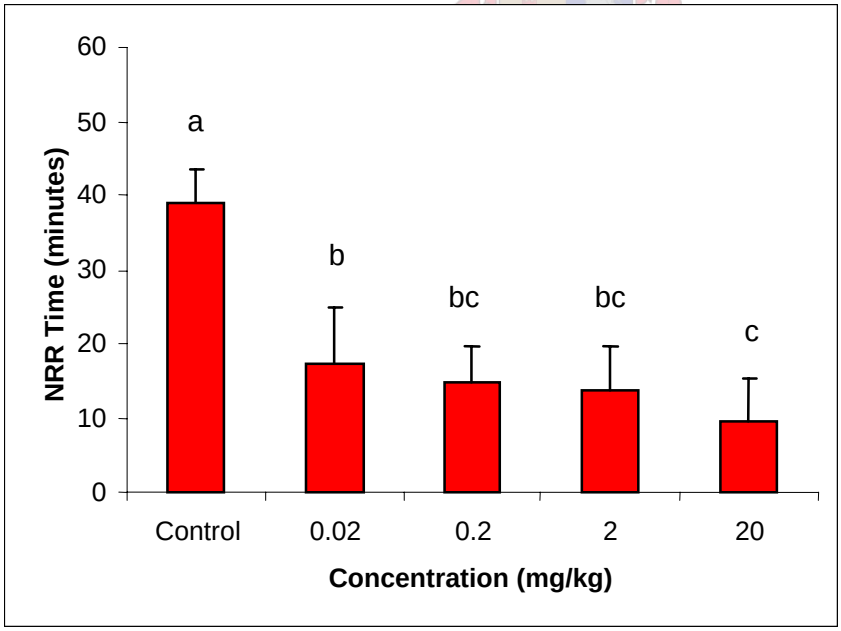

Figure 3.8 Mean neutral red retention time (minutes) ±SD of *E. fetida* exposed to different concentrations of chlorpyrifos in soil for 4 weeks (n=12; different letters on the error bars indicate that the means are significantly different among treatments (ANOVA; F=604.12; df=1,4; $p<0.05$)).

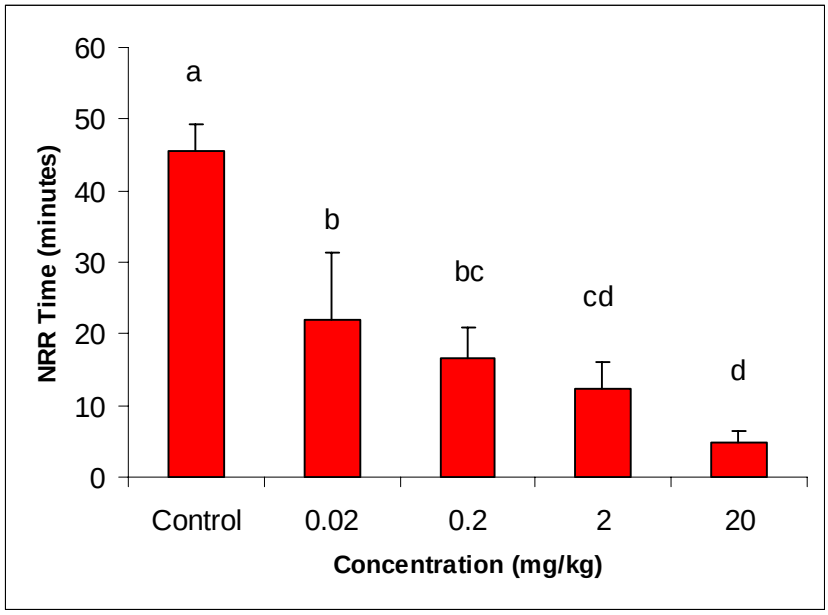

Figure 3.9 Mean neutral red retention time (minutes) ±SD of *E. fetida* exposed to different concentrations of copper oxychloride in soil for 4 weeks (n=6; different letters on the error bars indicate that the means are significantly different among treatments (ANOVA; F=442.95; df=1,4; p<0.05)).

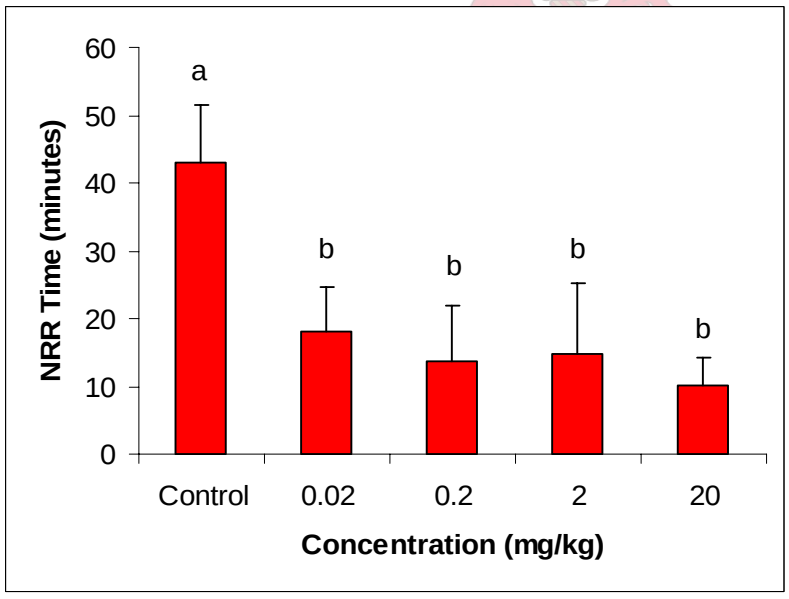

Figure 3.10 Mean neutral red retention time (minutes) ±SD of *E. fetida* exposed to different concentrations of a mixture of chlorpyrifos and copper oxychloride in soil for 4 weeks (n=12; different letters on the error bars indicate that the means are significantly different among treatments (ANOVA; F=372.74; df=1,4; p<0.05)).

Figure 3.9 illustrates the NRR times of the earthworms exposed to copper oxychloride. The control worms had significantly ($p<0.05$) higher NRR times than the exposed concentration treatments (see Table1(e.1)). The highest concentration treatment (20 mg/kg) had a significantly lower NRR time than the lowest concentration treatment (0.02 mg/kg). There was a dose response relationship shown by the NRR time. The NRR time of the 0.2 mg/kg concentration treatment was significantly higher than 20 mg/kg treatment, but not significantly different from the 0.02 mg/kg and the 2 mg/kg treatments. The NRR time of the 2 mg/kg concentration treatment was significantly different from the control and the 0.02 mg/kg treatments, but not significantly different from the other concentration treatments. The highest concentration treatment (20 mg/kg) differed significantly from all the concentration treatments, except the 2 mg/kg treatment.

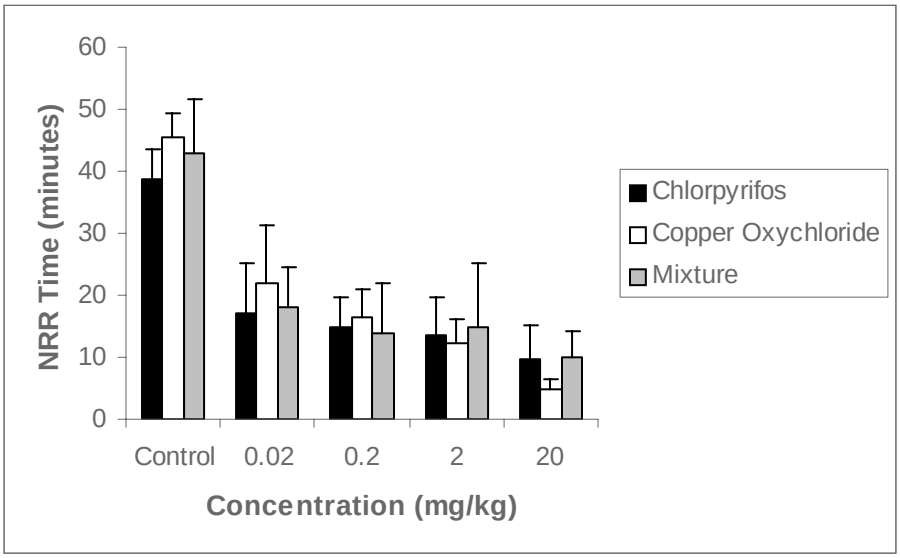

Figure 3.11 Mean neutral red retention time (minutes) ± SD of *E. fetida* exposed to different concentrations of a Chlorpyrifos (n=12), copper oxychloride (n=6) and a 1:1 mixture of chlorpyrifos and copper oxychloride (n=12) (see Tables 1(e.1 and e.2)).

The mixture of chlorpyrifos and copper oxychloride had an effect on the NRR time of the exposed worms (Figure 3.10). The control worms had a significantly ($p<0.05$) higher NRR time than the rest of the concentration treatments (see Table 1(e.1)). The exposed treatment groups did not differ significantly ($p>0.05$) from each other. Although there was no dose relationship exhibited by the NRR times of worms exposed to different

concentrations of a mixture of the two pesticides, the cells of the exposed worms were affected. The dose related effect on NRR time exhibited by the earthworms exposed to single pesticide exposures was not shown by the earthworms exposed to the mixture (Figure 3.11).

3.2.2.2 *Acetylcholinesterase activity*

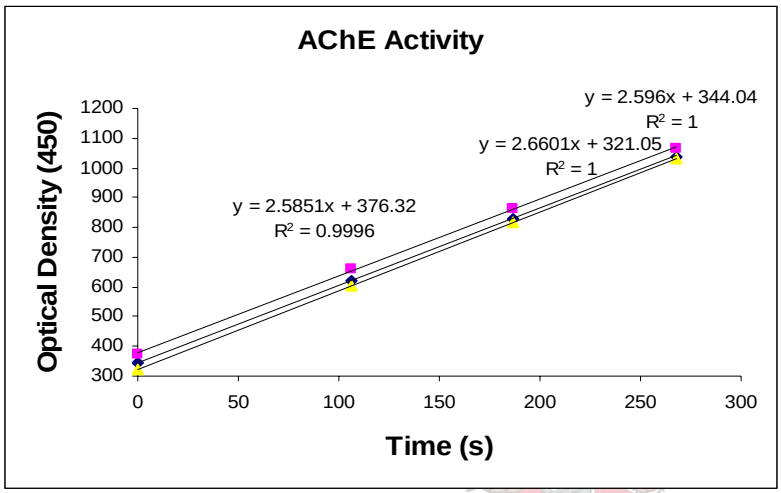

Figure 3.12 A linear curve of three readings (at 405nm) of the standard solution of whole earthworm (*E. fetida*) homogenate.

Table 3.1 Protein readings (Optical density) of 15 samples of whole worm homogenate of *E. fetida* as well as the standard.

Standard	**2.72**
Worm 1	**2.81**
Worm2	**3.16**
Worm 3	**2.90**
Worm 4	**3.05**
Worm 5	**2.94**
Worm 6	**3.08**
Worm 7	**3.07**
Worm 8	**3.00**
Worm 9	**3.01**
Worm 10	**2.78**
Worm 11	**3.14**
Worm 12	**3.06**
Worm 13	**3.06**
Worm 14	**2.89**
Worm 15	**2.76**

The three readings of the standard curve gave linear curves, which meant that the enzyme substrate was stable and the enzyme assay was working well (Figure 3.12). The OD values for the protein analysis of 15 worm samples and a standard were close (mean = 2.96 ± 0.14), and that meant that the samples were homogeneous (the ratio of buffer to worm mass was the same).

Figure 3.13 illustrates the relative acetylcholinesterase activity at 405 nm in whole bodies of earthworms exposed to different concentrations of chlorpyrifos. Control worms had a significantly ($p<0.05$) higher enzyme activity than the worms exposed to the highest concentration treatment (20 mg/kg) (see Table 1(f.2)). The control worms did not differ significantly ($p>0.05$) to the other concentration treatment worms, although the 0.2 mg/kg treatment worms had a slightly lower (but not statistically significant) enzyme activity. There was a noticeable dose relationship between the highest and the lowest exposure groups, although the enzyme activity of the 0.02 mg/kg and 2 mg/kg concentration treatments was slightly higher than the control, and did not differ significantly ($p>0.05$) from the control. As illustrated on Figure 3.14, earthworms exposed to different concentrations of copper oxychloride showed no significant differences ($p>0.05$) in acetylcholinesterase activity (see Table 1(f.2)). This implies that copper oxychloride did not have any effect on the acetylcholinesterase activity of the exposed worms.

Figure 3.15 represents the mean AChE activity of earthworms exposed to different concentrations of a binary mixture of chlorpyrifos and copper oxychloride. There is a noticeable dose relationship with control worms having a significantly ($p<0.05$) higher enzyme activity than the highest concentration treatment worms (20 mg/kg). The control worms did not differ significantly ($p>0.05$) from the other concentration treatments in enzyme activity. The highest concentration treatment had a significantly ($p<0.05$) lower enzyme activity than the rest of the concentration treatments. The 0.02 mg/kg concentration treatment had a slightly higher enzyme activity than the rest of the concentration treatments (even the control) although only significantly ($p<0.05$) different from the 20 mg/kg treatment.

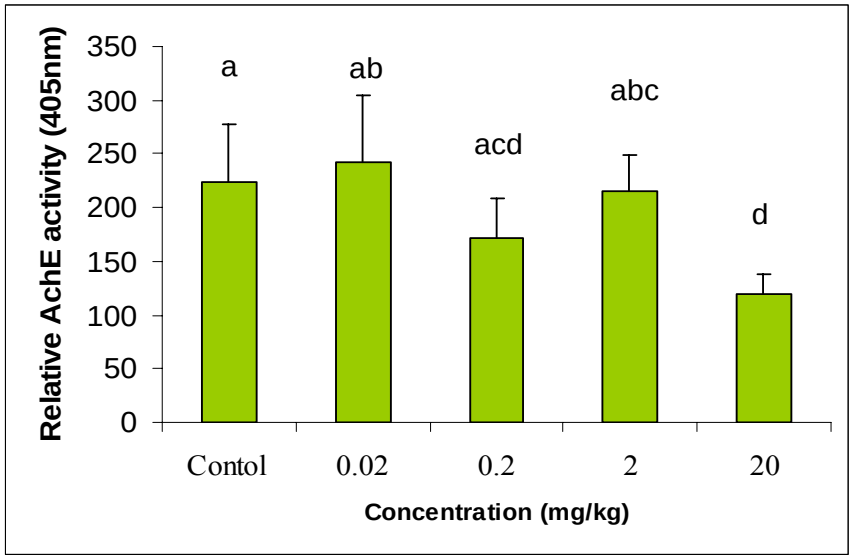

Figure 3.13 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of chlorpyrifos in soil for 4 weeks (n=12; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=832.74; df=1,4; p<0.05)).

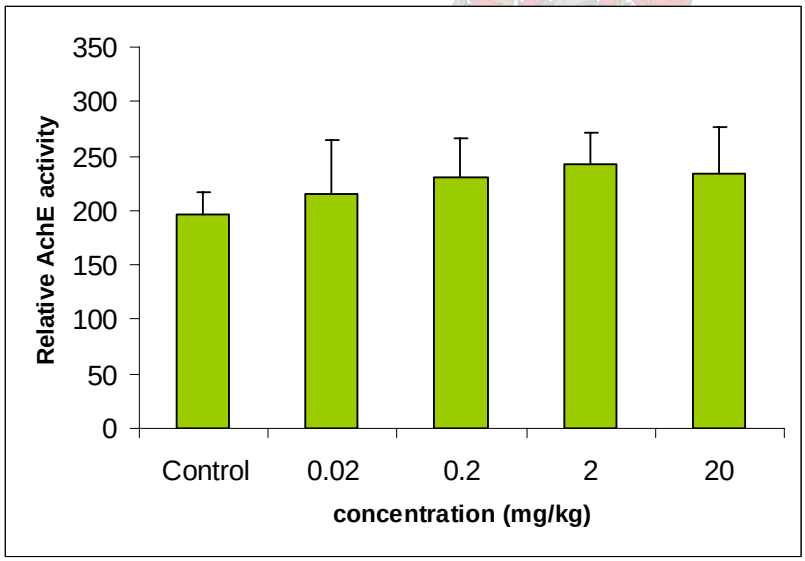

Figure 3.14 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of copper oxychloride in soil for 4 weeks (n=6).

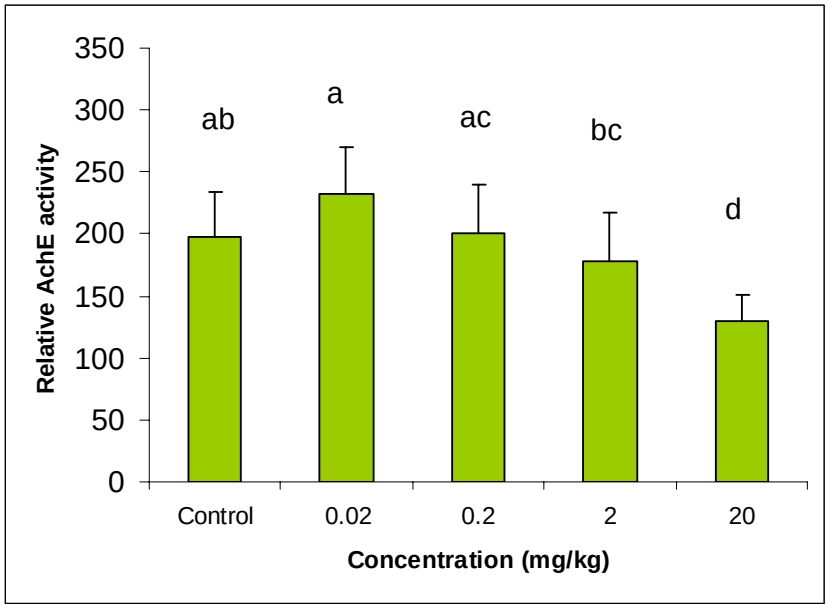

Figure 3.15 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of a 1:1 mixture of chlorpyrifos and copper oxychloride in soil for 4 weeks (n=12; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=1677.98; df=1,4; p<0.05)).

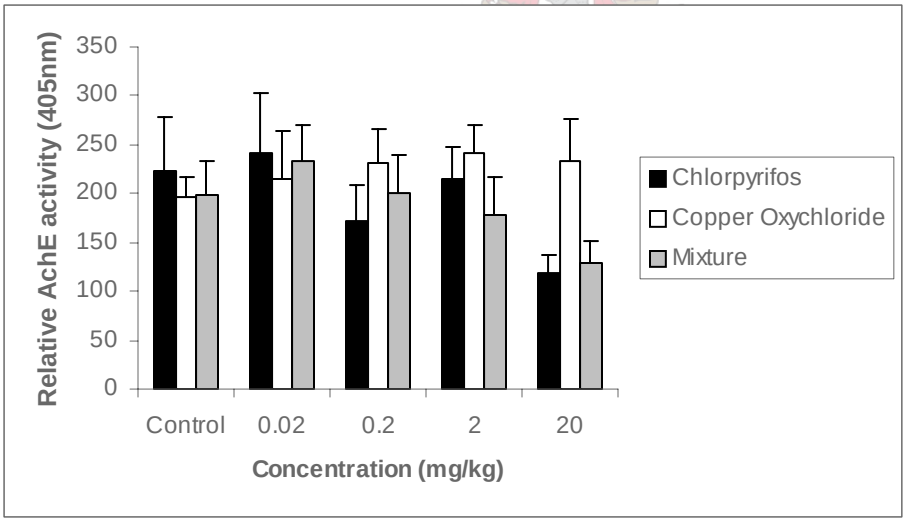

Figure 3.16 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* after 4 weeks of exposure to different concentrations of chlorpyrifos (n=12), copper oxychloride (n=6) and a 1:1 mixture of chlorpyrifos and copper oxychloride (n=12) in soil (see Tables 1(f.1and f.2)).

When comparing the results of the three pesticide treatments (Figure 3.16 and Tables 1(f.1 and f.2)) it can be seen that chlorpyrifos and the mixture had a noticeable dose-related effect with the control having a higher enzyme activity than the highest treatment concentration (20 mg/kg). No effect was found with the earthworms treated with copper oxychloride.

3.3 **Exposures of *E. fetida* in artificial groundwater**

3.3.1. *Chlorpyrifos and copper oxychloride*

The control treatment group of the earthworms exposed to chlorpyrifos showed no significant differences ($p>0.05$) to the exposed treatment groups, except for the highest concentration treatment (2 mg/l) (Figure 3.17 and Table 2(a.1)). The highest concentration treatment (2 mg/l) was also not significantly different ($p >0.05$) from the other exposed treatment groups (0.002 mg/l, 0.02 mg/l, 0.2 mg/l), which were not significantly different ($p>0.05$) from each other.

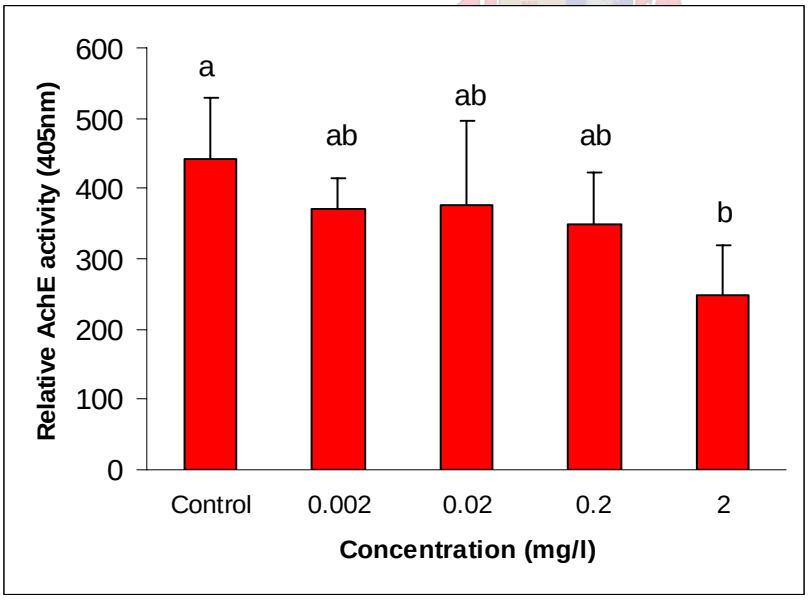

Figure 3.17 Mean relative acetylcholinesterase activity ±SD of *E. fetida* exposed to different concentrations of chlorpyrifos in artificial groundwater for 48 hours (n=6; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; $F=563$; $df=1,4$; $p<0.05$)).

Earthworms exposed to different concentrations of copper oxychloride showed no significant differences ($p>0.05$) among all the concentration treatment groups, including the control (Figure3.18 and Table 2(a.1)).

The results of the mixture exposure (Figure 3.19 and Table 2(a.1)) were similar to those of the chlorpyrifos exposure (Figure 3.17), with the control group differing significantly from the highest concentration treatment (2 mg/l), and the other exposure treatment groups (0.002 mg/l, 0.02 mg/l, 0.2 mg/l) not differing significantly from each other.

The results of the exposure to chlorpyrifos, copper oxychloride and the mixture thereof, in artificial groundwater (Figure 3.20) were somewhat similar to the results obtained in the soil (Figure 3.16) exposure in this study, with chlorpyrifos and the mixture showing a dose related effect and copper oxychloride showing no effect on enzyme activity. There was no interaction between copper oxychloride and chlorpyrifos in the mixture. Copper oxychloride showed no effect on AChE activity on its own or in the mixture with chlorpyrifos.

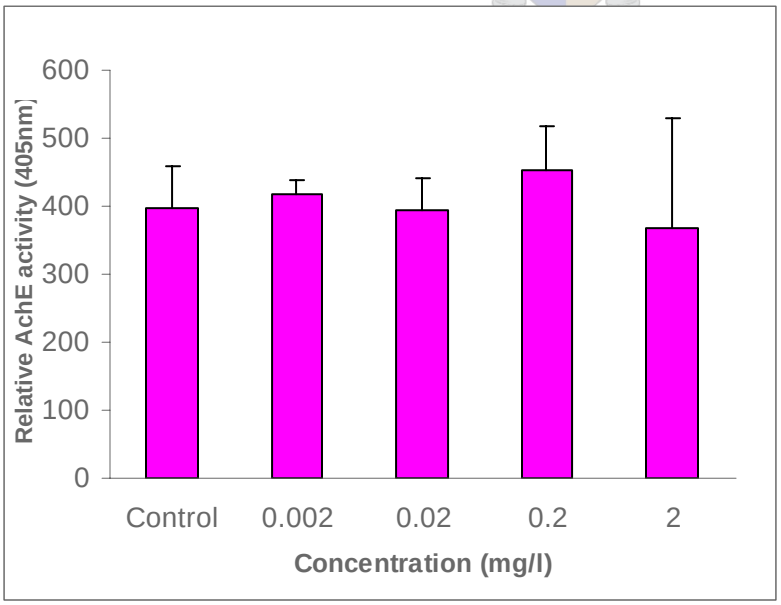

Figure 3.18 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of copper oxychloride in artificial groundwater for 48 hours; n=6.

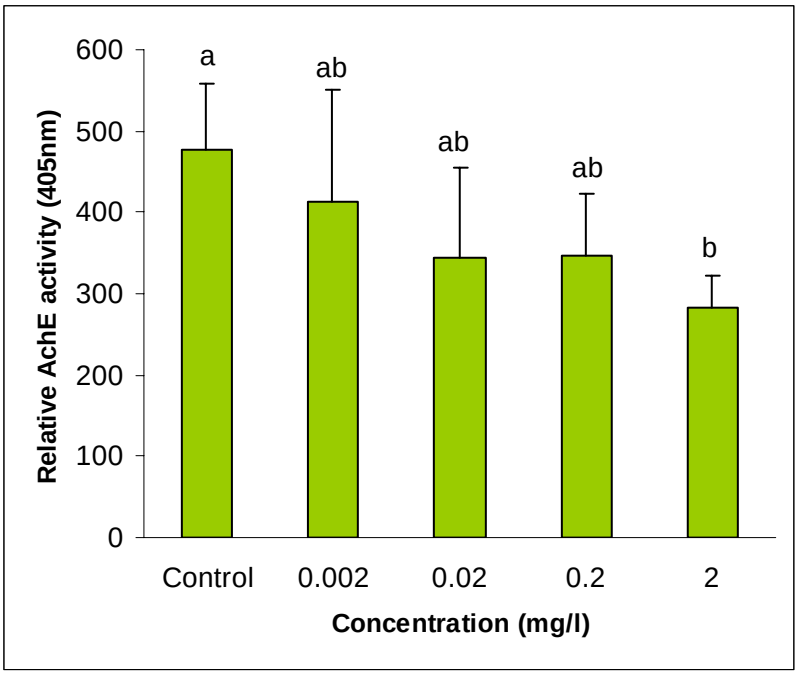

Figure 3.19 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of a 1:1 mixture chlorpyrifos and copper oxychloride in artificial groundwater for 48 hours (n=6; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=456.31; df=1,4; $p<0.05$)).

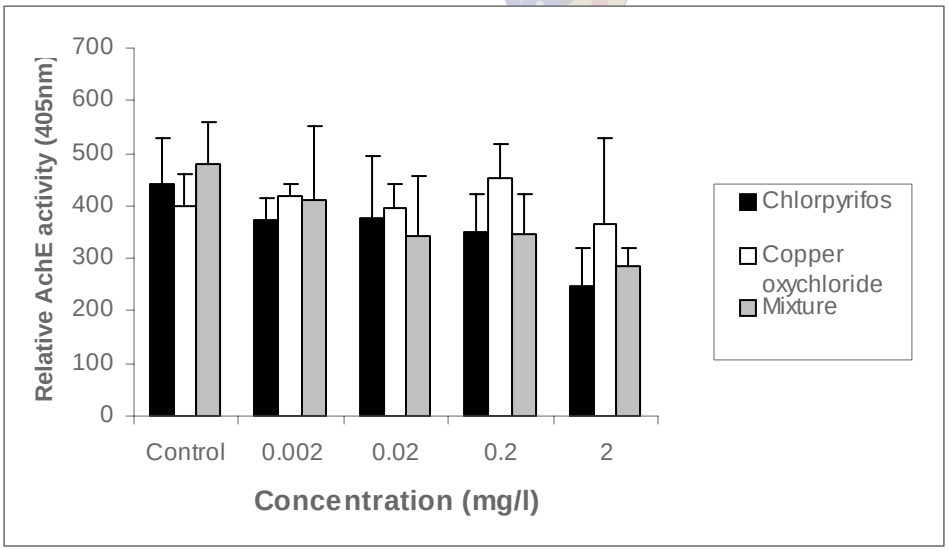

Figure 3.20 Mean relative acetylcholinesterase activity (at 405nm) of *E. fetida* exposed to different concentrations of chlorpyrifos (n=6), copper oxychloride (n=6), and a 1:1 mixture of the two pesticides (n=6) in artificial groundwater for 48hours (see Tables 2(a. 1 and a.2)).

3.3.2 *Chlorpyrifos and Azinphos-methyl*

Figure 3.21 illustrates the relative AChE activity of earthworms exposed to different concentrations of chlorpyrifos in artificial groundwater. The three lowest concentration treatments (control, 0.002 mg/l, and 0.02 mg/l) showed no significant differences ($p>0.05$) among each other. These three concentration treatments were significantly different ($p<0.05$) from the highest concentration treatment (2 mg/l), but not significantly different ($p>0.05$) from the 0.2 mg/l treatment. The 0.2 mg/l treatment was not significantly different from any of the other concentration treatments, including the control (see Table 2(b.2)).

Earthworms exposed to the following concentration treatments of azinphos-methyl; control, 0.002 mg/l and 0.02 mg/l; showed no significant differences ($p>0.05$) in AChE activity (Figure 3.22 and Table 2(b.2)). The 0.002 mg/l and 0.02 mg/l concentration treatments had a slightly higher enzyme activity than the control (not statistically significant), and a significantly higher ($p<0.05$) enzyme activity than the highest concentration treatments (0.2 mg/l and 2 mg/l). The control treatment had a significantly higher ($p<0.05$) enzyme activity than the 2 mg/l treatment, but not significantly different ($p>0.05$) from the 0.2 mg/l treatment. The 0.2 mg/l treatment had a significantly higher ($p<0.05$) enzyme activity than the 2 mg/l treatment.

The earthworms exposed to the lowest concentration treatments (control, 0.002 mg/l and 0.02 mg/l) of the mixture of chlorpyrifos and azinphos-methyl showed no significant differences ($p>0.05$) in enzyme activity (Figure 3.23 and Table 2(b.2)). These concentration treatments had significantly higher ($p<0.05$) enzyme activities than the highest concentration treatments (0.2 mg/l and 2 mg/l). The highest concentration treatments (0.2 mg/l and 2 mg/l) did not show any significant differences ($p>0.05$) in enzyme activity. There were therefore two distinct levels of enzyme activity, the high (control, 0.002 mg/l and 0.02 mg/l) and the low (0.2 mg/l and 2 mg/l), with the 2 mg/l treatment slightly lower in enzyme activity than the 0.2 mg/l treatment (although not statistically significant).

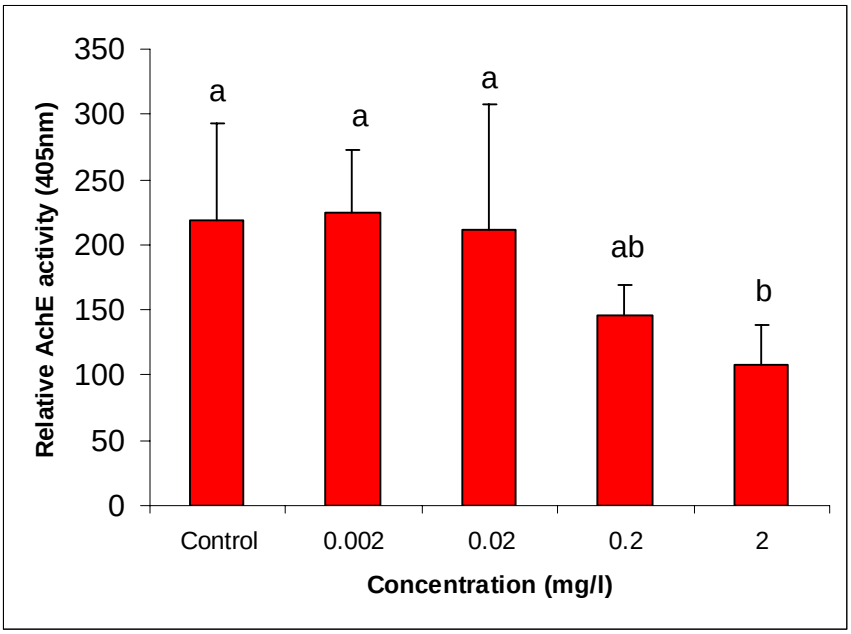

Figure 3.21 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E fetida* exposed to different concentrations chlorpyrifos in artificial groundwater for 48 hours (n=6; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=266.04; df=1,4; $p<0.05$)).

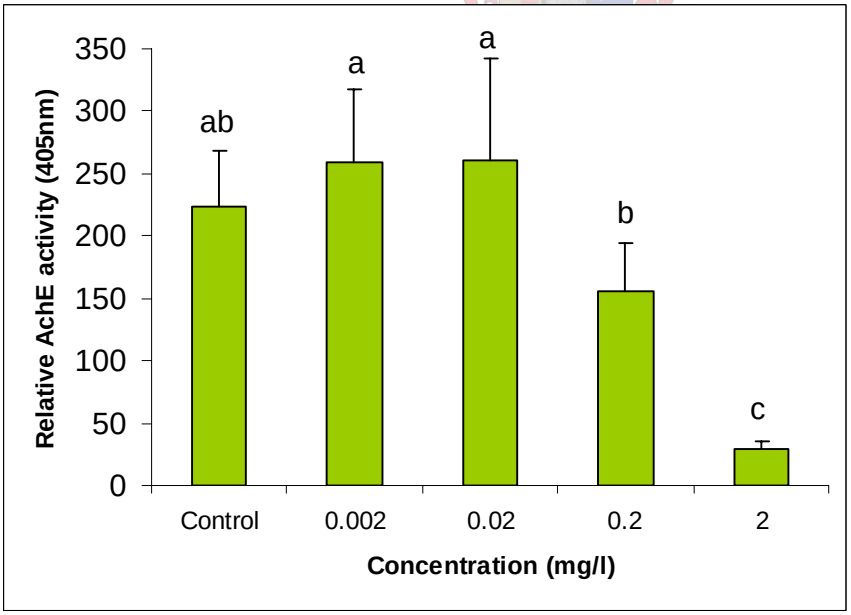

Figure 3.22 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of azinphos-methyl in artificial groundwater for 48 hours (n=8; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=507.51; df=1,4; $p<0.05$)).

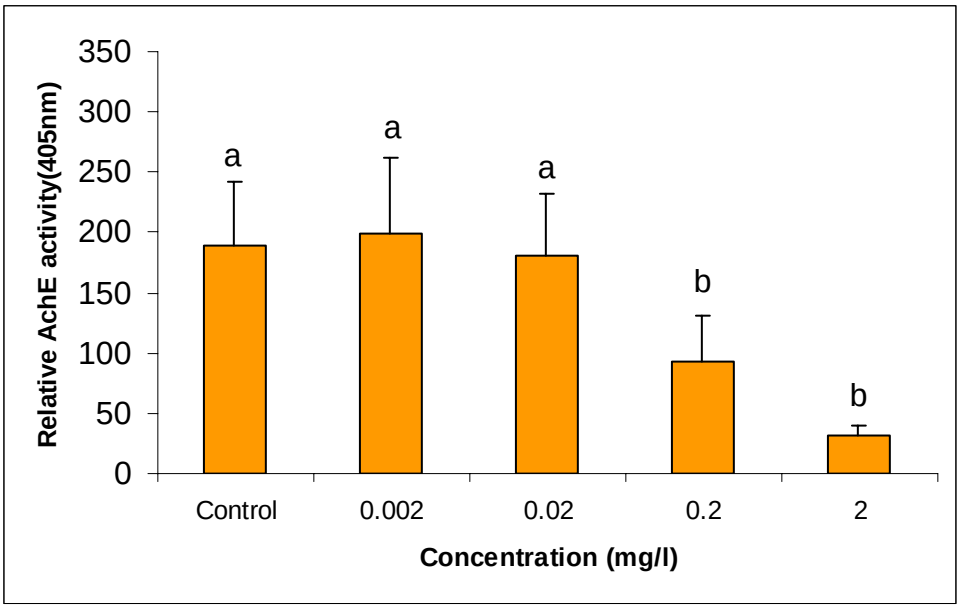

Figure 3.23 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of a 1:1 mixture of chlorpyrifos and azinphos-methyl in artificial groundwater for 48 hours (n=8; different letters on error bars indicate that the means are significantly different among treatment groups (ANOVA; F=350.82; df=1,4; $p<0.05$)).

Earthworms exposed to the lowest concentration treatments (control, 0.002 mg/l and 0.02 mg/l) of chlorpyrifos, azinphos-methyl and the mixture, showed no differences in enzyme activity. Earthworms from the 0.2 mg/l exposure concentrations of chlorpyrifos and azinphos-methyl singly, had slightly higher AChE activity values (146±24 and 156±39 (at 405 nm) respectively) than the mixture (93±38 (at 405 nm)), although this was not significantly different ($p>0.05$)(see Table 2(b.2)). Earthworms from the highest concentration treatment (2 mg/l) of chlorpyrifos showed a significantly higher ($p>0.05$) AChE value (108±30 (at 405 nm)) than azinphos-methyl and the mixture (28±7 and 31±8 (at 405 nm)) (see Table 2(b.2)). Azinphos-methyl on its own showed a significant difference ($p<0.05$) in AChE activity between the 0.2 mg/l and the 2 mg/l treatment, which was not shown by chlorpyrifos and the mixture. In all three pesticide treatments the dose relationship started showing from the 0.02 mg/l concentration treatment to the 2 mg/l concentration treatment.

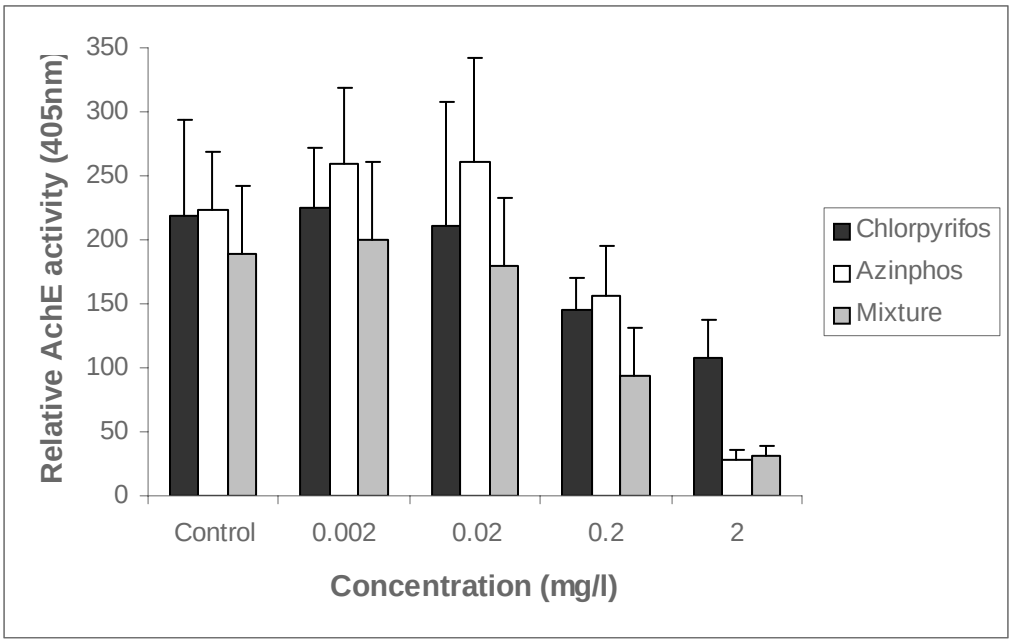

Figure 3.24 Mean relative acetylcholinesterase activity (at 405nm) of *E. fetida* exposed to different concentrations of chlorpyrifos (n=6), azinphos-methyl (n=8) and a 1:1 mixture of chlorpyrifos and azinphos methyl (n=8) in artificial groundwater for 48 hours (see Tables 2(b.1and b.2)).

Earthworms exposed to azinphos-methyl had a higher enzyme activity than those exposed to chlorpyrifos in all exposure treatments except the 2 mg/l treatment (Figure 3.24). The enzyme activity of earthworms exposed to the mixture was lower than that of earthworms exposed to the single components in all concentration treatments, except the highest concentration treatment (2 mg/l), where the earthworms exposed to the mixture had a slightly higher enzyme activity than those exposed to azinphos-methyl (Figure 3.24).

3.3.3 *Chlorpyrifos and Cypermethrin*

Earthworms exposed to chlorpyrifos singly showed some significant differences between concentration treatments (Figure 3.21 and Section 3.3.2). Earthworms exposed to different concentrations of cypermethrin showed no significant differences ($p>0.05$) in AChE activity (Figure 3.25 and 3.27). There were no significant differences in the AChE activity of earthworms exposed to different concentrations of a mixture of chlorpyrifos

and cypermethrin (Figure 3.26 and 3.27). All earthworms died in the 2 mg/l exposure treatment of the mixture of chlorpyrifos and cypermethrin, therefore the AChE activity could not be measured at that exposure treatment (Figure 3.26 and Table 2(c.1)).

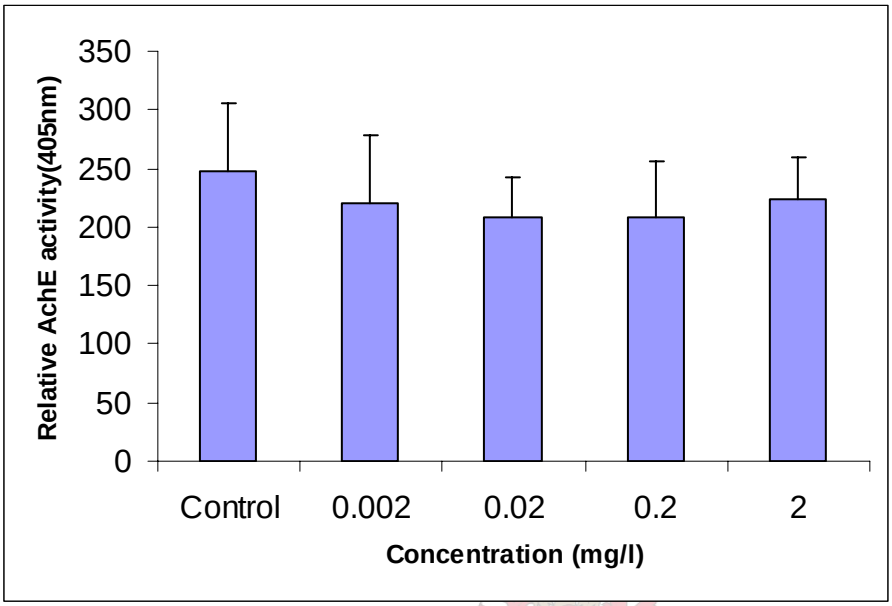

Figure 3.25 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of cypermethrin in artificial groundwater for 48 hours (n=8).

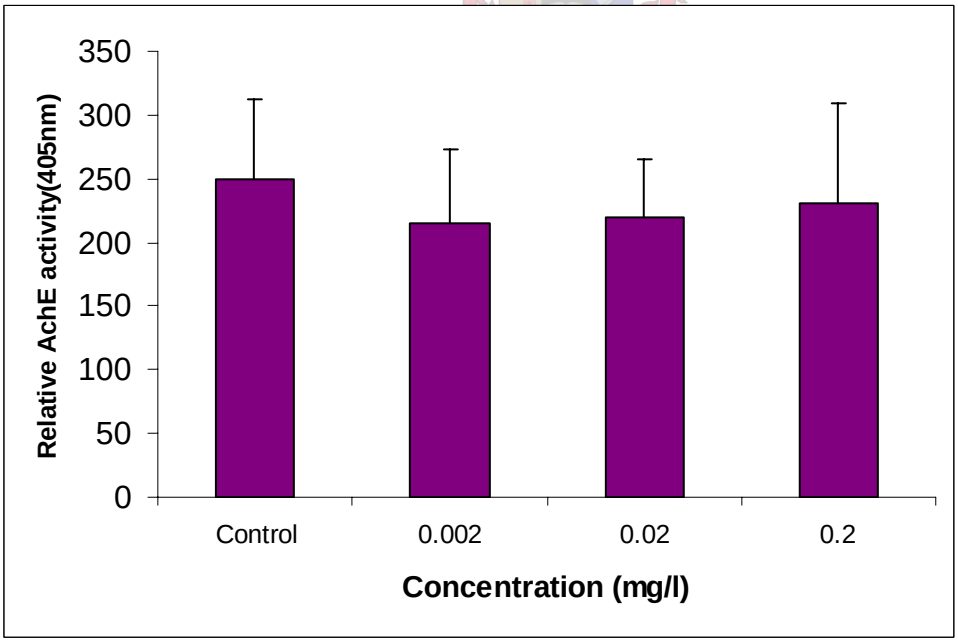

Figure 3.26 Mean relative acetylcholinesterase activity (at 405nm) ±SD of *E. fetida* exposed to different concentrations of a mixture of chlorpyrifos and cypermethrin in artificial groundwater for 48hours (n=8).

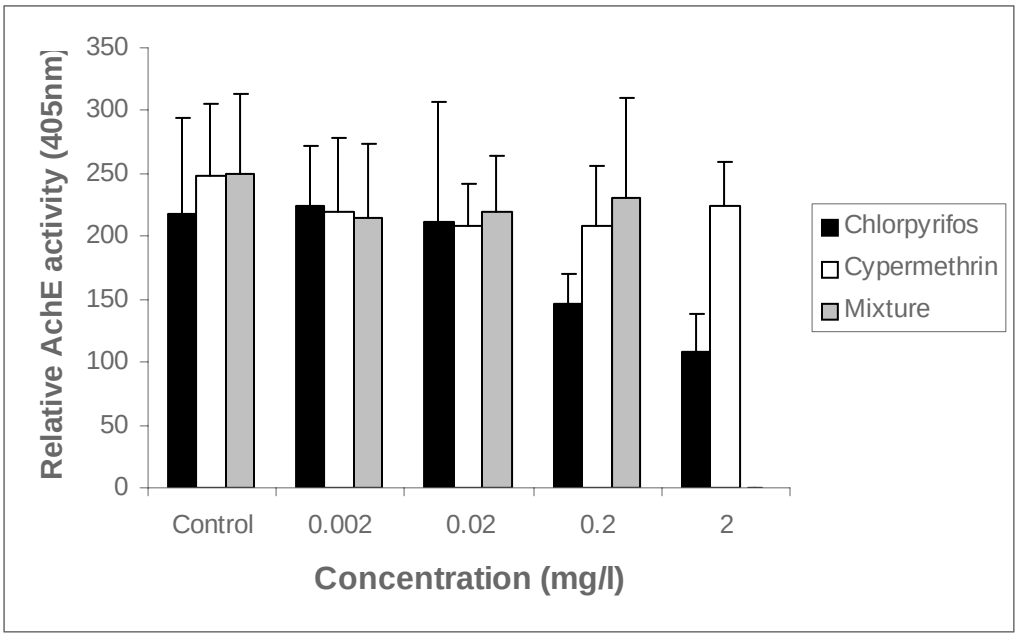

Figure 3.27 Mean relative acetylcholinesterase activity of *E. fetida* exposed to different concentrations of chlorpyrifos (n=6), cypermethrin (n=8), and a 1:1 mixture of chlorpyrifos and cypermethrin (n=8) (see Tables 2 (c.1and c.2)).

CHAPTER 4

DISCUSSION

4.1. Lifecycle parameters

4.1.1 *Growth*

With regard to biomass change as a measure of growth, earthworms from all pesticide exposure treatments survived, but lost weight (Figures 3.1-3.4). These worms were taken from compost, an ideal environment for *E. fetida*, where they were fed with fresh cattle manure. During the experimental period they were in artificial soil, which is a totally different substrate, and they were fed with moistened dry cattle manure. This change in substrate material and the difference in organic matter content of the two substrates, coupled with the change in feeding material could have affected the biomass of the earthworms, as was shown by control worms also losing weight. Spurgeon *et al* (1994), also found a decline in earthworm biomass in all their treatments (including the controls), and attributed that to the lack of suitable food in the OECD soil medium they also used. They then suggested that experiments should include animal manure in the test medium. However, even when manure is present, a large part of the diet in earthworms still consists of soil particles (Jager *et al* 2003).

The highest concentration treatment (20 mg/kg) of chlorpyrifos (Figure 3.1 and 3.4) significantly affected the growth (biomass change) of the earthworms. It is therefore evident that, although the LC_{50} of chlorpyrifos in adult *E. fetida* is reported to be 1077mg/kg (Eason *et al* 1999), concentrations as low as 20 mg/kg have negative sub-lethal effects on these organisms.

With regard to copper oxychloride (Figure 3.2 and 3.4), there was a slight effect exhibited by growth of the worms because the biomass change of the exposed treatment groups was slightly higher than that of the control, although not statically significant.

Maboeta *et al* (In press) also found no significant effect of copper oxychloride on biomass of adult *E. fetida* at a concentration of 73 mg/kg, which is much higher than the concentrations used in the present study. Where juveniles were however used for exposures, such as in the study of Helling *et al* (2000), growth of *E. fetida* was significantly reduced at a concentration of copper oxychloride as low as 3.3mg/kg. This could however be attributed to the differences in the ages of the exposed worms in the different studies. Juvenile earthworms are somewhat more sensitive to toxicants than adults as shown by Spurgeon and Hopkin (1996).

Another factor is that in the present study and that of Maboeta *et al* (In press), artificial soil was used as a substrate, instead of the cattle manure used in the other studies. Speciation of metals is influenced by soil characteristics such as organic matter content (Kiewiet and Ma 1991). The route of uptake in these studies could also have been different. In the previous studies, earthworms were fed on spiked cattle manure, while in the present study the cattle manure which was added as food for the worms was uncontaminated. This emphasizes the importance of oral uptake versus uptake through the skin, which influence bioavailability of the toxicant (Belfroid *et al* 1995). These factors therefore prohibit direct comparison of the results of the present study with those of Helling *et al* (2000) because of the expected difference in the bioavailability of the pesticide.

The mixture of chlorpyrifos and copper oxychloride (Figure 3.3 and 3.4) also did not have a statistically significant effect on biomass change (loss) compared to the control, although the exposed treatment groups had a slightly higher percentage biomass change than the control. There was therefore an interaction between the two pesticides in the mixture, as shown by the effects on biomass, because chlorpyrifos on its own showed a significant effect at 20 mg/kg, while this effect was not exhibited in the mixture (Figure 3.4). The presence of Cu ions at this concentration could therefore have had an antagonistic effect on chlorpyrifos. This indication would have to be followed up with a higher frequency of exposures at more concentrations, to come to a definite conclusion with regard to antagonism. Biomass change was not a sensitive endpoint in measuring

the effects of copper oxychloride and the mixture of chlorpyrifos and copper oxychloride in this study. The concentrations chosen for this study were probably too low to show effects on biomass change of adult *E. fetida*. Biomass change could be a sensitive parameter for measuring the effects of chlorpyrifos singly on these organisms, as is shown by the effect of the highest concentration treatment of this pesticide on this endpoint in this study. Chlorpyrifos as a single substance had a different effect on biomass change than in a mixture with copper oxychloride.

4.2.2 *Reproduction*

Earthworms exposed to chlorpyrifos had a significantly reduced cocoon production at the highest concentration treatment (20 mg/kg) (Figure 3.5) compared to the control and other lower exposure groups. Hatching success and number of hatchlings per cocoon were not affected by the presence of chlorpyrifos in the substrate (Figures 3.6 and 3.7). Cocoon production seemed to be more sensitive than other reproductive parameters for chlorpyrifos. Spurgeon *et al* (1994), also found cocoon production of *E. fetida* to be a sensitive measure of reproduction for heavy metals. Reinecke *et al* (2001), on the other hand, found effects of other substances on cocoon viability (hatching success and number of hatchlings per cocoon) to be a more sensitive endpoint for measuring sublethal effects than cocoon production. That is in contrast with what is found for chlorpyrifos in the present study, but their study was on the effects of heavy metals.

Copper oxychloride and the mixture of chlorpyrifos and copper oxychloride, had no effect on cocoon production, hatching success or the number of hatchlings per cocoon (Figures 3.5, 3.6 and 3.7). This is again in contrast to findings by Helling *et al* (2000) and Reinecke *et al* (2002), where significant negative effects on cocoon production were exhibited at a concentration of 3.3mg/kg of copper oxychloride. They also found from their studies a considerable impact of copper oxychloride on reproduction at a concentration of 10mg/kg, as shown by a reduced hatching success and number of hatchlings per cocoon. The impact of chlorpyrifos on cocoon production (at 20 mg/kg) was however not exhibited in the mixture of chlopryrifos and copper oxychloride. The

presence of copper oxychloride seemed to have inhibited the effect of chlorpyrifos. This finding would also have to be investigated further to verify its validity. Chlorpyrifos singly at the highest concentration showed an effect on cocoon production as a measure of reproduction, that was not shown by chlorpyrifos in a mixture with copper oxychloride (Figure 3.5).

4.2 Biomarkers

4.2.1 *Neutral red retention assay*

Chlorpyrifos exerted a dose related response on the neutral red retention times of the exposed earthworms (Figure 3.8). This is in agreement with the result found by Eason *et al* (1999) on the effect of chlorpyrifos on the lysosomal membrane integrity of the coelomocytes of *E. andrei*. Copper oxychloride also exhibited a dose related effect on the neutral red retention time of the exposed earthworms (Figure 3.9), which is in agreement with finding from a study by Reinecke *et al* (2002) on the effect of this fungicide on the lysosomal membrane integrity of the coelomocytes of *E. fetida*. The mixture of chlorpyrifos and copper oxychloride also exerted an effect on lysosomal membrane integrity as shown by the neutral red retention time, with the control having a significantly higher retention time than all the exposed groups, but in this case a dose related response was not found (Figure 3.10). This difference in the result of exposure to the mixture in comparison to that of single substances suggests an interaction of some kind between the two pesticides, which manifested at the cellular (membrane) level. The effect of the mixture of chlorpyrifos and copper oxychloride on NRR time was different from that of the single components.

The neutral red retention times of earthworms exposed in artificial groundwater were between 2 and 10 minutes, even for the control. These times were very short compared to those of earthworms exposed in soil with control earthworms having retention times longer than 40 minutes. According to Selgen (1985), the efficiency of NRR in the

lysosome is dependent on the efficiency of membrane bound proton pumps. Any event impairing this proton pump system will result in a lowered NRR time according to Svendsen and Weeks (1995) who also stated that various stressors can possibly have this effect. In the present study some steps, apart from the presence of pesticides, might have been stressful to the earthworms during the groundwater exposure. Earthworms were first starved for 48 hours, and then exposed to an aqueous medium in a beaker with no substrate to which they could adhere. Thus they were very active and were moving all the time, which probably cost them energy. These factors alone might have affected the integrity of the lysosomal membrane of the earthworm coelomocytes. As the NRR time for these worms were much shorter than the soil exposure worms, even for the control animals (no pesticide), it was decided not to use this biomarker for the artificial groundwater exposures.

4.2.2. *Acetylcholinesterase inhibition*

Compared to the control, copper oxychloride on its own had no effect on the AChE activity in both the soil and groundwater exposures (Figures 3.14 and 3.18). This is in agreement with findings by Scaps *et al* (1997) who concluded that AChE activity was not a sensitive biomarker of metal toxicity in their study of the impacts of cadmium and lead on cholinesterase and metabolic pathway enzyme activity on *E. fetida*. Chlorpyrifos and the mixture of chlorpyrifos and copper oxychloride did show a dose related effect on the enzyme activity of the exposed worms in the soil and groundwater exposures (Figures 3.16 and 3.20). The similarity of the effect of chlorpyrifos and the mixture on AChE activity could mean that it was only chlorpyrifos that affected AChE activity in the mixture. This implies that copper oxychloride in the mixture did not interact with chlorpyrifos to influence the effect on AChE activity in both exposure media. The mixture of chlorpyrifos and copper oxychloride did not affect AChE activity differently from the single components, both in soil and in groundwater.

Chlorpyrifos (Figures 3.21) and azinphos-methyl (Figure 3.22), both organophosphates, had a dose-related effect and affected the acetylcholinesterase activity of the exposed worms. The mixture of the two pesticides showed some interaction between the two pesticides, because at the highest concentration (2 mg/l) chlorpyrifos had a higher AChE activity than both azinphos-methyl and the mixture (Figure 3.24). At this concentration there seemed to be an additive effect exhibited by these pesticides on enzyme inhibition. At all the other concentration treatments the AChE activity of the mixture was lower than that of the single pesticides (Figure 3.24). Because these are both organophosphates, they act on the AChE enzyme as a target site, and in that way interact with each other in a mixture (Richardson *et al* 2001).

Cypermethrin had no effect on the AChE activity of the exposed worms in comparison to the control (Figure 3.25), probably because this enzyme is not the target site for pyrethroids. All the exposed earthworms died at the highest exposure concentration of the mixture of chlorpyrifos and cypermethrin (Figure 3.26). This is probably because pyrethroids are highly toxic and organophosphates have the ability to enhance pyrethroid toxicity (Ray and Foreshaw 2000). The mixture of chlorpyrifos and cypermethrin at the other exposure concentrations had no effect on the AChE activity of the exposed earthworms (Figure 3.26); this is different from the effect of chlorpyrifos alone (Figure 3.21) and means that cypermethrin could have antagonized the AChE inhibition effect on chlorpyrifos. Although pyrethroids and organophosphates are neurotoxins, they act on different sites in the nervous system. Pyrethroids act on voltage-dependent sodium and chloride channels in the axon while organophosphates act by inhibiting the AChE activity in the synapse of between the neurons (Costa 1988). These two target sites are both involved in the transmission of impulses (Schmidt-Nielsen 1990), and are possibly related in function. Chlorpyrifos as a single substance affected AChE differently from the mixture of chlorpyrifos and cypermethrin.

4.3 Conclusion

Growth and reproduction were not very sensitive parameters in measuring the effects of sublethal concentrations of copper oxychloride and the mixture of chlorpyrifos and copper oxychloride on adult *E. fetida* at the concentration range used for this study. In the present study there were only single applications of copper oxychloride and the mixture of chlorpyrifos and copper oxychloride. It is possible that the organisms had sufficient time during the exposure period to recover from any effects on growth and reproduction. The situation in the field is different, because there are several consecutive applications of pesticides per season throughout the year (Helling *et al* 2000). Chlorpyrifos as a single substance affected growth and reproduction slightly differently from the mixture of chlorpyrifos and copper oxychloride, by showing significant effects at the highest concentration treatment (Figures 3.4 and 3.5).

The NRR time proved to be a sensitive biomarker for chlorpyrifos and copper oxychloride singly and in a mixture in soil exposures. The mixture of the chlorpyrifos and copper oxychloride had a different effect on NRR time from the single components. The mixture of chlorpyrifos and copper oxychloride did not affect AChE activity any differently from single components, both in soil and in groundwater. Results obtained for AChE activity of the lowest exposure concentrations of chlorpyrifos were similar in soil and groundwater, although the highest exposure concentration (2 mg/kg or 2 mg/l) had a higher enzyme inhibition in water than in soil (Figure 3.13 and Figure 3.21).

Chlorpyrifos and azinphos-methyl in a mixture in groundwater showed an additive effect on AChE inhibition at the highest concentrations (Figure 3.24). Chlorpyrifos in a mixture with cypermethrin did not have the effect on AChE activity that it had as a single component (Figure 3.27), therefore cypermethrin antagonized the AChE inhibition of chlorpyrifos. Cypermethrin and chlorpyrifos singly were not toxic enough to kill the

earthworms at the highest concentration treatment, although in a mixture the earthworms died at this concentration. The AChE inhibition, as a specific biomarker of organophosphate toxicity, can be a valuable tool in assessing the effects of organophosphates and mixtures organophosphates with other pesticides on non-target soil invertebrates. It is also evident from the results of the present study that AChE inhibition cannot be used as a tool for assessing the effects of heavy metal containing pesticides.

Studies by various authors have established links between biomarkers and lifecycle parameters in response to a range of chemical concentrations (Svendsen and Weeks 1997 and Reinecke *et al* 2002). The results of this study did not indicate any links between the biomarker responses and lifecycle parameters. Although there were no effects on the lifecycle parameters, there were effects exhibited by the biomarkers. The results obtained in the present study support the use of biomarkers in assessing the pesticide effects on non-target soil invertebrates, and highlight the importance and the sensitivity of biomarkers in assessing effects at below organismal level. Organisms may not seem to be affected by pesticides because there are no effects exhibited observable on lifecycle parameters, although these organisms are affected at sub-cellular and biochemical level. Effects at these levels could manifest at higher levels of organization with time (Maboeta *et al* 2003).

It is recommended that in future experiments in OECD artificial soil medium, earthworms must be fed animal manure in excess, to take care of the weight loss problem, which could probably be due to inadequate amount of food, and lack of organic matter in the artificial soil medium. Earthworms could then be weighed over time (maybe weekly), instead of the beginning and the end of experiment, to see if there is any change in biomass over time. A higher range of concentrations could also be used to determine effects on lifecycle parameters. Lastly, the pesticide mixtures done in artificial groundwater could also be done in soil, to see if there are any effects on lifecycle parameters and to see if those effects can be linked to biomarker results.

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APPENDIX 1

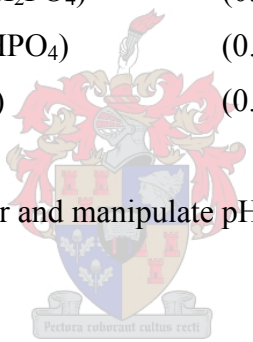
BIOMARKER TEST SOLUTIONS

A. Neutral Red Retention Assay

1. Earthworm Ringer Solution

Sodium Chloride ($NaCl_2$)	(0.414g)
Potassium Chloride (KCl)	(0.036g)
Calcium Chloride ($CaCl_2$)	(0.042g)
Magnesium Sulfate ($MgSO_4$)	(0.027g)
Potassium Hydrophosphate (KH_2PO_4)	(0.005g)
Sodium Hydrophosphate (Na_2HPO_4)	(0.004g)
Sodium Bicarbonate ($NaHCO_3$)	(0.035g)

Dissolve in 100ml of distilled water and manipulate pH to 7.3



2. Stock Solution

20 mg Neutral Red (Toluyne Red) [$C_{15}H_{17}N_4Cl$]
1 ml DMSO (Dimithylsulfoxide) [C_2H_6OS]
Mix well in small container (Eppendorf tube)

3. Working Solution

2.5 ml Ringer Solution
10 µl Stock Solution
Mix well

B. Acetylcholinesterase Activity

1. Phosphate Buffer

Na_2HPO_4 141.96 g/mol = 14.196g in 1000ml of distilled water

Set pH to 7 or 8 as desired with HCl and/or NaOH

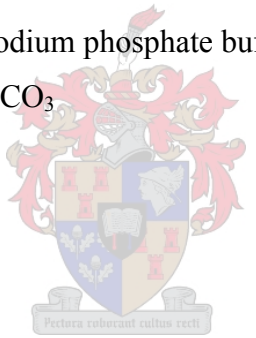
2. Acetylthiocholine Iodide

10.835 mg of Acetylthiocholine iodide dissolved in 0.5ml of pH7 Sodium phosphate buffer.

3. DTNB

9.9 mg DTNB in 2.5 ml pH7 Sodium phosphate buffer.

Dissolve and add 3.75 mg $NaHCO_3$



APPENDIX 2

EXPERIMENTAL DATA

1. IN SOIL

1.1 LIFECYCLE PARAMETERS

1.1.1(b) Biomass change of earthworms exposed to chlorpyrifos

Control n=40

Startmass	Endmass	Difference
0.5808	0.5219	0.0589
0.8769	0.3765	0.5004
0.7266	0.6008	0.1258
1.0532	0.5422	0.511
0.6955	0.662	0.0335
0.6224	0.4594	0.163
0.7031	0.4244	0.2787
0.7043	0.4554	0.2489
0.5226	0.4962	0.0264
0.6387	0.4224	0.2163
0.3891	0.3909	-0.0018
0.3839	0.4259	-0.042
0.2495	0.2697	-0.0202
0.2343	0.5353	-0.301
0.5204	0.3898	0.1306
0.4002	0.3527	0.0475
0.3152	0.2292	0.086
0.3966	0.4164	-0.0198
0.3709	0.4137	-0.0428
0.3376	0.2275	0.1101
0.2754	0.3459	-0.0705
0.3218	0.4528	-0.131
0.397	0.361	0.036
0.4155	0.4303	-0.0148
0.4104	0.5417	-0.1313
0.2541	0.3351	-0.081
0.5546	0.4464	0.1082
0.2837	0.3001	-0.0164
0.2914	0.2145	0.0769
0.2335	0.3946	-0.1611
0.44	0.3018	0.1382
0.2596	0.2962	-0.0366
0.3595	0.2462	0.1133

	0.3716	0.2944	0.0772
	0.4955	0.3849	0.1106
	0.3488	0.4535	-0.1047
	0.3603	0.1757	0.1846
	0.6314	0.281	0.3504
	0.3689	0.5503	-0.1814
	0.2465	0.364	-0.1175
Mean	**0.4510325**	**0.394568**	**0.056465**
SD	0.1891174	0.11138	0.1686449
%biomass change			**12.519054**

Chlorpyrifos n = 40

0.02 mg/kg

Startmass	Endmass	Difference
0.3241	0.3757	-0.0516
0.3245	0.2926	0.0319
0.3057	0.3053	0.0004
0.4069	0.2968	0.1101
0.414	0.3287	0.0853
0.4447	0.3036	0.1411
0.3129	0.4294	-0.1165
0.4091	0.3379	0.0712
0.345	0.3745	-0.0295
0.3387	0.3815	-0.0428
0.447	0.4053	0.0417
0.6103	0.3649	0.2454
0.3139	0.3041	0.0098
0.4891	0.4376	0.0515
0.4617	0.434	0.0277
0.7738	0.4609	0.3129
0.4074	0.4075	-1E-04
0.3937	0.6922	-0.2985
0.3987	0.409	-0.0103
0.4224	0.0908	0.3316
0.5674	0.4395	0.1279
0.8598	0.5405	0.3193
0.4122	0.4906	-0.0784
0.437	0.6989	-0.2619
0.3356	0.7481	-0.4125
0.6514	0.4196	0.2318
0.5229	0.4347	0.0882
0.5438	0.3638	0.18
0.2981	0.3944	-0.0963
0.4619	0.2629	0.199
0.4353	0.4685	-0.0332

0.2 mg/kg

Startmass	Endmass	Difference
0.5605	0.4762	0.0843
0.4721	0.4749	-0.0028
0.3894	0.4136	-0.0242
0.4628	0.4217	0.0411
0.555	0.3645	0.1905
0.5346	0.3507	0.1839
0.4318	0.3329	0.0989
0.4428	0.4055	0.0373
0.5286	0.468	0.0606
0.3888	0.4173	-0.0285
0.3854	0.4516	-0.0662
0.5231	0.4895	0.0336
0.3634	0.4495	-0.0861
0.33	0.3055	0.0245
0.4425	0.4784	-0.0359
0.5117	0.3851	0.1266
0.5075	0.4889	0.0186
0.4283	0.3252	0.1031
0.6314	0.4106	0.2208
0.5131	0.2856	0.2275
0.5113	0.3318	0.1795
0.4478	0.3875	0.0603
0.4942	0.3356	0.1586
0.4151	0.3867	0.0284
0.5817	0.4121	0.1696
0.3759	0.3959	-0.02
0.4048	0.4571	-0.0523
0.4921	0.3334	0.1587
0.5449	0.2376	0.3073
0.3133	0.3753	-0.062
0.3554	0.5085	-0.1531

	0.7814	0.5089	0.2725		0.2783	0.2728	0.0055
	0.4506	0.3353	0.1153		0.5012	0.3279	0.1733
	0.536	0.3329	0.2031		0.4681	0.6292	-0.1611
	0.3733	0.6995	-0.3262		0.4654	0.3905	0.0749
	0.2887	0.4482	-0.1595		0.7078	0.3728	0.335
	0.4179	0.8525	-0.4346		0.3863	0.4121	-0.0258
	0.7118	0.3814	0.3304		0.4538	0.3261	0.1277
	0.476	0.3033	0.1727		0.4943	0.3815	0.1128
	0.495	0.2583	0.2367		0.3928	0.3517	0.0411
Mean	**0.459993**	**0.420353**	**0.03964**	**Mean**	**0.462183**	**0.395533**	**0.06665**
SD	0.138723	0.147341	0.1971372	**SD**	0.086779	0.074818	0.11386551
%biomass change		**8.617532**		**%biomass change**		**14.42071**	

2 mg/kg

Startmass	Endmass	Difference
0.2234	0.3488	-0.1254
0.5544	0.5019	0.0525
0.2947	0.3449	-0.0502
0.5079	0.4348	0.0731
0.521	0.6104	-0.0894
0.6719	0.225	0.4469
0.3839	0.4311	-0.0472
0.2847	0.307	-0.0223
0.5395	0.3343	0.2052
0.3576	0.3527	0.0049
0.4885	0.4625	0.026
0.3753	0.5052	-0.1299
0.5913	0.4097	0.1816
0.444	0.3984	0.0456
0.2653	0.4227	-0.1574
0.3839	0.5242	-0.1403
0.5671	0.6086	-0.0415
0.4541	0.4038	0.0503
0.3677	0.3588	0.0089
0.4395	0.2463	0.1932
0.4449	0.2867	0.1582
0.3362	0.3231	0.0131
0.3731	0.3496	0.0235
0.3668	0.4518	-0.085
0.4282	0.3868	0.0414
0.2615	0.2689	-0.0074
0.3926	0.3692	0.0234
0.3289	0.3809	-0.052
0.4636	0.3116	0.152
0.3251	0.2646	0.0605

20 mg/kg

Startmass	Endmass	Difference
0.5596	0.3972	0.1624
0.4735	0.3803	0.0932
0.4715	0.4241	0.0474
0.5898	0.482	0.1078
0.508	0.5173	-0.0093
0.4863	0.3384	0.1479
0.5424	0.4553	0.0871
0.6873	0.3775	0.3098
0.6218	0.464	0.1578
0.4157	0.429	-0.0133
0.4152	0.3477	0.0675
0.3516	0.4621	-0.1105
0.7672	0.394	0.3732
0.4395	0.5285	-0.089
0.6128	0.3705	0.2423
0.383	0.4852	-0.1022
0.5002	0.367	0.1332
0.3609	0.3574	0.0035
0.3761	0.3885	-0.0124
0.4272	0.412	0.0152
0.7153	0.3655	0.3498
0.8385	0.3945	0.444
0.7207	0.4786	0.2421
0.553	0.436	0.117
0.6746	0.4885	0.1861
0.576	0.564	0.012
0.677	0.4924	0.1846
0.6214	0.5053	0.1161
0.4945	0.4919	0.0026
0.8002	0.47	0.3302

	0.3048	0.5951	-0.2903		0.6275	0.5211	0.1064
	0.3597	0.2759	0.0838		0.6851	0.3363	0.3488
	0.4169	0.4338	-0.0169		0.5974	0.5203	0.0771
	0.2772	0.3876	-0.1104		0.4768	0.4037	0.0731
	0.4538	0.2636	0.1902		0.7673	0.5022	0.2651
	0.3427	0.3017	0.041		0.6721	0.4288	0.2433
	0.2533	0.4171	-0.1638		0.5521	0.3524	0.1997
	0.6166	0.3668	0.2498		0.7813	0.4812	0.3001
	0.4606	0.3305	0.1301		0.5071	0.6775	-0.1704
	0.4026	0.3939	0.0087		0.5007	0.4201	0.0806
Mean	**0.40812**	**0.384758**	**0.0233625**	**Mean**	**0.570705**	**0.442708**	**0.1279975**
SD	0.106739	0.095672	0.1347767	**SD**	0.131203	0.072039	0.144811
%biomass change		**5.724419**		**%biomass change**		**22.42796**	

1.1.1(c) Biomass change of earthworms exposed to copper oxychloride

Control n=20

	Startmass	**Endmass**	**Difference**
	0.5136	0.3622	0.1514
	0.2703	0.4572	-0.1869
	0.4603	0.3056	0.1547
	0.3292	0.2698	0.0594
	0.3825	0.3156	0.0669
	0.6012	0.3701	0.2311
	0.497	0.3285	0.1685
	0.3665	0.3622	0.0043
	0.4991	0.3053	0.1938
	0.4767	0.5852	-0.1085
	0.3908	0.3965	-0.0057
	0.4649	0.408	0.0569
	0.4544	0.369	0.0854
	0.3876	0.3339	0.0537
	0.3943	0.4093	-0.015
	0.5034	0.3149	0.1885
	0.4041	0.3187	0.0854
	0.5055	0.2869	0.2186
	0.418	0.302	0.116
	0.4017	0.3397	0.062
Mean	**0.436055**	**0.35703**	**0.079025**
SD	0.075744	0.071391	0.107023
% biomass change			**18.12271**

Copper oxychloride n=20

0.02 mg/k	**0.2 mg/k**

g

Startmass	Endmass	Difference
0.6218	0.4098	0.212
0.3396	0.2608	0.0788
0.6647	0.5083	0.1564
0.4851	0.4078	0.0773
0.5464	0.3769	0.1695
0.4053	0.5155	-0.1102
0.496	0.5881	-0.0921
0.4241	0.4366	-0.0125
0.5198	0.3864	0.1334
0.8297	0.3682	0.4615
0.5482	0.4266	0.1216
0.7052	0.5026	0.2026
0.412	0.3905	0.0215
0.4722	0.4004	0.0718
0.3798	0.5983	-0.2185
0.5549	0.533	0.0219
0.4897	0.4477	0.042
0.4937	0.2813	0.2124
0.3471	0.3905	-0.0434
0.5793	0.3225	0.2568
0.51573	**0.42759**	**0.08814**
0.120245	0.089058	0.14678
17.09034		

Mean
SD
%biomass change

g

Startmass	Endmass	Difference
0.6785	0.3917	0.2868
0.8264	0.259	0.5674
0.8163	0.4767	0.3396
0.4034	0.4196	-0.0162
0.5683	0.6493	-0.081
0.5769	0.3664	0.2105
0.4744	0.411	0.0634
0.4146	0.3518	0.0628
0.3015	0.6069	-0.3054
0.5293	0.3111	0.2182
0.4627	0.3803	0.0824
0.6111	0.6093	0.0018
0.5379	0.2331	0.3048
0.3895	0.3789	0.0106
0.3385	0.3947	-0.0562
0.2975	0.2946	0.0029
0.3882	0.4363	-0.0481
0.2728	0.3256	-0.0528
0.4494	0.4099	0.0395
0.5053	0.2451	0.2602
0.492125	**0.397565**	**0.09456**
0.152725	0.113382	0.1894745
19.21463		

Mean
SD
%biomass change

2 mg/kg

Startmass	Endmass	Difference
0.9649	0.3236	0.6413
0.5085	0.4595	0.049
0.4171	0.3162	0.1009
0.3665	0.48	-0.1135
0.5494	0.5989	-0.0495
0.4273	0.2676	0.1597
0.5092	0.4289	0.0803
0.4803	0.3483	0.132
0.6508	0.3632	0.2876
0.4996	0.328	0.1716
0.4395	0.279	0.1605
0.3759	0.3796	-0.0037
0.333	0.3642	-0.0312
0.3536	0.4207	-0.0671
0.434	0.3771	0.0569

20 mg/kg

Startmass	Endmass	Difference
0.7512	0.5467	0.2045
0.5258	0.3267	0.1991
0.574	0.4528	0.1212
0.4351	0.2757	0.1594
0.396	0.4116	-0.0156
0.7968	0.4307	0.3661
0.2979	0.5337	-0.2358
0.5023	0.3007	0.2016
0.585	0.532	0.053
0.672	0.3828	0.2892
0.5306	0.3388	0.1918
0.2838	0.3522	-0.0684
0.2703	0.2261	0.0442
0.3676	0.2107	0.1569
0.3452	0.456	-0.1108

	0.394	0.3327	0.0613		0.4504	0.4297	0.0207
	0.3009	0.2809	0.02		0.2952	0.3915	-0.0963
	0.3165	0.3227	-0.0062		0.4147	0.2433	0.1714
	0.4533	0.2712	0.1821		0.385	0.3414	0.0436
	0.357	0.2796	0.0774		0.3081	0.2686	0.0395
Mean	**0.456565**	**0.361095**	**0.09547**	**Mean**	**0.45935**	**0.372585**	**0.086765**
SD	0.143879	0.081465	0.1575216	**SD**	0.151762	0.09952	0.1433201
%biomass change		**20.91049**		**%biomass change**		**18.88865**	

1.1.1(d) Biomass change of earthworms exposed to a mixture of chlorpyrifos and copper oxychloride

Control n=40

Startmass	Endmass	Difference
0.3381	0.3125	0.0256
0.3122	0.3479	-0.0357
0.2908	0.2716	0.0192
0.2132	0.6182	-0.405
0.3109	0.4609	-0.15
0.4625	0.3967	0.0658
0.4078	0.3758	0.032
0.4669	0.2894	0.1775
0.3615	0.2958	0.0657
0.3269	0.4304	-0.1035
0.3289	0.3613	-0.0324
0.2582	0.3642	-0.106
0.2533	0.2737	-0.0204
0.3077	0.352	-0.0443
0.356	0.2152	0.1408
0.3581	0.3944	-0.0363
0.3818	0.4308	-0.049
0.2433	0.2856	-0.0423
0.2951	0.3925	-0.0974
0.2732	0.3379	-0.0647
0.4427	0.4767	-0.034
0.5282	0.5132	0.015
0.6732	0.4731	0.2001
0.5945	0.5152	0.0793
0.5239	0.4644	0.0595
0.5808	0.4455	0.1353
0.3777	0.3603	0.0174
0.4382	0.3505	0.0877
0.6345	0.5105	0.124
0.6432	0.2888	0.3544

	0.8411	0.4951	0.346
	0.5669	0.5266	0.0403
	0.5371	0.3717	0.1654
	0.577	0.6339	-0.0569
	0.4469	0.4631	-0.0162
	0.4538	0.5308	-0.077
	0.5894	0.2743	0.3151
	0.8454	0.6772	0.1682
	0.5176	0.404	0.1136
	0.3166	0.3546	-0.038
Mean	**0.441878**	**0.408408**	**0.03347**
SD	0.156059	0.106416	0.140343
%biomass change			**7.574497**

Mixture: chlorpyrifos and copper oxychloride n = 40

0.02 mg/kg

Startmass	Endmass	Difference
0.376	0.2536	0.1224
0.5177	0.3003	0.2174
0.3409	0.3703	-0.0294
0.3374	0.5839	-0.2465
0.2713	0.3353	-0.064
0.3092	0.4253	-0.1161
0.3331	0.3882	-0.0551
0.2884	0.309	-0.0206
0.2482	0.319	-0.0708
0.3838	0.4161	-0.0323
0.541	0.2683	0.2727
0.3243	0.5062	-0.1819
0.3455	0.565	-0.2195
0.5803	0.3949	0.1854
0.2854	0.3693	-0.0839
0.3688	0.341	0.0278
0.2695	0.3152	-0.0457
0.2628	0.3566	-0.0938
0.2433	0.3476	-0.1043
0.2585	0.2566	0.0019
0.5044	0.5105	-0.0061
0.6775	0.656	0.0215
0.6955	0.3428	0.3527
0.7121	0.2815	0.4306
0.284	0.3299	-0.0459
0.3796	0.3038	0.0758
0.7578	0.1925	0.5653
0.3908	0.2938	0.097
0.4353	0.4787	-0.0434

0.2 mg/kg

Startmass	Endmass	Difference
0.4679	0.3749	0.093
0.277	0.3446	-0.0676
0.2287	0.3363	-0.1076
0.3564	0.3158	0.0406
0.366	0.4489	-0.0829
0.2512	0.3132	-0.062
0.3456	0.362	-0.0164
0.2623	0.2876	-0.0253
0.2741	0.2279	0.0462
0.4067	0.3047	0.102
0.221	0.2923	-0.0713
0.3238	0.347	-0.0232
0.2778	0.3063	-0.0285
0.2809	0.3385	-0.0576
0.4429	0.3872	0.0557
0.2792	0.3624	-0.0832
0.2492	0.4149	-0.1657
0.3007	0.2864	0.0143
0.3823	0.3198	0.0625
0.2796	0.3705	-0.0909
0.6768	0.3645	0.3123
0.5511	0.5089	0.0422
0.5912	0.2815	0.3097
0.7065	0.4259	0.2806
0.3295	0.3628	-0.0333
0.4323	0.5168	-0.0845
0.666	0.2595	0.4065
0.4045	0.4027	0.0018
0.4846	0.4698	0.0148

	0.395	0.571	-0.176		0.5126	0.3365	0.1761
	0.4846	0.4546	0.03		0.62	0.3566	0.2634
	0.5826	0.3849	0.1977		0.5034	0.3717	0.1317
	0.6271	0.378	0.2491		0.405	0.5794	-0.1744
	0.5383	0.4257	0.1126		0.5221	0.4558	0.0663
	0.6282	0.3652	0.263		0.3352	0.4984	-0.1632
	0.5575	0.4382	0.1193		0.6852	0.4317	0.2535
	0.4645	0.4413	0.0232		0.4945	0.377	0.1175
	0.4525	0.3691	0.0834		0.5646	0.3173	0.2473
	0.948	0.4176	0.5304		0.6598	0.2637	0.3961
	0.6632	0.2946	0.3686		0.5866	0.3681	0.2185
Mean	**0.451598**	**0.383785**	**0.0678125**	**Mean**	**0.42512**	**0.367245**	**0.057875**
SD	0.169197	0.099796	0.1954224	**SD**	0.146947	0.076921	0.1558233
%biomass change		**15.01614**		**%biomass change**		**13.6138**	

2 mg/kg

Startmass	Endmass	Difference
0.3574	0.3041	0.0533
0.3164	0.2832	0.0332
0.2897	0.4498	-0.1601
0.2939	0.2985	-0.0046
0.2014	0.6068	-0.4054
0.299	0.2425	0.0565
0.3836	0.3277	0.0559
0.3237	0.2944	0.0293
0.2904	0.2976	-0.0072
0.3733	0.4053	-0.032
0.2715	0.339	-0.0675
0.2822	0.3958	-0.1136
0.3093	0.3268	-0.0175
0.2303	0.2789	-0.0486
0.2571	0.2656	-0.0085
0.4253	0.3051	0.1202
0.2953	0.4579	-0.1626
0.3047	0.2638	0.0409
0.2089	0.2087	0.0002
0.3195	0.3072	0.0123
0.5632	0.3264	0.2368
0.2696	0.3422	-0.0726
0.3975	0.3488	0.0487
0.3071	0.3849	-0.0778
0.4629	0.369	0.0939
0.2742	0.3639	-0.0897
0.5152	0.2378	0.2774
0.4556	0.2386	0.217

20 mg/kg

Startmass	Endmass	Difference
0.5243	0.2565	0.2678
0.3318	0.5804	-0.2486
0.3806	0.4273	-0.0467
0.2839	0.382	-0.0981
0.3398	0.4092	-0.0694
0.3352	0.386	-0.0508
0.2551	0.4186	-0.1635
0.4039	0.396	0.0079
0.3266	0.3077	0.0189
0.336	0.3293	0.0067
0.3681	0.3926	-0.0245
0.3099	0.3739	-0.064
0.3406	0.2843	0.0563
0.438	0.409	0.029
0.3674	0.4506	-0.0832
0.2895	0.3229	-0.0334
0.345	0.4352	-0.0902
0.2268	0.3471	-0.1203
0.2084	0.2237	-0.0153
0.3137	0.2472	0.0665
0.6173	0.4242	0.1931
0.4307	0.201	0.2297
0.4178	0.3123	0.1055
0.4849	0.4062	0.0787
0.6084	0.3666	0.2418
0.4373	0.423	0.0143
0.4382	0.5068	-0.0686
0.3363	0.2885	0.0478

	0.469	0.2921	0.1769		0.6255	0.2932	0.3323
	0.2351	0.2161	0.019		0.5264	0.284	0.2424
	0.7141	0.5386	0.1755		0.3968	0.4078	-0.011
	0.6695	0.4214	0.2481		0.8552	0.382	0.4732
	0.56	0.5147	0.0453		0.3109	0.2503	0.0606
	0.5735	0.3741	0.1994		0.5031	0.523	-0.0199
	0.4614	0.295	0.1664		0.4488	0.315	0.1338
	0.4569	0.2065	0.2504		0.3754	0.2854	0.09
	0.6032	0.4595	0.1437		0.3919	0.2966	0.0953
	0.6856	0.3764	0.3092		0.6979	0.5834	0.1145
	0.6702	0.4672	0.203		0.438	0.2795	0.1585
	0.7876	0.2942	0.4934		0.5208	0.2528	0.268
Mean	**0.404108**	**0.343153**	**0.060955**	**Mean**	**0.414655**	**0.361528**	**0.0531275**
SD	0.156238	0.092296	0.1588644	**SD**	0.131531	0.091623	0.145575
%biomass change		**15.08386**		**%biomass change**		**12.81246**	

1.1.2(a) Reproduction of earthworms exposed to chlorpyrifos

Chlorpyrifos

	no. of cocoons	**no. hatched cocoons**	**% hatching success**	**no of hatchlings**	**hatchlings/cocoon**
Control	32	19	59.375	49	2.578947368
	39	26	66.66666667	47	1.807692308
	34	17	50	36	2.117647059
	34	22	64.70588235	50	2.272727273
Means	**34.75**	**21**	**60.18688725**	**45.5**	**2.194253502**
SD	2.986078811	3.915780041	7.457450207	6.454972244	0.321167458
0.02 mg/kg	27	18	66.66666667	37	2.055555556
	30	19	63.33333333	41	2.157894737
	29	16	55.17241379	35	2.1875
	42	23	54.76190476	41	1.782608696
Means	**32**	**19**	**59.98357964**	**38.5**	**2.045889747**
SD	6.782329983	2.943920289	5.95252628	3	0.184398953
0.2 mg/kg	33	15	45.45454545	38	2.533333333
	40	22	55	49	2.227272727
	45	27	60	51	1.888888889
	42	19	45.23809524	36	1.894736842
Means	**40**	**20.75**	**51.42316017**	**43.5**	**2.136057948**
SD	5.099019514	5.057996968	7.308336666	7.593857167	0.30847814

2 mg/kg	43	23	53.48837209	52	2.260869565
	35	17	48.57142857	39	2.294117647
	42	28	66.66666667	61	2.178571429
	47	25	53.19148936	55	2.2
Means	**41.75**	**23.25**	**55.47948917**	**51.75**	**2.23338966**
SD	4.991659711	4.645786622	7.790458521	9.287087811	0.053425779
20 mg/kg	17	8	47.05882353	22	2.75
	11	5	45.45454545	7	1.4
	28	18	64.28571429	49	2.722222222
	26	10	38.46153846	26	2.6
Means	**20.5**	**10.25**	**48.81515543**	**26**	**2.368055556**
SD	7.937253933	5.560275773	10.96834805	17.3781472	0.64865126

1.1.2(b) Reproduction of earthworms exposed to copper oxychloride

	no. of cocoons	no. hatched cocoons	% hatching success	no of hatchlings	hatchlings/cocoon
Control	35	16	45.71428571	37	2.3125
	36	19	52.77777778	37	1.947368421
Mean	**35.5**	**17.5**	**49.24603175**	**37**	**2.129934211**
SD	0.707106781	2.121320344	4.994643137	0	0.258187015
0.02 mg/kg	38	26	68.42105263	49	1.884615385
	35	12	34.28571429	25	2.083333333
Mean	**36.5**	**19**	**51.35338346**	**37**	**1.983974359**
SD	2.121320344	9.899494937	24.13732922	16.97056275	0.140514809
0.2 mg/kg	45	25	55.55555556	47	1.88
	32	16	50	24	1.5
Mean	**38.5**	**20.5**	**52.77777778**	**35.5**	**1.69**
SD	9.192388155	6.363961031	3.928371007	16.26345597	0.268700577
2 mg/kg	45	22	48.88888889	49	2.227272727
	32	13	40.625	31	2.384615385
Mean	**38.5**	**17.5**	**44.75694444**	**40**	**2.305944056**
SD	9.192388155	6.363961031	5.843451872	12.72792206	0.11125806
20 mg/kg	46	19	41.30434783	41	2.157894737

	28	8	28.57142857	18	2.25
Mean	**37**	**13.5**	**34.9378882**	**29.5**	**2.203947368**
SD	12.72792206	7.778174593	9.003533549	16.26345597	0.065128256

1.1.2(c) Reproduction of earthworms exposed to a mixture of chlorpyrifos and copper oxychloride

	no. of cocoons	hatched cocoons	% hatching success	no of hatchlings	hatchlings/cocoon
Control	22	14	63.63636364	31	2.214285714
	12	6	50	12	2
	33	20	60.60606061	44	2.2
	24	6	25	10	1.666666667
Mean	**22.75**	**11.5**	**49.81060606**	**24.25**	**2.020238095**
SD	8.61684397	6.806859286	17.54323663	16.21470526	0.255206555
0.02 mg/kg	21	10	47.61904762	20	2
	14	7	50	17	2.428571429
	28	21	75	53	2.523809524
	37	17	45.94594595	28	1.647058824
Mean	**25**	**13.75**	**54.64124839**	**29.5**	**2.149859944**
SD	9.831920803	6.396613687	13.67405746	16.34013464	0.405292219
0.2 mg/kg	16	12	75	22	1.833333333
	21	10	47.61904762	20	2
	33	18	54.54545455	44	2.444444444
	26	8	30.76923077	18	2.25
Mean	**24**	**12**	**51.98343323**	**26**	**2.131944444**
SD	7.257180352	4.320493799	18.30679789	12.11060142	0.269673442
2 mg/kg	19	11	57.89473684	21	1.909090909
	19	8	42.10526316	11	1.375
	28	19	67.85714286	38	2
	36	19	52.77777778	34	1.789473684
Mean	**25.5**	**14.25**	**55.15873016**	**26**	**1.768391148**
SD	8.185352772	5.6199051	10.72065534	12.35583533	0.276067716
20 mg/kg	15	5	33.33333333	7	1.4
	24	10	41.66666667	22	2.2
	36	17	47.22222222	34	2
	34	20	58.82352941	42	2.1
Mean	**27.25**	**13**	**45.26143791**	**26.25**	**1.925**

SD	9.708243919	6.782329983	10.69231905	15.23975065	0.359397644

1.2. BIOMARKERS

1.2.1(a) Neutral red retention time of earthworms exposed to chlorpyrifos (n = 12)

	Contol	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
	37	14	18	21	13
	40	28	20	13	9
	45	17	18	21	9
	45	21	10	13	9
	41	12	10	9	13
	37	17	9	9	9
	29	5	14	13	5
	33	24	26	13	5
	37	13	14	21	5
	41	5	14	5	5
	41	26	10	21	25
	40	25	14	5	9
Mean	**38.83333**	**17.25**	**14.75**	**13.66667**	**9.666667**
SD	4.60895	7.7942286	5.0294587	6.110101	5.613836

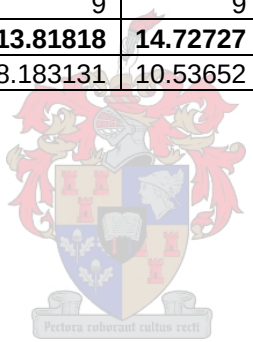
1.2.1(b) Neutral red retention time of earthworms exposed to copper oxychloride (n = 6)

Control	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
43	26	15	14	6
44	28	16	16	6
45	29	17	9	5
53	29	21	9	5
43	9	21	17	5
45	11	9	9	2

Mean	**45.5**	**22**	**16.5**	**12.33333**	**4.833333**
Stdev	3.781534	9.380832	4.460942	3.777124	1.47196

1.2.1(c) Neutral red retention time of earthworms exposed to a mixture of chlorpyrifos and copper oxychloride (n = 12)

	Control	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
	56	22	10	13	17
	37	16	9	5	5
	46	16	6	5	5
	53	16	8	22	6
	28	20	32	6	11
	48	14	26	8	8
	39	29	13	26	13
	44	5	17	36	9
	41	17	13	24	16
	40	25	9	8	12
	41	20	9	9	9
Mean	**43**	**18.18182**	**13.81818**	**14.72727**	**10.09091**
SD	8.530989	6.457124	8.183131	10.53652	4.134115



1.2.2(a)Acetycholinesterase activity of earthworms exposed to chlorpyrifos (n=12)

Contol	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
269	231	173	258	101
221	134	176	186	110
194	205	179	204	111
229	219	127	177	148
296	209	211	239	106
272	232	196	278	137
183	343	231	169	102

	186	372	149	217	110
	150	264	90	213	136
	310	244	192	187	125
	138	194	137	208	112
	241	262	196	251	124
Mean	**224.0833**	**242.4167**	**171.4167**	**215.5833**	**118.5**
SD	53.32988	61.34799	37.77005	32.9759	15.28219

1.2.2(b) Acetycholinesterase activity of earthworms exposed to copper oxychloride (n = 6)

Control	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
207	142	262	274	285
179	250	181	235	257
176	247	271	211	261
184	170	248	205	230
217	267	207	270	177
219	212	218	255	192
197	**214.6667**	**231.1667**	**241.6667**	**233.6667**
19.58571	49.62929	34.9137	29.51384	42.16001

1.2.2(c) Acetycholinesterase activity of earthworms exposed to a mixture of chlorpyrifos and copper oxychloride (n = 12)

Control	0.02 mg/kg	0.2 mg/kg	2 mg/kg	20 mg/kg
220	194	201	156	161
136	276	164	178	121
136	250	126	142	145
208	247	169	122	91
252	247	156	221	112
222	280	250	145	124
196	259	251	253	157
224	248	216	172	108
159	152	220	222	152

	202	201	196	184	127
	200	229	248	149	111
	216	212	203	194	142
Mean	**197.5833**	**232.9167**	**200**	**178.1667**	**129.25**
SD	36.06423	37.17638	40.17688	38.8279	22.05829

2. IN ARTIFICIAL GROUNDWATER

2.1 Acetylcholinesterase activity of earthworms exposed to chlorpyrifos and copper oxychloride (5 minute readings)

(a) *Chlorpyrifos:* *n = 6*

Control	0.002 mg/l	0.02 mg/l	0.2 mg/l	2 mg/l
537	335	438	474	316
513	322	360	382	196
398	407	424	295	337
458	422	514	313	275
295	397	162	271	162

	451	349	363	357	206
Mean	**442**	**372**	**376.8333**	**348.6667**	**248.6667**
StDev	87.11831	41.82822	119.5063	73.58442	70.90181

(b) ***Copper oxychloride****: n = 6*

	Control	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
	432	451	336	506	104
	372	389	377	344	237
	443	422	347	424	464
	470	429	430	517	476
	373	408	445	486	391
	301	412	430	432	528
Mean	**398.5**	**418.5**	**394.1667**	**451.5**	**366.6667**
StDev	61.76326	20.98333	47.02092	65.02846	163.6187

(c) ***Mixture (chlorpyrifos and copper oxychloride)****: n = 6*

	Control	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
	563	534	437	396	318
	444	588	421	436	300
	522	352	285	302	259
	560	448	170	235	228
	392	211	295	304	326
	385	343	451	403	270
Mean	**477.6667**	**412.6667**	**343.1667**	**346**	**283.5**
StDev	81.34535	138.5029	111.5176	77.29166	37.76639

2.2. Acetylcholinesterase activity of earthworms exposed to chlorpyrifos and azinphos-methyl (10 minute readings)

(a) ***Chlorpyrifos:*** *n = 6*

0mg/l	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
161	308	312	147	121
345	197	185	105	99
181	209	234	157	157
245	213	315	160	115
240	248	68	172	79
137	174	154	135	76

Mean	**218.1667**	**224.8333**	**211.3333**	**146**	**107.8333**
StDev	75.52593	47.33462	95.83249	23.64741	30.21534

(b) ***Azinphos-methyl****: n = 8*

	0mg/l	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
	208	297	110	128	22
	178	363	240	139	22
	248	290	293	175	29
	251	226	305	181	38
	191	263	210	119	29
	173	174	387	128	24
	305	205	289	235	26
	238	256	254	140	40
Mean	**224**	**259.25**	**261**	**155.625**	**28.75**
StDev	44.91897	59.13121	80.63321	39.11133	6.902381

(c) Mixture (chlorpyrifos and azinphos-methyl): *n = 8*

	0mg/l	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
	133	167	176	79	23
	117	134	125	58	33
	178	153	108	77	30
	149	184	138	43	50
	196	253	223	130	26
	262	176	233	90	25
	245	326	241	156	29
	230	201	199	113	31
Mean	**188.75**	**199.25**	**180.375**	**93.25**	**30.875**
StDev	53.76603	62.29825	51.72437	37.6516	8.408117

2.3. Acetylcholinesterase activity of earthworms exposed to chlorpyrifos and cypermethrin (10 minute readings)

(a) Cypermethrin*: n = 8*

0mg/l	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
263	283	184	281	231
344	179	165	180	228
288	181	228	157	223
215	192	222	240	227
149	245	183	168	173
231	219	273	251	187

	260	317	216	162	228
	236	145	196	230	295
Mean	**248.25**	**220.125**	**208.375**	**208.625**	**224**
StDev	56.70412	58.0823	33.97031	47.48515	36.05947

(b) Mixture (chlorpyrifos and cypermethrin)*: n = 8*

0mg/l	**0.002 mg/l**	**0.02 mg/l**	**0.2 mg/l**	**2 mg/l**
337	157	212	194	
292	251	201	159	
155	177	164	175	
190	172	169	178	
218	338	220	163	
212	186	261	311	
306	227	226	352	
283	213	301	309	
Mean	**249.125**	**215.125**	**219.25**	**230.125**	
StDev	63.96972	58.6404	45.41161	79.49921	

APPENDIX 3 **STATICTICAL TABLES FOR THE FACTORIAL ANOVA TEST** (Shaded cells: p<0.05)

1. EXPERIMENTS IN SOIL

1(a) Factorial ANOVA test for differences in biomass change

Table 1(a.1) Newman-Keuls test for Biomass change
Approximate Probabilities for Post Hoc Tests

Pesticide	**Concentr.**															
Chlorpyrifos	Control		0.966	0.980	0.908	0.796	0.988	0.991	0.988	0.993	0.983	0.867	0.996	0.982	0.958	0.989
Chlorpyrifos	0.02mg/kg	0.966		0.986	0.689	0.613	0.979	0.974	0.960	0.969	0.965	0.784	0.993	0.895	0.985	0.740
Chlorpyrifos	0.2mg/kg	0.980	0.986		0.939	0.804	0.950	0.985	0.984	0.992	0.960	0.889	0.977	0.996	0.889	0.997
Chlorpyrifos	2mg/kg	0.908	0.689	0.939		0.360	0.910	0.887	0.845	0.864	0.869	0.792	0.959	0.832	0.941	0.745
Chlorpyrifos	20mg/kg	0.796	0.613	0.804	0.360		0.836	0.761	0.690	0.424	0.850	0.228	0.758	0.824	0.779	0.797
Cu oxychl.	Control	0.988	0.979	0.950	0.910	0.836		0.973	0.981	0.994	0.849	0.833	0.783	0.995	0.970	0.996
Cu oxychl.	0.02mg/kg	0.991	0.974	0.985	0.887	0.761	0.973		0.875	0.982	0.973	0.787	0.959	0.996	0.985	0.995
Cu oxychl.	0.2mg/kg	0.988	0.960	0.984	0.845	0.690	0.981	0.875		0.982	0.980	0.725	0.965	0.993	0.982	0.991
Cu oxychl.	2mg/kg	0.993	0.969	0.992	0.864	0.424	0.994	0.982	0.982		0.997	0.746	0.984	0.996	0.990	0.994
Cu oxychl.	20mg/kg	0.983	0.965	0.960	0.869	0.850	0.849	0.973	0.980	0.997		0.768	0.887	0.992	0.970	0.992
Mixture	Control	0.867	0.784	0.889	0.792	0.228	0.833	0.787	0.725	0.746	0.768		0.914	0.800	0.899	0.752
Mixture	0.02mg/kg	0.996	0.993	0.977	0.959	0.758	0.783	0.959	0.965	0.984	0.887	0.914		0.999	0.984	0.999
Mixture	0.2mg/kg	0.982	0.895	0.996	0.832	0.824	0.995	0.996	0.993	0.996	0.992	0.800	0.999		0.997	0.907
Mixture	2mg/kg	0.958	0.985	0.889	0.941	0.779	0.970	0.985	0.982	0.990	0.970	0.899	0.984	0.997		0.997
Mixture	20mg/kg	0.989	0.740	0.997	0.745	0.796	0.996	0.995	0.991	0.994	0.992	0.752	0.999	0.907	0.997	

Table 1(a.2) Univariate tests of significance for biomass change

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2.05143	1	2.051433	82.50316	0.000000
Pesticide	0.09578	2	0.047890	1.92600	0.146821
Concentration	0.07827	4	0.019567	0.78691	0.534019
Pesticide*Concentration	0.21232	8	0.026539	1.06735	0.384652
Error	12.30813	495	0.024865		

1 (b) Factorial ANOVA test for differences in the number of cocoons

Table 1(b.1) Univariate tests of Significance for the number of cocoons

Effect		Degr. of Freedom	MS	F	p
Intercept	45984.05	1	45984.05	805.9298	0.000000
Pesticide	1285.08	2	642.54	11.2613	0.000167
Concentration	279.08	4	69.77	1.2228	0.318839
Pesticide*Concentration	788.72	8	98.59	1.7279	0.126408
Error	1997.00	35	57.06		

Table1(b.2) Newman-Keuls test for the number of cocoons
Approximate Probabilities for Post Hoc Tests

Pesticide	**Concentration**															
Chlorpyrifos	Control		0.659	0.977	0.944	0.317	0.904	0.957	0.990	0.973	0.983	0.466	0.519	0.514	0.449	0.452
Chlorpyrifos	0.02mg/kg	0.659		0.894	0.808	0.516	0.838	0.885	0.937	0.896	0.926	0.667	0.671	0.695	0.549	0.447
Chlorpyrifos	0.2mg/kg	0.977	0.894		0.778	0.134	0.977	0.979	0.809	0.968	0.962	0.248	0.377	0.321	0.384	0.510
Chlorpyrifos	2mg/kg	0.944	0.808	0.778		0.080	0.948	0.955	0.859	0.952	0.938	0.158	0.260	0.214	0.270	0.384
Chlorpyrifos	20mg/kg	0.317	0.516	0.134	0.079		0.299	0.258	0.198	0.178	0.252	0.718	0.885	0.838	0.926	0.880
Cu oxychl.	Control	0.904	0.838	0.977	0.948	0.299		0.872	0.988	0.961	0.968	0.455	0.539	0.516	0.494	0.546
Cu oxychl.	0.02mg/kg	0.957	0.885	0.979	0.955	0.258	0.872		0.988	0.944	0.936	0.410	0.516	0.480	0.489	0.570
Cu oxychl.	0.2mg/kg	0.990	0.937	0.809	0.859	0.198	0.988	0.988		1.000	0.968	0.342	0.483	0.424	0.485	0.609
Cu oxychl.	2mg/kg	0.973	0.896	0.968	0.952	0.178	0.962	0.944	1.000		0.809	0.310	0.434	0.384	0.430	0.542
Cu oxychl.	20mg/kg	0.983	0.926	0.962	0.938	0.252	0.968	0.936	0.968	0.809		0.408	0.531	0.485	0.516	0.616
Mixture	Control	0.466	0.667	0.248	0.158	0.718	0.455	0.410	0.342	0.310	0.408		0.930	0.841	0.970	0.948
Mixture	0.02mg/kg	0.519	0.671	0.377	0.260	0.885	0.539	0.516	0.483	0.434	0.531	0.930		0.872	0.936	0.930
Mixture	0.2mg/kg	0.514	0.695	0.321	0.214	0.838	0.516	0.480	0.424	0.384	0.485	0.841	0.872		0.968	0.952
Mixture	2mg/kg	0.449	0.549	0.384	0.270	0.926	0.494	0.489	0.485	0.430	0.516	0.970	0.936	0.968		0.778
Mixture	20mg/kg	0.453	0.447	0.510	0.384	0.880	0.546	0.570	0.609	0.542	0.616	0.948	0.930	0.952	0.778	

1(c) **Factorial ANOVA test for differences in hatching success**

Table 1(c.1) Newman-Keuls test for hatching success
Approximate Probabilities for Post Hoc Tests

Pesticides	**Concentration**															
Chlorpyrifos	Control		0.983	0.983	0.877	0.987	0.986	0.991	0.971	0.935	0.394	0.984	0.978	0.978	0.954	0.934
Chlorpyrifos	0.02mg/kg	0.983		0.972	0.644	0.983	0.980	0.985	0.944	0.925	0.379	0.977	0.945	0.960	0.872	0.923
Chlorpyrifos	0.2mg/kg	0.983	0.972		0.998	0.999	0.996	0.994	0.989	0.992	0.682	0.985	0.987	0.954	0.995	0.987
Chlorpyrifos	2mg/kg	0.878	0.644	0.998		0.999	0.999	0.999	0.992	0.992	0.645	0.999	0.996	0.996	0.974	0.991
Chlorpyrifos	20mg/kg	0.987	0.983	0.999	0.999		0.965	0.994	1.000	0.907	0.485	0.994	0.999	0.999	0.999	0.715
Cu oxychl	Control	0.986	0.980	0.996	0.999	0.965		0.974	0.999	0.966	0.580	0.954	0.998	0.999	0.999	0.911
Cu oxychl	0.02mg/kg	0.991	0.985	0.994	0.999	0.994	0.974		0.999	0.983	0.620	0.874	0.997	0.998	0.999	0.969
Cu oxychl	0.2mg/kg	0.971	0.944	0.989	0.992	1.000	0.999	0.999		0.995	0.702	0.998	0.848	0.935	0.967	0.993
Cu oxychl	2mg/kg	0.935	0.925	0.992	0.992	0.907	0.966	0.983	0.995		0.316	0.984	0.989	0.995	0.990	0.959
Cu oxychl	20mg/kg	0.394	0.379	0.682	0.645	0.485	0.580	0.620	0.702	0.316		0.641	0.623	0.703	0.629	0.539
Mixture	Control	0.984	0.977	0.985	0.999	0.994	0.954	0.874	0.998	0.984	0.641		0.996	0.996	0.998	0.965
Mixture	0.02mg/kg	0.978	0.945	0.987	0.996	0.999	0.998	0.997	0.848	0.989	0.623	0.996		0.959	0.958	0.986
Mixture	0.2mg/kg	0.976	0.960	0.954	0.996	0.999	0.999	0.998	0.934	0.995	0.703	0.996	0.959		0.988	0.992
Mixture	2mg/kg	0.954	0.871	0.995	0.974	0.999	0.999	0.999	0.967	0.990	0.629	0.998	0.958	0.988		0.989
Mixture	20mg/kg	0.934	0.923	0.987	0.991	0.715	0.911	0.969	0.993	0.959	0.539	0.965	0.986	0.992	0.989	

Table 1(c.2) Univariate Tests of Significance for hatching success

Effect	SS	Degr. of freedom	MS	F	p
Intercept	117294.8	1	117294.8	839.1921	0.000000
Pesticides	499.7	2	249.9	1.7877	0.182286
Concentrations	798.5	4	199.6	1.4282	0.245155
Pesticides*Concentrations	323.1	8	40.4	0.2890	0.965116
Error	4892.0	35	139.8		

1(d) **Factorial ANOVA test for differences in the number of hatchlings per cocoon**

Table 1(d.1) Univariate Tests of Significance for the number of hatchlings per cocoon

Effect	SS	Degr. of freedom	MS	F	p
Intercept	195.7738	1	195.7738	1869.369	0.000000
Pesticides	0.3954	2	0.1977	1.888	0.166474
Concentrations	0.1632	4	0.0408	0.390	0.814652
Pesticides*Concentrations	1.0030	8	0.1254	1.197	0.328931
Error	3.6655	35	0.1047		

Table 1(d.2) Newman-Keuls test for the number of hatchlings per cocoon
Approximate Probabilities for Post Hoc Tests

Pesticides	**Concentration**															
Chlorpyrifos	Control		0.993	0.974	0.988	0.964	0.999	0.992	0.708	0.974	0.971	0.994	0.868	0.995	0.834	0.981
Chlorpyrifos	0.02mg/kg	0.993		0.986	0.996	0.964	0.752	0.970	0.757	0.985	0.997	0.923	0.995	0.943	0.830	0.968
Chlorpyrifos	0.2mg/kg	0.974	0.986		0.996	0.974	1.000	0.992	0.749	0.987	0.994	0.992	0.959	0.988	0.855	0.984
Chlorpyrifos	2mg/kg	0.988	0.996	0.996		0.867	1.000	0.994	0.691	0.785	0.912	0.996	0.989	0.999	0.828	0.982
Chlorpyrifos	20mg/kg	0.964	0.964	0.974	0.867		0.992	0.943	0.424	0.816	0.925	0.960	0.961	0.985	0.586	0.891
Cu oxychl	Control	0.999	0.752	1.000	1.000	0.992		0.945	0.643	0.997	1.000	0.910	1.000	0.994	0.745	0.936
Cu oxychl	0.02mg/kg	0.992	0.970	0.992	0.994	0.943	0.945		0.684	0.976	0.995	0.892	0.995	0.980	0.696	0.825
Cu oxychl	0.2mg/kg	0.708	0.757	0.749	0.691	0.424	0.643	0.684		0.545	0.724	0.723	0.766	0.704	0.769	0.651
Cu oxychl	2mg/kg	0.974	0.985	0.987	0.786	0.816	0.997	0.976	0.545		0.921	0.984	0.976	0.994	0.705	0.946
Cu oxychl	20mg/kg	0.971	0.997	0.994	0.912	0.925	1.000	0.995	0.724	0.921		0.997	0.977	0.999	0.850	0.986
Mixture	Control	0.994	0.923	0.992	0.996	0.960	0.910	0.892	0.723	0.984	0.997		0.996	0.974	0.777	0.931
Mixture	0.02mg/kg	0.868	0.995	0.959	0.989	0.961	1.000	0.995	0.766	0.976	0.977	0.996		0.998	0.873	0.989
Mixture	0.2mg/kg	0.996	0.943	0.988	0.999	0.985	0.994	0.980	0.704	0.994	0.999	0.974	0.998		0.811	0.969
Mixture	2mg/kg	0.834	0.830	0.855	0.828	0.586	0.745	0.696	0.769	0.705	0.850	0.777	0.873	0.811		0.557
Mixture	20mg/kg	0.981	0.968	0.984	0.982	0.891	0.936	0.825	0.651	0.946	0.986	0.931	0.989	0.969	0.557	

1(e) **Factorial ANOVA test for differences in the neutral red retention time**

Table 1(e.1) Newman-Keuls test for neutral red retention time
Approximate Probabilities for Post Hoc Tests

P1= chlorpyrifos
P2=Cu oxychloride
P3=Mixture
C1=control
C2=0.02mg/kg
C3=0.2mg/kg
C4=2mg/kg
C5=20mg/kg

P1	C1		0.000008	0.000020	0.000010	0.000018	0.181844	0.000022	0.000032	0.000026	0.000015	0.082664	0.000009	0.000017	0.000012	0.000020
P1	C2	0.000008		0.702358	0.861095	0.267487	0.000017	0.765270	0.806813	0.850553	0.296749	0.000020	0.280518	0.810100	0.698193	0.002797
P1	C3	0.000020	0.702358		0.985641	0.663558	0.000026	0.689749	0.952047	0.994194	0.668879	0.000032	0.137506	0.574973	0.938043	0.032076
P1	C4	0.000010	0.861095	0.985641		0.574614	0.000012	0.776195	0.961283	0.938325	0.485878	0.000015	0.131718	0.894007	0.669209	0.037493
P1	C5	0.000018	0.267487	0.663558	0.574614		0.000020	0.162163	0.672276	0.584165	0.891881	0.000023	0.003754	0.357936	0.669038	0.121456
P2	C1	0.181844	0.000017	0.000026	0.000012	0.000020		0.000008	0.000010	0.000032	0.000018	0.423100	0.000022	0.000020	0.000015	0.000023
P2	C2	0.000022	0.765270	0.689749	0.776195	0.162163	0.000008		0.728194	0.802995	0.189515	0.000017	0.221164	0.852133	0.568921	0.000986
P2	C3	0.000032	0.806813	0.952047	0.961283	0.672276	0.000010	0.728194		0.770832	0.630440	0.000012	0.119385	0.825781	0.882723	0.046023
P2	C4	0.000026	0.850553	0.994194	0.938325	0.584165	0.000032	0.802995	0.770832		0.571870	0.000010	0.181800	0.837137	0.869300	0.025578
P2	C5	0.000015	0.296749	0.668879	0.485878	0.891881	0.000018	0.189515	0.630440	0.571870		0.000020	0.005300	0.380726	0.472434	0.211048
P3	C1	0.082664	0.000020	0.000032	0.000015	0.000023	0.423100	0.000017	0.000012	0.000010	0.000020		0.000008	0.000026	0.000018	0.000026
P3	C2	0.000009	0.280518	0.137506	0.131718	0.003754	0.000022	0.221164	0.119385	0.181800	0.005300	0.000008		0.291650	0.050639	0.000020
P3	C3	0.000017	0.810100	0.574973	0.894007	0.357936	0.000020	0.852133	0.825781	0.837137	0.380726	0.000026	0.291650		0.765456	0.005796
P3	C4	0.000012	0.698193	0.938043	0.669209	0.669038	0.000015	0.568921	0.882723	0.869300	0.472434	0.000018	0.050639	0.765456		0.076448
P3	C5	0.000020	0.002797	0.032076	0.037493	0.121456	0.000023	0.000986	0.046023	0.025578	0.211048	0.000026	0.000020	0.005796	0.076448	

Table 1(e.2) Univariate Tests of Significance for neutral red retention time

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	51107.12	1	51107.12	1192.585	0.000000
Pesticides	54.23	2	27.12	0.633	0.532739
Concentrations	18726.03	4	4681.51	109.243	0.000000
Pesticides*Concentrations	414.73	8	51.84	1.210	0.298245
Error	5571.03	130	42.85		

1(f) **Factorial ANOVA test for differences in acetylcholinesterase activity**

Table 1(f.1) Univariate Tests of Significance for AChE activity

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5240747	1	5240747	3158.624	0.000000
Pesticides	26839	2	13420	8.088	0.000491
Concentrations	60993	4	15248	9.190	0.000001
Pesticides*Concentrations	67768	8	8471	5.106	0.000016
Error	214035	129	1659		

Table1(f.2) Newman-Keuls test for AChE activity
Approximate Probabilities for Post Hoc Tests

P1= chlorpyrifos
P2=Cu oxychloride
P3=Mixture
C1=control
C2=0.02mg/kg
C3=0.2mg/kg
C4=2mg/kg
C5=20mg/kg

P1	C1		0.938405	0.129656	0.665745	0.000016	0.741278	0.881424	0.718860	0.899373	0.962007	0.661787	0.894877	0.611542	0.227834	0.000058
P1	C2	0.938405		0.018754	0.821151	0.000026	0.428035	0.852870	0.979177	0.969597	0.896744	0.403006	0.962934	0.435046	0.050403	0.000024
P1	C3	0.129656	0.018754		0.271568	0.020588	0.395026	0.238483	0.060508	0.018497	0.058989	0.543798	0.056021	0.593393	0.731566	0.032114
P1	C4	0.665745	0.821151	0.271568		0.000039	0.879484	0.962846	0.707961	0.770859	0.889681	0.796947	0.814773	0.707961	0.401023	0.000326
P1	C5	0.000016	0.000026	0.020588	0.000039		0.000689	0.000060	0.000015	0.000023	0.000021	0.000897	0.000018	0.000745	0.013678	0.596524
P2	C1	0.741278	0.428035	0.395026	0.879484	0.000689		0.805945	0.591339	0.408674	0.639105	0.976367	0.602608	0.987272	0.338482	0.003232
P2	C2	0.881424	0.852870	0.238483	0.962846	0.000060	0.805945		0.836068	0.816787	0.928786	0.660378	0.886338	0.456031	0.341948	0.000302
P2	C3	0.718860	0.979177	0.060508	0.707961	0.000015	0.591339	0.836068		0.950892	0.991144	0.527182	0.929136	0.507679	0.124372	0.000021
P2	C4	0.899373	0.969597	0.018497	0.770859	0.000023	0.408674	0.816787	0.950892		0.684314	0.379200	0.896744	0.403316	0.048699	0.000021
P2	C5	0.962007	0.896744	0.058989	0.889681	0.000021	0.639105	0.928786	0.991144	0.684314		0.596755	0.969597	0.608587	0.129354	0.000024
P3	C1	0.661787	0.403006	0.543798	0.796947	0.000897	0.976367	0.660378	0.527182	0.379200	0.596755		0.550905	0.902257	0.585211	0.004699
P3	C2	0.894877	0.962934	0.056021	0.814773	0.000018	0.602608	0.886338	0.929136	0.896744	0.969597	0.550905		0.549810	0.120416	0.000021
P3	C3	0.611542	0.435046	0.593393	0.707961	0.000745	0.987272	0.456031	0.507679	0.403316	0.608587	0.902257	0.549810		0.683585	0.004391
P3	C4	0.227834	0.050403	0.731566	0.401023	0.013678	0.338482	0.341948	0.124372	0.048699	0.129354	0.585211	0.120416	0.683585		0.034513
P3	C5	0.000058	0.000024	0.032114	0.000326	0.596524	0.003232	0.000302	0.000021	0.000021	0.000024	0.004699	0.000021	0.004391	0.034513	

2. ARTIFICIAL GROUNDWATER EXPERIMENTS

2(a) **Factorial ANOVA test for differences in AChE activity** *(Chlorpyrifos and copper oxychloride)*

P1= chlorpyrifos
P2=Cu oxychloride
P3=Mixture
C1=control
C2=0.002mg/l
C3=0.02mg/l
C4=0.2mg/l
C5=2mg/l

Table 2(a.1) Newman-Keuls test for AChE activity
Approximate Probabilities for Post Hoc Tests

P1	C1		0.814898	0.796498	0.663040	0.017231	0.829122	0.646574	0.881436	0.852910	0.817437	0.764883	0.833981	0.691467	0.681270	0.100156
P1	C2	0.814898		0.924887	0.891383	0.206185	0.954266	0.942514	0.901414	0.773210	0.917131	0.500653	0.930766	0.979740	0.956648	0.513951
P1	C3	0.796498	0.924887		0.945800	0.206735	0.905589	0.924809	0.735164	0.765280	0.978440	0.504644	0.895960	0.985773	0.974064	0.533127
P1	C4	0.663040	0.891383	0.945800		0.295774	0.924015	0.868455	0.899171	0.591331	0.725391	0.305709	0.870156	0.993686	0.958553	0.580312
P1	C5	0.017231	0.206185	0.206735	0.295774		0.113855	0.056567	0.118002	0.011027	0.202138	0.002310	0.066397	0.160073	0.233759	0.497060
P2	C1	0.829122	0.954266	0.905589	0.924015	0.113855		0.918977	0.932637	0.836620	0.970864	0.632545	0.782173	0.958214	0.945717	0.383191
P2	C2	0.646574	0.942514	0.924809	0.868455	0.056567	0.918977		0.964034	0.794865	0.948873	0.654088	0.909390	0.897209	0.886302	0.245074
P2	C3	0.881436	0.901414	0.735164	0.899171	0.118002	0.932637	0.964034		0.870130	0.949285	0.659543	0.930254	0.952634	0.933683	0.382261
P2	C4	0.852910	0.773210	0.765280	0.591331	0.011027	0.836620	0.794865	0.870130		0.766799	0.609706	0.871707	0.608418	0.603431	0.070842
P2	C5	0.817437	0.917131	0.978440	0.725391	0.202138	0.970864	0.948873	0.949285	0.766799		0.482458	0.945004	0.967414	0.913714	0.483433
P3	C1	0.764883	0.500653	0.504644	0.305709	0.002310	0.632545	0.654088	0.659543	0.609706	0.482458		0.707958	0.308292	0.309447	0.018684
P3	C2	0.833981	0.930766	0.895960	0.870156	0.066397	0.782173	0.909390	0.930254	0.871707	0.945004	0.707958		0.908287	0.893665	0.269413
P3	C3	0.691467	0.979740	0.985773	0.993686	0.160073	0.958214	0.897209	0.952634	0.608418	0.967414	0.308292	0.908287		0.955965	0.246063
P3	C4	0.681270	0.956648	0.974064	0.958553	0.233759	0.945717	0.886302	0.933683	0.603431	0.913714	0.309447	0.893665	0.955965		0.442437
P3	C5	0.100156	0.513951	0.533127	0.580312	0.497060	0.383191	0.245074	0.382261	0.070842	0.483433	0.018684	0.269413	0.246063	0.442437	

Table 2(a.2) Univariate Tests of Significance for AChE activity in chlorpyrifos and copper oxychloride

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	12907232	1	12907232	1652.443	0.000000
Pesticides	36571	2	18286	2.341	0.103225
Concentrations	189048	4	47262	6.051	0.000283
Pesticides*Concentrations	85594	8	10699	1.370	0.223678
Error	585825	75	7811		

2(b) **Factorial ANOVA test for differences in AChE activity** *(chlorpyrifos and azinphos methyl)*

Table 2(b.1) Univariate Tests of Significance for AChE activity in chlorpyrifos and Azinphos methyl

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	3070712	1	3070712	1102.044	0.000000
Pesticides	52918	2	26459	9.496	0.000174
Concentrations	469281	4	117320	42.105	0.000000
Pesticides*Concentrations	37699	8	4712	1.691	0.110455
Error	264706	95	2786		

Table 2(b.2) Newman-Keuls test for AChE activity
Approximate Probabilities for Post Hoc Tests

P1= chlorpyrifos
P2=Azinphos methyl
P3=Mixture
C1=control
C2=0.002mg/l
C3=0.02mg/l
C4=0.2mg/l
C5=2mg/l

P1	C1		0.968933	0.806609	0.139596	0.003514	0.834482	0.455577	0.539671	0.226171	0.000171	0.716209	0.775807	0.655580	0.000752	0.000159
P1	C2	0.968933		0.962277	0.119412	0.002403	0.976270	0.219189	0.398799	0.213576	0.000132	0.786037	0.888753	0.683931	0.000529	0.000119
P1	C3	0.806609	0.962277		0.185560	0.006100	0.892237	0.425434	0.480183	0.272910	0.000159	0.696774	0.665163	0.682659	0.001378	0.000133
P1	C4	0.139596	0.119412	0.185560		0.173431	0.106410	0.003783	0.003658	0.730262	0.000644	0.419981	0.317186	0.435384	0.145474	0.000550
P1	C5	0.003514	0.002403	0.006100	0.173431		0.002168	0.000187	0.000134	0.203917	0.027707	0.035798	0.017458	0.051050	0.601480	0.018636
P2	C1	0.834482	0.976270	0.892237	0.106410	0.002168		0.417338	0.546391	0.186992	0.000119	0.711963	0.810304	0.621429	0.000492	0.000171
P2	C2	0.455577	0.219189	0.425434	0.003783	0.000187	0.417338		0.950063	0.009711	0.000134	0.159148	0.268029	0.098654	0.000121	0.000132
P2	C3	0.539671	0.398799	0.480183	0.003658	0.000134	0.546391	0.950063		0.009658	0.000141	0.170084	0.295152	0.102330	0.000134	0.000134
P2	C4	0.226171	0.213576	0.272910	0.730262	0.203917	0.186992	0.009711	0.009658		0.000328	0.461712	0.401761	0.376043	0.119542	0.000298
P2	C5	0.000171	0.000132	0.000159	0.000644	0.027707	0.000119	0.000134	0.000141	0.000328		0.000123	0.000133	0.000127	0.058132	0.939360
P3	C1	0.716209	0.786037	0.696774	0.419981	0.035798	0.711963	0.159148	0.170084	0.461712	0.000123		0.706838	0.764166	0.011227	0.000123
P3	C2	0.775807	0.888753	0.665163	0.317186	0.017458	0.810304	0.268029	0.295152	0.401761	0.000133	0.706838		0.776671	0.004555	0.000121
P3	C3	0.655580	0.683931	0.682659	0.435384	0.051050	0.621429	0.098654	0.102330	0.376043	0.000127	0.764166	0.776671		0.019233	0.000128
P3	C4	0.000752	0.000529	0.001378	0.145474	0.601480	0.000492	0.000121	0.000134	0.119542	0.058132	0.011227	0.004555	0.019233		0.027379
P3	C5	0.000159	0.000119	0.000133	0.000550	0.018636	0.000171	0.000132	0.000134	0.000298	0.939360	0.000123	0.000121	0.000128	0.027379	

2(c) **Factorial ANOVA test for differences in AChE activity** *(chlorpyrifos and cypermethrin)*

Table 2(c.1) Newman-Keuls test for AChE activity
Approximate Probabilities for Post Hoc Tests

P1= chlorpyrifos
P2=cypermethrin
P3=Mixture
C1=control
C2=0.002mg/l
C3=0.02mg/l
C4=0.2mg/l
C5=2mg/l

P1	C1		0.999514	0.971925	0.166304	0.007280	0.952295	0.997720	0.997565	0.988895	0.997446	0.968392	0.919620	0.971401	0.998731	
P1	C2	0.999514		0.999403	0.221620	0.008356	0.716302	0.986607	0.999796	0.999445	0.978019	0.850038	0.999564	0.997767	0.860588	
P1	C3	0.971925	0.999403		0.138009	0.007667	0.947691	0.998447	0.994742	0.928414	0.998304	0.960009	0.899886	0.993603	0.998483	
P1	C4	0.166304	0.221620	0.138009		0.207040	0.043744	0.222613	0.040730	0.098661	0.202514	0.046097	0.153928	0.194825	0.174623	
P1	C5	0.007280	0.008356	0.007667	0.207040		0.000828	0.009535	0.003487	0.006376	0.007672	0.000845	0.007458	0.008375	0.005499	
P2	C1	0.952295	0.716302	0.947691	0.043744	0.000828		0.881808	0.961289	0.946407	0.850687	0.976914	0.954507	0.927462	0.547677	
P2	C2	0.997720	0.986607	0.998447	0.222613	0.009535	0.881808		0.999732	0.998949	0.897692	0.927462	0.998415	0.976914	0.987249	
P2	C3	0.997565	0.999796	0.994742	0.040730	0.003487	0.961289	0.999732		0.993468	0.999564	0.968452	0.996029	0.999210	0.999328	
P2	C4	0.988895	0.999445	0.928414	0.098661	0.006376	0.946407	0.998949	0.993468		0.998682	0.957051	0.974569	0.996646	0.998494	
P2	C5	0.997446	0.978019	0.998304	0.202514	0.007672	0.850687	0.897692	0.999564	0.998682		0.918437	0.998369	0.986370	0.977389	
P3	C1	0.968392	0.850038	0.960009	0.046097	0.000845	0.976914	0.927462	0.968452	0.957051	0.918437		0.967595	0.953842	0.802517	
P3	C2	0.919620	0.999564	0.899886	0.153928	0.007458	0.954507	0.998415	0.996029	0.974569	0.998369	0.967595		0.989733	0.998853	
P3	C3	0.971401	0.997767	0.993603	0.194825	0.008375	0.927462	0.976914	0.999210	0.996646	0.986370	0.953842	0.989733		0.996329	
P3	C4	0.998731	0.860588	0.998483	0.174623	0.005499	0.547677	0.987249	0.999328	0.998494	0.977389	0.802517	0.998853	0.996329		
P3	C5															

Table 2(c.2) Univariate Tests of Significance for AChE activity in chlorpyrifos and cypermethrin

Effect	SS	Degr. of Freedom	MS	F	p
Pesticides	27761.0	1	27761.00	8.617716	0.004247
Concentrations	21064.2	3	7021.40	2.179621	0.096073
Pesticides*Concentration	43416.2	7	6202.31	1.925355	0.074857
Error	283482.1	88	3221.39