THE EFFECTS OF ORGANOPHOSPHATE EXPOSURE ON NON TARGET TERRESTRIAL AND AQUATIC ORGANISMS FOLLOWING DIFFERENT EXPOSURE REGIMES: LINKING BIOMARKER RESPONSES AND LIFE-CYCLE EFFECTS

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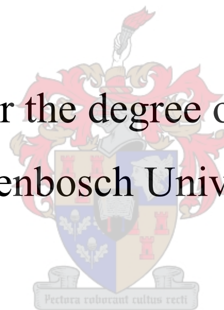

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ABSTRACT

The use of organophosphate pesticides is still an integral part of commercial farming activities and these substances have been implicated as a major source of environmental contamination in South Africa. Evidence exists that many non target animals in and around agricultural areas are at risk of being affected due to the mobile nature of pesticides and the intermittent nature of pesticide application. The extent to which non-target animals are affected by exposure to two organophosphates (azinphos-methyl and chlorpyrifos) was investigated through monitoring selected biomarker responses and life cycle effects under laboratory conditions in two selected test species. A representative species from both the aquatic and terrestrial environment was used as these two compartments of the environment are inevitably linked due to the mobility of pesticides from the area of application to surrounding areas. The earthworm *Eisenia fetida* was used as test organism in the terrestrial environment while the fish *Oreochromis mossambicus* served as representative of the aquatic environment. Juvenile life stages of both species were subjected to standard acute toxicity tests which showed that for both species, juvenile life stages were more sensitive to both pesticides than adults. It was also illustrated that azinphos-methyl is more toxic than chlorpyrifos to both species.

Both test species were also subjected to an intermittent exposure regime in order to assess the effects of repeated pesticide application on biomarker, life-cycle and behaviour responses. The results indicated that for similar exposure regimes, azinphos-methyl was more toxic to *E. fetida* than chlorpyrifos and detrimentally affected all endpoints investigated. The present study suggests that exposure concentration may have a more pronounced effect in inducing a toxic response than exposure interval, irrespective of the pesticide used. In addition to this, *E. fetida* was unable to avoid the presence of these pesticides in soil, even at concentrations as high as 50% of the LC_{50} value, indicating that the presence of pesticides in the soil pose a realistic threat to earthworms and other soil dwelling organisms. Biomarker responses, morphological effects and feeding behaviour was assessed for *O. mossambicus* and similar to the terrestrial toxicity experiments, there was evidence to suggest that in the case of an intermittent exposure scenario, azinphos-methyl was more hazardous than chlorpyrifos to this species. For the majority of endpoints that were investigated, it appeared that exposure interval played a more important role in inducing an effect than exposure concentration. At a

shorter exposure interval, the majority of endpoints showed no difference between higher and lower exposure concentrations, while at a longer exposure interval the effects of exposure concentration became evident. In addition, feeding behaviour was affected by pesticide exposure in a dose-dependent manner.

The present study yielded important results that improve the understanding of biological impacts of pesticide pollution on the environment. This can aid in optimising farming practices such as pesticide application not only in terms of eradicating the pest organisms, but also in terms of mitigating the environmental effects associated with large-scale pesticide use, thereby ensuring sustained biodiversity in these areas.

OPSOMMING

Die gebruik van organofosfaat plaagdoders is ‘n integrale deel van kommersiële landbou aktiwiteite maar hierdie middels is ook ‘n prominente bron van omgewingsbesoedeling in Suid-Afrika. Daar is bewys dat verskeie nie-teiken diere in en om landbouareas geaffekteer word weens die nie-statiese aard van plaagdoders in die omgewing, sowel as die herhalende aard van plaagdodertoediening. Die graad waartoe nie-teiken diere geaffekteer word deur die plaagdoders azinphos-metiel en chlorpyrifos is ondersoek deur die monitering van verskeie biomerkerresponse en lewenssiklus-effekte in geselekteerde toetsspesies binne ‘n beheerde laboratoriumomgewing. ‘n Verteenwoordigende spesie van beide die akwatiese en die terrestriële omgewing is gebruik aangesien hierdie twee dele van die omgewing onlosmaaklik verbind is weens die beweging van plaagdoders vanaf die area van toediening na omringende areas. Die erdwurm *Eisenia fetida* is gekies as toetsorganisme vir die terrestriële omgewing en die varswatervis *Oreochromis mossambicus* het gedien as verteenwoordigende spesie vir die akwatiese omgewing. Onvolwasse diere van beide spesies is onderwerp aan standaard akute toksisiteitstoetse en daar is gevind dat, vir beide spesies, onvolwasse diere meer sensitief vir die betrokke plaagdoders is as volwasse diere. Dit is ook gevind dat azinphos-metiel giftiger is as chlorpyrifos vir beide spesies.

Beide toetsspesies is ook onderwerp aan ‘n chroniese blootstellingsregime om die effek van herhaalde plaagdodertoediening op biomerker-, lewenssiklus- en gedragsresponse te ondersoek. Die resultate van die herhaalde blootstelling het aangedui dat vir soortgelyke blootstellingsregimes, azinphos-metiel giftiger is as chlorpyrifos vir *E. fetida* en dat beide middels alle eindpunte wat ondersoek is, nadelig affekteer. Die huidige studie toon ook bewyse dat blootstellingskonsentrasie ‘n meer prominente effek as blootstellingsinterval kan hê in die teweegbringing van ‘n toksiese respons. Verder was *E. fetida* nie in staat om die teenwoordigheid van die plaagdoders in grond te vermy nie, self nie by konsentrasies so hoog as 50% van die LC_{50} waarde nie. Laasgenoemde resultaat dui dus aan dat die aanwesigheid van plaagdoders in die grondomgewing ‘n realisitese bedreiging inhou vir erdwurms en ander grondorganismes.

Soortgelyk aan die terrestriële toksisiteitseksperimente, was daar getuienis vir die verhoogde toksisiteit van azinphos-metiel relatief tot chlorpyrifos vir *O. mossambicus.* Dit blyk dat

blootstellingsinterval ‘n meer prominente rol as blootstellingskonsentrasie speel in die teweegbringing van effekte vir die meerderheid van die eindpunte wat ondersoek is. In die geval van ‘n korter blootstellingsinterval het die meerderheid van eindpunte wat ondersoek is geen verskille getoon tussen ‘n hoër en ‘n laer konsentrasie nie, terwyl met ‘n langer blootstellingsinterval daar ‘n aanduiding was dat blootstellingskonsentrasie ‘n meer prominente rol gespeel het. Verder is gevind dat voedingsgedrag in *O. mossambicus* geaffekteer is op ‘n konsentrasie verwante manier.

Die huidige studie toon resultate wat ‘n belangrike bydrae kan lewer tot die begrip van die biologiese impakte van organofosfaat plaagdoders op die omgewing. Die resultate kan gebruik word vir die optimisering van boerderypraktyke soos plaagdodertoediening, sodat laasgenoemde effektief is vir die beheer van pes-organismes, maar ook die impakte van grootskaalse plaagdodertoediening kan minimaliseer en sodoende die biodiversiteit binne hierdie areas sal beskerm.

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CHAPTER ONE

INTRODUCTION

1.1 Pesticide use and human welfare

The use of pesticides has improved the quality of life for large sectors of the human population in two fundamental ways. Firstly, the use of pesticides for the control of disease vectors has significantly reduced the impact of diseases such as typhus and malaria in tropical and subtropical areas (Dikshith, 1991). Secondly, the yield of important food crops such as wheat and maize has increased significantly with the use of pesticides (Dinham, 1993). In contrast to the many short- and medium term benefits to the agricultural sector and playing a major role in controlling vector borne diseases, there is growing evidence that continued pesticide use is posing a risk to both human and environmental health (Lu, 1991; London et al., 2000; Schulz, 2004). Chronic or intermittent low dose exposures to pesticides are increasingly thought to be the cause of chronic health problems in humans, including reproductive, immunological, respiratory and carcinogenic effects (Maroni and Fait, 1993; Mansour, 2004).

The U.S. Environmental Protection Agency (USEPA) defines a pesticide as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (Ecobichon, 2001). The word "pesticide" can thus be seen as a generic name for a group of chemicals that are classified on the basis of the pattern of use and organism killed. In addition to the important agricultural pesticide classes that encompass insecticides, herbicides and fungicides, pest control agents are grouped as acaricides, larvicides, miticides, molluscides, rodenticides and scabicides (Ecobichon, 2001).

1.2 Adverse environmental effects associated with pesticide use

In contrast to many pollutants that are by-products of industrial activities, pesticides are pollutants that are deliberately applied to the environment (Walker et al., 1996). In some cases pesticides are applied directly to water bodies to control aquatic weeds, algae, pest fish, undesired invertebrates and insects. In most instances, however, they are applied to the terrestrial environment, mainly to combat agricultural pests associated with crop production

and from there are transported to the aquatic environment via different routes, mainly runoff and spray drift. The amount of pesticide that is transferred in this way depends on a number of factors such as the nature of the pesticide application, ambient weather conditions at the time of spraying, the time interval between pesticide application and heavy rainfall, the slope and soil type of the catchment area and whether vegetation buffer areas are present along rivers and dams (Wauchope, 1978, Schulz et al., 2001b).

Pesticide use is aimed at controlling hazardous pest organisms, but in many instances it also poses a threat to many species that are not considered to be environmental pests. As a result the concepts of "target" and "non target" organisms have been formulated (Nimmo, 1985). The term "target organism" refers to the pest organisms to be eradicated, while the term "non target organism" refers to beneficial organisms that are important for ecological functioning and are at risk of being affected by pesticide use. For example, in the aquatic environment, pesticides are mainly used for the control of undesired organisms such as mosquitoes, often with the result that nontarget organisms such as macroinvertebrates, fish and amphibians are also affected (Ward et al., 1995; Schultz and Liess, 1999). In the terrestrial environment, earthworms, mites and springtails are but a few examples of beneficial nontarget soil organisms that may be negatively affected by pesticides used for the control of pest organisms in agricultural areas (Booth et al., 1998; O'Halloran et al., 1999).

In addition to impacting negatively on beneficial soil biota, there is evidence that pesticide pollution affects numerous other non target species. An example of pesticides affecting non target vertebrate organisms is the deleterious effects that pesticide application has on a number of bird species. Pesticide use has been implicated in the decline in numbers and distribution ranges of several bird species that are common in farmland areas. This is either as a result of direct accidental poisoning of birds, but in most cases the decline can be linked to a pesticide-induced decline in the invertebrates that serve as food during the breeding season. Chick survival and invertebrate availability has been correlated for a number of bird species (Moreby et al., 2001). Eggshell thinning brought on by the organochlorine pesticide DDT and its metabolite DDE in a number of bird species is another example of pesticides affecting birds (Connell et al., 1999). Reyes et al. (2002) reported that pesticide runoff into marine aquaculture areas can partly explain the decrease in shrimp production along parts of the Mexican coastline, illustrating that even the marine environment is affected by terrestrial

pesticide use. Agricultural pesticide application has also been cited as a potential cause for the decline in amphibians but few studies have directly linked pesticide application to amphibian decline (Richards and Kendall, 2002). The most important problem resulting from adverse effects on non target species is that many of these organisms have an indispensable ecological role in the environment and their disappearance or decline may negatively impact on ecological processes and adversely affect food webs and other aspects of ecological community structure (Bretaud et al., 2000).

1.3 Development and classification of pesticides

1.3.1 Inorganic pesticides

Modern organic pesticides as is known today only became an integral part of agricultural production less than 100 years ago. Inorganic chemicals such as sulphur played a major role in combating agricultural pests until the 1930s, when the first synthetic organic pesticides, the dinitro compounds and thiocyanates, were introduced (Dikshith, 1991). As late as 1950, substantial amounts of inorganic chemicals were still in use, including calcium arsenate, copper sulphate, lead arsenate and sulphur (Klassen et al., 1982). With the exception of sulphur, these inorganic pesticides were almost completely displaced by synthetic organic pesticides in subsequent years (Plimmer, 2001).

1.3.2 Organic pesticides

The 1940s and 1950s were productive years in terms of synthetic organic chemistry and pesticide development (Ware, 1978). The development and large-scale commercial application of pesticides began in the early 1940s with the introduction of DDT, an organochlorine compound (Smith, 2001). The organochlorines are insecticides that consist of carbon, chlorine and hydrogen as basic molecules (Ware, 1978) and affect the neural transmission in organisms (Plimmer, 2001). DDT was first synthesized as early as 1874, but it was only when its insecticidal properties were discovered in 1939 that it was produced in large quantities (Dikshith, 1991). Later, when the long environmental half-life and adverse effects of DDT on human and environmental health became evident, restrictions on its use reduced the production volume and today DDT is no longer important as a commercial product (Connell et al., 1999; Wibe et al., 2004). The organochlorines have to a large extent been replaced by other groups of pesticides, but a few products, such as endosulfan, are still in use to control pests associated with cotton and deciduous fruit production (Broomhall, 2002). Organic pesticide groups that were subsequently developed to replace the

environmentally persistent organochlorines include the organophosphates, carbamates and pyrethroids (Carr et al., 1997).

1.3.2.1 Organophosphates

The organophosphates were first synthesized in 1945 and were partly the result of finding modifications of chemical warfare agents (such as sarin and soman) useful as insecticides (Walker et al., 1996; Hill, 2003). The organophosphates are all derived from phosphoric acid and have the highest vertebrate toxicity of all pesticides (Ware, 1978). The main mechanism by which the organophosphates exert a toxic effect is the inhibition of cholinesterases (ChEs), an important group of enzymes of the nervous system of both vertebrates and invertebrates (Ware, 1978; Walker and Thompson, 1991). Organophosphates are used on a large scale in the agricultural sector to control invertebrate pests associated with fruit and cotton production. Other uses include spraying of nesting sites of birds, such as certain finch species that are considered agricultural pests and controlling disease vectors such as mosquitoes. Commercially important organophosphates include parathion, malathion, monocroptophos, diazinon, methyl parathion and chlorpyrifos. Organophosphates comprise more than one third of the registered pesticides presently on the world market (Hill, 2003).

1.3.2.2 Carbamates

The carbamate group of insecticides are all derivatives of carbamic acid and were first synthesized in the early 1930s (Walker et al., 1996). Interest in the development of carbamates was renewed in the mid-1950s when there was a need for insecticides having anticholinesterase activity with less mammalian toxicity than the organophosphates that were in use at the time. Similar to the organophosphates, carbamates also exert a toxic effect through the inhibition of ChEs but in general they are less toxic to mammals than organophosphates (Ware, 1978; Ecobichon, 2001). Onset of toxic effects and recovery from carbamate exposure is generally faster than for organophosphates, as carbamates are direct ChE inhibitors that do not require metabolic activation (Hill, 2003). Carbamates are mostly used to combat agricultural pests, but are also used to control household insects such as cockroaches. Commercially important carbamates include carbaryl, methomyl and propoxur, better known as Baygon®.

1.3.2.3 Pyrethroids

The pyrethroids are a relatively new class of pesticides and are synthetic materials analogous to the natural insecticide pyrethrin. Investigations into the chemical structure of natural

pyrethrin were started in the 1920s, but it was only after the 1970s that intensive efforts were made to develop synthetic pyrethroids (Kaneko and Miyamoto, 2001). The mode of action of pyrethroids is similar to that of the organochlorines, namely disruption of ion channels of cell membranes, and they are generally more toxic to insects than to mammals (Shaw and Chadwick, 1998). Natural pyrethrin, obtained from the chrysanthemum species *Chrysanthemum cinerariaefolium*, is not useful for agricultural purposes because of its high production costs and instability in sunlight (Ware, 1978; Plimmer, 2001). In contrast, the synthetic pyrethroids are very stable in sunlight and are generally effective against most agricultural pests when used at a low application rate. The most common pyrethroid pesticides are permethrin (Ambush®) and fenvalarate (Pydrin®).

1.4 Characteristics and mechanism of action of organophosphates

1.4.1 Chemical structure

Generally, *organophosphate* is used as a generic term to include all of the insecticides containing phosphorus. Organophosphates are esters which all have the same basic structure as indicated in Figure 1.1.

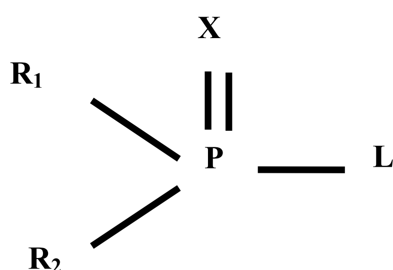


Figure 1.1: The basic molecular structure of organophosphorus pesticides (Ware, 1978)

The letter L (Figure 1.1) represents a reactive and variable group and is the so-called "leaving group". This is the substituent that is replaced when the organophosphate phosphorylates acetylcholinesterase, the primary target enzyme in the nervous system of organisms. The leaving group is also usually the most susceptible to hydrolysis and thus breakdown. The letters R1 and R2 (Figure 1) represents less reactive groups and are most often alkoxy groups, but may also be alkyl-, aryl, alkylthio-, or alkylamino groups. The letter X (Figure 1) represents either oxygen or sulphur (Moriarty, 1999; Chambers et al., 2001). Malathion and dimethoate are examples of organophosphates containing sulfur while monocroptophos and dichlorvos are examples containing oxygen. The organophosphates can be subdivided into

three classes, namely the aliphatic, phenyl, and heterocyclic derivatives based on their chemical structure (Ware, 1978). The environmental half-lives of organophosphate pesticides vary between the different pesticides and may also vary between different formulations of the same pesticide (Hill, 2003).

1.4.2 Physical properties

The physical and chemical characteristics of organophosphate pesticides are significant for determining their activity and their eventual effects on ecosystems. Organophosphates are mostly broken down by UV radiation and soil microorganisms and are generally regarded as non-persistent in the environment (Hill, 2003). The physical properties of these pesticides, such as partition coefficient, adsorption coefficient and water solubility, are to a large extent determined by the chemical structure of the compound and may differ by an order of magnitude between different compounds. For example, the water solubility of azinphos-methyl is only 2mg/l, while it is 2500mg/l for dimethoate (Nimmo, 1985). Substances with high water solubility tend not to adsorb to organic compounds and are more likely to disperse in the environment. The heterocyclic organophosphate group, including products such as diazinon, is generally the most complex and usually has longer-lasting residues in the environment than many of the aliphatic or phenyl derivatives such as monocroptophos and parathion (Ware, 1978).

1.4.3 Mechanism of action of organophosphates

The main mechanism of action of the organophosphates is the inhibition of cholinesterases, a group of enzymes critical to the normal functioning of the nerves in animals with complex nervous systems (Heath, 1961; O'Brien, 1967; Connell et al., 1999). In these organisms, synapses exist between neurons or between neurons and muscle or gland cells. In these synapses, cell communication is made possible through a neurotransmitter, such as acetylcholine or butyrylcholine, which is released by the presynaptic neuron (Figure 1.2). When an impulse is generated, this neurotransmitter changes the resting potential in the plasma membrane of the receptive segment of the postsynaptic cell, creating an action potential in that cell which in turn continues the transmission of the impulse (Costa, 1988; Miller and Harley, 1999). When the nerve impulse reaches the end bulb of the axon, it causes storage vesicles containing the neurotransmitter to release the neurotransmitter through exocytosis into the synaptic cleft. When released, the neurotransmitter binds with the receptor protein in the postsynaptic membrane, causing a depolarization similar to that of the

presynaptic cell and allowing the impulse to continue its path. Once the neurotransmitter has crossed the synaptic cleft, an enzyme (such as acetylcholinesterase in the case of the neurotransmitter acetylcholine) quickly inactivates it, thus returning the cell to its original resting state (Costa, 1988; Miller and Harley, 1999). In the event of organophosphate exposure, the pesticide binds to the active site on the cholinesterase enzyme, resulting in a stable, unreactive inhibited enzyme. This in turn causes an accumulation of free, unbound acetylcholine at the nerve ending and thus a continual stimulation of electrical activity (Costa, 1988; Miller and Harley, 1999).

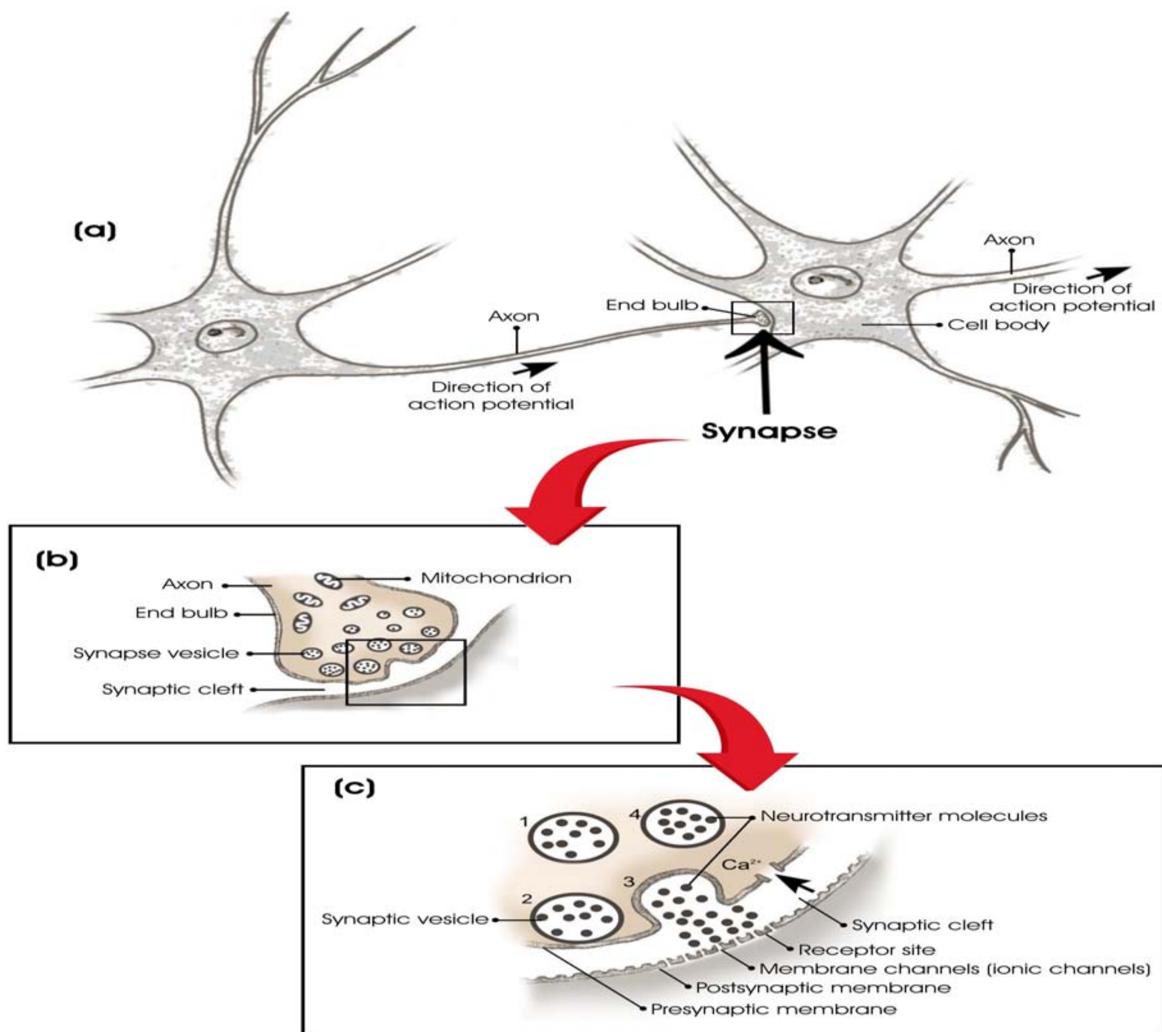


Figure 1.2: Chemical transmission across a synapse. (a) Pre- and postsynaptic neurons with end bulb. (b) Enlarged view of the end bulb containing synaptic vesicles. (c) Enlargement of a portion of the end bulb showing exocytosis. The sequence of events in neurotransmitter release is: (1) a synaptic vesicle containing neurotransmitter approaches the plasma membrane; (2) due to the influx of calcium ions, the vesicle fuses with the membrane; (3) exocytosis occurs; and (4) the vesicle reforms and begins to fill with more neurotransmitter. (Original artwork from Miller and Harley, 1999, redrawn by Judith Piek).

Cholinesterases are widely distributed throughout the animal kingdom, mainly associated with nerve tissue and represent a subclass of "B" esterases that specifically hydrolyse cholinesters (Walker and Thompson, 1991). Cholinesterases belong to a group of enzymes termed "serine hydrolases" because the amino acid serine is found at the active site of these enzymes. Esterases are classified into two main classes: (a) the cholinesterases that include acetylcholinesterase (substrate specificity for acetylcholine) and butyrylcholinesterase (substrate specificity for acetylcholine and butyrylcholine); and (b) the "unspecified" carboxylesterases that can hydrolyse a wide range of esters (Walker and Thompson, 1991).

Not all organophosphates are direct cholinesterase inhibitors. Many organophosphate compounds, typically those belonging to the phosphorothioate group, must be metabolically converted to its oxygen analog in order to become an active inhibitor of cholinesterase (O'Brien, 1967). The generally used pesticide chlorpyrifos is an example of an organophosphate that needs to be metabolically activated in order to inhibit the target enzyme (Strauss and Chambers, 1995; Hernandez et al., 1998). Metabolic activation of many organophosphates results in these substances becoming more toxic than the parent compound. Additionally, organophosphates may be converted to nontoxic/noninhibitory compounds through a series of reactions within the organism involving the microsomal mixed-function oxidase (MFO) enzyme system (Gallo and Lawryck, 1991).

Typical symptoms associated with organophosphate poisoning in mammals depend on the severity of exposure, but generally include loss of coordination, excessive lacrimation and salivation, abdominal cramps, fatigue, nausea and involuntary muscle contractions (Hill, 2003). Severe organophosphate intoxication may lead to mortality, usually the result of respiratory failure or cardiac arrest (O'Brien, 1967). With most organophosphorus insecticides, enzyme inhibition is irreversible and recovery is dependent on the synthesis of new enzyme. Therefore the symptoms associated with OP poisoning may persist for some time after exposure (Habig and D'Guilio, 1991).

1.5 Assessment of the effects of organophosphates on biological systems

Pesticide pollution resulting from agricultural practices, and the consequential negative effects on non target species, is a problem characteristic of various types of farming activities in most parts of the world (Booth et al., 1998; Sancho et al., 2000; Rendón-von Osten, 2005).

Quantifying the extent of this phenomenon has proven to be problematic due to the large variety of pesticides in use and the intermittent nature of pesticide application which makes sampling and detection of these substances difficult. In addition, field conditions are not static and environmental variables are known to affect pesticide behaviour. As a result, pesticide residues in the environment tend to show substantial variation in space and time (Van Wijngaarden et al., 1996).

Many organophosphate and carbamate pesticides have relatively short environmental half-lives and the nature of pesticide pollution is often transient, especially in the aquatic environment as many water bodies, such as rivers, are not static (Schulz, 2001). This makes detection of pesticide residues by analytical chemistry techniques complicated, as concentrations may fall below detection limits within hours to days after entering the aquatic system (Phillips et al., 2002). Pesticides can also be highly mobile, especially in the aquatic environment, which makes the time of sampling critical for detecting the presence of a given pesticide. The chemical analyses of abiotic samples (water, sediment, soil etc.) are generally sensitive and accurate to detect the presence of pesticides in the environment, but cannot provide adequate information on potential impacts of contaminants on biological systems. An alternative is the study of physiological responses in living organisms to assess and predict the extent to which this toxicant can affect a given population or ecosystem (Van Gestel and Van Brummelen, 1996; Connell et al., 1999).

Contaminants affect organisms through exerting stress, and in order to predict future impacts of pollutant-induced stress it is necessary to define the stressor and categorize the stress response in the organism or population. A stressor is defined as any physical, chemical or biological entity that can induce an adverse response in an organism (Landis and Yu, 1995). If the organism is unable to acclimatise or adapt to the stressor, physiological, morphological or reproductive changes may occur. This is generally as a result of repartitioning of energy by diverting energy reserves to cope with the increased energy demand associated with stress and away from processes such as growth and reproduction (Calow, 1991; Gibbs et al., 1996; Connell et al., 1999). Decreased growth and reproduction may alter species abundance and may affect the diversity of communities, and therefore are ecologically relevant endpoints to a toxic response (Vermeulen et al., 2001). Biological responses to stress on sub-cellular level, such as changes in the activity of certain enzymes, normally precede effects on whole animal level, such as mortality, growth and reproduction. When monitoring contaminant-

induced stress at sub-cellular or cellular level, it is possible to detect adverse responses before undesirable effects such as reduced growth or mortality become present at population level (Gil and Pla, 2001). Using cellular or molecular level biological responses to detect contaminant-induced stress is referred to as the biomarker approach and many studies have clearly demonstrated the usefulness of biomarkers for the fast and sensitive detection of chemical stresses within organisms (Van Gestel and van Brummelen, 1996; Walker et al., 1996; Chambers et al., 2002).

1.6 Biomarkers

A biomarker is a measurable biological parameter measured at sub-organismal level that changes in response to xenobiotic exposure and other environmental or physiological stressors and can be an indicator of toxicant exposure (Chambers et al., 2002). Biomarkers can broadly be divided into two classes, namely specific and non-specific (Peakall, 1992). A specific biomarker is a response induced by a specific toxicant or class of toxicants. An example from the present study would be the inhibition of cholinesterase activity by organophosphate pesticides. Non-specific biomarkers on the other hand, are not limited to a single toxicant and such biomarker responses can be induced by several different toxicants (Peakall, 1992). An example from the present study would be the neutral red retention time of lysosomes, a biomarker of membrane integrity which thus gives an indication of cellular damage. Biomarkers are often used as instruments for environmental assessment in the field, either as biomarkers of exposure or biomarkers of effect (Walker et al., 1996). Biomarkers of exposure are indicators that an organisms or population has been exposed to a toxicant. The biomarker response, however, may not necessarily be directly related to the toxicant's specific mode of action and may not be predictive of the degree to which the organism or population may be affected. Biomarkers of effect are predominantly associated with the toxicant's mode of action and are sufficiently well characterized to relate the degree of biomarker response to the degree to which the organism or population will be affected (Chambers et al., 2002).

As biomarkers indicate a response due to an environmental chemical that is already present, they have the disadvantage of only being useful in retrospective risk assessment. However, as they are measured at cellular or sub-cellular level, they are highly sensitive and serve as an early warning system of pollution-induced stress. Thus, by monitoring cellular, molecular

and biochemical changes (e.g. gene-expression patterns, DNA integrity, activities of specific enzymes, structure and function of organelles, etc.), the potential harm of an agent can be assessed before more severe consequences such as reproductive failure or mortality occurs (Lam and Wu, 2003).

1.7 Agrichemical use and environmental pesticide pollution in South Africa

The use of agricultural chemicals is widespread in South Africa and this country is the main market for pesticides in sub-Saharan Africa (Dinham, 1993). Herbicides, insecticides and fungicides are the three most important groups of agrichemicals used (London, 1992). The two main consumers of these chemicals are the maize farming and deciduous fruit farming sectors. Maize is predominantly farmed in the central and northern parts of the country and uses a large amount of herbicides for weed control (De Klerk, 1989). Deciduous fruit is produced throughout South Africa, but production is centered in the Western Cape with more than 440km^2 of growing area representing 82% of the orchards in South Africa (London and Myers, 1995; DFTP Statistics, 2004). Deciduous fruit is produced under intensive farming conditions, characterized by a high level of mechanization, substantial labour input and the routine use of large amounts of pesticides and fertilizers. Agricultural chemical usage in this sector is high due to the fact that the economic value of the crop is often reduced to zero on infestation by insects. In the deciduous fruit farming sector as a whole, insecticides account for approximately 25% of total agrichemical usage, with organophosphates being the most important group and comprising more than 50% of insecticide use by weight (London and Myers, 1995). Other important pesticides for this sector include organochlorines, pyrethroids and insect growth regulators (IGRs) (London and Myers, 1995).

Spray drift and runoff originating from orchards has been proven to be an important source of pesticide pollution in both the terrestrial and aquatic environment in South Africa (Schultz, 2001a; Dabrowski et al., 2002; Teske et al., 2002; Schultz, 2004). Important functional groups of soil biota, such as different species of earthworms, are present in these areas and it has been shown that they are adversely affected by the presence of pesticides in an around agricultural land (Reinecke and Reinecke, 2007b). Earthworm migration and/or mortality as a result of the indiscriminate use of pesticides may lead to a long-term reduction in soil fertility with possible effects on crop production. Runoff- and spray drift related pesticide pollution has also been detected in some rivers flowing through orchard areas in the Western

Cape (Thiere and Schultz, 2004). These rivers are home to diverse invertebrate communities (Thiere and Schultz, 2004) and many endemic and highly threatened fish species (Skelton, 2001) which may be adversely affected by the presence of pesticides in surface waters.

As background to the present study, the use of pesticides in the Western Cape deciduous fruit production sector was determined through evaluating spray programs containing details of pesticide use for apple and pear orchards for the 2004/2005 season. Spray programs were obtained from farmers, sales representatives from Terason (the main pesticide supplier to the sector) and personnel from the Agriculture Research Council (ARC). From these spray programs a summary was made in terms of:

i) Which organophosphates are sprayed on a regular basis?
ii) When during the fruit production cycle are they sprayed?
iii) What is the time interval between applications of the same / different substances?

It was concluded that the most commonly used organophosphates are chlorpyrifos, prothiophos, azinphos-methyl and methyl parathion. These pesticides are predominantly applied against infestations of codling moth (*Cydia pomonella*), and mealy bugs (*Planococcus spp*.). The application interval is generally every two weeks, but can range from 11 to 40 days, depending on the severity of the infestation (Schulz et al., 2001a, 2001b). Spraying normally commences towards the end of winter (July/August) and may continue well into the summer months (February/March) (Thiere and Schultz, 2004). In some cases the specific time and duration of spraying is optimised for the nature and severity of the pest infestation. The majority of pesticide application, however, is routine spraying based on pest control programs, which often results in excessive pesticide application as the pesticides are sprayed irrespective of the severity of the pest infestation (London and Myers, 1995).

1.8 Selection of pesticides for the present study

Based on the data obtained from spraying programs of farmers, two organophosphates were selected as the focus of this study. These are Dursban© EC, an emulsifiable concentrate with chlorpyrifos as active ingredient and Azinphos WP, a wettable powder containing azinphos-methyl as active ingredient. These two pesticides are both heterocyclic organophosphate derivatives, but show substantial variation in chemical and toxicological properties and are

thus expected to exhibit different biological effects. A summary of the most important chemical characteristics of both these substances are presented in Table 1.1, followed by a general discussion on each.

Table 1.1: Chemical characteristics of the organophosphates azinphos-methyl and chlorpyrifos

Property	Azinphos-methyl	Chlorpyrifos
Chemical name (IUPAC)	S-(3,4,dihydro-4-oxobenzo (1,2,3)-triazin-3-ylmethal-O,O-dimethyl phosphorodithioate	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
Chemical formula	$C_{10}H_{12}N_3O_3PS_2$	$C_9H_{11}Cl_3NO_3PS$
Molecular weight	317.3	350.62
CAS registry no.	86-50-0	2921-88-2
Water solubility	2 mg/l at 25 ^oC	30 mg/l at 25 ^oC
Partition coefficient	Not available	4.6990
Adsorption coefficient	1000	6070

1.8.1 Azinphos-methyl

Azinphos-methyl is one of the most toxic organophosphate insecticides, causing cholinesterase inhibition for up to several weeks (Gallo and Lawryck, 1991). According to the World Health Organization (WHO) pesticide classification system, which is based on acute dermal and oral toxicity in the adult rat, azinphos-methyl belongs to class IB – those pesticides regarded as highly hazardous (WHO, 2004). It is a broad-spectrum non-systemic insecticide that acts mainly as a contact and stomach poison and is used primarily as a foliar application against leaf feeding insects. In South Africa it is used on a large scale in the deciduous fruit industry, mainly for the control of codling moth (*Cydia pomonella*), Eastern fruit moth (*Grapholita molesta*), Bryobia mites (*Bryobia praetiosa*) and boll worm (*Heliothis armigera*) (Nexus Spray Manual, 2003).

The persistence of azinphos-methyl in soils is variable, but is generally low under field conditions (Wauchope et al., 1992). It is fairly immobile in soils as it adsorbs strongly to soil particles and has low water solubility. Azinphos-methyl also has low leaching potential and

is therefore unlikely to contaminate ground water (Wauchope et al., 1992). The most important breakdown processes are biodegradation, degradation by UV light and hydrolytic decomposition. In water, azinphos-methyl is subjected to rapid degradation by sunlight and micro organisms, with a half-life of up to two days (Tanner and Knuth, 1995).

The relatively rapid breakdown of azinphos-methyl in both soil and water indicates a low accumulation potential in the environment. Therefore repeated applications of this pesticide should theoretically not pose a major threat to non target organisms, provided the time that passes between repeated exposures is long enough to allow the affected organisms to recover from the effects induced by the high acute toxicity of this pesticide.

1.8.2 Chlorpyrifos

According to the WHO pesticide classification system, chlorpyrifos is ranked in class II and regarded as only moderately hazardous (WHO, 2004). It was originally used as a vector control agent for the control of mosquitoes, but is no longer registered for this use in most parts of the world (Hill, 2003). It now finds its main application in the agriculture sector and is used in controlling a variety of pest insects as stated by the British Crop Protection Council in The Pesticide Manual (2000). In the South African agriculture industry chlorpyrifos is mainly used in the fruit industry and is regularly sprayed on table grapes, citrus and deciduous fruits to control arthropod pests (Reinecke and Reinecke, 2007b).

Chlorpyrifos is a moderately persistent pesticide in soils (Howard, 1991). The half-life in soil is normally between 60 and 120 days, but can range from two weeks to over one year (Howard, 1991; Wauchope et al., 1992). Adsorbed chlorpyrifos is subjected to degradation by soil microorganisms, UV light and chemical hydrolysis. In general, chlorpyrifos adsorbs strongly to soil particles and is not readily soluble in water (Racke, 1992; Wauchope et al., 1992). The principal metabolite of chlorpyrifos, TCP (3,5,6-trichloro-2- pyridinol), adsorbs weakly to soil particles and appears to be moderately mobile and persistent in soils (US EPA, 1989). The type of formulation used largely determines the concentration and persistence of chlorpyrifos in water (US EPA, 1986). The pesticide adheres readily to sediments and suspended organic matter (US EPA, 1986). The primary loss of chlorpyrifos from water is through the process of volatilisation and volatility half-lives of 3.5 and 20 days have been estimated for pond water (Racke, 1992). The photolysis half-life of chlorpyrifos was found to be three to four weeks under midsummer conditions in the USA (Schimmel et al., 1983).

In water with a pH of 7 at a temperature of 25° C, the half-life was found to vary between 35 and 78 days (Howard, 1991).

In relation to azinphos-methyl, chlorpyrifos has a substantially longer half-life both in water and in soil. This longer environmental half-life, coupled with repeated pesticide application, indicates a greater accumulation potential in the environment. This implies an increased risk to non target organisms, which may be exposed for longer periods of time due to the pesticide persisting in the water or soil. This risk is exacerbated by repeated pesticide application which results in an intermittent exposure scenario where there will be little or no recovery of the affected organisms if the exposure interval is relatively short, thus not allowing the pesticide concentration in the environment to be reduced to low enough levels where it is not harmful to non target species. In terms of acute toxicity and short term exposures however, chlorpyrifos poses less of a risk than azinphos-methyl due to its lower acute toxicity.

1.9 Selection of test organisms for the present study

Although organophosphates are primarily applied to the terrestrial environment, substantial evidence exists for the movement of these chemicals to the aquatic environment (Bretaud et al., 2000; Schulz, 2004). The aquatic environment presents a fundamentally different exposure situation from the soil environment. In some cases, the half life of contaminants is shorter in water than in soil. As mentioned, this is especially true for the organophosphates, as these pesticides are readily broken down by UV radiation and therefore may persist for longer in the soil environment. This was reported for chlorpyrifos by Poletika et al. (2002). Another very important difference between an aquatic and terrestrial exposure scenario is the bioavailability of the compound in the two compartments of the environment. In soils, many contaminants are mainly bound to the soil particles, thus reducing/affecting their bioavailability to soil dwelling organisms. Soil characteristics, such as moisture content and pH have been proven to be important variables affecting bioavailability of contaminants (Bauer and Römbke, 1997, Connell et al., 1999; Spurgeon et al., 2005). In contrast, most contaminants in the aquatic environment are readily bioavailable, even if these are bound to suspended particles.

The dynamics of exposure duration for the aquatic environment is also likely to differ from the terrestrial environment. In water, the contaminant will disperse throughout the water

body, while in the soil the contaminant, depending on the ambient conditions, is more likely to remain fairly localized (Connell et al., 1999; Reinecke and Reinecke, 2007b). This has two important implications: firstly, a contaminant entering a water body will disperse, thus diluting the concentration of the toxic compound while in the soil the compound is more likely to remain localized and may persist in a high concentration in a selective part of the soil environment. Secondly, when a heterogeneous distribution of a toxicant occurs in the terrestrial environment, a possible avoidance response would allow the animal to move away from areas where the toxicant is present. Due to a potentially homogenous distribution of a toxicant in aquatic medium, the usefulness of an avoidance response, if it exists, would be doubtful.

Due to the mobile nature of organophosphate pesticides, it is clear that organisms in both the terrestrial and aquatic compartment of the environment are at risk of pesticide exposure. These two compartments of the environment are inextricably linked and a study on the environmental effects of pesticide pollution on organisms should include representative species for both of these compartments. During the present study, the earthworm *Eisenia fetida* was used as representative of the soil environment, whilst the freshwater cichlid fish *Oreochromis mossambicus* was selected as representative of the aquatic environment. Neither of the two selected test species is native to the Western Cape, but was selected because the use of indigenous species is limited by their unsuitability to laboratory conditions and the difficulty of obtaining adequate numbers of animals. Juvenile animals were used because growth and maturation, two of the endpoints that were investigated in the present study, necessitated this. It is also well known that newborn and younger animals are in most cases more sensitive to toxicants than older or mature animals (Matsumura, 1975; Rozman et al., 2001). In the case of the two selected test species, adequate literature exists regarding the effects of the selected pesticides on adults individuals, but information on juvenile animals are lacking. Knowing the effects of contaminants on juvenile animals is critical, as survival rates of juveniles play a significant role in recruitment and determination of the future population structure (Fogarty et al., 1991; Heath et al., 1997). Therefore, any factor significantly affecting juvenile survival can adversely impact on the entire population.

1.9.1 Earthworms as test organisms for evaluation of the terrestrial environment

Several reasons exist for selecting an earthworm as a representative species for the terrestrial environment. Earthworms are one of the most ecologically relevant groups of soil biota,

representing 60-80% of total animal biomass in soil (Fitzgerald et al., 1997). The main function of soil organisms is the breakdown and conversion of organic matter into inorganic compounds and the mobilization of nutrients. Soil organisms, especially earthworms, play an integral role in maintaining soil fertility as they contribute to the different processes of decomposition and in the process affect soil aeration, water transport and soil structure (Lee, 1985; Scott-Fordsmand and Weeks, 2000). Additionally, earthworms form part of complex food webs both in the soil and in the broader environment.

As earthworms are in direct contact with the soil, they are extremely vulnerable to soil contaminants, making them ideal for assessing the effects of terrestrial pollutants and serving as biomonitors of soil quality (Bouche, 1992; Reinecke and Reinecke, 2004). The morphology, taxonomy and physiology of many earthworms are well known and many species are easy to culture and handle, making them easy to use in both laboratory and field tests (Edwards and Bohlen, 1996). For these reasons, earthworms have gained acceptance for use in ecotoxicological tests to assess the effects of pollutants on soil-dwelling organisms (Karnak and Hamelink, 1982; Zang et al., 2000). The earthworm *Eisenia fetida* was used as test organism in the present study. This species is regularly used for ecotoxicological testing and is one of the species prescribed by the OECD for the testing of chemicals (OECD, 1984a; OECD, 2004). *E. fetida* is a compost worm occurring in the litter layer and is not a typical soil dwelling species, but its susceptibility to chemicals resembles that of soil inhabiting species (Kula and Larink, 1997).

1.9.2 Fish as test organisms for evaluation of the aquatic environment

Fish are an integral component of most aquatic ecosystems and are therefore relevant organisms for toxicity testing and biomonitoring studies (Slabbert et al., 2004; Kadye, 2008). The understanding of fish responses to the uptake of contaminants has high ecological relevance as exposure to contaminants may induce behavioural or physiological changes, possibly impairing vital functions such as feeding, reproduction or predator avoidance (Little and Finger, 1990; Ballesteros et al., 2009). A number of species are routinely used in biomarker studies and biomonitoring programs and standardised protocols exist for determining both the acute and chronic effects of pollutants in the aquatic environment (OECD, 1984b; OECD, 1992). The relevant OECD guidelines stipulate that the choice of test fish is at the discretion of the laboratory concerned, provided that it fulfils the criteria set down by the organisation. An indigenous species that fulfilled all these requirements was the

freshwater cichlid fish *Oreochromis mossambicus*, commonly known as the Mozambique tilapia. This species was selected as it is known to occur in the study area and has been proven to be a suitable species for ecotoxicological studies (Brackenbury and Appleton, 1997; Shailaja and D'Silva, 2003).

1.10 Selection of endpoints measured in the present study

1.10.1 Inhibition of cholinesterase activity

The inhibition of cholinesterase activity serves as a reliable biomarker both of exposure and of effect of organophosphates (Coppage and Braidech, 1976; Fulton and Key, 2001; Chambers et al., 2002; Vioque-Fernández et al., 2009). It is well accepted that 20% or greater inhibition of AChE in birds, fishes and invertebrates indicates exposure to organophosphate insecticides (Mayer and Ellersiek, 1986). More recently however, there is growing evidence that is AChE activity is also affected by other neurotoxic contaminants including heavy metals and organochlorines (Gill et al., 1990a, 1990b; Sturm et al., 1999; Beauvais et al., 2001; Petraglio et al., 2008). The persistence of ChE inhibition in organisms following organophosphate exposure has been the subject of a number of studies and results indicate that the time for enzyme recovery is a function of the degree of initial inhibition and therefore the nature of the pesticide exposure. This is likely because the recovery of enzyme activity is largely the result of de novo synthesis of enzyme and the greater the degree of inhibition, the more enzyme synthesis is required (Fulton and Key, 2001). As a substantial part of this project focused on the effects of intermittent pesticide exposure, the pattern of ChE inhibition following multiple exposures to a given pesticide was investigated in both *E. fetida* and *O. mossambicus*.

1.10.2 Neutral red retention time

The lysosomal membrane stability assay for use in earthworms was developed by Weeks and Svendsen (1996). The principle of this assay is the ability of the lysosomes of healthy cells to absorb and retain a vital dye, while the same dye will leak out of damaged lysosomes into the surrounding cytosol. The time the dye takes to leak out of the lysosomes is proportional to the integrity of the lysosome membrane and cells with more damage will have a shorter retention time of the dye than cells with less damage. The lysosomal membrane stability assay has been used extensively for assessing the effects of contaminants on earthworms and various authors have found a relationship between the neutral red retention time (NRRT) and

various sublethal endpoints such as growth and reproduction in earthworms (Scott-Fordsmand et al., 1998; Reinecke and Reinecke, 1999; Booth et al., 2001; Reinecke et al., 2002; Maboeta et al., 2004). Booth et al. (2001) reported that the NRRT assay is sensitive to pesticides even at field application rates and that it is sensitive enough to be utilized as a biomarker of organophosphate pollution in the environment. In fish, the NRRT assay has been successfully used on a fish cell line to determine the acute cytotoxic effects of organophosphates. The results from these studies validated the use of this assay on fish cell lines to determine cytotoxicity of organophosphorus compounds (Li and Zhang, 2001; Babín and Tarazona, 2005).

1.10.3 Life cycle parameters and morphological effects

In order for biomarker studies to have ecological relevance, the biomarker response should be linked to relevant life cycle parameters and morphological effects in order to establish a possible relationship between the two endpoints. Reproduction is of particular importance in ecotoxicological assessments because of its influence on population dynamics (Spurgeon et al., 1994). Growth and general body condition can be affected by the presence of contaminants. Contaminant-induced stress may cause energy reserves to be utilized to cope with the increased energy demand associated with stress and away from processes such as growth and reproduction (Calow, 1991; Gibbs et al., 1996). On a morphological level, the function and structure of certain organs such as the gonads and liver may be affected in vertebrates. This normally manifests as histological abnormalities (Wester et al., 2002; da Veiga et al., 2002; Fanta et al., 2003) or atypical organ mass relative to total body mass, which may influence the functionality of the organ (Corsi et al., 2003; Khallaf et al., 2003).

1.10.4 Behavioural effects

Conducting acute and chronic toxicity tests for potentially toxic substances is costly as well as time and labour intensive. The use of behaviour responses, such as avoidance behaviour, has been proposed as a quick screening tool for preliminary assessment of toxicity, especially in the soil environment (Slimak, 1997; Loureiro et al., 2005; Lukkari et al., 2005). Results of avoidance tests using *E. fetida* in nine soils in three independent laboratories correlated well with *Eisenia* reproduction tests (Achazi, 2002) which further supports avoidance tests as a useful tool for ecological risk assessment of soils (Van Zwieten et al., 2004). In the event of a heterogeneous distribution of a toxicant in the aquatic medium, it is possible that organisms can detect and avoid the substance. Generally however, it is unlikely that

toxicants in the aquatic environment would have a heterogeneous distribution, making the usefulness of an avoidance response doubtful. A number of aquatic avoidance tests have been developed and used with some success, but these are generally complicated and require fairly elaborate test protocols (Yeardley et al., 1996). Behavioural responses such as differences in swimming behaviour or modification of feeding response are therefore more suitable behaviour endpoints to investigate in aquatic organisms. Feeding response is a behaviour endpoint that is has been shown to be influenced by toxicant exposure (Grippo and Heath, 2003; Roex et al., 2003). It is also an ecologically relevant endpoint, as any depression of feeding rate will have implications on the energy budget and thus on growth, reproduction and survival of the individual. Subsequently, this may lead to adverse effects at population and/or community level (Kumar and Chapman, 1998).

1.11 Hypothesis, aims and objectives

Null hypothesis:

Two null hypotheses were formulated for the present study:

- Firstly, juvenile animals are not expected to be more sensitive to organophosphate pesticides than adults following acute exposures.
- Secondly, exposure to organophosphate pesticides will not result in negative effects on non-target organisms. Various exposure regimes, comprising of different exposure concentrations and intervals, are unlikely to influence the selected endpoints, namely biomarker responses, growth, reproduction and behaviour, in different ways.

Hypothesis:

Two hypotheses were formulated for the present study:

- Firstly, juvenile animals are expected to be more sensitive to organophosphate pesticides than adults following acute exposures.
- Secondly, organophosphate pesticides will negatively affect non-target organisms and for the endpoints investigated namely biomarker responses, growth, reproduction and behaviour. A more pronounced response is expected at higher treatment concentrations and treatment interval is expected to play a less pronounced role than treatment concentration in inducing a response.

Objective:

This project was a laboratory-based study to determine the effects of organophosphates on selected non target organisms in both the terrestrial and the aquatic compartments of the environment. Through using biomarkers, coupled with selected life-cycle parameters, the effects on test organisms were assessed both at sub-cellular and whole-organism level. The objective was to establish links between biomarker responses and morphological and life-cycle effects under controlled laboratory conditions, and to determine the sensitivity of juvenile animals to the selected pesticides.

Specific aims:

1. To determine the acute toxicity of the two selected organophosphate pesticides, azinphos-methyl and chlorpyrifos, to juvenile life stages of *E. fetida* and *O. mossambicus* and compare these to values obtained from the literature of adult animals of the same species.

2. To investigate the effects of the two pesticides on different levels of biological organisation, including sub-cellular responses such as cholinesterase inhibition, and whole-organism level effects such as growth and reproduction.

3. To investigate the effects of different exposure concentration and exposure interval combinations to determine the extent to which non-target animals are affected by the two different pesticides, and to determine the role that treatment concentration and interval plays in inducing a toxic response.

CHAPTER TWO

MATERIALS AND METHODS

2.1 General biology, taxonomy and culture of experimental animals

2.1.1 Earthworms – *Eisenia fetida*

The earthworm *E. fetida* is an epigeic litter dwelling worm of palaearctic origin which now has an almost worldwide distribution (Simms and Gerard, 1985). It is most common in soils with a high organic content (Spurgeon et al., 2000) and requires high moisture levels of between 70 and 80% for optimal growth and reproduction (Venter and Reinecke, 1988). This species reaches sexual maturity between 60 and 80 days post hatching and cocoon production starts about four days after mating. Cocoon production is continuous and the hatching time varies between 14 and 44 days (Venter and Reinecke, 1988). The life cycle of *E. fetida* is presented in Figure 2.1.

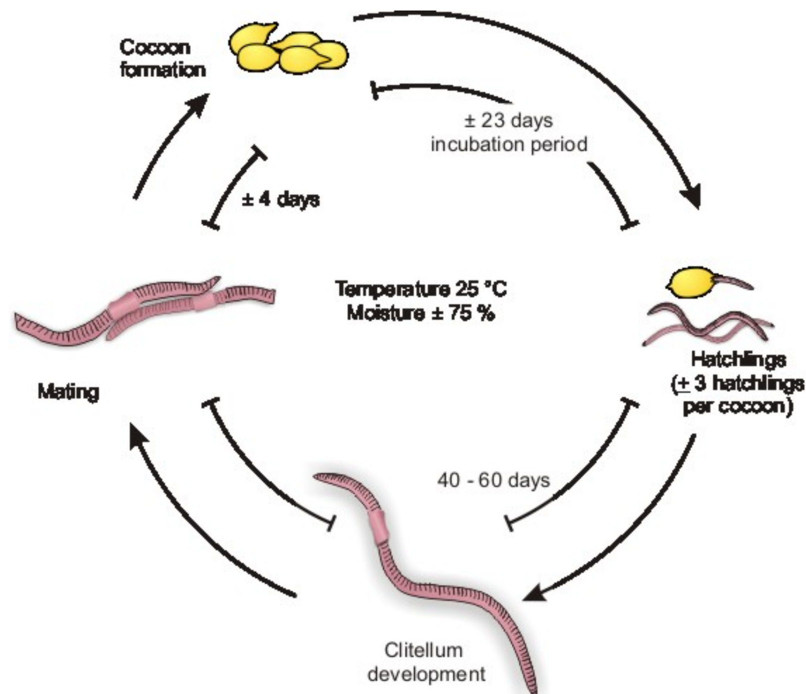


Figure 2.1: The life cycle of the earthworm *E. fetida* (Adapted from Venter and Reinecke, 1988. Figure used with kind permission from Dr. R.A. Maleri).

The taxonomy of *E. fetida*:

Phylum:	Annelida
Class:	Clitellata
Subclass:	Oligochaeta
Order:	Opisthophora
Suborder:	Lumbricida
Superfamily:	Lumbricoidea
Family:	Lumbricidae (Rafinesque – Schmalz, 1815)
Subfamily:	Lumbricinae (Rafinesque – Schmalz, 1815)
Genus:	*Eisenia* (Malm, 1877)
Species:	*Eisenia fetida* (Savigny, 1826)

For the present study, *E. fetida* specimens were collected from an uncontaminated compost heap on Middelvlei wine farm near the town of Stellenbosch. These animals were cultured in plastic containers in a climate controlled room at 20°C and 65% humidity. The containers were filled with cattle manure combined with organic material from the areas where the animals were sampled. Throughout the study, the worms were fed on a weekly basis with fresh cattle manure obtained from the University of Stellenbosch Experimental Farm at Welgevallen. Manure was only collected from animals not exposed to feed additives and medication. To obtain a synchronised culture of worms, the culture containers were inspected once every four weeks for cocoons. The cocoons were washed and allowed to hatch in distilled water. Hatchlings were collected on a daily basis and all individuals that hatched in a single week were pooled and transferred into aged medium from the culture containers and taken to be as of similar age. Hatchlings were also fed with cattle manure on a biweekly basis until they reached the correct age to be used for the experiments. Pre-clitellate individuals of 30-40 days old were used for exposures, unless stated otherwise.

2.1.2 Fish - *Oreochromis mossambicus*

The tilapia *O. mossambicus* is indigenous to Southern Africa and naturally occurs in the northern and eastern parts of South Africa (Skelton, 2001). The Mozambique tilapia is a mouth brooder, with the female parent incubating the fertilized eggs in her buccal cavity until several days after hatching (Popma and Masser, 1999). The total number of eggs produced is a function of body size with one to two eggs produced per gram of bodyweight (Trewavas, 1983; Popma and Lovshin, 1994). Most tilapia species, including *O. mossambicus* reach

sexual maturity at six months or earlier (Mair and Abella, 1997), with sexual maturity in tilapia species being a function of age, size and environmental conditions (Baroiller and Jalabert, 1989). The life cycle of a typical mouth brooder, such as *O. mossambicus* is presented in Figure 2.2.

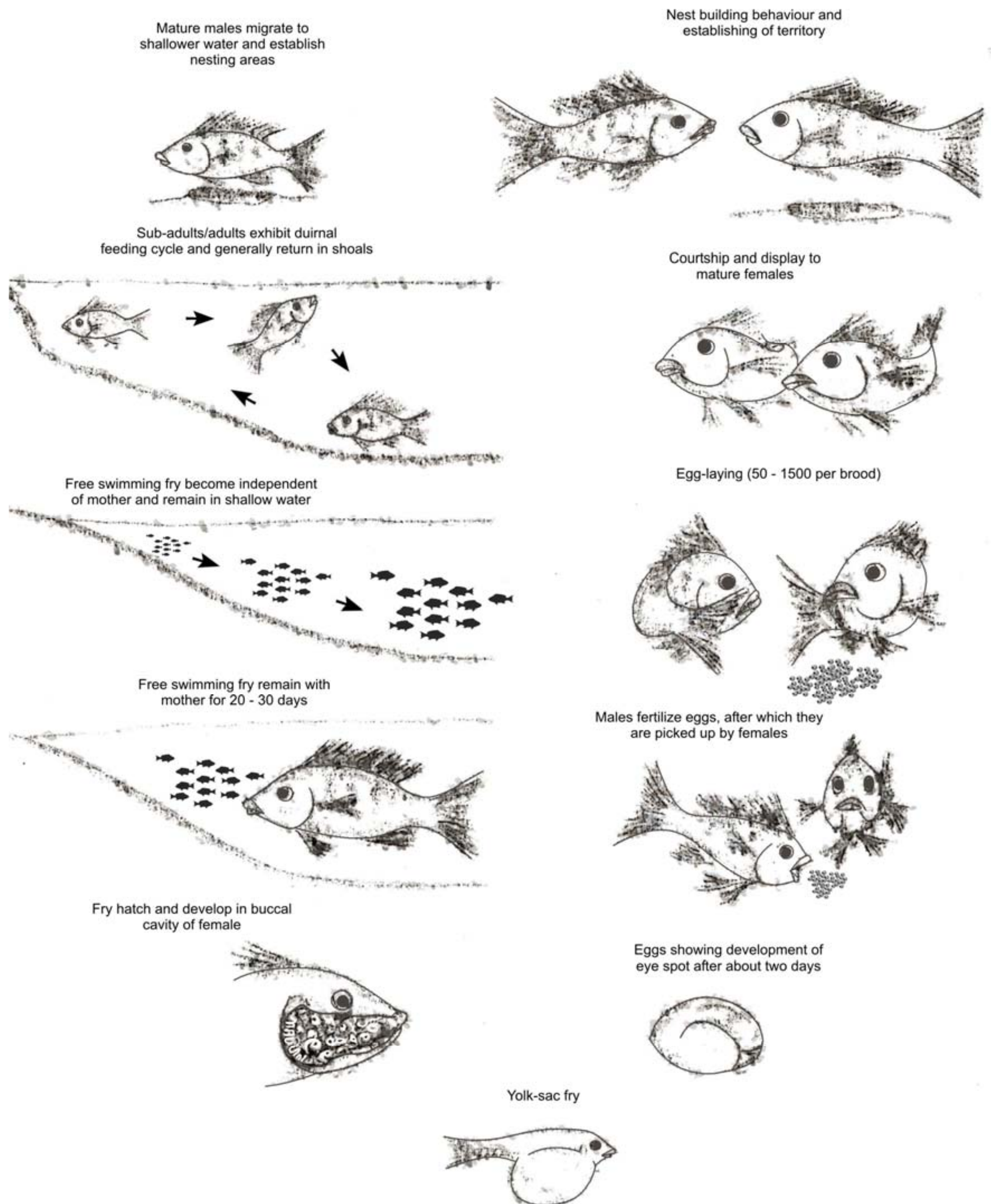


Figure 2.2: The life cycle of the freshwater fish *O. mossambicus* (Pullen and Lowe-McConnel, 1982, adapted and redrawn by Judith Piek)

The taxonomy of *O. mossambicus*:

Phylum:	Osteichthyes
Class:	Actinopterygii
Super-order:	Acanthopterygii
Order:	Perciformes
Family:	Ciclidae
Genus:	*Oreochromis* (Günther, 1889)
Species:	*Oreochromis mossambicus* (Peters, 1852)

The fish used in the present study was obtained from the Division of Aquaculture at the University of Stellenbosch. Subpopulations of *O. mossambicus* were originally collected from different sites throughout Southern Africa and maintained in an aquaculture research facility at the experimental farm of the University of Stellenbosch. Details on the exact sampling locations of the animals are presented in the work of Hall (2001). Breeding stock for the present study was kept in a temperature controlled recirculation system. Fish were stocked at a ratio of 1 male to 4 females and the breeding ponds were inspected twice weekly for females carrying eggs. When a female was observed to be carrying eggs, the eggs were carefully removed from her mouth and allowed to hatch in the laboratory in aerated 10L glass aquaria filled with matured tap water maintained at 28°C. All fish (brood stock and juveniles) were fed twice daily on commercially available tilapia food obtained from Nutrex Pty. Ltd. Floating pellets were fed to the larger fish while swim-up fry (first-feeding fry that have absorbed the yolk-sac) were maintained on a powder food diet. As with the earthworms, tilapia juveniles between four and five weeks old were used for experiments.

2.2 Experimental design

2.2.1 Exposure media

Earthworms were exposed in artificial soil as described in the toxicity testing guidelines of the Organization for Economic Cooperation and Development (OECD, 1984). This test involves exposing earthworms to a toxicant in a defined mixture of silica sand (70%), kaolin clay (20%) and peat moss (10%). The sand was obtained from Consol Glass Pty. Ltd (Cape Town), and the clay from Serina Kaolin Pty. (Cape Town). Pindstrup sphagnum peat moss imported from Denmark was used. The peat moss was dried for a period of 24-36 hours in a drying oven prior to use. These ingredients were weighed, mixed in the correct proportions

(70% sand: 20% peat moss: 10% clay) and moistened to ±35% by weight with distilled water. Calcium carbonate ($CaCO_3$) was added to adjust the pH of the soil to between 5.5 and 6.5. The prepared soil was then incubated for 24 hours before mixing in the pesticide and starting the experiment. Glass jars containing 750g of artificial soil were used for all exposures. Soil moisture and pH was determined at the onset of every experiment and monitored on a weekly basis using a Sartorius MA 45 moisture meter and a Crison Micro pH meter. Three replicates were conducted for each experiment and 10 worms used per exposure in each replicate, unless stated otherwise. If mortality of more than 10% (one individual per replicate) was observed in the control group, the experiment was terminated and repeated (OECD, 1984).

Fish were exposed in glass jars/tanks in the laboratory (in the case of acute exposures) or in a recirculation system (in the case of intermittent exposures). Acute aquatic exposures were conducted as described by the relevant OECD procedure (OECD, 1992). Glass jars with a volume of 2.5L were used for all acute exposures and fish were stocked at densities lower than the maximum loading of 1.0g fish/litre prescribed by the OECD (OECD, 1992). All jars were filled with reconstituted water made with analytical grade chemicals obtained from Merck Chemicals. Reconstituted water was made up using 25 g NaCl and 8 g $NaHCO_3$ per 100 litre of distilled water (Hurter, 2002). This was used as an alternative to tap water to ensure minimal variation in water quality. Similar as with the terrestrial exposures, if mortality of more than 10% (one individual per replicate) was observed in the control group, the experiment was terminated and repeated (OECD, 1992).

All jars and tanks for fish were aerated and temperature controlled. A temperature of $28 \pm 1\ ^{o}C$ was maintained and water quality parameters (temperature, dissolved oxygen, pH and conductivity) was measured at the onset and completion of each acute experiment and monitored on a daily basis in the case of intermittent exposures. The recirculation system where the intermittent exposures were conducted consisted of circular 400L tanks, with 40L glass tanks filled with matured tap water used for the actual exposures. Fish were moved from the 400L tanks to the exposure tanks and allowed to acclimatise for six hours before the pesticide was administered. Following a 24-hour exposure period they were returned to the original tanks until the follow-up exposure.

2.2.2 Exposure concentrations and intervals

Acute and intermittent exposures were conducted for both the terrestrial and aquatic sections of this project using the test organisms described in Section 2.1. For the acute exposures, a range finding test was conducted for each pesticide and the result of this range finding test was used to select the concentration range for the final exposure to determine the LC_{50} value. The concentrations for the range finding test were determined from relevant literature. The range finding tests for earthworms ranged from 20-100mg/kg for azinphos-methyl and 31.25-500mg/kg for chlorpyrifos. For fish, two range-finding concentrations were used for each pesticide. The concentrations were 0.5 and 0.1 mg/l for azinphos-methyl and 1.0 and 0.5 mg/l for chlorpyrifos.

Exposure concentrations for the intermittent exposures, which lasted for 12 weeks, were selected based on the LC_{50} values that were determined from the acute exposures. For both pesticides, two fractions of the LC_{50} were randomly selected, as choosing a field-relevant concentration was difficult due to the large variation in pesticide concentrations measured in the field and reported in the literature. For the aquatic exposures, 10% and 50% of the LC_{50} value of each pesticide was used, while for terrestrial exposures the corresponding concentrations were 5% and 25% of the LC_{50} value. For each pesticide concentration, two treatment intervals were tested, namely a 14-day treatment interval and a 28-day treatment interval. These were also chosen randomly as pesticide application intervals can be variable, as was discussed in Section 1.7.

In summary, each pesticide was tested at four different concentration/interval combinations in both the aquatic and terrestrial environment. The exposure concentrations and intervals for both the terrestrial and aquatic exposures are presented in Table 2.1 and 2.2. In both tables the last column contains abbreviations for each of the treatment groups specifying the pesticide, treatment concentration and treatment interval for that specific group. These abbreviations were used in the following chapters to present and discuss the results. The explanation of abbreviations is as follows. For example: Exposure to azinphos-methyl (AZP) at 50% of LC_{50} value for an exposure interval of 14 days = AZP50/14.

Table 2.1: Summary of treatment regimes for *E. fetida* in terms of exposure concentrations and intervals for intermittent azinphos-methyl and chlorpyrifos treatments.

Pesticide	Exp. Conc. (fraction of LC_{50} value*)	Exp. Interval (days)#	Abbreviation
Azinphos-methyl (AZP)	1.27 mg/kg (5%)	14	AZP5/14
		28	AZP5/28
	6.35 mg/kg (25%)	14	AZP25/14
		28	AZP25/28
Chlorpyrifos (CPF)	4.64 mg/kg (5%)	14	CPF5/14
		28	CPF5/28
	23.20 mg/kg (25%)	14	CPF25/14
		28	CPF25/28

* AZP LC_{50} value: 25.34 mg/kg; CPF LC_{50} value: 92.83 mg/kg (Results from present study, refer to Table 3.1 and 3.2 from Chapter 3.)

Number of days between consecutive pesticide applications

Table 2.2: Summary of treatment regimes for *O. mossambicus* in terms of exposure concentrations and intervals for intermittent azinphos-methyl and chlorpyrifos treatments.

Pesticide	Exp. Conc. (fraction of LC_{50} value*)	Exp. Interval (days)#	Abbreviation
Azinphos-methyl (AZP)	0.0007 mg/l (10%)	14	AZP10/14
		28	AZP10/28
	0.00035 mg/l (50%)	14	AZP50/14
		28	AZP50/28
Chlorpyrifos (CPF)	0.005 mg/l (10%)	14	CPF10/14
		28	CPF10/28
	0.025 mg/l (50%)	14	CPF50/14
		28	CPF50/28

* AZP LC_{50} value: 0.007 mg/l; CPF LC_{50} value: 0.05 mg/l (Results from present study, refer to Table 3.3 and 3.4 from Chapter 3.)

Number of days between consecutive pesticide applications

2.2.3 Pesticides

Commercial preparations of the pesticides azinphos-methyl (trade name: Azinphos WP®) and chlorpyrifos (trade name: Dursban EC®) were obtained from Terason Pty. Ltd., and diluted to the correct concentration of active ingredient using distilled water. Exposure concentrations and subsequent calculations were based on the amount of active ingredient (AI) in the commercial preparation. All pesticide mixtures were made up immediately prior to starting every experiment.

2.2.4 Chemicals and disposables

All chemicals and reagents used for the biomarker assays were obtained from Sigma-Aldrich or Biorad Laboratories, unless stated otherwise. Disposable items (micropipette tips, micro plates and microcentrifuge tubes) were obtained from Whitehead Scientific.

2.3 Biomarker protocols

2.3.1 Preparation of tissue homogenates for ChE assay

Tissue preparation was done immediately after completion of the relevant exposure. The earthworms were sacrificed by putting them in a -80°C freezer for 30 minutes and thawing them on ice for the subsequent tissue preparation. After weighing the earthworm (after thawing), it was transferred to a glass vial kept on ice. Cold homogenizing buffer was added in a ratio of 1:4 (w/v) and the animals were homogenized using a Polytron Kinematika tissue homogenizer. Phosphate buffered saline (PBS) was used as homogenizing buffer. The crude homogenate was centrifuged at 13 000 rpm for 30 minutes at 4 ^{o}C (Reinecke and Reinecke, 2007a, 2007b) in a Biofuge centrifuge from Heraeus Instruments. The supernatant was removed and stored in micro centrifuge tubes on ice where after it was frozen at-80°C until determining the ChE activity and protein content. Fish were sacrificed by severing of the spinal cord with a dissection scalpel. Fish brain tissue was prepared in a similar way to the earthworm tissue, with the exception of the centrifugation step. The crude homogenate was centrifuged at 11 000 rpm for 10 minutes at 4 ^{o}C (Ibrahim et al., 1988).

The supernatant was removed and stored in micro centrifuge tubes on ice where after it was frozen at -80°C until determining the AChE activity and protein content. The protein content of different samples was determined to allow differences in the protein content of individual samples to be taken into account when determining the AChE activity of these samples.

Tissue samples from male and female fish were pooled for analysis of AChE activity as no literature was found to suggest that the normal activity of this enzyme, and the sensitivity to inhibition by organophosphates, differed between the two genders.

2.3.2 Determination of protein content of samples

The protein content of samples was determined using a protein determination solution from Biorad Laboratories. This method, based on the original methods of Bradford (1976), relies on the binding of the dye Coomassie Brilliant Blue G-250 to the protein in the sample. The free dye has absorption maxima at 470 and 650nm, but for the protein-dye complex this shifts to 595nm. The amount of absorption is proportional to the amount of protein present in the sample (Bradford, 1976). A protein standard curve was constructed using bovine serum albumen (BSA) Fraction V (Roche Chemicals). Protein concentrations ranging from 0.1-1.0 mg/ml was pipetted in triplicate into a microtiter plate, using a total of 20µl sample volume per well. To this was added 180µl of Biorad reagent that consisted of Coomassie Brilliant Blue dye in phosphoric acid and methanol. Absorbance was determined at 595 nm using a Multiskan micro plate reader after incubating the sample for 10 minutes at room temperature. From these values, a standard curve was constructed and the protein content of the samples could be linked to the optical density (OD) values. The standard curve is presented in Figure 2.3. A secondary protein standard was prepared using a pooled batch of untreated animals. This secondary protein standard was analyzed on every plate and was used to monitor assay conditions and reagents between plates analyzed at different times.

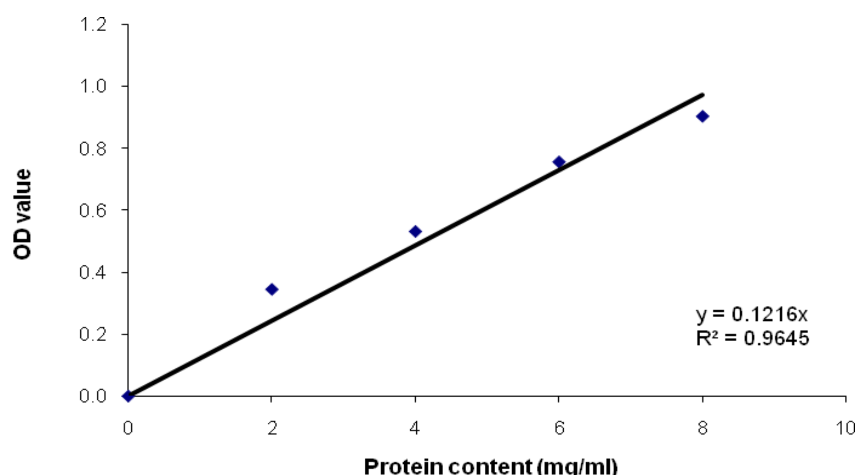
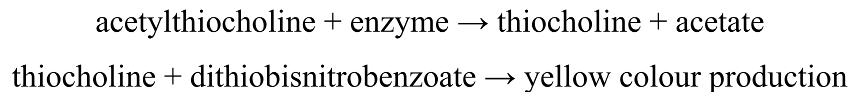


Figure 2.3: A protein standard curve using bovine serum albumin (BSA) Fraction V as standard.

2.3.3 Determination of cholinesterase activity of tissue homogenates

Cholinesterase activity was determined according to the method of Ellman et al. (1961) adapted for microtiter plates. The Ellman method is based on monitoring the activity of acetylcholinesterase in terms of the yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. The following two-step reaction forms the basis of the Ellman assay (Ellman et al., 1961):



acetylthiocholine + enzyme → thiocholine + acetate

thiocholine + dithiobisnitrobenzoate → yellow colour production

In this assay, acetylthiocholine is used as the substrate with which the enzyme reacts, producing a product that interacts with the colourant. Enzyme activity can therefore be determined by monitoring the change in absorption over time, using the 412 nm filter in a spectrophotometer. The tissue homogenate prepared from the exposed animals is the enzyme source. The assay was carried out in the following steps:

1. Microcentrifuge tubes containing the frozen supernatant were removed from the -80°C freezer and placed in a Styrofoam box with ice in order to thaw. At the same time, the DTNB (5-thio(2-nitrobenzoic acid)) and acetylthiocholine solutions were prepared and kept on ice. (Concentrations for these, as well as for the phosphate buffer, are presented in Appendix 1). The phosphate buffer was prepared in advance and stored in the fridge, storage time not exceeding one week.

2. After the solutions were prepared and the supernatants have thawed, all reagents were pipetted into a 96-well plate. Every sample was pipetted in triplicate and the mean of the three readings was used for calculating enzyme activity. The acetylthiocholine was added last as this initiates the reaction. The following quantities were added to each well using a multi tip micropipette:

- 75µl phosphate buffer
- 50µl reagent (DTNB)
- 25µl homogenate
- 50µl substrate (acetylthiocholine iodide)

3. As soon as the substrate is added, the reaction starts and the plate was put into the plate reader immediately and a reading taken. Following this, a reading was taken every 15 seconds for 5 minutes.

4. Upon completion of the Ellman assay and the Biorad protein determination, as described in section 2.3.2, the values were used to determine the enzyme activity in the samples. Enzyme activity was calculated as the amount of product formed per minute per gram protein in the sample. This was done in the following manner:

Calculation of specific enzyme activity (after Fisher, 2000):

$$\text{Enzyme activity} = ((\Delta A/\text{min}) \times \text{MEC}) / \text{PC}$$

Where:
ΔA = change in absorbance of sample
MEC = molar extinction coefficient of DTNB reagent
PC = protein content of sample

2.3.4 Neutral red retention time assay

Neutral red retention time of earthworm coelomocytes was determined according to the lysosomal membrane stability assay developed for earthworms by Weeks and Svendsen (1996). Earthworms were removed from the soil and gently washed in distilled water to remove all soil particles. Cells were collected from earthworms through puncturing the body wall just behind the clitellum with a sterile needle and drawing 20µl of the coelomic fluid into a syringe containing 20µl earthworm Ringer solution (Reagents and quantities are presented in Appendix 1). The contents of the syringe was then placed on two clean microscope slides and placed in a moisture chamber for 30 seconds to allow the ameboid cells to attach to the surface of the slide. During this time a working solution of Toluyene Red stain was prepared by mixing 5µl stock solution (See Appendix 1) with 1.25ml Ringer solution. This working solution was replaced every hour and with every new slide that was prepared in order to prevent crystal formation. Twenty microliters of the working solution was added to the cell suspension on the slide and covered with a cover slip, where after the counting procedure started using the 400x magnification of a light microscope.

Cells from each slide were randomly counted at two-minute intervals, distinguishing between stained (red/pink cytosol) and unstained (transparent cytosol) cells. In order to distinguish between stained and unstained cells, the diaphragm of the microscope is used to allow more or less light to penetrate the slide. Stained cells are still visible under high light intensity, while unstained cells are not. Counting is continued at two-minute intervals until 50% of the cells that were counted were stained ones. This time was recorded as the neutral red retention time of the lysosomes in the sample.

2.4 Measurement of morphological and reproductive parameters

2.4.1 Growth, maturation and reproduction in *E. fetida*

These parameters were monitored on a biweekly basis during an intermittent 12-week exposure to the different exposure regimes of each of the selected pesticides. Juvenile *E. fetida*, aged between 30 and 40 days old, obtained from synchronized laboratory cultures, were used. Animals in the selected age category were sorted in order to obtain a homogenous population in terms of size and weight with no significant differences between the start weights of the different groups. Every group was weighed at the start of the experiment, using an electronic laboratory balance, and every 14 days thereafter until the end of the 12-week exposure period. As there were no statistically significant differences between the mean start weights of the different groups, growth was expressed in terms of the end weight of the animals instead of weight change.

Maturation was recorded throughout the exposure time by observing clitellum development of the worms every time they were weighed. In the final two weeks of the experiment the soil from the exposure container was inspected for the presence of cocoons. These were counted, placed in 24-well multiwell plates in distilled water and left to hatch to determine cocoon viability and number of hatchlings per cocoon. Cocoon viability was defined as the number of cocoons yielding hatchlings relative to the total number of cocoons that were produced. A cocoon was considered to be non-viable if it had not hatched within six weeks after being produced. Non-viable cocoons were observed to be different in appearance to viable cocoons and often had an opaque light yellow colour compared to a deeper and darker yellow observed for viable cocoons.

2.4.2 Length and mass measurements of *O. mossambicus* and calculation of organ indices

O. mossambicus juveniles of six weeks old were weighed and measured, using a ruler and a laboratory balance, and randomly divided into groups of 20. These groups were arbitrarily divided into treatment and control groups. Growth measurements were recorded separately for males and females at the end of week 12 of the exposure. The reason for this is the fact that male and female *O. mossambicus* have different growth rates (Mair et al., 1997) and by measuring and analyzing the data for the two genders together, very high variation will be observed and pesticide-induced differences in growth parameters may be overlooked.

Upon completion of the exposure, fish were sedated by immersing them in icy water for 30 seconds before length and mass measurements were taken. Body length was taken as the standard length, thus the size of the fish from the snout to the end of the body, but excluding the caudal fin. After the morphological measurements were taken, fish were sacrificed as described in Section 2.3.1. Immediately following this, the brain was dissected out and placed in ice cold homogenizing buffer and tissue was prepared as described in Section 2.3.1. The liver and gonads were dissected out of the body cavity and weighed using a laboratory balance. The liver somatic index (LSI), gonadosomatic index (GSI) and condition factor (CF) was then calculated in the following way:

LSI = (liver weight / total body mass) x 100

GSI = (gonad weight / total body mass) x 100

$CF = W / L^3$, where W is the mass and L the body length

2.5 Behavioural studies

2.5.1 Burrowing and avoidance behaviour in *E. fetida*

Avoidance behaviour experiments were conducted using standard OECD artificial soil and following the method described by Yeardley et al. (1996) and Van Zwieten et al. (2004). Prepared OECD soil was placed in rectangular plastic boxes measuring 20x10cm and each box contained 550g soil with a moisture content of 35- 40% and pH of between 5.5 and 6.5. The soil depth in the exposure containers was approximately 5cm. Ten worms were used per exposure and every experiment had five replicates unless otherwise stated.

Before testing for a possible avoidance response of *E. fetida* towards the two organophosphates, worms were exposed to clean soil in the test containers and the dispersal of the worms was monitored. This was done to ensure that worms dispersed randomly in the absence of a toxicant. Ten worms were placed in exposure containers containing only clean soil and allowed to burrow undisturbed for a period of 48 hours. Following this, the soil in the exposure box was divided into two equal parts by pulling a metal spatula through the midline of the box and inserting a plastic divider. By doing this, any worms in the middle of the box were extracted from the soil. These worms were judged as not to be on either side of the box and were not counted. The two portions of soil were removed separately from the box and the number of worms on each side of the divider was counted. This was done to ensure that in the absence of a toxicant the worms dispersed into the medium randomly and without favouring a side. It is important to establish that worms did not naturally exhibit behaviour which could be mistaken for avoidance behaviour and thus cause false positive results. This experiment had ten replicates, using 100 worms in total.

Following this preliminary dispersal behaviour experiment, the same experimental design was followed for the avoidance tests, only this time the worms were offered a choice between clean soil and soil contaminated with a pesticide. Similar containers were used, but prior to putting the soil into the boxes, one half was spiked with either chlorpyrifos or azinphos methyl at 25% of the LC_{50} value. The two soils were then placed on either side of the box and the divider removed. The box was then lightly tapped to settle the soil and 10 worms were placed on the soil surface along the centre line of each replicate box and left to burrow. The boxes were covered with perforated cling film and placed in a 20°C climate room for 48h as described by Yeardley et al. (1996). Five replicates were done with 10 worms per replicate. The results were determined as described above. If an avoidance response was observed at 25% of the LC_{50} value, the experiment was repeated using 5% of the LC_{50} value. If an avoidance response was not noted, the exposure concentration was increased to 50% of the LC_{50} value, in order to give an indication of whether the worms did not avoid the pesticide at all or whether avoidance was concentration dependent.

In addition to investigating avoidance response in *E. fetida*, the effect that both acute and intermittent pesticide exposure had on the worm's ability to burrow and work the soil was investigated. Following the 14-day acute exposure to both pesticides, all surviving worms from treated and control groups were placed on clean soil and any attempts at burrowing was

noted. Burrowing activity was quantitatively investigated during the intermittent exposure by recording the burrowing time of treated and control worms at two-week intervals during the 12-week experiment using a stopwatch. Every two weeks the worms were removed from the soil and placed in a jar containing distilled water to prevent desiccation, while more pesticide was mixed into distilled water was added to the 14 day exposure intervals and the soil was placed back into the jar. Soil from the control groups and the groups subjected to a 28 day exposure interval received distilled water only. After returning the soil to the jar, the worms were placed on the soil surface. Burrowing ability was quantified as the time taken for the worms to burrow away from the soil surface, as described in the work of Stenersen (1979). This time was recorded at 14-day intervals during the 12-week intermittent exposure. Burrowing times were compared between the various treated and control groups at the start and end of the exposure period. Burrowing time of each individual treatment group at the beginning and end of the experiment was also compared.

2.5.2 Feeding behaviour in *O. mossambicus*

Larger fish than those used for the acute toxicity tests were used for the feeding trial as the use of larger fish (2-3cm total length) permitted more accurate observation of feeding response. Fish were maintained in the laboratory for a period of two weeks in order to acclimatise and become used to the movements of the observer around the tank. This was done in order to avoid handling-related stress influencing the feeding response of the animals during the actual feeding trial. A preliminary experiment was done where untreated fish were offered an excess of food and the number of food particles that were consumed in a five-minute period was determined. This amount of food was offered in subsequent experiments. Feeding behaviour was recorded for solitary fish, and then for fish in groups of two or three individuals per tank to determine whether the number of fish present affected feeding behaviour.

In order to determine the food consumption of treated and control fish, two fish per tank were exposed to two concentrations (10% and 50% of the LC_{50} value) of each pesticide for a period of 24 hours. Following this exposure, fish were transferred to individual tanks containing clean water. After being allowed to recover from handling stress for two hours, the fish were offered a limited amount of food (10 pellets) and feeding response (i.e. the time it took for the fish to actively attempt to consume the food particles) was observed. After the initial observation period, an additional 10 food pellets were offered and the number of

pellets remaining after 60 minutes was recorded. Feeding behaviour was thus quantified in terms of feeding response and food consumption.

2.6 Statistical analysis

2.6.1 Acute toxicity tests

Data from the final exposure ranges were analysed using the EPA probit program version 1.5. In cases where the data was not suitable for use with the probit model, the Trimmed Spearman Karber method was used to calculate the LC_{50} value.

2.6.2 Intermittent toxicity tests

Means and standard deviations, where applicable, were calculated using the MS Excel software program. Data was analysed for statistical differences between treatments using theStatistica Version 7 (StatSoft 2004) software program. All data was tested for normality using the Kolgomorov-Smirnoff test and parametric data was analysed by using a one-way ANOVA followed by post hoc analysis using Fisher's LSD test to determine whether differences between various treatments groups existed. If data was nonparametric, it was log transformed and tested again for normality. If the transformed data was parametric, it was analysed in the same way as other parametric data. If the transformed data was still nonparametric, the original untransformed data was analysed using a Kruskall Wallis ANOVA to test for differences, followed by post hoc analysis using a multiple comparison of mean ranks for all groups.

2.6.3 Avoidance behaviour tests

For the avoidance response experiments, a hypothesis was formulated stating that animals will not be able to avoid pesticide contaminated soil and therefore the same number of animals will be present in both the control and contaminated soils. Data obtained from these experiments was tested using a chi-squared test to compare the observed and expected number of animals in the two soils and to determine whether an avoidance response was present.

CHAPTER THREE

RESULTS

3.1 Effects of organophosphates on earthworms

3.1.1 Acute exposures

At the end of the range finder experiment, which consisted of five azinphos-methyl concentrations ranging from 20-100mg/kg, 30% mortality was observed in the 20 mg/kg treatment group and 100% mortality in all the other treated groups. In the 100 mg/kg treatment group the earthworms did not attempt to burrow and died on the soil surface within the first 24h of the exposure period. No mortality was observed in the control group. The final exposure range used consisted of six concentrations ranging between 10-40 mg/kg azinphos methyl. The experiment was conducted in triplicate (n=30) unless stated otherwise and the results analysed using Finney's Probit Analysis. The results and the derived LC_{50} value are presented in Table 3.1.

Table 3.1: Acute toxicity of azinphos-methyl to juvenile *E. fetida* after a 14-day exposure period. The probit method of data analysis was used.

Exposure concentration (mg/kg)	No of worms exposed*	% mortality
0.0	40	0
10.0	40	0
15.0	40	13
20.0	40	15
25.0	30	47
30.0	20	70
40.0	10	100
Probit analysis estimates		
LC_{50}		25.34 mg/kg
95% Lower Confidence		23.42 mg/kg
95% Upper Confidence		27.92 mg/kg

* More than 30 individuals were used when a surplus of worms of the selected size range was available. Less individuals were used when the majority of animals died before the end of the exposure time.

The range finder test for chlorpyrifos treatments also consisted of five exposure concentrations, ranging from 31.25-500 mg/kg. Earthworms exposed to 125, 250 and 500 mg/kg did not tunnel into the soil and showed an avoidance response by crawling to the side of the jar and not burrowing into the soil at the beginning of the experiment. All these animals were dead by day seven of the experiment. All animals in the 31.25 mg/kg and 62.5 mg/kg exposure groups tunnelled away from the surface and by the end of the 14-day exposure period there were no mortalities in the groups exposed to 31.25 mg/kg and 62.5mg/kg and the control group. Based on the results of the range finding test, a final exposure range was selected with seven concentrations ranging between 31.25 mg/kg and 125 mg/kg. The experiment was conducted in triplicate and the results analysed using the Spearman-Karber method of data analysis, as the data distribution was not suited to Probit Analysis. The results and the derived LC_{50} value are presented in Table 3.2.

Table 3.2: Acute toxicity of chlorpyrifos to juvenile *E. fetida* after a 14-day exposure period. The Spearman Karber method of data analysis was used.

Concentration (mg/kg)	No of worms exposed*	% mortality
0.00	30	0
31.25	20	0
50.00	40	5
62.50	30	0
75.00	28	11
87.50	38	32
100.00	38	66
125.00	20	100
	Probit analysis estimates	
LC_{50}		92.83 mg/kg
95% Lower Confidence		80.30 mg/kg
95% Upper Confidence		116.42 mg/kg

* More than 30 individuals were used when a surplus of worms of the selected size range was available. Less individuals were used when the majority of animals died before the end of the exposure time or when no mortality was observed.

3.1.2 Intermittent exposures

The abbreviations used hereafter for the different treatment groups were based on the nature of the treatment which that specific group was subjected to as was explained in Section 2.2.2.

3.1.2.1 Mortality

No mortality was observed in the control group or any of the groups of earthworms exposed to chlorpyrifos, irrespective of exposure concentration or interval. There was also no mortality in the two groups exposed to azinphos-methyl at a 28-day treatment interval. Mortality occurred in the two azinphos-methyl treated groups subjected to a 14-day exposure interval (AZP5/14 and AZP25/14). Mortality of one, three and two worms respectively was observed for the three AZP5/14 replicates by the end of the 12 week exposure period. All animals from the AZP25/14 treated group died by week six of the exposure period and therefore no data on any sublethal endpoints exist for this group.

3.1.2.2 Effects on life-cycle parameters

a) Growth

The mean start weight calculated among all groups was 0.882±0.093g. There were no statistically significant differences between the mean start weights of the different groups ($F=0.654$; $p>0.05$; Appendix 2: Table 1) and growth was therefore expressed in terms of the end weight of the animals instead of weight change. Following exposure to azinphos-methyl, statistically significant differences in mean end weight were observed between all azinphos-methyl treatment groups and the control group ($F=24.96$; $p<0.05$ Appendix 2: Table 2a,b) with the exception of the AZP5/28 treatment group which was not different from the control group ($p>0.05$). The mean end weight for the three surviving azinphos-methyl treated groups were statistically significantly different from each other ($p<0.05$), with the AZP5/14 group having the lowest mean end weight. These results are presented in Figure 3.1.

A decrease in feeding response after the first pesticide treatment was observed in all treated animals compared to the control, but this decrease was not quantified. A decrease in feeding response was deducted by noting that some of the food offered to the treated animals remained uneaten, compared to the control animals which consumed all food that was offered to them. The decrease in feeding response appeared more prominent in the AZP5/14 group than in the two groups subjected to a 28-day treatment interval. The latter two groups

consumed some food, while feeding response was absent in the AZP5/14 group and these animals stopped feeding completely during the latter half of the exposure period.

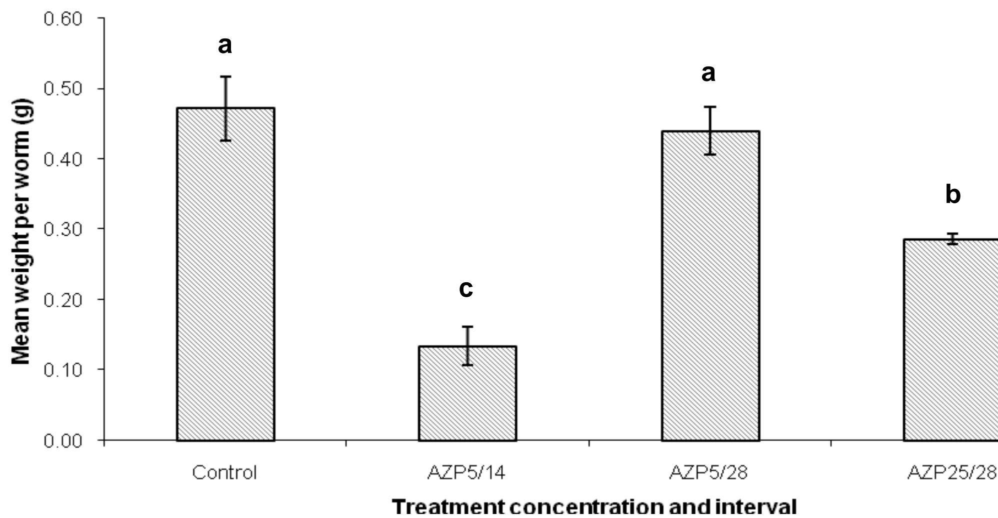


Figure 3.1: Mean end mass of *E. fetida* following an intermittent 12-week exposure to azinphos-methyl at different exposure concentrations and intervals (n>24 in all groups). Error bars indicate standard deviations. Different letters denote statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

In contrast to the azinphos-methyl exposures, worms from the chlorpyrifos treatments gained weight during the exposure period and no reduction in feeding response was observed in any of the treated groups compared to the control group. Statistical analysis indicated that all chlorpyrifos treated groups were different from the control in terms of end weight ($F=24.96$; $p<0.05$ Appendix 2: Table 2a,b), but no differences existed between the four different chlorpyrifos treated groups ($p>0.05$). The lowest mean end weight was recorded for the CPF25/14 group but this was not statistically significantly lower than the other three treatment groups. Figure 3.2 present the end weights of the different chlorpyrifos treated groups and the control group.

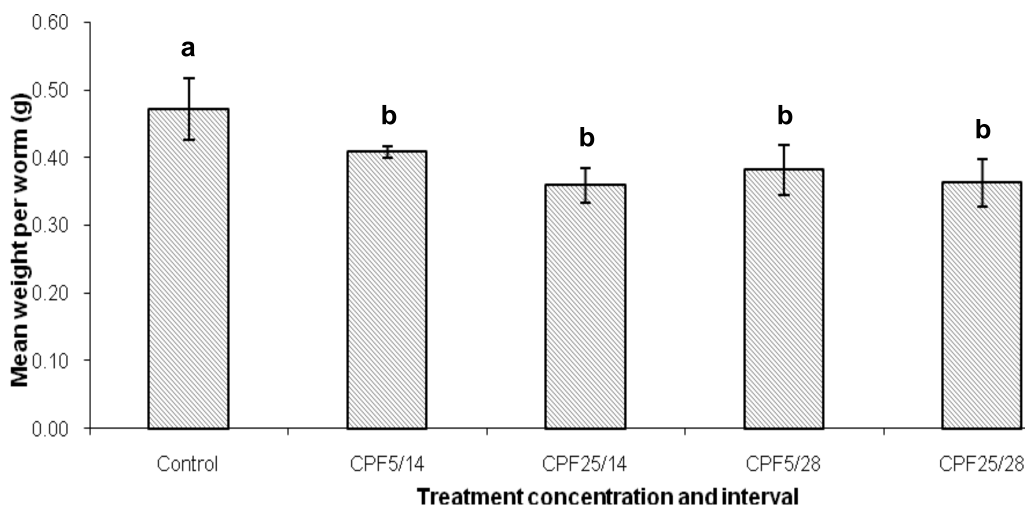


Figure 3.2: Mean end mass of *E. fetida* following an intermittent 12-week exposure to chlorpyrifos at different exposure concentrations and intervals (n=30 in all groups). Error bars indicate standard deviations. Different letters denote statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

b) Maturation and reproduction

All control animals from both the azinphos-methyl and chlorpyrifos experiments were sexually mature with fully developed clitella by the end of the exposure period and cocoon production was observed in the last two weeks (week 10 to week 12) of both pesticide treatments. A total of 308 cocoons was produced by all the control worms, with a mean of 3.2±1.03 cocoons per worm (Table 3.4). Reproduction in the azinphos-methyl treated groups that survived to the end of the experiment was severely reduced compared to the controls in terms of cocoon production. Maturation was affected in animals subjected to a 14-day exposure interval with no worms in the AZP5/14 treatment showing any sign of clitellum development and as a result no cocoons were produced. This was not observed in the 28-day treatment interval groups and more than 80% of the worms in these groups were fully clitellate by Week 10 of the experiment. In spite of sexual maturation and normal clitellum development, reproduction was absent in the A25/28 group (Table 3.3).

The only group exposed to azinphos-methyl that was able to reproduce was the AZP5/28 group. This group produced a total of 15 cocoons with a mean cocoon production per worm of 0.5±0.2 cocoons. A statistically significant difference was observed in cocoon production between this group and the control group ($F=11.610$, $p<0.05$; Appendix 2: Table 3.1a, b).

Cocoon viability for the AZP5/28 group was 88.7±10.3%. There was no difference (p>0.05 Appendix 2: Tables 3.1 - 3.2) between the treatment and control groups in terms of cocoon viability (88.7% vs. 97.4%) and mean number of hatchlings per cocoon (3.1 vs. 2.6). Results for all reproductive endpoints recorded for the azinphos-methyl treated groups and controls are summarized in Table 3.3.

Table 3.3: Reproductive success of *E. fetida* following an intermittent 12-week exposure to azinphos-methyl at different exposure concentrations and intervals. Mean values are presented in the last three columns ± standard deviations. Values significantly different from the control are indicated by an asterisk (*). For treatment concentration and interval abbreviations see Table 2.1.

Treatment	Total worms#	Total cocoons	Non-viable cocoons	Cocoon viability (%)	Cocoons per worm	Hatchlings per cocoon
Control	97	308	8	97.4 ±3.41	3.2 ±1.03	2.6± 0.2
AZP5/14	16	0	0	0	0	0
AZP5/28	30	15	2	88.7±10.3	0.5± 0.2*	3.1± 1.2
AZP25/28	30	0	0	0	0	0

This value refers to the total number of worms that survived to the end of the exposure period.

Reproduction was also affected in the worms exposed to chlorpyrifos. Maturation appeared to be unaffected by exposure to this pesticide as all animals in all four treated groups were fully clitellate by the end of the exposure period. Cocoons were produced in all treated groups but cocoon production was statistically significantly reduced in all treated groups relative to cocoon production in the control group (F=11.610; p<0.05; Appendix 2: Table 3.1a,b). More cocoons were produced in the two groups exposed to the lower chlorpyrifos concentration (CPF5/14 and CPF5/28) than the two groups exposed to the higher concentration (CPF25/14 and CPF25/28) as can be seen in Table 3.4. However, the difference observed in the numbers of cocoons produced by the different chlorpyrifos treated groups was not statistically significant (p>0.05; Appendix 2: Table 3.2 - 3.3). No statistically significant differences were observed between treatments in terms of cocoon viability (ranging from 88.7% to 92.0% for treated groups vs. 97.2% for control groups) and number of hatchling per cocoon (ranging from 2.1-2.4 for treated groups vs. 2.6 for the control

group). Results for all reproductive endpoints recorded for chlorpyrifos treated groups are summarized in Table 3.4.

Table 3.4: Reproductive success in *E. fetida* following an intermittent 12-week exposure to chlorpyrifos at different exposure concentrations and intervals. Mean values are presented in the last three columns ± standard deviations. Values significantly different from the control are indicated by an asterisk (*). For treatment concentration and interval abbreviations see Table 2.1.

Treatment	Total worms#	Total cocoons	Non-viable cocoons	Cocoon viability (%)	Cocoons per worm	Hatchlings per cocoon
Control	97	308	8	97.4 ± 3.41	3.2 ±1.03	2.6 ± 0.2
CPF5/14	30	44	4	92.0 ± 7.5	1.5 ± 0.5*	2.4 ± 0.3
CPF25/14	30	17	2	88.7 ± 12.7	0.6 ± 0.5*	2.1 ± 0.2
CPF5/28	30	48	4	92.0 ± 8.0	1.6 ± 0.3 *	2.3 ± 0.2
CPF25/28	30	19	3	89.3 ± 10.1	0.6 ± 0.5 *	2.3 ± 0.3

This value refers to the total number of worms that survived to the end of the exposure period.

3.1.2.3 Biomarker responses

a) Cholinesterase activity

Cholinesterase activity (micromoles of product (thiocholine) formed per minute per microgram of protein in the sample) showed large variation between individual animals in the same treatment group for both treated and control groups. Enzyme activity in the three azinphos-methyl treated groups was inhibited by more than 90% compared to the control. The group exposed to the lower concentration of azinphos-methyl at a 14-day interval (AZP5/14) was the most severely affected with less than 1% enzyme activity compared to the control. Enzyme activity for more than 50% of the animals in this group was completely inhibited. All treated groups showed statistically significant differences from the control group (H=107.161; $p<0.05$; Appendix 2: Table 4a) but no statistically significant differences in enzyme inhibition were observed between the treated groups ($p>0.05$; Appendix 2: Table 4b). These results are presented in Figure 3.3.

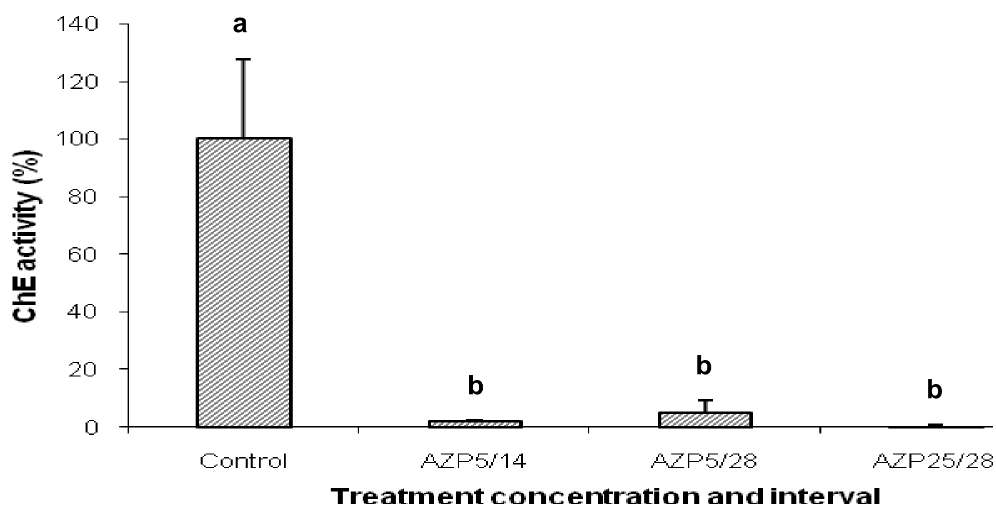


Figure 3.3: Cholinesterase activity in specimens of *E. fetida* following an intermittent 12-week exposure to azinphos-methyl at different exposure concentrations and intervals (n>16 for all groups). Error bars indicate standard deviations. Different letters denote statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

Large variation was also observed in cholinesterase activity of the earthworms exposed to chlorpyrifos. The various treated groups had enzyme activity ranging from 16.4 to 36.7% of control activity. The two groups exposed to the higher pesticide concentration (CPF25/14 and CPF25/28) had significantly lower enzyme activity than the control ($p<0.05$; Appendix 2: Table 4a, b). Enzyme activity of the two groups exposed to the lower pesticide concentration (CPF5/14 and CPF5/28) had intermediate enzyme activity which did not differ significantly from the control or from the two groups exposed to a higher concentration ($p>0.05$) as can be seen in Figure 3.4. The lowest enzyme activity was observed in the CPF25/14 group (16.4%) and the highest in the CPF5/28 group (36.7%).

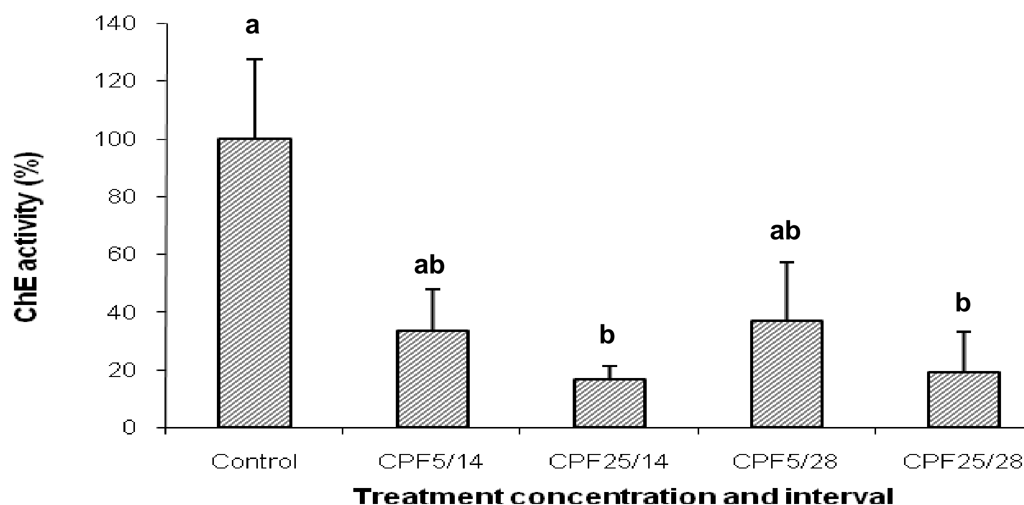


Figure 3.4: Cholinesterase activity in specimens of *E. fetida* following an intermittent 12-week exposure to chlorpyrifos at different exposure concentrations and intervals (n>16 for all groups). Error bars indicate standard deviations. Different letters denote statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

b) Neutral red retention time

The neutral red retention time was determined at the end of the experimental period within 48h of completing the exposure. Fourteen animals were sampled from the control group and cells obtained from these animals had a mean retention time of 32 minutes. Between 10 and 20 animals, depending on the time available, were analysed from each of the various pesticide treated groups. Cells from all azinphos-methyl treated groups had significantly shorter neutral red retention times than the control, varying from 9 minutes for the AZP25/28 group to 12 minutes for the AZP5/28 group. The results for all azinphos-methyl treated groups were different from the control (H=154.45; $p<0.05$; Appendix 2: Table 5a) and there was no statistically significant difference between the two lower concentration treatment groups (AZP5/14 and AZP5/28), which had retention times of 9 and 11 minutes, respectively. Similarly, there was no statistically significant difference between the AZP5/14 group and the AZP25/28 group, while a statistically significant difference was observed between the two groups subjected to azinphos-methyl at a 28-day treatment interval ($p<0.05$; Appendix 2: Table 5b). The mean neutral red retention times of the various treated groups, along with standard deviations are presented in Figure 3.5.

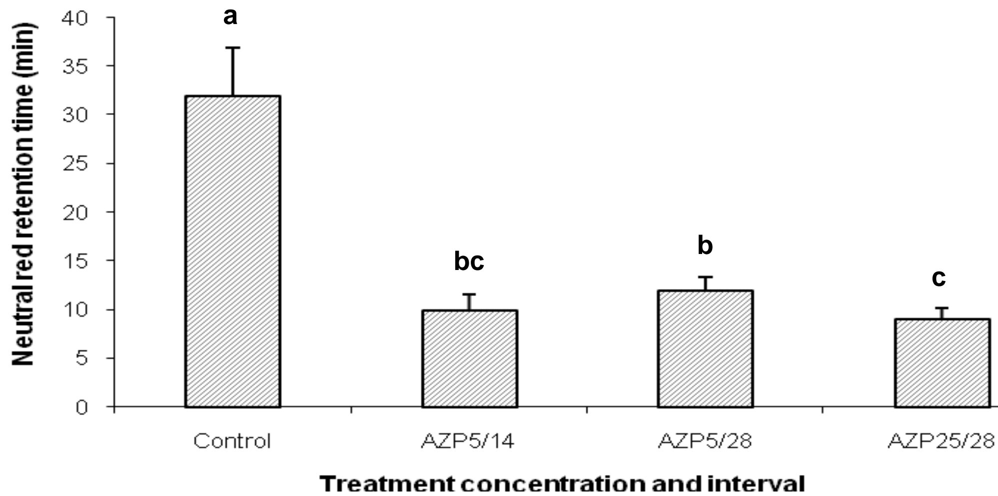


Figure 3.5: Mean neutral red retention times of *E. fetida* coelomocytes following an intermittent 12-week exposure to azinphos-methyl at different exposure concentrations and intervals. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

Between 10 and 20 animals were analysed from each of the different chlorpyrifos treated groups. Cells from these animals had significantly shorter neutral red retention times than the control group, varying from 12 minutes for the CPF25/14 group to 18 minutes for the CPF5/28 group ($H=154.45$; $p<0.05$; Appendix 2: Table 5b). The only chlorpyrifos treated group that was not statistically significantly different from the control was the CPF5/28 group ($p>0.05$). The three other groups (CPF5/14, CPF25/14 and CPF25/28) were statistically significantly different from the control but not from each other ($p>0.05$). The mean neutral red retention times of the various treated groups, along with standard deviations are presented in Figure 3.6.

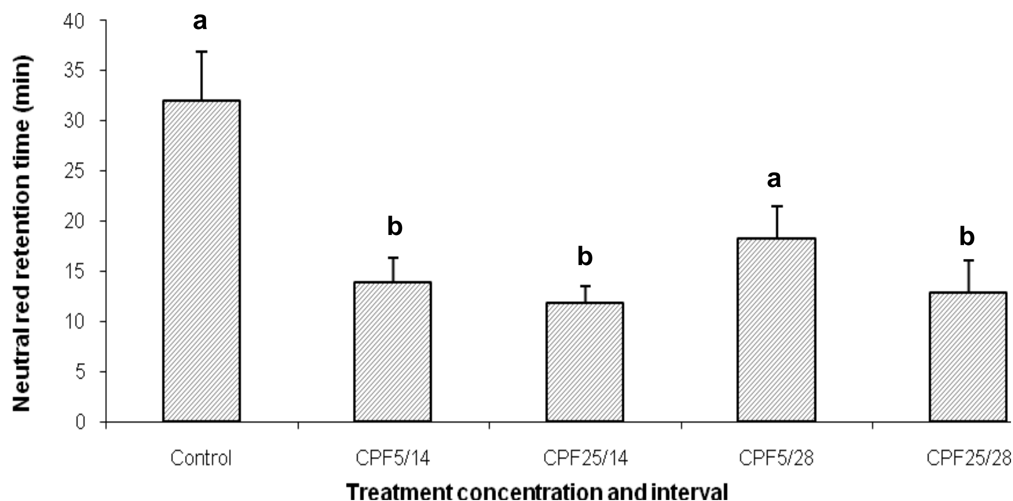


Figure 3.6: Mean neutral red retention times of *E. fetida* coelomocytes following an intermittent 12-week exposure to chlorpyrifos at different exposure concentrations and intervals. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.1.

3.1.3 Behaviour responses and other effects

During the execution of the acute exposures, behaviour responses and morphological changes in the earthworms were noted but not quantified and are described in sections (a) and (b) below. Quantitative measurements were made for burrowing behaviour during the intermittent exposure experiment and avoidance response experiments. These are described in this section under points (c) and (d) below.

a) Morphological changes observed following acute exposures

Animals were inspected on day 7 and day 14 of the exposure period. Both pesticides induced prominent morphological changes in some of the treated animals, with the changes being more evident at higher concentrations and a larger number of animals were also affected at the higher concentrations. Some of the animals exposed to azinphos-methyl were swollen and thick and excreted large amounts of bright yellow coelomic fluid when handled. Lesions on the body wall were observed both macroscopically and microscopically in some individuals from all azinphos-methyl treatment concentrations. At lower concentrations, the majority of the treated animals were tightly coiled into balls, whereas the animals exposed to higher concentrations were contracted into a short (<2cm) stiff body shape and did not show any reaction when touched, apart from coelomic fluid excretion.

The morphological changes observed in worms exposed to chlorpyrifos were markedly different from those exposed to azinphos-methyl. In contrast to the animals exposed to azinphos-methyl, the animals exposed to higher concentrations of chlorpyrifos became thin and limp and showed general body wall degeneration. Some animals appeared to have detached some of their anterior body segments. Movements were sluggish when they were gently poked with a spatula on different areas of the body and most animals showed no avoidance response when touched.

b) Behaviour effects observed following acute exposures

At the end of the 14-day exposure period, all control worms made active attempts to burrow and disappeared from the soil surface within two minutes, while treated animals from both azinphos-methyl and chlorpyrifos exposures either showed no burrowing activity during this time or only partly disappeared from the soil surface. Burrowing activity was more impaired in earthworms exposed to azinphos-methyl than earthworms exposed to chlorpyrifos.

c) Burrowing behaviour following intermittent exposures

Burrowing time in the control group stayed constant throughout the exposure period and no statistically significant differences were observed between the burrowing times of these earthworms at the beginning and at the end of the exposure period ($F=1.33$; $p>0.05$; Appendix 2: Table 6a). For both the azinphos-methyl and chlorpyrifos exposures, no differences in burrowing times for the different treated groups were observed at the beginning of the experiment ($p>0.05$), and these burrowing times were also not different from those of the control worms at the beginning of the experiment ($p>0.05$).

For the azinphos-methyl treatments, the burrowing times of the three azinphos-methyl treated groups were statistically significantly different from the burrowing time of the control group at the end of the exposure period ($H=49.938$; $p<0.05$ Appendix 2: Table 6b, c). No statistically significant difference was observed between the two 28-day treatment interval groups (AZP5/28 and AZP25/28) in terms of end burrowing time ($p<0.05$), while both these groups were different from the AZP5/14 group ($p<0.05$). Mean burrowing times for the different pesticide treated groups are presented in Figure 3.7. The AZP5/14 group had the longest burrowing time of all azinphos-methyl treated groups at the end of the exposure period. The AZP25/14 group was omitted from the statistical analysis, as the last data point

recorded for this group was in week six of the experiment, when animals were severely affected by the presence of the pesticide and burrowing time exceeded 10 minutes. All animals in the latter treatment group also subsequently died as indicated in Section 3.1.2.1.

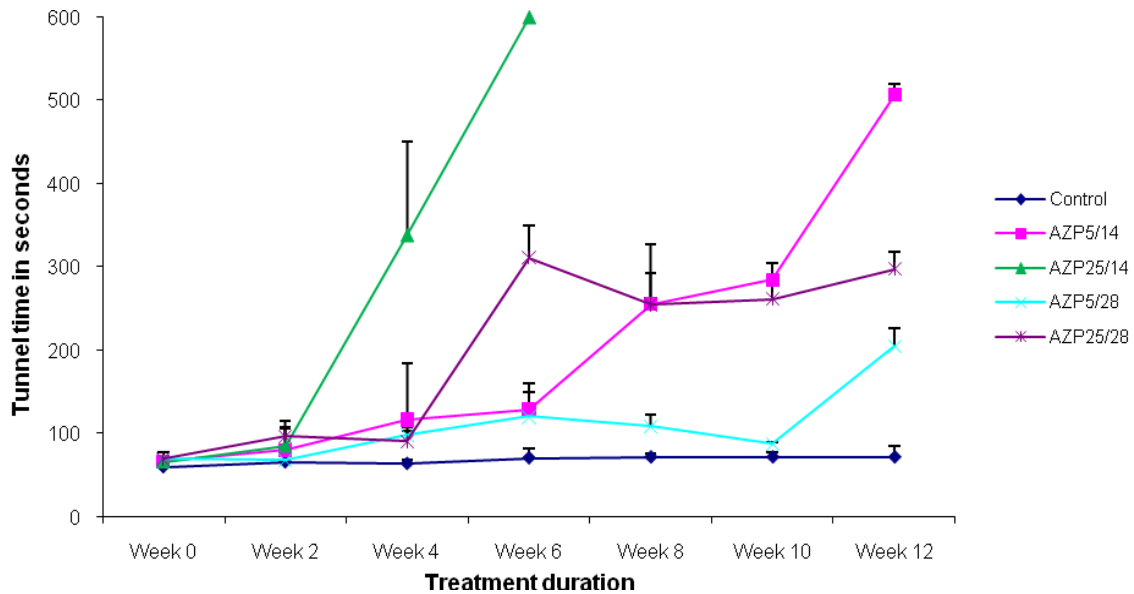


Figure 3.7: Mean burrowing time of *E. fetida* specimens at 14-day intervals following an intermittent 12-week exposure to azinphos-methyl at different exposure concentrations and intervals. Error bars indicate standard deviations. The last data point for the AZP25/14 treatment was recorded at Week 6 of the exposure and 100% mortality was subsequently observed for this group. For treatment concentration and interval abbreviations see Table 2.1.

The burrowing time of the chlorpyrifos treated earthworms did not differ from the burrowing times of the control group at the start of the exposure period. Burrowing times of the earthworms exposed to chlorpyrifos increased with exposure time in a dose dependent manner, but no statistically significant differences were observed between burrowing times of the exposed animals and the control animals at the end of the study ($F=1.33$; $p>0.05$ Appendix 2: Table 6a). The longest burrowing time was recorded for the CPF25/14 group and the shortest for the CPF5/14 group. A graph of the mean burrowing time of all animals exposed to chlorpyrifos is presented in Figure 3.8.

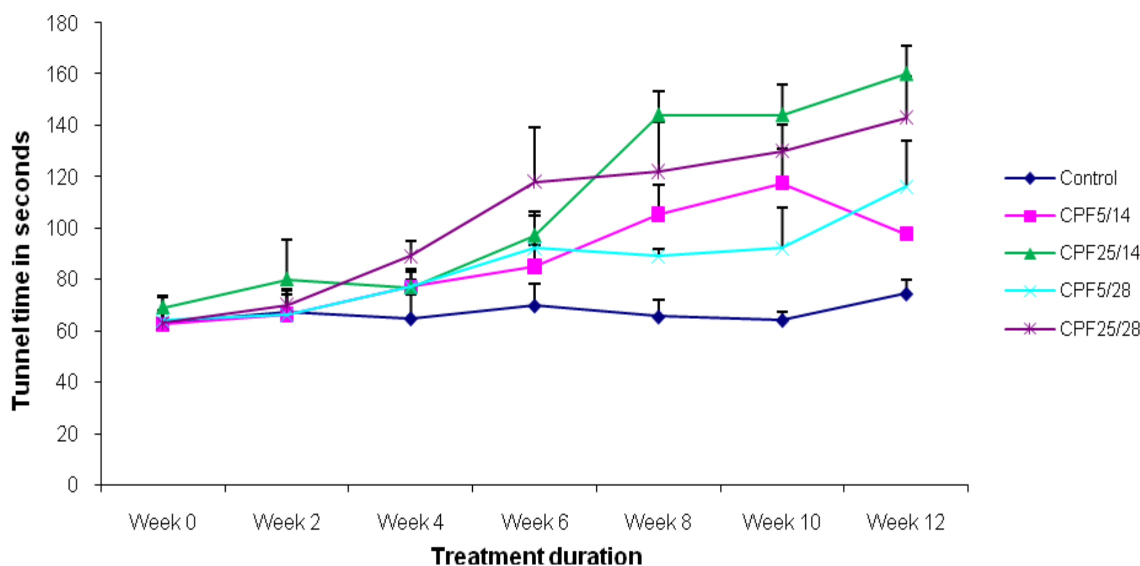


Figure 3.8: Mean burrowing time of *E. fetida* specimens at 14-day intervals following an intermittent 12-week exposure to chlorpyrifos at different exposure concentrations and intervals. Error bars indicate standard deviations. For treatment concentration and interval abbreviations see Table 2.1.

d) Avoidance behaviour

In the preliminary behaviour experiment, a total of 48 animals occurred on the one side of the container and 52 on the other side following the 48h exposure period. Statistical analysis of the results indicate that *E. fetida* dispersed randomly in the selected test soil with no evidence of the animals having a preference for either side of the exposure container ($p>0.05$).

Subsequently *E. fetida* was exposed to 6 and 12 mg/kg azinphos-methyl for an exposure period of 48h. In both experiments the animals were allowed to choose between clean soil and soil contaminated with the pesticide. The two concentrations that were used represent 25% and 50% of the LC_{50} value (25.34 mg/kg) that was reported in Table 3.1. Five replicates with 10 animals each were used for both the 6 and 12 mg/kg exposures. At the end of the exposure time, 21 animals in total occurred in the azinphos-methyl contaminated soil and 29 in total in the clean soil in the 6 mg/kg exposure. In the 12mg/kg exposure, 22 animals in total occurred in the azinphos-methyl contaminated soil and 28 in total in the clean soil as indicated in Table 3.5. Although more animals occurred in the clean soil at both concentrations, the difference was not statistically significant ($p>0.05$ Appendix 2: Table 7) and there is no statistical evidence for the presence of an avoidance response at the two azinphos-methyl concentrations that were tested here.

The concentrations for the chlorpyrifos exposures that represented 25% and 50% of the LC_{50} value (92.83 mg/kg as indicated in Table 3.2) were 23 and 46 mg/kg. The same number of animals and a similar experimental design was used as in the previous azinphos-methyl experiment. In the 23 mg/kg exposure, 28 animals were found in the clean soil and 22 in the contaminated soil. The corresponding numbers of animals for the 46 mg/kg exposure was 26 animals in the clean soil and 24 animals in the chlorpyrifos contaminated soil (Table 3.5). More animals were found in the clean soil at both concentrations, but the differences were not statistically significant ($p>0.05$ Appendix 2: Table 7) and there is no statistical evidence for the presence of an avoidance response at the two chlorpyrifos concentrations that were tested here.

Table 3.5: Result of avoidance experiment using two sublethal fractions of both azinphos-methyl and chlorpyrifos. The lower concentrations (6 mg/kg and 23 mg/kg) refer to 25% of the LC_{50} value of each pesticide and the higher concentrations (12 mg/kg and 46 mg/kg) to 50% of the LC_{50} value. (AZP = azinphos-methyl and CPF = chlorpyrifos).

Treatment	No of animals in clean soil	No of animals in contaminated soil	p-value*
AZP: 6 mg/kg	29	21	p>0.05
AZP:12 mg/kg	28	22	p>0.05
CPF: 23 mg/kg	28	22	p>0.05
CPF: 46 mg/kg	26	24	p>0.05

*The p-values greater than 0.05 indicate that there was no significant difference in the number of animals in the contaminated soil versus the clean soil for any of the concentrations tested here.

3.1.3 Summary

In order to place the results that were obtained in context and to highlight where significant differences occurred, a summary of the results for the intermittent exposures are presented in Tables 3.6 and 3.7.

Table 3.6: Summary of results for all the different endpoints measured in the earthworm *E. fetida* following intermittent exposure to azinphos-methyl for a period of 12 weeks. Different letters denote statistically significant differences between treatment groups.

Endpoint	Treatment regime in terms of pesticide concentration and interval				
	Control	AZP5/14	AZP25/14	AZP5/28	AZP25/28
Mortality	None	20%	100%	None	None
End weight	a	c	---	a	b
ChE activity	a	b	---	b	b
Neutral red retention	a	bc	---	b	c
Cocoon production	a	b	---	b	b
Cocoon viability	a	a	---	a	a
Hatchlings/cocoon	a	a	---	a	a
Burrowing time	a	b	---	b	b

--- indicates that no data was available for this group due to 100% mortality as indicated.

Table 3.7: Summary of results for all the different endpoints measured in the earthworm *E. fetida* following intermittent exposure to chlorpyrifos for a period of twelve weeks. Different letters denote statistically significant differences between treatment groups.

Endpoint	Treatment regime in terms of pesticide concentration and interval				
	Control	CPF5/14	CPF25/14	CPF5/28	CPF25/28
Mortality	None	None	None	None	None
End weight	a	b	b	b	b
ChE activity	a	ab	b	ab	b
Cocoon production	a	b	b	b	b
Neutral red retention	a	b	b	a	b
Cocoon viability	a	a	a	a	a
Hatchlings/cocoon	a	a	a	a	a
Burrowing time	a	ab	b	ab	b

3.2. Effects of organophosphates on fish

3.2.1 Acute exposures

Fish were exposed to range finding concentrations of 0.5 and 0.1 mg/l azinphos-methyl. These concentrations almost immediately resulted in hyperactive behaviour in the treated fish and caused 100% mortality within the first hour of exposure during the 24h exposure period. The final azinphos-methyl exposure range consisted of six concentrations ranging between 0.004 and 0.010mg/l. The experiment was conducted in triplicate and the results analysed using Finney's Probit Analysis. The results and the derived LC_{50} value are presented in Table 3.8.

Table 3.8: Acute toxicity of azinphos-methyl to juvenile *O. mossambicus* following an exposure period of 24 hours. The probit method of data analysis was used.

Exposure concentrations (mg/l)	No of fish exposed	% mortality
0.000	30	0
0.004	30	3
0.005	30	30
0.006	30	40
0.008	30	63
0.010	30	95
0.015	30	100
	Probit analysis estimates	
LC_{50}		0.007 mg/l
95% Lower Confidence		0.006 mg/l
95% Upper Confidence		0.008 mg/l

For the chlorpyrifos treatments, the fish were exposed to range finding concentrations of 1.0 and 0.5 mg/l chlorpyrifos. These concentrations almost immediately resulted in hyperactive behaviour in the exposed fish and caused 100% mortality within the first hour of exposure during the 24h exposure period. The final exposure range for the chlorpyrifos treatment consisted of six concentrations that ranged between 0.005 and 0.250mg/l. The experiment was conducted in triplicate and the results analysed using the Spearman-Karber method of data analysis. The results and derived LC_{50} value are presented in Table 3.9.

Table 3.9: Acute toxicity of chlorpyrifos to juvenile *O. mossambicus* following a 24 hour exposure period. Data was analysed using the Spearman-Karber method.

Concentration (mg/l)	Number of fish exposed	Percentage mortality
0.000	30	0
0.005	30	10
0.010	30	10
0.025	30	13
0.050	30	43
0.100	30	97
0.250	30	100
Spearman - Karber estimates:		
Spearman-Karber trim		10.00%
LC_{50}:		0.05mg/l
95% Lower Confidence:		0.04mg/l
95% Upper Confidence:		0.06mg/l

3.2.2 Intermittent exposures

The abbreviations used hereafter for the different treatment groups were based on the nature of the treatment which that specific group was subjected to as presented in Section 2.2.2.

3.2.2.1 Mortality

No mortality was observed during intermittent exposure experiments in either the control or the treated groups.

3.2.2.2 Growth parameters

Fish were weighed and measured individually at the beginning of the experiment and the mean start weight was 0.831±0.170 g and the mean start length was 288±17 mm total length. Statistically significant differences were not found between the start weights of the different treatment groups ($H=7.33$; $p>0.05$; Appendix 2: Table 8) or the start length ($F=0.936$; $p>0.05$ $p>0.05$; Appendix 2: Table 9) and thus growth was expressed in terms of end length and weight of the fish. The data for the morphological parameters (growth and organ-somatic

indices) are presented separately for the two genders as male and female fish have different growth rates as discussed in Section 2.4.2.

a) End length

In the case of male individuals of the azinphos-methyl treated groups, statistically significant differences in end length were observed between the control group and all treated groups ($F=26.08$; $p<0.05$; Appendix 2: Table 10a), with the exception of the AZP50/28 group ($p>0.05$). There was a statistically significant difference ($p<0.05$) between the two groups subjected to a 28-day exposure interval (AZP10/28 and AZP50/28) but no statistically significant difference between the two groups subjected to a 14-day treatment interval (AZP10/14 and AZP50/14), irrespective of treatment concentration ($p>0.05$; Appendix 2: Table 10b). In the case of females, end length of only the groups exposed to a 14-day treatment interval (AZP10/14 and AZP50/14) was statistically significantly different from the control group ($p<0.05$) and these two groups were not statistically different from each other ($p>0.05$; Appendix 2: Table 10c). Both the groups exposed to a 14-day treatment interval were significantly different ($p<0.05$) from the two 28-day interval groups (AZP10/28 and AZP50/28). The two 28-day treatment interval groups were not statistically significantly different from each other ($p>0.05$). These results are presented in Figure 3.9.

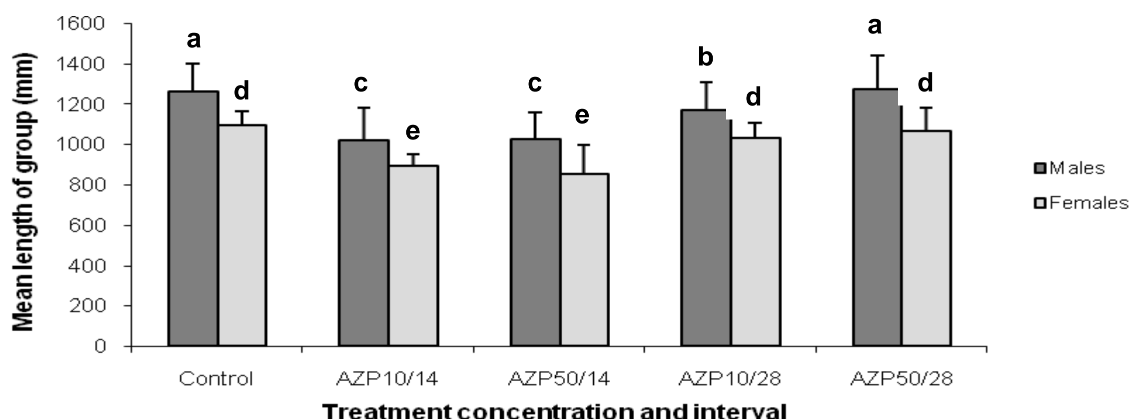


Figure 3.9: Mean end lengths of male and female *O. mossambicus* specimens following an intermittent 12-week exposure to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

For the chlorpyrifos treated groups, a very similar trend to the results for the azinphos-methyl experiment was found. In the case of male fish, for the parameter end length, all chlorpyrifos treated groups were statistically significantly different from the control group (F=26.082; p<0.05; Appendix 2: Table 10a). There was no statistically significant difference between the two groups subjected to a 14-day treatment interval (CPF10/14 and CPF50/14), irrespective of treatment concentration (p>0.05). The two groups subjected to chlorpyrifos exposure at a 28-day treatment interval (CPF10/28 and CPF50/28) were statistically significantly different from each other (p<0.05; Appendix 2: Table 10b). In the case of female fish, for the parameter end length, all chlorpyrifos treated groups were statistically significantly different from the control group (p<0.05). There was no statistically significant difference (p>0.05; Appendix 2: Table 10c) between the two groups subjected to a 14-day treatment interval (CPF10/14 and CPF50/14). The two groups subjected to a 28-day treatment interval (CPF10/28 and CPF50/28) were statistically significantly different from each other (p<0.05), and there was no statistically significant difference between the CPF50/28 group and the two groups subjected to a 14-day treatment interval (p>0.05). These results are presented in Figure 3.10.

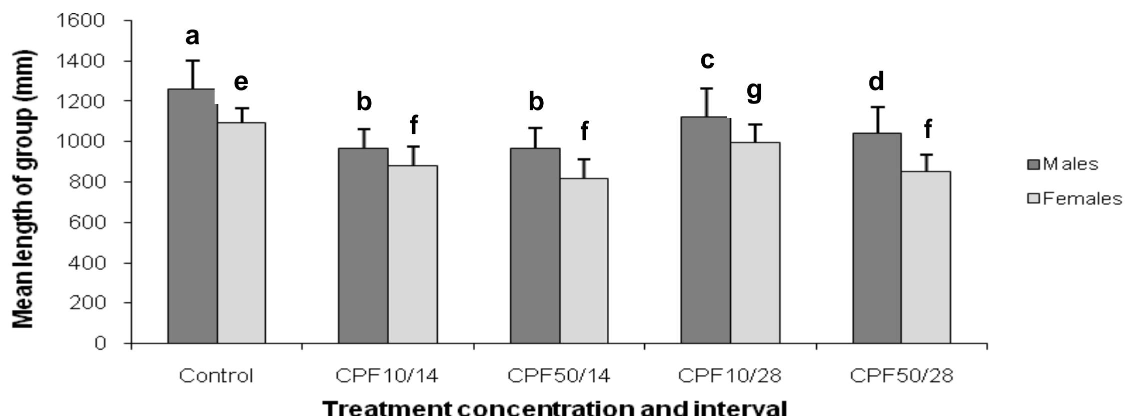


Figure 3.10: Mean end lengths of male and female *O. mossambicus* specimens following an intermittent 12-week exposure to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

b) End weight

In the case of male individuals of the azinphos-methyl treated groups, statistically significant differences in end weight were observed between the control group and all the treated groups ($F=13.892$; $p<0.05$; Appendix 2: Table 11a, b), with the exception of the AZP50/28 group which was not significantly different from the control ($p>0.05$). The two groups subjected to a 28-day treatment interval (AZP10/28 and AZP50/28) were not statistically significantly different from each other ($p>0.05$), irrespective of treatment concentration. The two groups subjected to a 14-day treatment interval (A10/14 and AZP50/14) were statistically significantly different from the control group and from each other, as well as from the two 28-day treatment interval groups ($p<0.05$). In the case of female fish, no statistically significant differences were observed between the two groups subjected to a 28-day treatment interval (AZP10/28 and AZP50/28), irrespective of concentration and these two groups were not different from the control group ($p>0.05$; Appendix 2: Table 11c). The two groups subjected to a 14-day treatment interval were statistically significantly different from the control group and from the two groups subjected to a 28-day treatment interval ($p<0.05$), but not from each other ($p>0.05$). These results are presented in Figure 3.11.

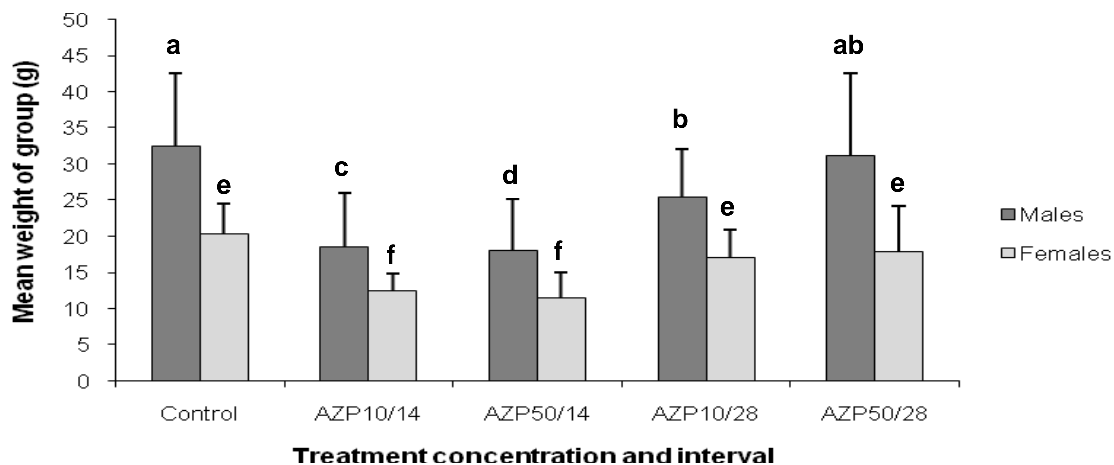


Figure 3.11: Mean end weights of male and female *O. mossambicus* specimens following an intermittent 12-week exposure to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

In the case of male individuals of the chlorpyrifos treated groups, all treated groups were statistically significantly different from the control group ($p<0.05$; Appendix 2: Table 11a, b).

The two groups subjected to a 14-day treatment interval (CPF10/14 and CPF50/14) were not statistically significantly different from each other, irrespective of treatment concentration (p>0.05). The same was true for the two groups exposed to a 28-day treatment interval (CPF10/28 and CPF50/28). Both groups subjected to a 14-day treatment interval were statistically significantly different from the two groups subjected to a 28-day treatment interval (p<0.05). In the case of female fish, all chlorpyrifos treated groups were observed to be statistically significantly different from the control (p<0.05 Appendix 2: Table 11c), but no statistically significant differences were observed between the four chlorpyrifos treatment groups (p>0.05) as presented in Figure 3.12.

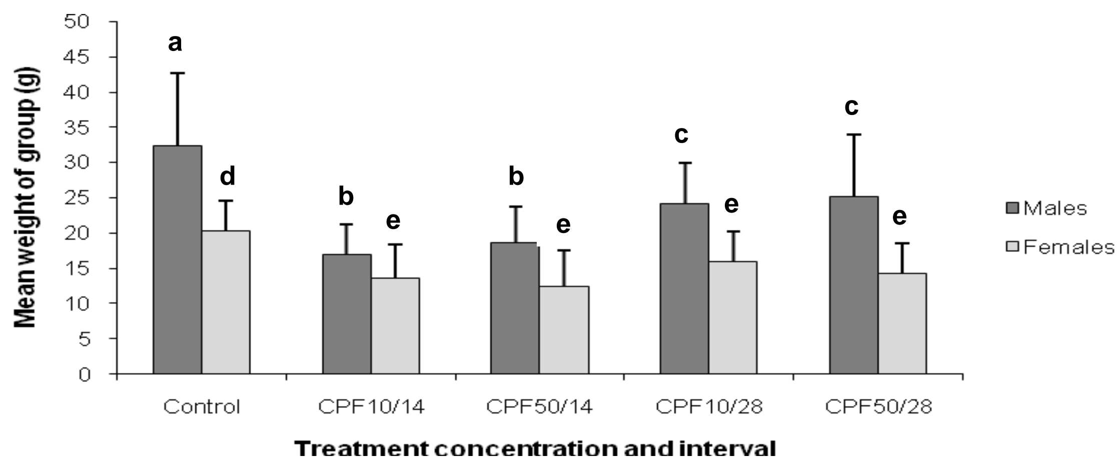


Figure 3.12: Mean end weights of male and female *O. mossambicus* following an intermittent 12-week exposure to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

c) Condition factor

In the case of male fish of the <u>azinphos-methyl</u> treatments, there were statistically significant differences in condition factor among the various groups (H=102.962; p<0.05 Appendix 2: Table 12a, b). The two groups subjected to a 28-day treatment interval (AZP10/28 and AZP50/28) were not statistically significantly different from each other and also not different from the control group (p>0.05). The two groups subjected to a 14-day treatment interval (AZP10/14 and AZP50/14) were statistically significantly different from the control group (p<0.05) but not from each other (p>0.05). These two groups were also not statistically significantly different from the AZP10/28 group (p>0.05).

In the case of female fish in the azinphos-methyl treated groups, there was no statistically significant difference between the two groups exposed to a 28-day interval (AZP10/28 and AZP50/28) and these two groups were also not different from the control group (p>0.05; Appendix 2: Table 12c). The two groups subjected to a 14-day treatment interval (AZP10/14 and AZP50/14) were statistically significantly different from the control group, but not different from each other and also not different from the AZP10/28 group (p>0.05). These results are presented in Figure 3.13.

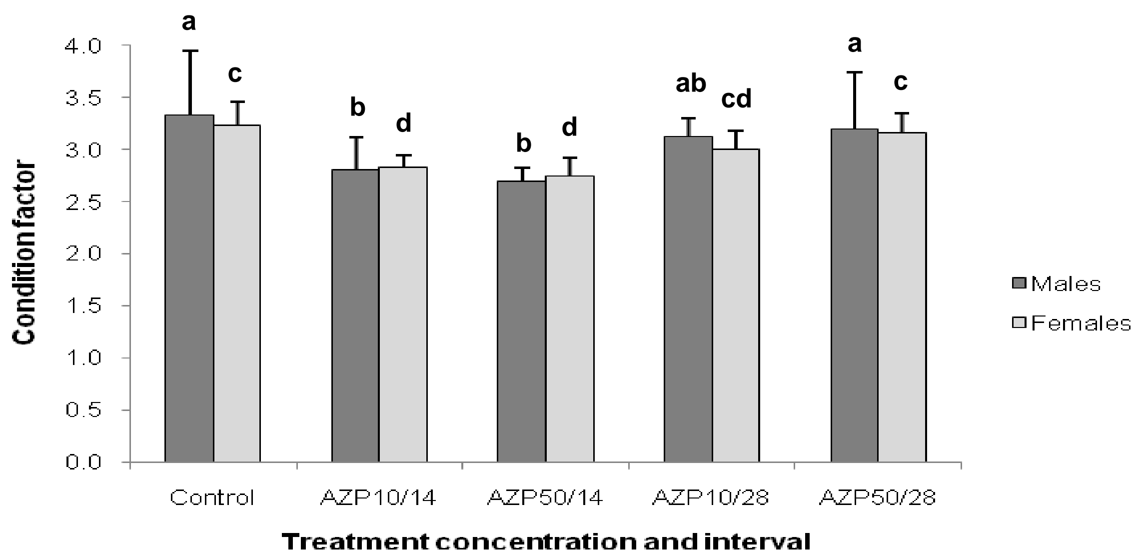


Figure 3.13: Condition factor of male and female *O. mossambicus* specimens following intermittent exposure to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

In the case of male fish of the chlorpyrifos treated groups, condition factor of the CPF10/14 and C50/28 groups were not statistically significantly different from the control group (p>0.05), while the CPF50/14 and CPF10/28 groups were (p<0.05 Appendix 2: Table 12a, b). There were no statistically significant differences in condition factor of male fish between the four chlorpyrifos treated groups (p>0.05). In the case of female fish, no statistically significant differences in condition factor were observed between any of the chlorpyrifos treated groups (p>0.05 Appendix 2: Table 12c). The treated groups were also not significantly different from the control (p>0.05), with the exception of the CPF10/28 group, which was statistically significantly different from the control group (p<0.05) but not from

any of the treated groups. The data for condition factor of the chlorpyrifos treated fish is presented in Figure 3.14.

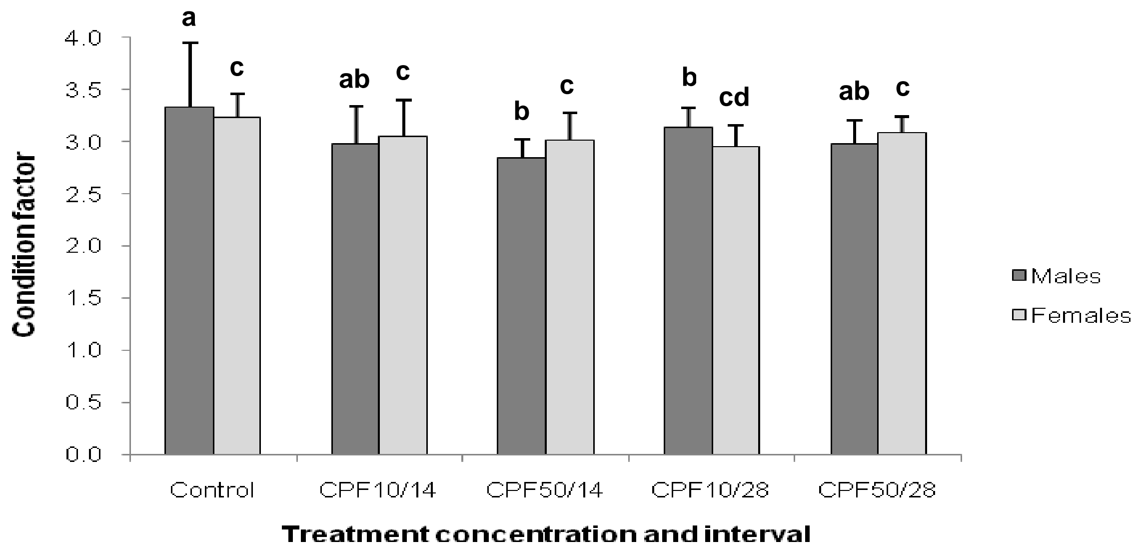


Figure 3.14: Condition factor of *O. mossambicus* specimens following intermittent exposure to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

3.2.2.3 Organ-somatic indices

a) Liver somatic index (LSI)

In the case of male fish, statistically significant differences in the liver somatic index were observed between the control group and all azinphos-methyl treated groups (F=19.411; p<0.05; Appendix 2: Table 13a, b), with the exception of the AZP10/28 group which was not statistically significantly different from the control group (p>0.05). There was no statistically significant difference between the two groups subjected to a 14-day exposure interval (AZP10/14 and AZP50/14), irrespective of treatment concentration (p>0.05). Similarly, there was no statistically significant difference between the two groups subjected to a 28-day exposure interval (AZP10/28 and AZP50/28), irrespective of treatment concentration (p>0.05).

In the case of female fish, the only treated group that was not statistically significantly different from the control group was the AZP10/28 group (p>0.05; Appendix 2: Table 13c). There was no statistically significant difference between the two groups subjected to a 14-day

treatment interval (AZP10/14 and AZP50/14), irrespective of treatment concentration ($p>0.05$). There was also no difference between the two groups subjected to a 28-day treatment interval (AZP10/28 and AZP50/28), irrespective of treatment concentration ($p>0.05$). There were statistically significant differences between the two groups subjected to a 14-day treatment interval and the two groups subjected to a 28-day treatment interval ($p<0.05$). Data on the mean LSI for all azinphos-methyl treatment groups and the control group is presented in Figure 3.15.

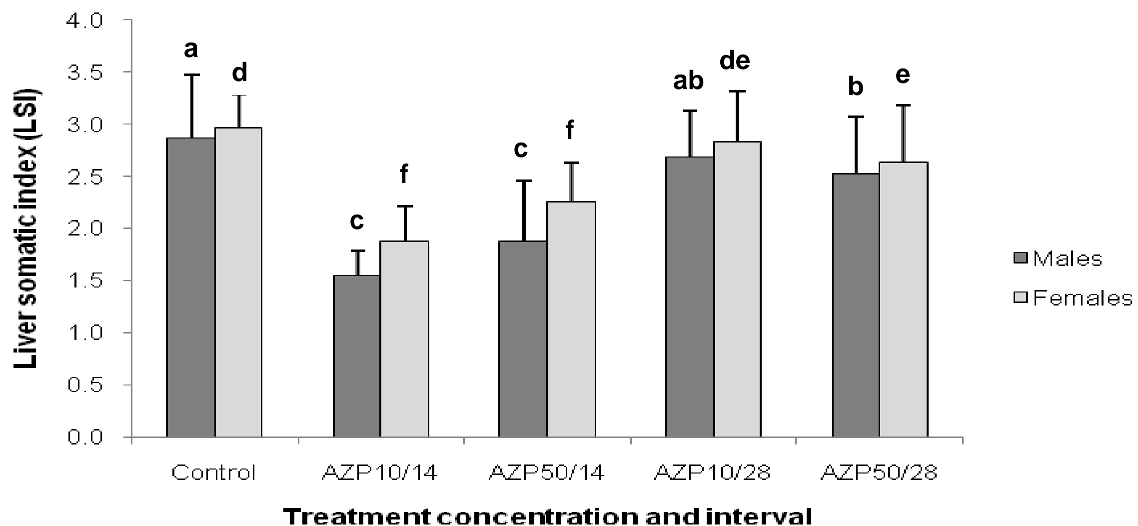


Figure 3.15: Liver somatic index (LSI) of male and female *O. mossambicus* specimens following intermittent exposure to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

For male individuals of the chlorpyrifos treatment groups, the liver somatic index of the two groups subjected to a 28-day interval (CPF10/28 and CPF50/28) was statistically significantly different from the control ($p<0.05$; Appendix 2: Table 13a, b) but not from each other ($p>0.05$). There was no significant difference between the two groups subjected to a 14-day treatment interval ($p>0.05$), and these two groups were not different from the control group ($p<0.05$). A similar trend was evident in female fish exposed to chlorpyrifos. The two groups subjected to a 28-day treatment interval were statistically significantly different from the control ($p<0.05$ Appendix 2: Table 13c) but not from each other ($p>0.05$). There was no statistically significant difference between the two groups subjected to a 14-day treatment interval and these two groups were not significantly different from the control group

(p>0.05). Data on the mean liver somatic index for all chlorpyrifos treated groups and the control group are presented in Figure 3.16.

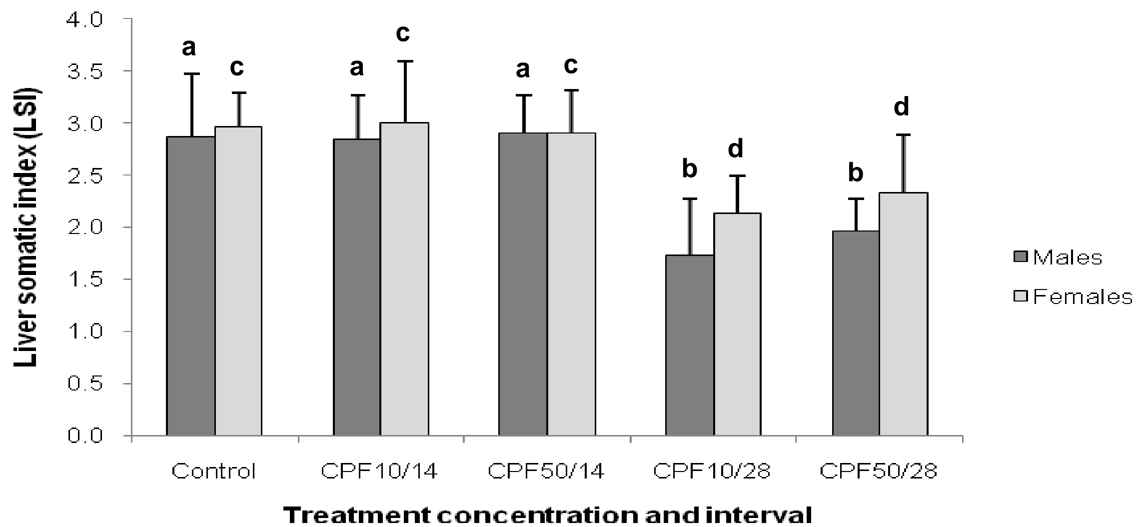


Figure 3.16: Liver somatic index (LSI) for male and female *O. mossambicus* specimens following intermittent exposure to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

b) Gonadosomatic index (GSI)

Different stages of gonadal development were observed in animals from the same treatment groups. Female fish had ovaries that ranged from mature, with large developed oocytes visible macroscopically, to immature ovaries that were small and where no developing oocytes could be observed. Similarly, the testes of some males appeared well-developed and mature while others were immature, with a thin and threadlike appearance. Very high variation existed in gonadal development in animals of the same gender from the same treatment group, based on visual observation of the gonads and the GSI value. For this reason, GSI data was analysed as separate datasets for the two genders. Statistical analysis indicated that no significant differences in gonadosomatic index existed among treated groups and between the treated groups and the control (p>0.05). This was the case for both the azinphos-methyl and chlorpyrifos treated groups and for both males (H=14.169; p>0.05; Appendix 2: Table 14a, b) and females (H=16.412; p>0.05; Appendix 2: Table 14c, d).

Results for gonadosomatic index of the azinphos-methyl and chlorpyrifos treated animals are presented in Figure 3.17 and 3.18.

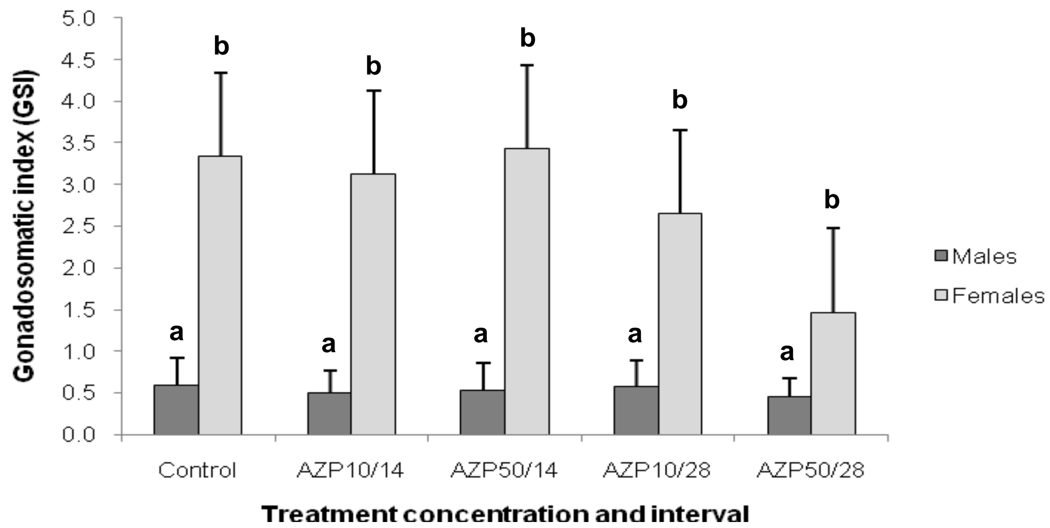


Figure 3.17: Gonadosomatic index (GSI) of male and female *O. mossambicus* specimens following intermittent exposure to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

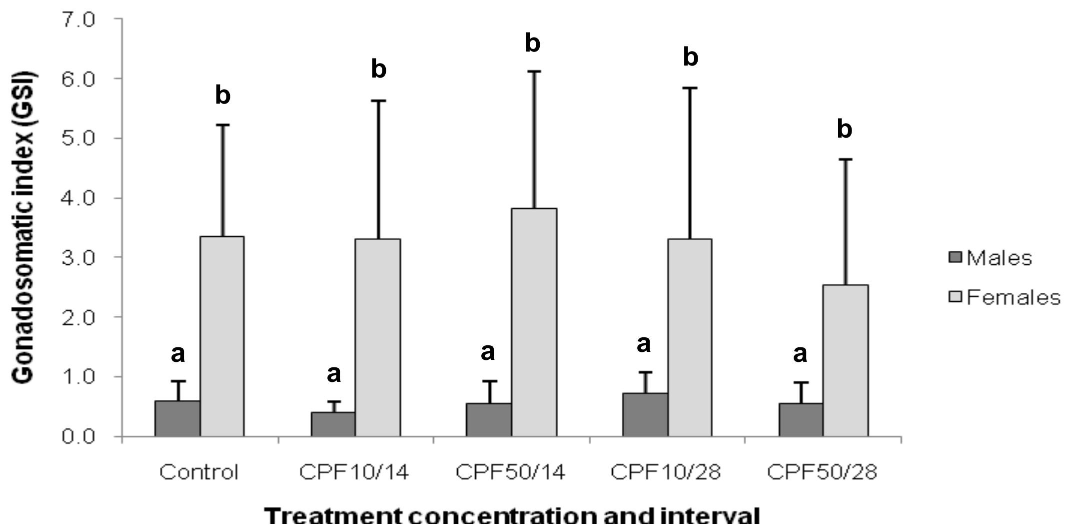


Figure 3.18: Gonadosomatic index (GSI) of male and female *O. mossambicus* specimens following intermittent exposure to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

3.2.2.4 Biomarker responses

a) Acetylcholinesterase activity

Acetylcholinesterase activity for the two genders were analysed together as explained in Section 2.3.1. Statistical analysis indicated significant differences between various treated groups and the control group ($H=64.581$; $p<0.05$ Appendix 2: Table 15a, b). For fish exposed to azinphos-methyl, enzyme activity was reduced relative to the control for all treated groups ($p<0.05$). There was a significant difference ($p<0.05$) between the two groups subjected to a 14-day exposure interval, with the AZP50/14 group having statistically significantly lower enzyme activity than the AZP10/14 group. There was no statistically significant difference ($p>0.05$) between the two groups subjected to a 28-day treatment interval (AZP10/28 and AZP50/28) and these two groups were also not significantly different from the AZP10/14 group ($p>0.05$). Data on the mean AChE activity for all azinphos-methyl treatment groups are presented in Figure 3.19.

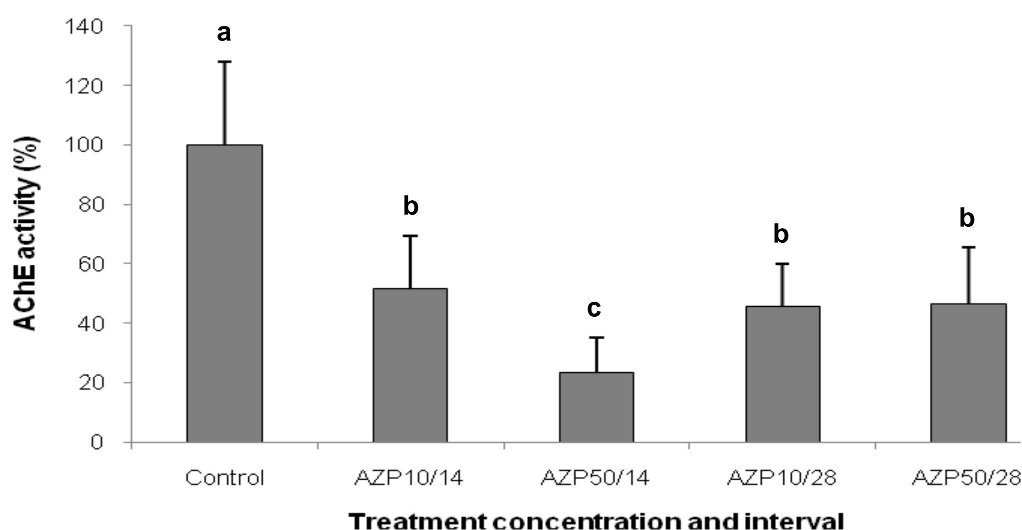


Figure 3.19: Brain acetylcholinesterase activity of *O. mossambicus* specimens exposed to azinphos-methyl at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

For the chlorpyrifos exposures, all pesticide treated groups were statistically significantly different from the control ($p<0.05$; Appendix 2: Table 15a, b) but no significant differences existed between the four chlorpyrifos-exposed groups, irrespective of treatment concentration or interval ($p>0.05$). Data on the mean AChE activity for all chlorpyrifos treatment groups are presented in Figure 3.20.

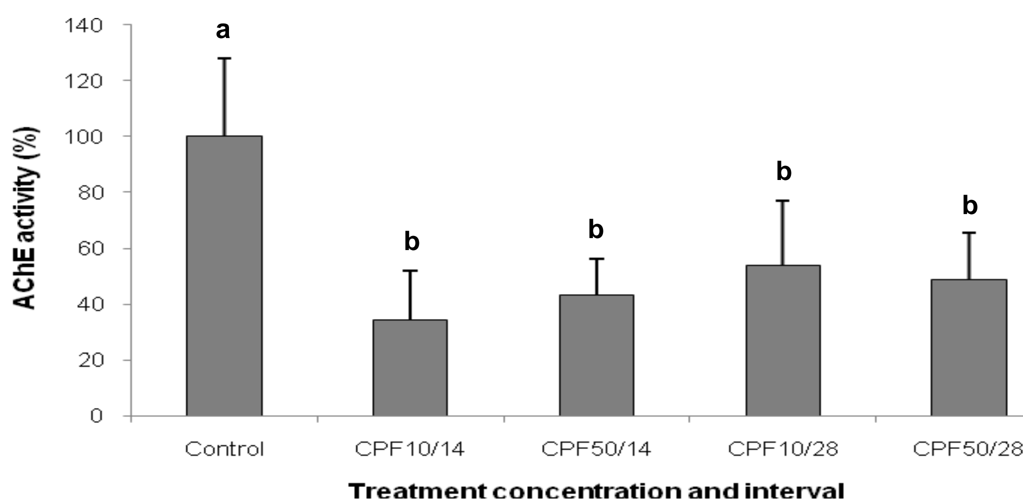


Figure 3.20: Brain acetylcholinesterase activity of *O. mossambicus* specimens exposed to chlorpyrifos at different treatment regimes. Error bars denote standard deviations. Different letters indicate statistically significant differences. For treatment concentration and interval abbreviations see Table 2.2.

3.2.3 Behavioural responses and other effects

Behavioural responses and morphological changes in the fish were noted but not quantified during the execution of the acute exposures. These are described in this section under points (a) and (b). Quantitative measurements were made for feeding behaviour experiments where feeding response time and total food consumption was investigated as endpoints following exposure to two different concentrations of azinphos-methyl and chlorpyrifos. The results of these experiments are described in this section under points (c) and (d).

a) Morphological effects observed after acute exposures

Morphological effects were noted after 1, 12 and 24 hours of exposure to each of the two pesticides. After the first hour of exposure, behaviour was aberrant as discussed in (b), but obvious morphological changes were absent. After 12 hours of exposure to both pesticides, fish in the higher exposure groups (>0.008mg/l for azinphos-methyl and >0.05mg/l for chlorpyrifos) exhibited a contracted, distorted body shape and this was followed by signs of paralysis, darkened body colouration and loss of equilibrium. Fish exposed to lower concentrations of both pesticides showed similar symptoms, with the exception of the distorted body shape, and the symptoms were generally less severe and only a few individuals were affected after 24 hours.

b) Behavioural effects observed after acute exposures

Behavioural responses were noted after 1, 12 and 24 hours of exposure to each of the two pesticides. Fish exposed to the range finding concentrations of 1.0 and 0.5 mg/l active ingredient for both pesticides were immediately agitated when exposed to the pesticide and behaviour was characterized by frantic darting around the tank and extreme hyperventilation, followed by death within the first 60 minutes of the exposure. Behaviour during the final exposures for both pesticides was characterised by inactivity and lying on the bottom of the tank or clustering at the water surface, interspersed by short bursts of hyperactivity every few minutes. After 24 hours of azinphos-methyl exposure, the few surviving fish in the 0.008 to 0.010 mg/l exposure groups exhibited similar behaviour effects to those observed in the range finding experiment. These effects were present after 12 hours of exposure and progressively intensified until the end of the 24h exposure period. The symptoms of the fish exposed to lower concentrations (0.004-0.006 mg/l) did not appear to be worse after 24 hours of exposure compared to 12 hours after exposure.

Chlorpyrifos treatment at 0.250 and 0.100 mg/l resulted in aberrant behaviour after one hour and 100% mortality occurred in the 0.250 mg/l group within 12 hours. Behaviour in these two groups was characterized by a loss of equilibrium, spiralling swimming behaviour and hyperventilation. Fish were also generally immobile followed by short bursts of hyperactivity. Fish in these groups were also typically clustering at the water surface or lying on the bottom of the tank. All individuals in these groups were affected. Fish exposed to lower concentrations of chlorpyrifos (0.005-0.05 mg/l) showed similar behaviour alterations as the fish exposed to higher concentrations, but symptoms were less severe and fewer individuals (less than 50% of the total number of fish in the exposure group) were affected.

c) Feeding response time

The preliminary experiment indicated the number of untreated fish per tank had an effect on the feeding behaviour. When only a single fish was present, feeding response was slow and the first feeding attempt was only observed 20-36 seconds after the food was given. In contrast, when two or three fish were present per tank, fish attempted to feed within 3-9 seconds after the food was made available. For the final experiment, two fish were used per tank with six tanks in each of the three replicates. The two concentrations for azinphos-methyl treatment were 0.0007 mg/l and 0.0035 mg/l, and the corresponding concentrations for chlorpyrifos were 0.005 mg/l and 0.025 mg/l. (These values represent 10% or 50% of the

LC_{50} value of both pesticides.) Statistical analysis indicated that all treated groups had significantly longer response times that the control (H=79.719; $p<0.05$ Appendix 2: Table 16a, b). The only exception to this was the group exposed to chlorpyrifos at 10% of the LC_{50} value (0.005 mg/l), which was not different from the control group ($p>0.05$). There was a statistically significant difference in feeding response time between the groups exposed to the lower and higher concentration for both pesticides ($p<0.05$). These results are presented in Figure 3.21.

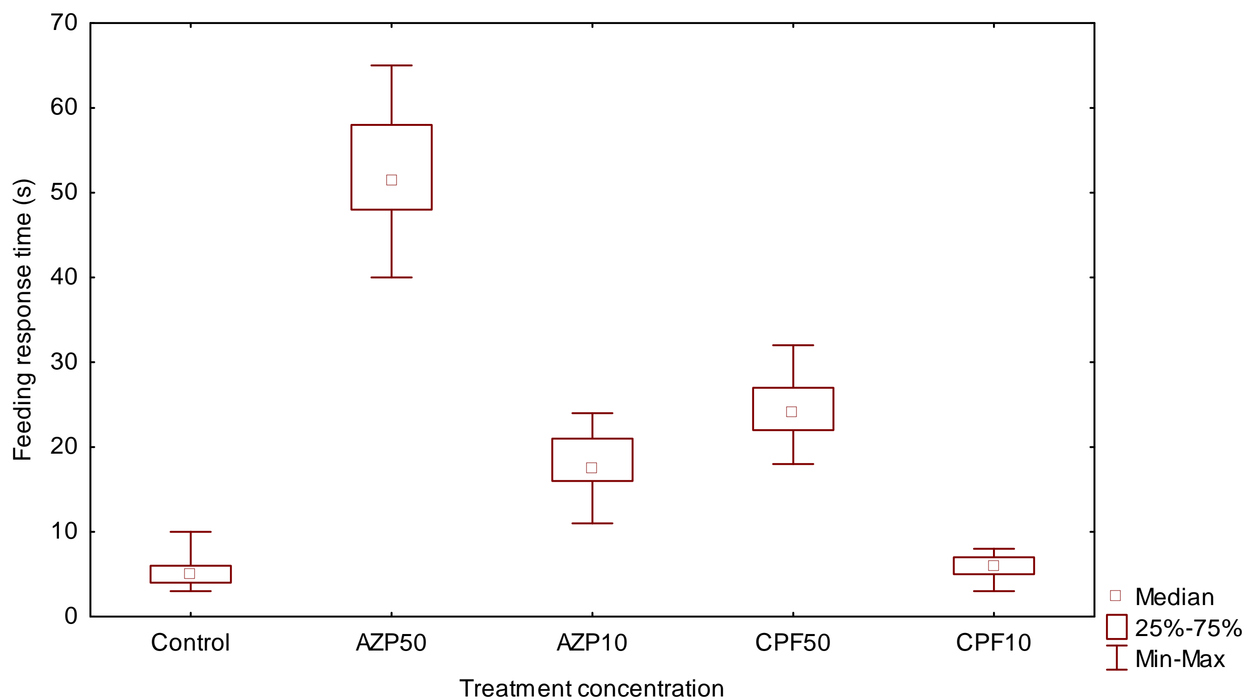


Figure 3.21: Feeding response time of *O. mossambicus* specimens following a 24h exposure to two sublethal concentrations (10% and 50% of the LC_{50} value) of azinphos-methyl and chlorpyrifos. The actual concentrations used were 0.007 mg/l and 0.0035 mg/l for azinphos-methyl and 0.005 mg/l and 0.025 mg/l for chlorpyrifos.

d) Food consumption

Statistical analysis indicated significant differences between the various treated groups and the control group (H=58.266; $p<0.05$ Appendix 2: Table 17a, b). All treated groups, with the exception of the group exposed to chlorpyrifos at 10% of the LC_{50} value (0.005 mg/l), consumed significantly less food particles than the control group ($p<0.05$). There were no statistically significant differences between the two groups exposed to azinphos-methyl at

10% and 50% of the LC_{50} value (0.0007 mg/l and 0.0035 mg/l) and these two groups were also not different ($p>0.05$) from the group exposed to chlorpyrifos at 50% of the LC_{50} value (0.025 mg/l). Fish exposed to chlorpyrifos at 50% of the LC_{50} value consumed significantly less pellets than fish exposed to 10% of the LC_{50} value ($p<0.05$). This data is presented in Figure 3.22.

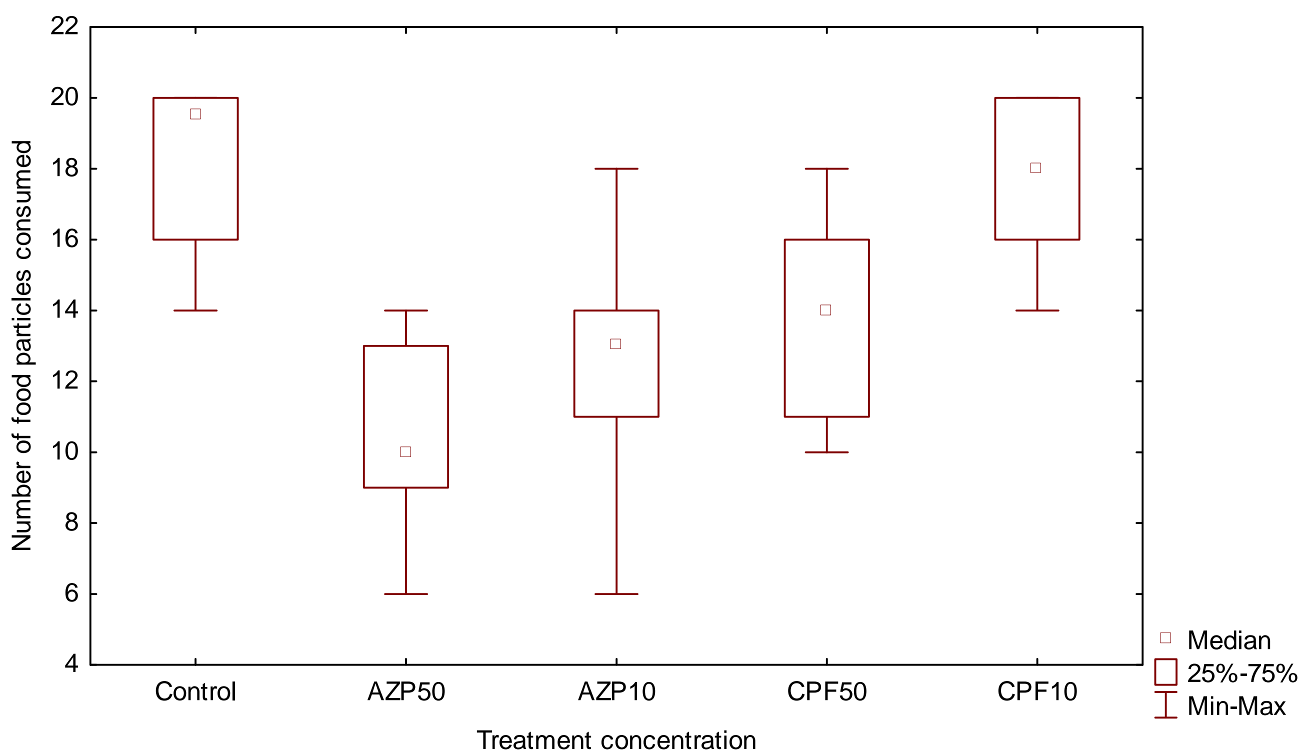


Figure 3.22: Food consumption of *O. mossambicus* specimens following a 24h exposure to two sublethal concentrations (10% and 50% of the LC_{50} value) of azinphos-methyl and chlorpyrifos. The actual concentrations used were 0.007 mg/l and 0.0035 mg/l for azinphos-methyl and 0.005 mg/l and 0.025 mg/l for chlorpyrifos.

3.1.2.4 Summary

A number of endpoints were investigated following the 12-week intermittent exposure period and a summary of the results are presented in Table 3.10 and 3.11.

Table 3.10: Summary of data for the various endpoints measured in the fish *O. mossambicus* following intermittent exposure to azinphos-methyl for a period of 12 weeks. Different letters denote statistically significant differences between treatment groups. The letters M and F after each endpoint refers to males and females respectively.

Endpoint	Treatment regime in terms of pesticide concentration and interval				
	Control	AZP10/14	AZP50/14	AZP10/28	AZP50/28
End weight – M	a	c	d	b	ab
End weight – F	a	b	b	a	a
End length – M	a	c	c	b	a
End length – F	a	b	b	c	c
Condition factor – M	a	b	b	ab	a
Condition factor – F	a	bc	c	ab	ab
Liver somatic index – M	a	b	b	ac	c
Liver somatic index – F	a	b	b	ac	c
Gonadosomatic index – M	a	a	a	a	a
Gonadosomatic index – F	a	a	a	a	a
AChE activity	a	b	c	b	b

Table 3.11: Summary of data for the various endpoints measured in the fish *O. mossambicus* following intermittent exposure to chlorpyrifos for a period of 12 weeks. Different letters denote statistically significant differences between treatment groups. The letters M and F after each endpoint refers to males and females respectively.

Endpoint	Treatment regime in terms of pesticide concentration and interval				
	Control	CPF10/14	CPF50/14	CPF10/28	CPF50/28
End weight – M	a	b	b	c	c
End weight – F	a	b	b	b	b
End length – M	a	b	b	c	d
End length – F	a	b	b	c	b
Condition factor – M	a	ab	b	b	ab
Condition factor – F	a	ab	ab	b	ab
Liver somatic index – M	a	a	a	b	b
Liver somatic index – F	a	a	a	b	b
Gonadosomatic index – M	a	a	a	a	a
Gonadosomatic index – F	a	a	a	a	a
AChE activity	a	b	b	b	b

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1: Toxicity of organophosphates to *E. fetida*

The present study aimed to investigate the effects of selected organophosphate pesticides on *E. fetida* as non target organism, on various levels of biological investigation following different exposure regimes. As a starting point, the study investigated the acute toxicity of chlorpyrifos and azinphos-methyl on juvenile animals and compared it to values from literature. The results of this are discussed in greater detail in section (a) but the results obtained disproves the null hypothesis which stated that juvenile animals are not expected to be more sensitive to organophosphate pesticides than adults following acute exposures. Regarding the investigation of the effects of intermittent pesticide exposure on a number of sublethal endpoints, the results indicate that organophosphate pesticides negatively affected *E. fetida* for the majority of the endpoints investigated namely biomarker responses, growth, reproduction and behaviour. These are discussed in greater detail in the rest of section 4.1. Strong evidence is presented throughout this section for higher exposure concentrations inducing more severe effects, and exposure interval playing a less pronounced role than exposure concentration in inducing an effect. This is in alignment with the second hypothesis that was proposed at the start of the study, which stated that a more pronounced response is expected at higher treatment concentrations and treatment interval is expected to play a less pronounced role than treatment concentration in inducing a response.

a) Acute toxicity and mortality

Based on the LC_{50} values obtained from the acute exposures during the present study, azinphos-methyl appears to be about four times more toxic than chlorpyrifos to juvenile specimens of the earthworm *E. fetida* (Tables 3.1 and 3.2). Acute toxicity tests for adult earthworms did not form part of the present study and therefore the values obtained for juvenile animals were compared to values reported for adults in the literature. The Pesticide Manual (2000), published by the British Crop Protection Council, reports the LC_{50} values for adult individuals of the species *E. fetida* as 215 mg/kg for chlorpyrifos and 59mg/kg for azinphos methyl. These values are much higher than the corresponding values of 93 mg/kg

and 25 mg/kg obtained for *E. fetida* in the present study, presenting evidence that juvenile earthworms are more sensitive to both pesticides than adults.

In contrast to the value presented in The Pesticide Manual (2000), Zhou et al. (2007) reported a LC_{50} value of 91.8 mg/kg for adults of the closely related species *E. andrei* for chlorpyrifos, which corresponds well to the value of 93 mg/kg observed for juveniles in the present study. These two conflicting values in the literature regarding the acute toxicity of chlorpyrifos to adult earthworms of the genus *Eisenia* makes it difficult to comment on the sensitivity of juvenile animals, in relation to adults, for this pesticide. If taking the LC_{50} value of 215 mg/kg, published in The Pesticide Manual (2000) as being representative of the toxicity of chlorpyrifos to this species, it is clear that both pesticides are more toxic to juvenile *E. fetida* than to adults of this species. As one of the main routes of uptake of the pesticides is through the body wall of the earthworms, the increased sensitivity of juvenile earthworms can partly be explained by the larger surface-area-to-volume ratio, and thus higher potential uptake rate, of juvenile earthworms in relation to adult worms. A higher rate of toxicant uptake due to a larger body surface area in relation to volume was suggested by Spurgeon et al. (2000) to explain the increased zinc sensitivity of the smaller earthworm species *Lumbricus rubellus* in relation to the larger species *Lumbricus terrestris*.

According to Matsumura (1975) the detoxification mechanisms of juvenile animals in general may not be as well-developed as those of adult animals. If it is assumed that this is the case for earthworms, there may be a biochemical basis for increased sensitivity of juvenile animals. One of the most important enzyme systems involved in the oxidation of endogenous and exogenous substances in both vertebrates and invertebrates, is the cytochrome P-450 dependent monooxygenase, or the mixed-function oxidase (MFO) system (Hyne and Maher, 2003). This system is a universally distributed highly conserved enzyme system known to be induced by a range of xenobiotic compounds (Livingstone, 1990). By altering the chemical structure of compounds, cytochrome P-450 enzymes may render some substances non-toxic or, in contrast, drastically increase the toxicity of others (Hyne and Maher, 2003). An example of the latter is the bioactivation of organophosphates to their oxygen analogs, thereby increasing their anticholinesterase effects (Hernandez et al., 1998). In earthworms, evidence exists for the presence of cytochrome P-450 monooxygenases (Stenersen et al, 1992; Eason et al., 1998), but whether the activity is different in adult and juvenile worms has not yet been established. This requires further investigation and therefore it is difficult to

determine the role that these enzymes play in attempting to explain the increased sensitivity of juvenile animals in relation to adults.

b) Toxicity and mortality following intermittent exposures

As the intermittent exposure experiment was designed to test sublethal pesticide concentrations, mortality was not defined as endpoint in this experiment and therefore the treatment concentrations were chosen to be well below those expected to be lethal. No mortality was observed in any of the chlorpyrifos treatment groups. In contrast, mortality was observed in some azinphos-methyl treated groups, with 100% mortality occurring in the group exposed to 25% of the LC_{50} value at a 14-day interval (AZP25/14), by week six of the exposure. The mortality that was observed in this group, compared to the 100% survival of the animals in the corresponding chlorpyrifos treatment (CPF25/14), confirms the higher toxicity of azinphos-methyl relative to chlorpyrifos to juvenile *E. fetida*. This result may also present evidence for higher cumulative toxicity of azinphos-methyl compared to chlorpyrifos following multiple exposures. This presumed higher cumulative toxicity is likely the result of either higher acute toxicity or a longer half-life in the soil, or a combination of the two (Connell et al., 1999). The environmental half-lives of the two pesticides are discussed later when presenting the rest of the data from the intermittent exposure experiment. It must be noted that a longer half-life in the exposure medium could have resulted in bioaccumulation of the pesticide in the organisms, which over time could have resulted in mortality, as was observed in the azinphos-methyl treatments.

c) Morphological changes

After the acute exposures to both pesticides, prominent morphological changes were observed in the treated earthworms. Effects observed in the chlorpyrifos treated worms following the acute exposures corresponded to those observed by Venkateswara Rao et al. (2003a) when exposing *E. fetida* to chlorpyrifos during a filter paper contact test. These effects include structural damage to the prostomium, necrosis of the body wall and severe damage to the circular and longitudinal muscles of the body. Additionally, Reddy and Rao (2008) observed bloody lesions and fragmentation of the anterior segments of the body when exposing *E. fetida* to the organophosphate profenofos. Similar effects were observed in the present study following acute exposure of the animals to chlorpyrifos and are described in Section 3.1.3. No comparative literature describing the effects of acute azinphos-methyl on *E. fetida* was found.

Regarding the intermittent exposures to these two pesticides, earthworms exposed to chlorpyrifos did not have any external lesions visible macroscopically. Only the earthworms exposed to azinphos methyl showed morphological changes resembling those observed following the acute exposures as was described in Section 3.1.3. Significant macroscopical changes appear to be readily induced in earthworms by high concentrations of certain organophosphates, and if these injuries are very severe it may result in mortality of the affected animals, as was observed in some of the azinphos-methyl treatments.

d) Acetylcholinesterase activity

Significant AChE inhibition following organophosphate exposure has been shown to occur in a number of vertebrate and invertebrate species at concentrations well below the LC_{50} value (Day and Scott, 1990; Booth et al., 2001; De Mel and Pathiratne, 2005). The present study yielded important results regarding the effect of pesticide exposure interval and exposure concentration on the degree of cholinesterase inhibition in earthworms following multiple exposures. A reduction in enzyme activity was observed in both azinphos-methyl and chlorpyrifos treated groups at the concentrations tested here, as indicated in Figures 3.3 and 3.4. Large inter-individual variation in enzyme activity between control animals was also observed in the present study, which is consistent with the results of Walker (1995), Collange et al. (2010) and Gonzáles Vejares et al. (2010). The results indicated that, when administered at a similar treatment regime in terms of treatment concentration and treatment interval, azinphos-methyl is a more potent cholinesterase inhibitor than chlorpyrifos in *E. fetida*. All animals exposed to azinphos-methyl had less than 10% enzyme activity relative to the control at the end of the exposure period, with the group exposed to 25% of the LC_{50} value at a 28-day interval (AZP25/28) being the most severely affected, with less than 1% activity (Figure 3.3). These results present evidence that treatment concentration plays a more prominent role than treatment interval in inducing enzyme inhibition. This is further confirmed by the results of the intermittent chlorpyrifos exposure where only the two groups exposed to the higher pesticide concentration (CPF25/14 and CFP 25/28) had significantly lower enzyme activity than the control group (Figure 3.4). The very low enzyme activity (<1% of control value) observed for the AZP25/28 treatment group further illustrates that *E. fetida* can survive high levels of cholinesterase inhibition and that almost complete inhibition of cholinesterase does not inevitably result in mortality. All worms from this group survived to the end of the 12-week exposure period. A similar result was also

observed by Booth et al. (1998) who reported that the earthworm *Aporrectodea caliginosa* was able to survive an 87% reduction in ChE activity following exposure to chlorpyrifos.

The link between cholinesterase inhibition and mortality in *E. fetida* was investigated by Stenersen (1979) who found that the length of the exposure has important consequences in terms of mortality. Nearly complete cholinesterase inhibition followed by a rapid recovery in the absence of a toxicant resulted in a limited toxic response, whereas less inhibition and a slower recovery resulted in a more severe toxic response. This could offer an explanation for the mortality observed in the two groups treated at a 14-day interval with azinphos-methyl during the present study. The degree of initial enzyme inhibition in the 14-day treatment interval groups was comparable to the inhibition that occurred in the 28-day treatment interval groups after the first exposure as the treatment concentration was the same. However, the ambient pesticide concentration in the soil was most likely higher in the 14-day treatment interval groups for the duration of the exposure period due to the shorter exposure interval between successive treatments. As a result recovery was most likely not possible in these groups and therefore mortality resulted. In spite of near-complete enzyme inhibition in the 28-day treatment interval groups, mortality did not occur due to a longer time between consecutive treatments which allowed adequate recovery time. The length of time between consecutive exposures is important as recovery of ChE activity following organophosphate exposure is generally slow in earthworms (Aamodt et al., 2007; Rault et al., 2008; Collange et al., 2010). Recovery is largely dependent on the synthesis of new enzyme molecules due to the fact that organophosphate pesticides form a stable irreversible bond with the target enzyme (Fulton and Key, 2001). Spontaneous reactivation of the phosphorylated enzyme in earthworms is generally very low (Rodriguez and Sanchez-Hernandez, 2007).

For the chlorpyrifos treatments, exposure concentration also played a more prominent role in inhibiting ChE activity than exposure interval. This is evident from the results presented in Figure 3.4, where only in the two groups exposed to a higher pesticide concentration the enzyme activity was significantly inhibited relative to the control. Cholinesterase activity in the two groups exposed to a lower concentration was not different from the control, nor was it different from the two groups exposed to a higher concentration. In the study of Rault et al. (2008), it was also illustrated that exposure concentration played a significant role in the pattern of ChE inhibition and recovery in the earthworm species *Allobophora chlorotica* and *A. caliginosa* following exposure to ethyl-parathion. Exposure concentration playing a more

prominent role than exposure interval in reducing AChE activity was also observed following organophosphate exposure in other animals. This was illustrated in mice by Long et al. (2006). When exposing mice to 10 and 20 mg/kg of the organophosphate dimethoate on three consecutive days, these authors observed that AChE inhibition increased progressively with successively repeated exposures, but that this effect was relatively small compared to the effects of dose.

Results observed during the present study therefore illustrate the potent cholinesterase-inhibiting ability of azinphos-methyl relative to chlorpyrifos in earthworms and gives some clarity to the different roles that exposure interval and concentration play in inducing the observed effect. Reduced acetylcholinesterase activity has been linked to a number of behavioural effects in a number of species (Beauvais et al., 2000, Scott and Sloman, 2004; Jensen et al., 1997). A number of behaviour effects were investigated in this study and the link between ChE inhibition and behaviour will be discussed later.

e) Neutral red retention time

In the present study neutral red retention time (NRRT), a biomarker of cellular damage, was affected in all treatment groups for both pesticides, with the exception of one chlorpyrifos treated group which was not different from the control group (Figures 3.5 and 3.6). The only group where the NRRT was not affected was the CPF5/28 group which was exposed to chlorpyrifos at the lower concentration (4.64 mg/kg) and longer interval (28 days). Booth and O'Halloran (2001) investigated NRRT in the earthworm *A. caliginosa* and concluded that this biomarker was sensitive enough to detect the presence of chlorpyrifos at a predicted environmental concentration of 4mg/kg following a four week laboratory exposure. Similar results were reported for the neutral red retention assay by the work of Booth et al. (2001) following chlorpyrifos exposures using mesocosms. In contrast to these results, the present study shows that for *E. fetida*, the NRRT was not affected by a chlorpyrifos concentration of 4.64 mg/kg following a 12-week exposure period. A likely explanation for the conflicting results presented by these two studies and the present study is the differences in the experimental design between the two studies and more importantly, that different earthworm species with apparent different sensitivities to chlorpyrifos, were used as test organisms.

The studies of Booth and O'Halloran (2001) and Reinecke and Reinecke (2007a) reported clear dose-response effects on NRRT following exposure to organophosphates in laboratory

and microcosm studies. All exposure groups in the present study (except CPF5/28) showed significantly shorter retention times than the control, and a dose-related response is observed for azinphos-methyl groups subjected to a longer (28-day) treatment interval, as indicated in Figure 3.5. In the chlorpyrifos exposures, concentration did not play a significant role in affecting the neutral red retention time in the two groups exposed using a shorter (14-day) treatment interval. In contrast, a dose-related response was evident for the groups exposed using a longer (28-day) treatment interval. Here only the group exposed to the higher pesticide concentration (CPF25/28) had a significantly shorter neutral red retention time than the control, as can be seen in Figure 3.6. This indicates that at a longer exposure interval, concentration becomes an important factor in inducing a toxic response. From these results, it is difficult to accurately determine the different effects that exposure interval and exposure concentration plays in inducting an effect on the NRRT of *E. fetida*, but there is some evidence that treatment concentration plays a more important role.

An important observation from the NRRT data is the short retention time of the control worms. The mean NRRT for untreated *E. fetida* individuals from the present study was 32 minutes, while Maboeta et al. (2004) reported much longer retention times (>60 minutes) for control animals of this species. Similar retention times for untreated earthworms were also reported for the earthworm *A. caliginosa* by Booth and O'Halloran (2001) and Reinecke and Reinecke (2007a). There may be more than one explanation for the observed low NRRT of the control worms, but a likely reason could be the fact that in the present study, the unexposed worms were subjected to experimental conditions that included being in nutrient poor artificial soil, limited access to food and the inability to disperse normally. These conditions were abnormal and perhaps suboptimal to the worms to a minor degree, so that they were not affected at morphological level, but only at cellular level. This chronic stress induced by being in suboptimal conditions for an extended period of time (12 weeks) then manifested as a reduced NRRT time, with this response being a biomarker of general stress.

f) Avoidance behaviour

A preliminary behaviour experiment in the present study indicated that *E. fetida* will disperse randomly in the selected test soil in the absence of a toxicant and that any avoidance response observed can therefore be attributed to the presence of the pesticide. From the results it is evident that *E. fetida* is unable to avoid the presence of the selected organophosphate pesticides in artificial soil, even at the relatively high concentrations tested here. This was

also found by Hodge et al. (2000) who observed that the earthworm *A. caliginosa* was unable to avoid chlorpyrifos and diazinon at field application rates as well as at higher concentrations. In contrast, Loureiro et al. (2005) found that *E. andrei* exhibited an avoidance response to the organophosphate dimethoate at a concentration of 40mg/kg, a concentration higher than used in the present study. Similarly, Zhou et al. (2007) reported an avoidance response by *E. andrei* to chlorpyrifos at concentrations of 40 and 60 mg/kg. These studies present evidence for a concentration-dependent avoidance response in this species.

The presence and severity of an avoidance response is often influenced by the concentration of the toxicant in the soil, according to Slimak (1997) and Van Zwieten et al. (2004). Wentsel and Guelta (1988) observed that *L. terrestris* was able to avoid copper and zinc at higher (35 mg/kg) but not lower concentrations (17 mg/kg). Similarly, Loureiro et al. (2005) reported that for the carbamate pesticides carbendazim and benomyl, an avoidance response was evident at concentrations over 10 mg/kg. In the case of benomyl, the degree of avoidance was influenced by the concentration of benomyl present in the soil. Results from the present study indicate that the avoidance response of *E. fetida* to chlorpyrifos and azinphos-methyl is not concentration related for the concentrations tested here. It appears that a threshold concentration exists at which earthworms from the genus *Eisenia* start to avoid organophosphates and, based on literature this value is around 40 mg/kg (Loureiro et al., 2005; Zhou et al., 2007). Avoidance of very high concentrations of chlorpyrifos (125-500 mg/kg) was observed in the present study during the range finding test for the acute exposure described in section 3.3.1. This supports the literature indicating an avoidance response to organophosphates at concentrations exceeding the apparent threshold value of 40 mg/kg.

An avoidance response induced only at relatively high concentrations (>40mg/kg), as appears to be the case for organophosphates, may not be of value to the animal as the concentrations inducing an avoidance response likely exceed those resulting in life-cycle effects, such as reduced reproduction. For example, in the study of Løkke and Van Gestel (1998), the No Observable Effects Concentration (NOEC) values for effects on reproduction in *E. fetida* were lower than the concentrations that are reported for avoidance in the study of Loureiro et al. (2005). Similarly, Hodge et al. (2000) illustrated that *A. caliginosa* did not avoid chlorpyrifos and diazinon at concentrations that resulted in effects on growth, maturation and reproduction in this species, as illustrated by Booth and O'Halloran (2001). These studies

illustrate that the affected animals appear unable to avoid organophosphate concentrations that are high enough to affect reproduction and may result in population level effects.

Earthworm avoidance response to contaminated soils is therefore only valuable for determining the potential risk of a specific contaminant to terrestrial biota if the animals can detect and avoid the contaminant at concentrations below the Lowest Observable Effects Concentration (LOEC) value for higher level effects such as reproductive impairment. This does not seem to be the case for organophosphates, making the usefulness and ecological relevance of using an avoidance behaviour test as screening tool for pesticide contaminated soils questionable.

g) Burrowing behaviour

In addition to inducing an avoidance response, contaminants can induce a number of other behaviour modifications in earthworms. In the present study, this was observed when investigating the impaired burrowing ability of the animals surviving acute exposures to azinphos-methyl and chlorpyrifos, as described in section 3.1.3 (b). The results of the acute as well as the intermittent exposure experiments indicated that both pesticides affected burrowing success, but that this effect was more pronounced in the worms exposed to azinphos-methyl than those individuals exposed to chlorpyrifos (Figure 5.7 and 5.8). All groups intermittently exposed to chlorpyrifos, irrespective of treatment regime, retained the ability to burrow and all worms were able to burrow away from the soil surface. Even though the burrowing time of chlorpyrifos treated animals at the end of the intermittent exposure period was longer than at the start, and longer than the control time, the differences were not statistically significant.

In contrast to the results of the chlorpyrifos exposure, the behaviour modifications observed in the azinphos-methyl treated animals were more prominent and these animals took a significantly longer time to burrow away from the soil surface than the control worms. Exposure interval played a more important role than exposure concentration in detrimentally affecting burrowing success, as the AZP5/14 group had the longest burrowing time at the end of the experiment. Little and Finger (1990) who studied behavioural indicators of sublethal toxicity in the aquatic environment indicated a relationship between locomotor activity and fitness-related parameters such as the ability to search for food and avoid predators,

suggesting locomotion as an ecologically valuable biomarker. Reinecke and Reinecke (2007a) presented evidence that in the earthworm *A. caliginosa*, the presence of organophosphate pesticides may cause the worms to cease burrowing activity and go into estivation and suggested that the triggering of such a response could have an important impact on growth and reproduction, eventually resulting in population level effects.

Haque and Ebing (1983), evaluating the toxicity of a variety of pesticides to *Lumbricus terrestris* and *E. fetida,* observed that behaviour disturbances caused by insecticides were more severe than those caused by fungicides or herbicides. This is likely due to the fact that many insecticides, such as organophosphates and carbamates, are potent cholinesterase inhibitors, whereas most fungicides and herbicides are not. Therefore organophosphates can influence earthworm behaviour not only by causing an avoidance response following detection of the chemical, but also through inhibiting normal functioning of the nervous system. Fábián and Petesen (1994) observed that the springtail *Folsomia fimetaria* exposed to dimethoate remained motionless or exhibited uncoordinated motion. Jensen et al. (1997) illustrated a clear correlation between acetylcholinesterase activity, dimethoate exposure dose and alterations in locomotor activity in the carabid beetle *Pterostichus cupreus*. Exposure to relatively high concentrations of organophosphates may cause immobility through ChE inhibition, reducing the worm's ability to burrow and work the soil as was illustrated in the present study and observed in *L. terrestris* by An der Län and Aspök (1962). Gupta and Sundararaman (1991) also demonstrated a correlation between cholinesterase inhibition and a loss of burrowing ability in the earthworm *Pheretima posthuma* following exposure to carbaryl, a cholinesterase inhibiting carbamate.

The present study presents clear evidence that the pesticides azinphos-methyl and chlorpyrifos have a negative effect on burrowing ability following single and repeated exposure to the same substance, but that the effects induced by azinphos-methyl is far more severe. This finding has ecological relevance, as any earthworm that is unable to burrow no longer has the ability to move away from suboptimal conditions or forage effectively. Reduced mobility likely played a role in the lowered food intake observed in the azinphos-methyl exposed worms as described in section 3.1.3 (a), as these animals were potentially less able to find and utilise the available food source. This is important as access to sufficient food is of prime importance for maintaining a high reproduction rate in earthworms

(Reinecke et al., 1990). The present study presents evidence for a link between impaired burrowing ability and feeding depression. This is observed in the animals exposed to azinphos-methyl which experienced impaired burrowing ability and also exhibited feeding depression. In contrast, the worms exposed to chlorpyrifos, which were not significantly affected in terms of burrowing ability, also did not show a decreased feeding response. The animals exposed to azinphos-methyl that was unable to feed effectively also suffered severe reproductive impairment and for both pesticides a clear dose response effect can be observed for reproduction. Impaired mobility is therefore likely to indirectly impact on reproductive success and lead to future population level effects. There is strong evidence for a link between behaviour responses and higher level effects such as growth, maturation and reproduction in the present study.

h) Growth

When assessing the effect of different treatment regimes (different combinations of exposure intervals and exposure concentrations) on growth, different results were obtained following exposure to the two pesticides. Mean end weights of earthworms exposed to the chlorpyrifos treatments were lower than the control end weight in all four treatment groups (Figure 3.1). No differences were however observed between the different treatment groups subjected to different concentrations and exposure intervals which illustrates that the different treatment concentration and interval combinations tested did not affect growth differently. In contrast, the work of Reinecke and Reinecke (2007a) illustrated a concentration dependent effect on weight loss in adult *A. caliginosa* exposed repeatedly to different concentrations of chlorpyrifos ranging from 0.5 to 8.0 µg/kg, using a 14-day exposure interval. The animals exposed to concentrations of 2.5 and 8.0 µg/kg of chlorpyrifos lost progressively more weight after each application and end weight of the highest (8.0 µg/kg) treatment group was significantly lower than the control or the other treated groups exposed to lower concentrations of chlorpyrifos. This result illustrates, in contrast to the present study, that exposure concentration can play a significant role in inducing an effect, especially at a relatively short exposure interval of 14 days. It must be noted however, that the study of Reinecke and Reinecke (2007a) used pesticide concentrations far lower than those used in the present study, thus potentially allowing for some degree of recovery to take place between consecutive exposures. The high exposure concentrations used in the present study can likely explain the lack of a concentration-related response following intermittent exposure to

chlorpyrifos. Exposure to azinphos-methyl resulted in significant differences in effects on growth in the various treatments. A shorter treatment interval of 14 days was lethal to animals exposed to the higher concentration and detrimentally affected growth in the animals exposed to the lower concentration. At the longer exposure interval of 28 days, growth was affected at the higher azinphos-methyl concentration but not at the lower concentration. Yasmin and D'Souza (2007) observed a similar result when exposing *E. fetida* to the organophosphate dimethoate. These authors exposed the animals to pesticide concentrations 0.4 and 1.6 mg/kg for a period of 28 days and reported significant weight loss only in the higher concentration.

The impaired growth observed in some of the azinphos-methyl treatments in the present study can possibly be explained by the effect that the presence of the pesticide had on feeding behaviour. No reduction in feeding response was observed in any of the control groups, with all food being consumed, while a reduction in feeding response was evident in two of the three azinphos-methyl treated groups (AZP5/14 and AZP25/28). No feeding depression was observed in the AZP5/28 group and, as indicated above, the end weight of this group was not different from the control. Impaired feeding efficiency causes a reduction in available energy to maintain metabolic and physiological functions, as well as leaving less energy available to cope with the physiological demands of being exposed to a toxicant (Roex et al., 2003). As the exposed animal is in a state of stress, energy is likely to be diverted away from growth and reproduction and shunted to coping with the effect of a chemical stressor and maintaining essential body functions (Calow, 1991; Gibbs et al., 1996). This is especially important for juvenile earthworms as this will reduce the amount of energy available for growth and maturation, and will result in slower maturation and a delay in, or even absence of, cocoon production (Booth and O'Halloran, 2001).

The differences in growth effects observed in the results for different exposure intervals for the azinphos-methyl treatments and the absence of any similar effect in chlorpyrifos treatments can partly be explained in terms of acute toxicity and environmental half-life of the two pesticides. The higher acute toxicity of azinphos-methyl relative to chlorpyrifos to *E. fetida* is described in The Pesticide Manual (2000) and was illustrated by the results of the acute toxicity tests conducted in the present study (Table 3.1 and 3.2). This higher acute toxicity of azinphos-methyl relative to chlorpyrifos, coupled with a potentially shorter half-life of azinphos-methyl in soil can likely explain why animals exposed to a shorter exposure

interval were more severely affected relative to animals exposed to a longer exposure interval. A short environmental half-life, coupled with a long exposure interval would potentially allow the pesticide to break down between consecutive applications, therefore allowing the affected animals to recover to a degree. The half-life of the two pesticides in artificial soil was not determined in this study, but according to literature azinphos-methyl generally has a shorter half-life than chlorpyrifos in soil (Wauchope et al., 1992; Knuth et al., 2000). A shorter environmental half-life would mean that the pesticide breaks down relatively quickly; potentially allowing animals to recover in between exposures if sufficient time is allowed. A short environmental half-life could explain why there was no difference observed between the AZP5/28 treatment and the control as these animals had adequate recovery time after being exposed to a relatively low pesticide concentration. Furthermore, the severe effects observed at a 14-day treatment interval is indicative of an inadequate recovery time and that the effects of repeated exposure can be cumulative if the exposure interval is relatively short.

i) Reproduction

Reproduction, in terms of the number of cocoons that were produced, was impaired in all treatment groups for both pesticides (Table 3.3 and 3.4). For the chlorpyrifos treatments, all treated groups reproduced and treatment concentration played a more significant role than treatment interval in affecting cocoon production. This is evident from the fact that cocoon production in the lower concentration groups was approximately twice as high as in the higher concentration groups. For the azinphos-methyl treated groups only the AZP5/28 group was able to reproduce and cocoon production in this group was significantly inhibited relative to the control group. No differences were observed between any of the treatment groups in terms of cocoon viability, or number of hatchlings per cocoon. These findings are in accordance with some of the results of Bustos-Obregon and Goicochea (2002) who reported that the organophosphate parathion affected reproduction in *E. fetida* in several ways. These authors reported effects on sperm production, number of cocoons produced and numbers of hatchlings produced. In addition, histological damage of the gonads and genotoxic effects were reported by them. Sorour and Larink (2001) reported abnormalities induced by benomyl, a carbamate fungicide, in spermatogenesis of *E. fetida*. These abnormalities included abnormal cytophore ultrastructure, and malformation of the spermatogonia, spermatids and spermatozoa. As carbamates and organophosphates both act as cholinesterase inhibitors, it should be considered that it is possible that similar

reproductive effects as those described by Sorour and Larink (2001) following carbamate exposure, could be induced by the organophosphate pesticides used in the present study. If this was the case, it would partly explain the reproductive impairment that was observed. This proposed similarity in reproductive effects at cellular level between carbamates and organophosphates is purely speculative and can not presently be substantiated by literature.

Yasmin and D'Souza (2007) investigated the effects of two concentrations of dimethoate (an organophosphate insecticide), carbendazim (a carbamate fungicide) and glyphosate (a herbicide) on reproduction in *E. fetida*. Dimethoate only affected reproduction at a higher concentration (1.6 mg/kg), while carbendazim affected reproduction at both concentrations (0.8 and 3.2 mg/kg) and glyphosate had no effect on reproduction at treatment concentrations of 2 and 8 mg/kg. These results illustrate that reproductive impairment induced by various agrichemicals may be dose-specific. The chlorpyrifos treatments in the present study present evidence for a dose-specific effect on cocoon production (Table 3.5) as more cocoons were produced in the groups exposed to a lower treatment concentration, irrespective of treatment interval. Reproduction in the azinphos-methyl treated groups was near-absent and this can likely be attributed to the effect that the presence of the pesticide had on maturation rate. Very few individuals in the azinphos-methyl exposures reached maturity and therefore only these animals were able to reproduce. In contrast, all chlorpyrifos treated individuals reached sexual maturity and the impaired reproduction cannot be ascribed to the pesticide affecting maturation could possibly have been caused by reproductive impairment at the level of gamete production, as was described by Bustos-Obregon and Goicochea (2002) and Sorour and Larink (2001). The results of the chlorpyrifos exposures indicate that maturation time was less sensitive to pesticide exposure than cocoon production, which conforms with the results of Booth and O'Halloran (2001) who found similar results in *A. caliginosa* following exposure to chlorpyrifos.

The results of the present study presents evidence that both these pesticides, even when present at relatively low concentrations, can potentially affect earthworm populations due to directly affecting reproduction by impairing maturation and subsequently cocoon production. The lack of maturation observed in the azinphos-methyl treated animals could be attributed to the impaired food intake observed in these worms. Lowered food consumption leads to less available energy, which in turn causes metabolic energy to be shunted away from non-essential physiological functions such as maturation and reproduction. This is further

confirmed by the fact that neither a reduction in food consumption or impaired maturation rate was observed in the chlorpyrifos treated animals.

4.2 Toxicity of organophosphates to *O. mossambicus*

This section of the study aimed to address a number of questions similar to those that were proposed when investigating the effects of organophosphates on *E. fetida* as representative of non target organisms in the soil environment. As with the acute toxicity experiments with *E. fetida*, there was evidence from the present study to suggest that juvenile *O. mossambicus* is more sensitive to the organophosphates in question than adult specimens from the same and closely related species, as discussed in section 4.2 (a). When considering the effects of various intermittent exposure regimes on a number of physiological and morphological endpoints, it is evident that intermittent pesticide exposure has a significant effect on these endpoints, as was hypothesized, and these effects are discussed and quantified in greater detail in section 4.2. The broad trend that appears evident is that exposure interval play a more significant role in inducing effects than exposure concentration, and that a dose-related response was not observed for many of the endpoints investigated. This is in contrast to most of the results observed for the experiments conducted on *E. fetida* and is not in alignment with the hypothesis that was proposed, i.e. that exposure interval is expected to play a less pronounced role in inducing an effect and that a more severe effect will be observed at a higher exposure concentration.

a) Acute toxicity and mortality

The results of the acute aquatic toxicity tests illustrate the higher toxicity of azinphos-methyl relative to chlorpyrifos to juvenile *O. mossambicus*. The LC_{50} values were 0.05 mg/l for chlorpyrifos and 0.007 mg/l for azinphos-methyl following an exposure period of 24h, as presented in Tables 3.6 and 3.7. Comparing these values to those reported for adult life stages of this species, as presented in the literature, is difficult due to the fact that most of the acute toxicity tests conducted on adult fish by other authors used a 96h exposure period. Therefore, for both pesticides, a comparable LC_{50} value for adult *O. mossambicus* following a 24h exposure period could not be determined from literature. El-Refai et al. (1976) however reported a LC_{50} value of 0.139 mg/l for chlorpyrifos for adult specimens of the closely related species *Oreochromis niloticus* following a 24h exposure period. Some comparative information for juveniles of other fish species are available and in this regard juvenile *O. mossambicus* seems to be more sensitive to chlorpyrifos than juvenile Japanese medaka (*Oryzias latipes*) and less sensitive than juvenile fathead minnows (*Pimephales promelas*). LC_{50} values of 0.30 mg/l and 0.02 mg/l are reported for these two species respectively

(Holcombe et al., 1982; Rice et al., 1997). It is interesting to note that the LC_{50} value of azinphos-methyl that was determined in the present study (0.007 mg/l) is the same as the LC_{50} value determined by Ferrari et al. (2004) for juvenile rainbow trout (*O. mykiss*), a species that is generally regarded as more sensitive to contaminants that *O. mossambicus*. The increased sensitivity of a number of salmon and trout species relative to many other fish species is clearly illustrated by Harris et al. (2000) for the pesticide azinphos-methyl.

The species *O. niloticus* and *O. mossambicus* are closely-related, and if assuming that the LC_{50} value for *O. mossambicus* will be fairly similar to LC_{50} value reported by El Refai et al. (1976) for *O. niloticus*, there is evidence that juvenile animals could be more sensitive to chlorpyrifos than adults. Oruç (2010) presented evidence for this by reporting a lower LC_{50} value (0.098 mg/l versus 0.150 mg/l) for juvenile than for adult *O. niloticus* following a 96h exposure to chlorpyrifos. A possible explanation for this is that younger fish possibly have lower levels of acetylcholinesterase activity, or are deficient in the enzymes needed for detoxification of anticholinesterase compounds, as proposed by Chandrasekara and Pathiratne (2007). Carboxylesterases are a class of "B" esterases (similar to acetylcholinesterase) that are present in fish (Arufe et al., 2007) and which are also inhibited by organophosphates. These enzymes also play a role in the detoxification of certain organophosphate pesticides through hydrolysis of the ester bonds in the pesticide (Jokanovic, 2001). In addition to playing a role in the detoxification, Clement (1984) and Arufe et al. (2007) suggested that carboxylesterases also provide a protective mechanism against the effects of anticholinesterase agents, such as organophosphates, by irreversibly binding to the insecticide and therefore preventing it from reaching the primary target, acetylcholinesterase.

If carboxylesterase activity in juvenile animals is different from that of adults, it could potentially influence the AChE inhibition by organophosphate pesticides and therefore the acute toxicity of cholinesterase-inhibiting compounds (Li and Fan, 1997). This was illustrated to be the case in mammals by Padilla et al. (2000) who reported that young rats were 5-fold more sensitive to chlorpyrifos than adults. These authors also indicated that rats detoxify chlorpyrifos and its oxon by binding to carboxylesterases and hydrolysis by A-esterases. It was shown that the young rat is deficient in both these detoxification enzymes, which likely explain the increased sensitivity of the juvenile rat versus the adult rat. While rats and fish belong to completely different taxonomic groups, the cholinesterases are a conserved enzyme system among different vertebrate groups (Viarengo et al., 2007).

Therefore the observed differences in sensitivity between juvenile and adult fish may be attributed to the same difference in enzyme activity that explained the age related sensitivity observed in rats by Padilla et al. (2000).

There is limited literature describing the age- and size related effects on the activity of these enzymes in fish, but the available literature suggests the opposite of what was found in mammals. Arufe et al. (2007) reported that both cholinesterase and carboxylesterase activities in the gilthead sea bream (*Sparus aurata*) showed a consistent increase between yolk-sac fry stage and three days post hatching. Similarly, Phillips et al. (2002) evaluated ChE activity through the larval stages of walleye (*Stizostedion vitreum*) and found that the ChE activity increased about 2.5 fold during the first 19 days post-hatch. In addition to this, several studies have illustrated that brain AChE activity is lower in older fish than in younger fish (Sturm et al., 1999; Beauvais et al., 2000; Flammarion et al., 2002). Thus there is no evidence in the literature to suggest that the higher sensitivity observed in juvenile tilapia in the present study relative to adult tilapia in the study of El-Refai et al. (1976) is due to differences in enzyme activity.

Differences in sensitivity of juvenile and adult animals could also be the result of differences in uptake rates of toxicants and thus differences in body loads, resulting in differences in effects. In adult fish, gill and gut epithelia are major routes of uptake (Randall et al., 1996; de la Torre et al., 2000), while in larval and juvenile fish the skin is also a respiratory interface through which toxicant can enter the organism (Kane et al., 2005). Therefore, the uptake rate of contaminants in juveniles may be far higher than the rate in adult fish of the same species due to physiological and morphological differences affecting the uptake of toxicants. Differences in uptake rate determine the amount of pesticide that eventually reach the target organ(s) and induce a toxic response. Physiological and morphological differences between juvenile and adult *O. mossambicus* may therefore present a likely explanation for the differences in sensitivity to acute exposures and observed LC_{50} values as these differences likely affect the uptake rate and body burden of the toxicant.

It is important to relate acute toxicity values to environmentally realistic values for the given toxicant. In this regard, several authors have investigated the degree of pesticide contamination in the environment, for example in surface waters of the Western Cape Province of South Africa, as a result of agricultural activities (London et al., 2000; Schulz

and Peall, 2001; Schulz et al., 2001b; Dabrowski et al., 2002; Dabrowski et al., 2006). These authors reported environmental concentrations of chlorpyrifos in these waters to be in the range of 0.001-1.62µg/l and azinphos-methyl concentrations to be in the range of 0.02-4.3 µg/l. In all the above-mentioned studies the maximum pesticide concentrations detected in water samples from the environment was lower than the 24h LC_{50} value that was determined for *O. mossambicus* in the present study. The acute values determined in the present laboratory study therefore indicate that environmental values should not cause mortality in *O. mossambicus* and other resident fish species occurring in areas at risk of exposure to organophosphate pollution.

However, an important factor that has to be taken into account is the fact that a large amount of pesticides that are mobilized into water sources as a result of runoff adheres to suspended particles in the water column. Pesticide concentrations reported for water samples may therefore give an underestimation of the amount of pesticide present. The level of chlorpyrifos associated with suspended particles was found to vary between 94-924µg/kg and the levels of azinphos-methyl varied between 0.9-1247µg/kg (Schulz, 2001; Schulz et al., 2001a, 2001b; Schulz, 2004; Thiere and Schulz, 2004). The effects of particle bound azinphos-methyl and chlorpyrifos on rainbow trout AChE activity was investigated by Sturm et al. (2007). These authors determined that the No Observable Effects Concentration (NOEC) value for AChE inhibition by particle bound pesticides was 20-200 times higher than the amount of suspended particle-bound pesticide that was measured in the environment. These results suggests that particle bound pesticides are unlikely to present a significant toxicological risk to aquatic biota. In the same study it was determined that the concentrations of environmentally relevant organophosphates measured in the water column are close to the NOEC values, suggesting significant ecotoxicological risk to fish inhabiting water bodies in agricultural areas (Sturm et al., 2007). In addition to inhibiting AChE activity, low level chlorpyrifos exposure has been illustrated to induce persisting neural development impairment in fish (Eddins et al., 2010). Therefore, the repetitive nature of low level pesticide application may have a cumulative effect, resulting in severe sublethal effects impacting on the fitness and ultimately survival of non-target aquatic species (Hill, 2003; Scott and Sloman, 2004).

b) Morphological and behavioural effects following acute exposures

Both the fish exposed to azinphos-methyl and to chlorpyrifos exhibited morphological and behavioural effects different from control fish, as described in Section 3.1.2.3 (a) and (b). The effects that were observed included signs of paralysis, as well as a darkened body colouration and a distorted body shape i.e. abnormal lateral bending to either side. These effects were similar to those observed in *O. latipes* following sublethal exposure to chlorpyrifos by Rice et al. (1997). The latter authors also reported that the time until initial onset of morphological effects were shorter at higher chlorpyrifos concentrations, similar to what was observed during the present study. Subcutaneous bleeding at both the base of the pectoral and caudal fins of the fish was observed during the present study, while in the study of Rice et al. (1997) bleeding was only reported in the caudal area. Holcombe et al. (1982) and Jarvinen et al. (1983) also reported similar effects such as abnormal lateral bending and a distorted body shape in fathead minnows (*Pimephales promelas*) and rainbow trout (*Onchorynchus mykiss*), similar to the effects reported in the present study. Tilton et al. (2010) reported that reduced swimming rates and severe muscle twitching occurred in a dose dependent manner in the zebrafish (*Danio rerio*) following a 24h chlorpyrifos exposure at 35-220 µg/l. Gül (2005) reported that following exposure of *O. niloticus* to chlorpyrifos-methyl, exposed fish were pale and discoloured in comparison to control fish. In contrast, the pesticide-exposed fish in the present study were darker than control fish.

The behavioural responses observed during the present study following acute exposures of juvenile *O. mossambicus* to chlorpyrifos corresponded to those responses observed for *O. niloticus* exposed to lethal and near-lethal concentrations of the organophosphate chlorpyrifos-methyl (Gül, 2005). These effects were also consistent with those observed by Venkateswara et al. (2003b) in juvenile *O. mossambicus* exposed to lethal concentrations of chlorpyrifos. No evidence of behavioural alterations similar to those induced by azinphos-methyl in *O. mossambicus* during the present study could be found in the literature, but the behaviour effects were to a large extent similar to those observed in fish exposed to the organophosphate pesticides chlorpyrifos, monocroptophos and dimethoate (De Mel and Pathiratne, 2004; Venkateswara Rao, 2004; Kavitha and Venkateswara Rao, 2007). Pan and Dutta (1998) showed that organophosphates are potent cholinesterase inhibitors and indicated that behavioural modifications, such as reduced swimming stamina may be caused by the inhibition of these enzymes. Evidence for this was presented by Tilton et al. (2010) who reported a dose dependent positive correlation that approached statistical significance

between AChE inhibition and swimming behaviour in *D. rerio*. These authors also stated that this effect may reduce the survival ability of juvenile animals by affecting feeding success and possibly affecting predator avoidance behaviour. Acetylcholinesterase inhibition presents a likely explanation for the behaviour effects, observed during the present study and the link between the activity of this enzyme and various behaviour endpoints is discussed under sections (c) and (d) in this chapter.

c) Feeding behaviour

Feeding behaviour is an endpoint that may be modified by exposure to a number of different pollutants, including heavy metals (Grippo and Heath, 2003), industrial chemicals (Wibe et al., 2004), and pesticides (MacRury and Johnson, 1999). The effects of organophosphates on fish feeding has been investigated in a number of species and both increased and decreased feeding activity has been illustrated following exposure to a number of different organophosphate pesticides. For example, following exposure to parathion, Banas and Sprague (1986) reported decreased food consumption in *O. mykiss*, while Roex et al. (2003) reported in increase in food consumption in *D. rerio*. Impairment of feeding behaviour following organophosphate exposure was also illustrated during the present study. Both feeding behaviour endpoints under investigation (feeding response time and food consumption) were affected following a single 24h exposure to azinphos-methyl and chlorpyrifos at two sublethal concentrations (Figures 3.23 and 3.24). The only group where pesticide exposure had no effect on feeding behaviour was the group exposed to the lowest chlorpyrifos concentration (10% of the LC_{50} value). It is evident that treatment concentration played a major role in inducing the observed changes in feeding response, shown by statistically significant differences between the two treatment concentrations for both pesticides.

The underlying mechanism of feeding impairment or modification of feeding behaviour may differ between various toxicants or classes of toxicants and is likely to be caused by a combination of factors. According to Scott and Sloman (2004) these may include damage to sensory organs or receptors, impairment of neuronal and neuromuscular function, impairment of chemoreception, or other physiological or biochemical responses or effects. The concentration-related response observed during the present study could be explained by the inhibition of acetylcholinesterase activity, with more inhibition occurring following exposure to a higher concentration, ultimately resulting in more feeding impairment. Fanta et al.

(2003) reported that following exposure to methyl parathion either through contaminated water or food, the catfish *Corydoras paleatus* showed reduced swimming ability which in turn affected their ability to obtain and consume food particles. This was confirmed by the study of Kavitha and Venkateswara Rao (2008) who reported a loss of feeding response in *G. affinis* following acute exposure to chlorpyrifos. This loss of feeding response was coupled with a reduction in swimming activity and severe inhibition of AChE. In the work of Castro et al. (2004), feeding response was used as an endpoint in a bioassay using the guppy *Poecilia reticulata* as test species. These authors observed a significant correlation between AChE inhibition and reduced food intake along a contamination gradient at the test sites. Kumar and Chapman (1998) reported significant feeding impairment and resultant weight loss in the eastern rainbow fish *Melanotaenia duboulayi* following a three week protenofos exposure. Similarly, Banas and Sprague (1986) and Pavlov et al. (1992) illustrated decreased food consumption in rainbow trout and bream (*Abramis brama* L.) following exposure to organophosphate pesticides.

Food intake is a broad measurement of energy input and reduced energy input will affect growth and possibly reproductive fitness (Grippo and Heath, 2003). In the *Oreochromis* genus, the number of eggs produced by a female is related to body size (Trewavas, 1983; Popma and Lovshin, 1994) and it is likely that impaired growth can result in reduced reproductive fitness. Cleveland and Hamilton (1983) observed growth effects induced by the organophosphate defoliant DEF in the salmon and catfish species *Salmo gairdneri* and *Ichtalurus punctatus* following a chronic exposure of 90 days, but did not investigate feeding behaviour alongside growth. As feeding behaviour was investigated only after a single exposure in the present study, the effects of intermittent organophosphate exposure on feeding behaviour cannot be discussed.

d) Acetylcholinesterase activity

In the present study, both azinphos-methyl and chlorpyrifos significantly affected AChE activity in the brain of juvenile fish following intermittent exposures. Following exposure to azinphos-methyl, the activity of this enzyme was reduced by more than 50% in all treatments. The lowest activity (23% of control activity) was observed in the AZP50/14 treatment group (Figure 3.21). This result was expected as this group was subjected to the most severe treatment both in terms of exposure concentration and exposure interval. In spite of substantial AChE inhibition, there was no mortality in any of the treatment groups. The link

between AChE inhibition and mortality in fish is not clear but substantial variation in sensitivity exists between species according to Fulton and Key (2001). In a review by the latter authors regarding acetylcholinesterase inhibition in estuarine fish, the relationship between AChE inhibition and other symptoms of organophosphate toxicity was investigated. The main finding was that AChE inhibition levels exceeding 70% was associated with mortality in most fish species, but that selected species, such as mummichogs (*Fundulus heteroclitus*) are able to tolerate remarkably high levels of AChE inhibition without associated mortality. A similar result was reported by Tilton et al. (2010) who illustrated that zebrafish can tolerate AChE inhibition of up to 86% without associated mortality. The present study provides evidence that *O. mossambicus* is also tolerant of high levels of AChE inhibition as no mortality occurred even when AChE activity was inhibited by almost 80% following a 12 week exposure period. In accordance with this, Venkateswara Rao et al. (2003b) observed up to 90% AChE inhibition in *O. mossambicus* following exposure to profenofos without associated mortality, and presented evidence for complete recovery of inhibition within 28 days. Similarly, Chandrasekerara and Pathiratne (2007) illustrated that the related species *O. niloticus* is also able to tolerate AChE inhibition exceeding 80% without mortality. The results of these authors, as well as the results from the present study, presents evidence that selected species from the genus *Oreochromis* are not particularly sensitive to organophosphate exposure, thus potentially limiting their use for field experiments.

The persistence of AChE inhibition in fish tissue following organophosphate exposure has been the subject of a number of studies and is important for the present study where experimental animals were subjected to various intermittent exposure regimes. The time between successive exposures could potentially allow some degree of recovery to take place, which could influence the final degree of enzyme inhibition at the end of the exposure period. Morgan et al. (1990) exposed two groups of juvenile Atlantic salmon (*Salmo salar*) to sublethal concentrations of fenitrothion using two different exposure regimes i.e. for 7 days continuously, or for 24 hours each on day one and on day eight, thus allowing for a seven day recovery period between exposures in the second exposure scenario. In the seven-day continuous exposure, inhibition increased with increasing insecticide concentrations in a typical dose-response manner. In the intermittent 2x24h exposure, total inhibition at the end of the exposure period was less and recovery was faster than for the group that was continuously exposed. In a similar study by Karen et al. (1998) *F. heteroclitus* individuals

were repeatedly exposed to chlorpyrifos for 6h exposure periods. One group of fish were exposed daily for four consecutive days while another group was exposed weekly for four consecutive weeks. The fish exposed on a daily basis had higher inhibition of brain AChE activity than the fish exposed weekly. The daily exposure regime exhibited cumulative inhibition, whereas for the weekly exposure scenario, AChE activity at four weeks was similar to that observed after two weeks. This suggests that the weekly intervals allowed sufficient time for recovery of enzyme activity, whereas daily exposures did not. Chandrasekara and Pathiratne (2007) subjected *O. niloticus* to single and repeated exposures of chlorpyrifos and monitored recovery of AChE activity. It was observed by these authors that enzyme recovery was slower in fish that were subjected to more than one exposure in relation to fish exposed only once. The same authors also reported that after 14 days of allowing fish to recover in clean water, the levels of AChE activity had not recovered to pre-exposure levels, suggesting an inadequate recovery period.

The results of the above-mentioned studies indicate that the time for enzyme recovery is a function of the degree of inhibition and therefore the nature (concentration and duration) of the pesticide exposure. This is likely because the recovery of enzyme activity is largely the result of de novo synthesis of enzyme and the greater the degree of inhibition the more enzyme synthesis is required (Fulton and Key, 2001). In the present study, exposure concentration played an important role in inhibiting AChE following exposure to both pesticides, but there is evidence from the results that at a longer exposure interval, the effect of treatment concentration becomes negligible, indicating that there is a degree of enzyme recovery. This can be clearly seen in the azinphos-methyl exposures, where there was a significant difference in AChE inhibition following a higher and a lower exposure concentration when animals were exposed at a 14-day exposure interval. In contrast, there was no significant difference in AChE inhibition between fish exposed to similar concentrations at a 28-day instead of a 14-day interval (Figure 3.21). For the corresponding chlorpyrifos treatments, the concentration dependent inhibition observed at a shorter interval was not detected. The reason for this is not completely clear, but the most likely explanation is that at high concentrations, azinphos-methyl is a more potent cholinesterase inhibitor than chlorpyrifos. Evidence for this can be seen when comparing enzyme activity in the two groups exposed to the higher pesticide concentration (50% of thee LC_{50} value) at a 14-day treatment interval. Fish exposed to azinphos-methyl using this treatment regime (AZP50/14)

only had 23% of control enzyme activity, while fish exposed to chlorpyrifos (CPF50/14) had 43% of control enzyme activity.

The relationship between AChE inhibition and various sublethal effects in various fish species has been extensively investigated. Cripe et al. (1984) exposed sheepshead minnows (*Cyprinidon variegatus*) to the organophosphate pesticides EPN and Guthion during life-cycle toxicity tests and concluded that swimming stamina was affected at concentrations higher than those affecting reproduction. AChE activity was depressed at all exposure concentrations, but swimming activity was only affected when AChE inhibition exceeded 80%. Similarly, Beauvais et al. (2000) clearly illustrated the link between impaired swimming activity and reduced AChE activity in juvenile rainbow trout (*Onchorynchus mykiss*) following exposure to diazinon and malathion. Exposure of coho salmon *Oncorhynchus kisutch* to different chlorpyrifos concentrations ranging between 0.6 and 2.5 mg/l reduced spontaneous swimming at all concentrations tested (Sandahl et al., 2005). Van Dolah et al., 1997 reported reduced swimming stamina in juvenile red drum (*Sciaenops ocellatus*) after a six-hour exposure to 12µg/l azinphos-methyl. Swimming stamina and swimming activity was not investigated in the intermittent exposure part of the present study, but results from the above-mentioned literature suggest that swimming performance in *O. mossambicus* could potentially have been affected, especially in the groups that had a high level of AChE inhibition (>75%), such as the group exposed to azinphos methyl at the higher exposure concentration and shorter exposure interval (AZP50/14).

Impairment of swimming activity may directly impact on survival due to reduced predator avoidance behaviour or impaired feeding ability according to Little and Finger (1990) and Scott and Sloman (2004). Impaired swimming ability was observed in the present study following acute exposures, as described in Section 3.1.2.3 (b) and this may in a field situation increase the risk of predation for juvenile fish. De Silva and Samayawardhena (2002) illustrated that juvenile guppy (*Poecilia reticulata*) exhibited abnormal swimming behaviour and signs of paralysis following chlorpyrifos exposure at concentrations as low as 0.5 µg/l - 2.0 µg/l, which is in the same range as measured environmental concentrations in water bodies in deciduous fruit producing areas of the Western Cape (London et al., 2000; Schulz and Peall, 2001; Schulz et al., 2001b; Dabrowski et al., 2002; Dabrowski et al., 2006). A link between low level organophosphate exposure, AChE inhibition and ecologically relevant behaviour such as predator avoidance has not been proven in the present study but is very

likely, as was suggested in the above-mentioned literature. More research is needed in this field to validate this link under laboratory conditions prior to investigating it in a field relevant situation.

e) Growth and condition factor

Growth and condition factor are two physiological parameters which are known to differ between sexes in many fish species, including *O. mossambicus*. In this species, male animals grow faster and to a larger size than females (Mair and Abella, 1997). For this reason growth and condition factor was investigated separately for the two sexes. For the growth parameters measured during the present study (end length and end weight) male and female fish from all treatments, with the exception of males in the AZP50/28 treatment group, were affected by intermittent exposure to azinphos-methyl and chlorpyrifos. The general trend was that exposure interval played a more pronounced role than exposure concentration in inducing growth effects in both sexes in *O. mossambicus*, especially in the 14-day treatment interval groups. Here there were no differences in end length and end weight between the higher and lower treatment concentrations for both pesticides and for both sexes. All 14-day treatment interval groups were different from the control for both pesticides and both sexes, for the parameters end weight and end length (Figures 3.11 to 3.14). A possible explanation for the lack of a concentration-dependent effect during the shorter (14-day) exposure interval, is the possibility that the lower concentration (10% of the LC_{50} value) presents a threshold level for the amount of pesticide that can be metabolically activated in the limited (24h) exposure time.

Both azinphos-methyl and chlorpyrifos belong to the phosphorothioate group of organophosphates containing the thiono moiety (=S) in the chemical structure (Hernandez et al., 1998). The substitution of the =S for =O on the phosphorus atom through the process of oxidation increases the toxicity of the pesticide (O'Brien, 1967). The oxidation of azinphos-methyl and chlorpyrifos occur through metabolic activation by the P-450 enzyme system to form azinphos-methyl-oxon and chlorpyrifos-oxon which exert a toxic effect (Strauss and Chambers, 1995). As only the metabolically activated form of the pesticide is toxic, it is this fraction of the total amount of pesticide that is present that can induce physiological and morphological effects. Therefore, in spite of much more toxicant being available for potential uptake and metabolization in the higher concentration (50% of the LC_{50} value) exposure, the amount of metabolically activated pesticide that can exert a toxic effect will be

limited by the amount of metabolising enzymes present in the liver. When several different processes are involved (in this case uptake, transport, metabolic activation etc.) it is the rate-limiting step that will determine the overall effect (Hermens, 1991). Alternatively, if the lower pesticide concentration does not present a threshold to the amount of pesticide that can be metabolically activated, it would imply that more metabolically active compound would reach the target organs in the higher exposure concentration. In this case, the amount of metabolically active compound produced in both the higher and lower exposure concentrations exceed the amount that is low enough to allow adequate recovery to take place between consecutive exposures. This means that the no observable effects concentration (NOEC) threshold for growth effects is exceeded in both concentrations at a 14-day treatment interval, resulting in the manifestation of growth effects for both treatment groups.

When considering the 28-day treatment interval groups for both pesticides, there was no clear trend regarding the effects of concentration and treatment interval on growth, apart from the fact that in most cases the 28-day treatment interval groups were significantly different from the 14-day treatment interval groups in terms of end length and weight. In some cases there were significant differences between the two treatment groups exposed to a higher and a lower concentration at a 28-day interval, but this was not consistently observed in all treatment groups. The data presents evidence that, in the case of a longer exposure interval of 28 days, the induction of growth effects is dependent on exposure concentration. Again, if the lower pesticide concentration does not present a threshold to the amount of pesticide that can be metabolically activated (and therefore produce physiological and morphological effects), it would mean that more metabolically active compound would reach the target organs in the higher exposure concentration, possibly inducing a more pronounced effect. In contrast to the 14-day treatment interval, there is a longer time (28 days) between successive exposures, thus allowing for recovery to take place. It is possible that the animals exposed to a lower concentration at a longer exposure interval, may recover to such a degree that the effects of the toxicant may not manifest at whole-organism level, thus explaining the absence of growth effects in the AZP10/28 and CPF10/28 groups.

There may be a number of mechanisms by which intermittent exposure to organophosphate pesticides can induce effects at whole-organism level, such as a change in growth rate. The most likely mechanisms are the impairment of feeding behaviour and changes in total energy metabolism of the animal (Roex et al., 2003). A reduction in food consumption and an increase in feeding response time were illustrated in the present study as discussed in Section

4.2 (c). As food intake is a rough indication of available energy, a reduction in food intake will necessarily lead to a reduction in available energy and most likely manifest as reduced growth over an extended period of time. Exposure to toxicants such as pesticides can also change a number of metabolic processes, resulting in changes in the total energy metabolism of the animal. Evidence for pesticide-induced changes in energy metabolism was presented by Begum and Vijayaraghavan (1999) who investigated the effects of the organophosphate Rogor on carbohydrate metabolism in the catfish *Clarias batrachus*. It was found that exposure to sublethal doses of this insecticide can disrupt carbohydrate metabolism in this species. The result of this is reduced oxidative metabolism in the muscle tissue which, over a period of time, can contribute to changes in growth rate. Additionally, if pesticide exposure does not alter metabolic processes directly, metabolic tradeoffs may exist between detoxification and other normal processes (Scott and Sloman, 2004). This was suggested by Handy et al. (1999) who subjected rainbow trout to chronic copper exposure and monitored swimming behaviour and endpoints related to metabolic rate. While the endpoints related to metabolic rate (ventilation rate, oxygen consumption, and serum chemistry) were not affected by copper exposure during the above-mentioned study, the exposed fish spent significantly less time performing normal swimming behaviour, which in turn may affect other behaviour endpoints such as feeding behaviour.

As condition factor is a function of weight and length, this endpoint followed more or less the same trend as was observed for the latter two parameters (Figures 3.15 and 3.16). Condition factor is an index based on the length-weight relationship of the fish and can be indicative of the health status of the whole body of the fish related to both environmental availability of food and internal energy resources of the animal (Wijeyaratne and Pathiratne, 2006). Condition factor can also be used to investigate the effects of contaminants (Kleinkauf et al., 2004), both under controlled laboratory conditions and during field investigations. Relating condition factor to ecotoxicological stress can be difficult due to the number of factors, both exogenous (e.g. temperature stress) and endogenous (e.g. stage of the reproductive cycle), that can influence this index. Additionally, there is conflicting evidence in the literature regarding whether condition factor is a sensitive enough endpoint to detect environmentally relevant levels of contaminants. For example, Wijeyaratne and Pathiratne (2006) found no differences in condition factor of *Rasbora caverii* collected from rice field associated water bodies that were exposed to organophosphate pesticides. De la Torre et al. (2005) confirmed this result when investigating the field applicability of condition factor in the fish

Cnesterodon decemmaculatus. These authors compared the condition factor of field-caught fish from polluted and reference sites and presented evidence that condition factor may not be a sensitive enough morphological response to measure low level contaminant induced stress in natural environments. Humphrey et al. (2007) also reported no difference in condition factor of wild-caught barramundi (*Lates calcarifer*) collected from five different estuaries, subjected to various degrees of anthropogenic impacts, near Queensland, Australia.

In contrast, Ozmen et al. (2006) reported significant differences in condition factor of carp *Cyprinus carpio* collected from control and contaminated areas of Karakaya Dam Lake in Turkey. This lake is subjected to industrial, agricultural and sewage pollution in different areas of the lake and the lowest condition factor was measured in fish from areas subjected to industrial and sewage pollution. Even though condition factor showed good results for potential field application in the present study and in the study of Ozmen et al. (2006), this response in fish need more evaluation under field conditions before it can be routinely used in ecotoxicological investigations.

f) Liver somatic index (LSI) and gonadosomatic index (GSI)

Tissue somatic indices are commonly reported in ecotoxicological studies on fish because of the relative ease of determination and the fact that certain indices, such as the liver somatic index, can be a predictor of adverse health in fish (Adams and McLean, 1985). Abnormal index values are in some cases associated with contaminant exposure, especially to hormonally active or endocrine disrupting compounds (EDCs) capable of affecting liver and gonad weight either through activating certain metabolising enzymes or interfering with the hormonal regulation of the reproductive organs (Sloof et al., 1983; Corsi et al., 2003; Kime 1997). In a review article on endocrine disrupting pesticides, McKinlay et al. (2008) listed a number of organophosphates as having endocrine disrupting properties, including affecting the expression of oestrogen-responsive genes, preventing thyroid hormone-receptor binding and antagonizing androgen activity. Neither azinphos-methyl nor chlorpyrifos was listed as EDCs, but Andersen et al., (2002) found chlorpyrifos to induce weak responses in estrogenicity assays. Similarly, Oruç (2010) reported that chlorpyrifos may act as an EDC in *O. niloticus* by decreasing serum estrogen and testosterone levels. No conclusive evidence could be found indicating whether azinphos-methyl has endocrine disrupting potential.

In the present study, liver somatic index (LSI) was investigated to determine whether organophosphate exposure affected liver function to the extent that it would manifest on morphological level. Many organophosphate pesticides, including azinphos-methyl and chlorpyrifos, are metabolically activated in the liver and consequently the liver is the organ that is likely to suffer serious morphological alterations in fish exposed to these pesticides (Anees, 1978; Gill et al., 1988; Fanta et al., 2003). Pesticide exposure significantly reduced the LSI of fish in all azinphos-methyl treatments with the exception of both the male and female fish exposed to azinphos-methyl at the lower concentration and longer exposure interval i.e. the AZP10/28 treatment (Figure 3.17). The reason for this is likely that the relatively low exposure concentration, coupled with a long exposure interval, allowed sufficient time for the fish to metabolise the pesticide and recover from pesticide-induced effects before these could manifest on morphological level. The azinphos-methyl concentration for the fish exposed to azinphos-methyl at the higher concentration and longer exposure interval i.e. the AZP50/28 treatment, however, apparently exceeds the NOEC value for inducing morphological changes in the liver of *O. mossambicus* as the LSI of both males and females of the AZP50/28 group is significantly different from the control. There were no differences in the LSI value between the groups exposed to azinphos methyl at both the higher and lower exposure concentrations at the longer exposure interval i.e. the AZP10/28 and AZP50/28 groups. This indicates that a more severe effect was induced by a higher pesticide exposure concentration, but the effect is still less pronounced than for the two groups who were exposed to a shorter (14-day) treatment interval. Both males and females from the latter two groups (AZP10/14 and AZP50/14) had LSI values significantly lower than the control group and the 28-day treatment interval groups, indicating that treatment interval was more important in affecting LSI than the treatment concentration. Treatment concentration only played a significant role during a longer (28 day) exposure interval.

In contrast to the azinphos-methyl treatments, where the 14-day interval exposure regime resulted in a significantly lower LSI value than the control (Figure 3.17), no differences were observed for the 14-day treatment interval for the chlorpyrifos exposures. For these exposures, only the 28-day treatment interval resulted in a lower LSI than the control (Figure 3.18). The reason for these contrasting results following exposure to chemically related compounds is not clear. Histological analysis of the liver tissue would likely have elucidated the mechanism behind the effect that pesticide exposure had on the structure of the liver, which could in turn have aided in interpretation of the results from the present study. The

detection of liver abnormalities induced by organophosphates through the use of standardised histological techniques has been illustrated by a number of authors, but in most cases this was not investigated alongside the LSI (Fanta et al., 2003; Capkin et al., 2006; de Melo et al., 2008). Therefore the link between histological abnormalities and the LSI requires further investigation and clarification.

Toxicological investigation of the liver is complicated as a range of abnormalities can be induced by various chemicals, and these are induced by a number of different mechanisms (Varanka et al., 2001; Williams and Iatropoulos, 2002; de Melo et al., 2008). In addition to being the body's primary detoxification organ and playing an essential role in metabolising and excreting toxic substances in the body (Hinton and Lauren, 1990), the liver of lower vertebrates such as fish and amphibians play a critical role in biochemical pathways such as vitellogenin synthesis (Hurter, 2002). Substances which act as endocrine disruptors therefore can affect the liver in several ways, including influencing the LSI. Contaminants can induce a number of effects on the liver, but the reduced LSI observed in some of the treated animals in the present study can possibly be attributed to a degree of liver necrosis induced by intermittent exposure to the pesticides, although no macroscopic lesions of the liver were observed. Ozmen et al. (2006) investigated the effects of water pollution on *C. carpio* in Karakaya Dam Lake in Turkey and reported that carp collected from areas subjected to industrial and sewage pollution had lower LSI values than carp collected from reference sites. In contrast, van der Oost et al. (1996) observed an increase in LSI values in fish collected from contaminated field sites. De la Torre et al. (2005) investigated the LSI in the freshwater fish *C. decemmaculatus* collected from polluted and reference field sites and a significantly higher LSI value was reported for the most contaminated site, whereas the LSI from a less polluted site did not differ from the control value. These authors suggested that the higher LSI may be the result of liver enlargement due to a compensatory proliferation process. From the literature, it would appear that an increased LSI value is generally associated with areas with a higher pollution load, but the results from the present study do not agree with this trend. Without histological investigation of the liver structure it is difficult to explain the reduced LSI observed in the present study, but the results can most likely be attributed to the pesticides having a necrotic effect on the liver.

The gonadosomatic index (GSI) can provide valuable information about the reproductive health of an organism and can be strongly influenced by gender and gonadal development

stage. Lower GSI values in fish are generally associated with gonads in immature stages of development, while higher GSI values are typical of more mature gonads (Hinck et al., 2007). Very high variation was observed for the GSI values for both the treated and the control fish in the present study and no statistically significant differences were observed between treated and control groups for both males and females following treatment to both azinphos-methyl and chlorpyrifos (Figure 3.19 and 3.20). At the end of the exposure period, fish with various stages of gonadal development, ranging from immature to fully mature, was observed in the control group as well as all the treatment groups as discussed in section 3.2.2.3 (b). When considering female GSI values it can be seen that the lowest values were measured in the AZP50/28 and CPF 50/28 treatment groups, but this was not statistically lower than any of the other groups. Due to the large variation that exists in the GSI values of both the control and treated fish of both genders, it is extremely difficult if not impossible to quantify the effect that pesticide exposure had on the GSI and therefore on gonadal development. The large variation in GSI values observed consistently in all groups can most likely be explained by the fact that the fish were old enough by the end of the exposure time for some individuals to have reached sexual maturity. Sexual maturity in most fish species, including *O. mossambicus*, is characterised by an increase in the size and mass of the gonads, especially in female animals. As this phenomenon is naturally highly variable even among animals of the same age, natural variation in GSI values due to the onset of sexual maturity may have masked the effects of pesticide exposure. For the present study, gonadosomatic index is not a sensitive enough parameter to detect the effects of organophosphates on gonadal development, mainly due to the variation in the degree of sexual maturity of fish at the end of the exposure period.

In contrast to the present study where juvenile fish were used, other authors have illustrated the usefulness of investigating the gonadosomatic index to detect reproductive effects in adult fish following exposure to sublethal concentrations of organophosphates. In the catfish *Channa punctatus*, reduced testicular weight, delayed sperm formation, changes in previtellogenic and vitellogenic oocytes and decrease in the diameter of stage III (mature) oocytes was reported in adult animals following a 120-day exposure to fenitrothion (Mani and Saxena, 1985; Saxena and Mani, 1987). Ram and Sathyanesan (1986) exposed adult male and female *C. punctatus* to 20 ppt cythion (50% malathion, 50% organic solvents) for six months and observed an increase in oocyte degeneration in females, which resulted in retarded ovarian growth and a lower GSI value. In males, spermatogenesis was arrested and

necrotic spermatocytes were apparent. These responses were correlated with fewer and less active gonadotropin-producing cells in the pituitary and the authors speculated that reduced gonadotropin levels might have contributed to the observed abnormalities. In the freshwater perch (*Anabas testudineus*) a reduction in GSI values was reported following a 90-day exposure to methyl parathion at a concentration of 0.106 µg/l (Choudhury et al., 1993). Khallaf et al. (2003) illustrated a correlation between female GSI and the presence of contaminants (heavy metals and pesticides) in field caught *O. niloticus* from a polluted agricultural site. Similarly, Singh and Singh (2008) reported a reduced GSI value in the catfish species *Rita rita* and *Mystus tengara* and the carp species *C. carpio* and *Labeo rohita* in pesticide-polluted agricultural areas relative to reference areas in Northern India. These studies illustrate that the GSI is a sensitive endpoint to measure during field studies when analysing adult animals, but the present study indicate that this parameter is not really a suitable endpoint to monitor when studying juvenile or sub-adult animals.

In the present study, histological examination of the gonads would be necessary to detect and quantify reproductive abnormalities that would likely have manifested as a result of organophosphate exposure. Examples of histological abnormalities in the gonads of fish that can be attributed to organophosphate pollution include abnormally small oocytes in the grass goby (*Zosterisessor ophiocephalus*) and irregular disintegrated cytoplasm in oocytes of the grey mullet (*Mugil cephalus*) as reported by Corsi et al. (2003). Severe histological damage to the gonadal structure in the bluegill (*Lepomis macrochirus*) was observed following sublethal exposures to diazinon (Dutta and Meijer, 2003). Goodman et al. (1979) illustrated a reduction in the number of eggs spawned by sheepshead minnows (*Cyprinodon variegatus*) continuously exposed to the organophosphate diazinon but did not correlate this with GSI values. Oruç (2010) evaluated steroids profiles of *O. niloticus* alongside the GSI following chlorpyrifos exposure and reported that while the GSI was not affected, significant effects were observed in terms of serum estrogen and testosterone, indicating disruption of the reproductive system.

4.3 General comparison between the effects of organophosphates in the terrestrial and aquatic environments and summary of results

The results from the present study illustrate a mechanistic link between pesticide-induced effects at various levels of biological organization, from biomarker and behaviour responses to whole-organism effects. This was illustrated in a model species from both the terrestrial

and aquatic environment and unequivocal evidence is presented for the potential harmful effects that pesticide pollution may have on non target organisms at risk of exposure in agricultural areas. The results of the acute toxicity tests indicated that mortality as a result of exposure to environmentally relevant pesticide concentrations are unlikely, but that repetitive exposure to some organophosphate pesticides may over time result in an acutely toxic response, as was observed following the intermittent exposure of the terrestrial test species *E. fetida* to azinphos-methyl. The acute tests further indicated that azinphos-methyl is more toxic than chlorpyrifos to both test species, and in both the case of *O. mossambicus* and *E. fetida* there was evidence that juvenile animals are more sensitive to these pesticides than adults. When considering the behavioural effects that were investigated, it was observed that ecologically important behaviour endpoints, such as feeding and locomotion, can be affected both after single and repeated exposures to both organophosphates in both species.

When considering the results of the intermittent exposures, significant detrimental effects following pesticide exposure were observed for the majority of endpoints that were investigated in both species, and valuable information is presented in terms of the impact that exposure interval has relative to exposure concentration in inducing effects in the aquatic and the terrestrial test species. The general trend observed following intermittent exposure of *O. mossambicus*, an aquatic test species, to the two pesticides, is that exposure interval plays a greater role than exposure concentration in inducing effects following an intermittent exposure scenario. This is in contrast to the general trend that was observed following the intermittent exposure of *E. fetida*, a terrestrial test species, to the two pesticides. In the latter case there was some evidence that exposure concentration played a greater role than exposure interval in inducing effects following an intermittent exposure scenario. This observation can most likely be attributed to the fundamental differences in exposure situation between the aquatic and the terrestrial environment both under the present experimental conditions and in the environment.

In the aquatic environment, exposure of non target organisms to organophosphate pesticides are generally transient as the pesticides are either diluted (in the case of a static water body), or simply washed downstream, as will likely be the case for a non-static water body such as a river or stream. This implies a pulse-type exposure scenario for aquatic organisms, where animals will be exposed to the pesticide for relatively short periods, followed by longer intervals where there is no toxicant present. During these periods, the affected organisms can

recover from the effects of the pesticide and a longer exposure interval would allow more time for recovering from the effect of the toxicant, therefore explaining why exposure interval played such a prominent role in inducing effects in the present study. This result is important when considering the nature of pesticide application and thus the nature of the exposure situation in the field environment. While the actual exposure time may be relatively short due to the mobile nature of pesticides in the aquatic environment, the pesticide application interval is often also short. This potentially leaves little time for recovery of the affected organisms between consecutive pesticide applications, highlighting the need for a longer pesticide application interval under field conditions. Exposure concentration would likely play a more important role in inducing effects in aquatic organisms in the event of a longer actual exposure time. The latter would be the case if the pesticide has a relatively long environmental half-life and is present in a static water body where consecutive pesticide applications would result in accumulation of the pesticide in the exposure medium. The environmental half-life of a pesticide is to a large extent determined by the various processes by which the pesticide is broken down in a specific environment. Sunlight/UV radiation and microbial degradation play an important role in the breakdown of organophosphate pesticides. For this reason organophosphates will break down relatively quickly in water where sunlight penetration is high and as a result organophosphate pesticides rarely accumulate in the aquatic environment.

When considering the terrestrial environment, there is strong evidence from the present study that exposure concentration may play a more important role than exposure interval in inducing effects in soil organisms. This can be attributed to the fact that the soil environment presents a fundamentally different physical environment from the aquatic environment. In soil, organophosphate pesticides are mostly bound to soil particles and will therefore remain localized in the soil without being diluted, as would be the case in the aquatic environment. The pesticide breakdown processes in soil are also different from water. Sunlight/UV radiation cannot penetrate into the soil and therefore any pesticide that has penetrated the soil will be broken down primarily by the process of microbial degradation. The static nature of organophosphate pesticides in the soil and the potentially slower breakdown rate contributes to these pesticides accumulating in the soil environment to a much greater degree than in the aquatic environment. In the present study, the higher exposure concentration would over the duration of the exposure period result in a higher ambient pesticide concentration in the soil, which would explain why higher pesticide concentrations resulted in more severe effects on

the test organisms. This also presents an explanation for the mortality observed in *E. fetida* following repeated sublethal exposure and the absence of any such effects in *O. mossambicus*, the aquatic test species.

4.4 Conclusion

Low levels of contaminants, such as pesticides, that are present in ecosystems as a result of anthropogenic activities may affect the behaviour, physiology, growth, reproduction and ultimately survival of many non target species. In the natural environment however, animals are exposed not only to chemical stressors, but also to non-chemical stressors such as climate, food shortages, pathogens and other environmental variables (Spurgeon et al., 2005). These variables can interact directly or indirectly with pollutants in a number of ways, such as changing the bioavailability of the substance or changing the biology or behaviour of organisms and therefore changing their sensitivity to the toxicant. The results obtained from the present study give valuable information regarding the effects of pesticide exposure in both the aquatic and the terrestrial environment. Therefore the results warrant further investigation in the form of an appropriate field trial to validate the effects that were observed following the laboratory-based exposures. Such a field trial however, did not form part of the planning of the project and was considered beyond the scope of the present study. Intensive agricultural practices such as deciduous fruit production are reliant on the use of a variety of agrichemicals, but if agriculture is to be ecologically and economically sustainable, an integrated approach to pest management is critical. This includes the optimisation of spray programs not only in terms of eradicating the pest organisms, but also in terms of mitigating the environmental effects associated with large-scale pesticide use. The present study presents valuable evidence on the environmental effects of various pesticide application regimes and where appropriate, this type of information should be used in the compilation of spray programs that are used in industry. Appropriate monitoring strategies are critical to protect sensitive non target species in agricultural areas against the potential adverse effects of contaminants.

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APPENDIX 1

1. **<u>Preparation of reagents for determination of AChE activity</u>**

1.1 Preparation of phosphate buffer (Na_2HPO_4)

Dissolve 14.196 g of Na_2HPO_4 in 1L of distilled deionized water. Set pH to 7 using HCl or NaOH.

1.2 Preparation of DTNB (5-thio(2-nitrobenzoic acid)) solution

Dissolve 39.6 mg of DTNB in 10 ml phosphate buffer and add 15 mg $NaHCO_3$

1.3 Preparation of acetylthiocholine solution

Dissolve 108.35 mg acetylthiocholine in 5 ml phosphate buffer

2. **<u>Preparation of reagents for determination of Neutral Red Retention Time</u>**

2.1 Preparation of earthworm Ringer solution (for 1L of Ringer)

Mix the following chemicals into 1L of distilled deionised water:

Chemical	Quantity (g)
Sodium chloride ($NaCl_2$)	4.144
Potassium chloride (KCl)	0.355
Calcium chloride ($CaCl_2$)	0.418
Magnesium sulfate ($MgSO_4$)	0.271
Potassium hydrophosphate (KH_2PO_4)	0.054
Sodium hydrophosphate (Na_2HPO_4)	0.043
Sodium Bicarbonate ($NaHCO_3$)	0.353

2.2 Preparation of stock solution Toluyene Red stain

20 mg Toluyene Red ($C_{15}H_{17}N_4CL$)

1ml DMSO (C_2H_6OS)

Mix well in microcentrifuge tube

2.3 Preparation of working solution Toluyene Red stain

2.5 ml Ringer solution

10 µl stock solution

Mix well and renew every hour to prevent crystal formation

APPENDIX 2: STATISTICAL ANALYSIS

Table 1: Statistical analysis (Anova) of weight data of *E. fetida* at start of intermittent exposure experiment

	Analysis of Variance Marked effects are significant at p < .05000							
Variable	SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
Start weight	0.000407	7	0.000058	0.001869	21	0.000089	0.653845	0.707488

Table 2(a): Statistical analysis (Anova) of weight data of *E. fetida* at end of intermittent exposure experiment

	Analysis of Variance Marked effects are significant at p < .05000							
Variable	SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
End weight	0.232660	7	0.033237	0.027955	21	0.001331	24.967610	0.000000

Table 2(b): Post-hoc statistical analysis (Fisher's LSD Test) of weight data of *E. fetida* at end of intermittent exposure experiment

	LSD Test; Variable: End weight Marked effects are significant at p < .05000							
Treatment	{1} M=.47198	{2} M=.40859	{3} M=.35864	{4} M=.38225	{5} M=.36202	{6} M=.13469	{7} M=.44025	{8} M=.28655
Control {1}		0.015344	0.000117	0.001220	0.000163	0.000000	0.200679	0.000002
CPF5/14 {2}	0.015344		0.108380	0.386614	0.132887	0.000000	0.300062	0.001446
CPF25/14 {3}	0.000117	0.108380		0.436784	0.901744	0.000001	0.012284	0.042124
CPF5/28 {4}	0.001220	0.386614	0.436784		0.504368	0.000000	0.065071	0.009099
CPF25/28 {5}	0.000163	0.132887	0.910744	0.504368		0.000010	0.015789	0.034167
AZP5/14 {6}	0.000000	0.000000	0.000001	0.000000	0.000001		0.000000	0.000441
AZP5/28 {7}	0.200679	0.300062	0.012284	0.065071	0.015789	0.000000		0.000150
AZP25/28 {8}	0.000002	0.001446	0.042124	0.009099	0.034167	0.000441	0.000150	

Table 3.1(a): Statistical analysis (Anova) of data for cocoon production of *E. fetida* at end of intermittent exposure experiment.

Variable	Analysis of variance Marked effects are significant at p < .05000							
	SS Effects	df Effects	MS Effects	SS Error	df Error	MS Error	F	p
Cocoon production	34.0931	5	6.81861	11.158	19	0.58728	11.61054	0.00003

Table 3.1(b): Post-hoc statistical analysis (Fisher's LSD Test) of data for cocoon production of *E. fetida* at end of intermittent exposure experiment

Treatment	LSD Test; Variable: Cocoon production Marked effects are significant at p < .05000					
	{1} M=2.9932	{2} M=2.5003	{3} M=2.8097	{4} M=2.3989	{5} M=2.8253	{6} M=2.5573
Control {1}		0.002596	0.000046	0.004711	0.000061	0.000034
CPF5/14 {2}	0.002596		0.166602	0.833527	0.198680	0.138864
CPF25/14 {3}	0.000046	0.166602		0.115079	0.916267	0.916267
CPF5/28 {4}	0.004711	0.833527	0.115079		0.138864	0.094849
CPF25/28 {5}	0.000061	0.198680	0.916267	0.138864		0.833527
AZP5/28 {6}	0.000034	0.138864	0.916267	0.094849	0.833527	

Table 3.2: Statistical analysis (Anova) of data for number of hatchlings per cocoon of *E. fetida* at end of intermittent exposure experiment

Variable	Analysis of variance Marked effects are significant at p < .05000							
	SS Effects	df Effects	MS Effects	SS Error	df Error	MS Error	F	p
Hatchlings per cocoon	1.9335	5	0.386693	3.05764	19	0.160928	2.402887	0.07539

Table 3.3: Statistical analysis (Anova) of data for cocoon viability of *E. fetida* at end of intermittent exposure experiment

Variable	Analysis of variance Marked effects are significant at p < .05000							
	SS Effects	df Effects	MS Effects	SS Error	df Error	MS Error	F	p
Cocoon viability	326.9400	5	65.388	1080.9	19	56.88947	1.14939	0.36933

Table 4 (a): Statistical analysis (Kruskal-Wallis Anova) of data for ChE activity of *E. fetida* at end of intermittent exposure experiment

Depend.: ChE activity	Kruskal-Wallis ANOVA by Ranks: ChE activity Independent (grouping) variable: Treatment Kruskal-Wallis test: H (7, N=125) = 107.1611 p = 0.000		
	Code	Valid N	Sum of Ranks
Control	101	23	2602.000
AZP5/14	102	5	118.000
AZP5/28	103	20	596.000
AZP25/28	104	15	160.000
CPF5/14	105	14	1150.000
CPF25/14	106	16	948.000
CPF5/28	107	17	1398.000
CPF25/28	108	15	903.000

Table 4(b): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of data for ChE activity of *E. fetida* at end of intermittent exposure experiment

Depend.: ChE activity	Multiple Comparisons p values (2-tailed); ChE activity Independent (grouping) variable: Treatment Kruskal-Wallis test: H (7, N=125) = 107.1611 p = 0.000							
	Control R:113.13	AZP5/14 R:23.600	AZP5/28 R:29.800	AZP25/28 R:10.667	CPF5/14 R:82.143	CPF25/14 R:59.250	CPF5/28 R:82.235	CPF25/28 R:60.200
Control		0.000015	0.000000	0.000000	0.325546	0.000138	0.214775	0.000300
AZP5/14	0.000015		1.000000	1.000000	0.053880	1.000000	0.041049	1.000000
AZP5/28	0.000000	1.000000		1.000000	0.000947	0.430301	0.000321	0.392625
AZP25/28	0.000000	1.000000	1.000000		0.000003	0.005333	0.000001	0.005064
CPF5/14	0.325546	0.053880	0.000947	0.000003		1.000000	1.000000	1.000000
CPF25/14	0.000138	1.000000	0.430301	0.005333	1.000000		1.000000	1.000000
CPF5/28	0.214775	0.041049	0.000321	0.000001	1.000000	1.000000		1.000000
CPF25/28	0.000300	1.000000	0.392625	0.005064	1.000000	1.000000	1.000000	

Table 5(a): Statistical analysis (Kruskal-Wallis Anova) of data for Neutral Red Retention Time (NRRT) of *E. fetida* at end of intermittent exposure experiment

Depend.: NRRT	Kruskal-Wallis ANOVA by Ranks: NRRT Independent (grouping) variable: Treatment Kruskal-Wallis test: H (7, N=252) = 154.4556 p = 0.000 Code	Valid N	Sum of Ranks
Control	101	28	6642.500
CPF5/14	102	36	4789.000
CPF25/14	103	36	3541.000
CPF5/28	104	36	6737.500
CPF25/28	105	36	4209.500
AZP5/14	106	20	1054.500
AZP5/28	107	36	3915.000
AZP25/28	108	24	989.000

Table 5(b): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of data for Neutral Red Retention Time (NRRT) of *E. fetida* at end of intermittent exposure experiment

Multiple Comparisons p values (2-tailed); NRRT
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (7, N=252) = 154.4556 p = 0.000

Depend.: NRRT	Control R:237.23	CPF5/14 R:133.03	CPF25/14 R:98.361	CPF5/28 R:187.15	CPF25/28 R:116.93	AZP5/14 R:52.725	AZP5/28 R:108.75	AZP25/28 R:41.208
Control		0.000000	0.000000	0.179147	0.000000	0.000000	0.000000	0.000000
CPF5/14	0.000000		1.000000	0.045655	1.000000	0.002185	1.000000	0.000049
CPF25/14	0.000000	1.000000		0.000007	1.000000	0.693558	1.000000	0.081924
CPF5/28	0.179147	0.045655	0.000007		0.001222	0.000000	0.000141	0.000000
CPF25/28	0.000000	1.000000	1.000000	0.001222		0.044410	1.000000	0.002261
AZP5/14	0.000000	0.002185	0.693558	0.000000	0.044410		0.163821	1.000000
AZP5/28	0.000000	1.000000	1.000000	0.000141	1.000000	0.163821		0.012254
AZP25/28	0.000000	0.000049	0.081924	0.000000	0.002261	1.000000	0.012254	

Table 6(a): Statistical Analysis (Anova) of data for burrowing times of *E. fetida* at start of intermittent exposure.

Variable	Analysis of Variance Marked effects are significant at p < .05000							
	SS Effects	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Start time	314.5022	7	44.92888	773.4333	23	33.62754	1.336074	0.27868

Table 6 (b): Statistical analysis (Kruskal-Wallis Anova) of data for burrowing times of *E. fetida* at start and end of intermittent exposure. (S = start of experiment; E = End of experiment - after 12 weeks exposure)

Depend.: Burrowing time	Kruskal-Wallis ANOVA by Ranks: Burrowing time Independent (grouping) variable: Treatment Kruskal-Wallis test: H (15, N=62) = 49.93822 p = .0000		
	Code	Valid N	Sum of Ranks
Control - S	101	10	122.000
AZP5/14 - S	102	3	65.000
AZP5/28 - S	103	3	72.000
AZP25/28 - S	104	3	76.000
CPF5/14 - S	105	3	42.000
CPF25/14 - S	106	3	78.000
CPF5/28 - S	107	3	51.000
CPF25/28 - S	108	3	42.000
Control - E	109	10	321.500
AZP5/14 - E	110	3	183.000
AZP5/28 - E	111	3	165.000
AZP25/28 - E	112	3	174.000
CPF5/14 - E	113	3	123.000
CPF25/14 - E	114	3	154.000
CPF5/28 - E	115	3	136.000
CPF25/28 - E	116	3	148.000

Table 6.2(c): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of data for burrowing time of *E. fetida* at end of intermittent exposure to azinphos-methyl

Depend.: Burrowing time	Multiple Comparisons p values (2-tailed); Burrowing time Independent (grouping) variable: Treatment Kruskal-Wallis test: H (15, N=62) = 49.93822 p = 0.0000								
	Control - S R:12.200	AZP5/14 - S R:21.833	AZP5/28 - S R:24.000	AZP25/28 - S R:25.333	CPF5/14 - S R:14.000	CPF25/14 - S R:26.000	CPF5/28 - S R:17.000	CPF25/28 - S R:14.000	Control - E R:32.150
Control - S		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP5/14 - S	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP5/28 - S	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP25/28 - S	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000
CPF5/14 - S	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000
CPF25/14 - S	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000
CPF5/28 - S	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000
CPF25/28 - S	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000
Control - E	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	
AZP5/14 - E	0.004769	0.941016	1.000000	1.000000	0.170392	1.000000	0.338170	0.170392	1.000000
AZP5/28 - E	0.037636	1.000000	1.000000	1.000000	0.645785	1.000000	1.000000	0.645785	1.000000
AZP25/28 - E	0.013810	1.000000	1.000000	1.000000	0.338170	1.000000	0.645785	0.338170	1.000000
CPF5/14 - E	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
CPF25/14 - E	0.118090	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
CPF5/28 - E	0.632818	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
CPF25/28 - E	0.212182	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000

AZP5/14 - E R:61.000	AZP5/28 - E R:55.000	AZP25/28 - E R:58.000	CPF5/14 - E R:41.000	CPF25/14 - E R:51.333	CPF5/28 - E R:45.333	CPF25/28 - E R:49.333
0.004769	0.037636	0.013810	1.000000	0.118090	0.632818	0.212182
0.941016	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
0.170392	0.645785	0.338170	1.000000	1.000000	1.000000	1.000000
1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
0.338170	1.000000	0.645785	1.000000	1.000000	1.000000	1.000000
0.170392	0.645785	0.338170	1.000000	1.000000	1.000000	1.000000
1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
1.000000		1.000000	1.000000	1.000000	1.000000	1.000000
1.000000	1.000000		1.000000	1.000000	1.000000	1.000000
1.000000	1.000000	1.000000		1.000000	1.000000	1.000000
1.000000	1.000000	1.000000	1.000000		1.000000	1.000000
1.000000	1.000000	1.000000	1.000000	1.000000		1.000000
1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	

Table 7: Results of chi-squared analysis of data from two-chamber avoidance tests using two concentrations of azinphos-methyl and chlorpyrifos.

Exposure: AZP 25% of LC_{50} value (6.35 mg/kg)		
	Contaminated	Clean
Observed	28	22
Expected	25	25
p=	0.396	

Exposure: CPF 25% of LC_{50} value (23.3 mg/kg)		
	Contaminated	Clean
Observed	22	28
Expected	25	25
p=	0.396	

Exposure: AZP 50% of LC_{50} value (12.7 mg/kg)		
	Contaminated	Clean
Observed	21	29
Expected	25	25
p=	0.258	

Exposure: CPF 50% of LC_{50} value (46.6 mg/kg)		
	Contaminated	Clean
Observed	24	26
Expected	25	25
p=	0.777	

Table 8: Statistical analysis (Kruskal-Wallis Anova) of start weight of *O. mossambicus* at start of intermittent exposure experiment.

Depend.: Start weight	Kruskal-Wallis ANOVA by Ranks: Start weight Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=30) = 9.463933 p = .3047 Code	Valid N	Sum of Ranks
Control	101	6	108.500
AZP10/14	102	3	61.000
AZP50/14	103	3	55.000
AZP10/28	104	3	13.000
AZP50/28	105	3	29.000
CPF10/14	106	3	51.000
CPF50/14	107	3	48.000
CPF10/28	108	3	37.000
CPF50/28	109	3	62.000

Table 9: Statistical analysis (Anova) of start length of *O. mossambicus* at start of intermittent exposure experiment.

Variable	Analysis of variance Marked effects are significant at p < .05000 SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
Start length	2070.00	8	258.75	5800	21	276.19	0.936853	0.50784

Table 10 (a): Statistical analysis (Anova) of end length data of *O. mossambicus* at end of intermittent exposure experiment

Variable	Analysis of variance Marked effects are significant at p < .05000							
	SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
End length	628.1630	17	36.95076	439.0157	348	1.416712	26.08206	0.00000

Table 10 (b): Post-hoc statistical analysis (Fisher's LSD Test) of end length data of *O. mossambicus* at end of intermittent exposure experiment
Data for male fish only

Treatment	LSD Test; Variable: End length Marked effects are significant at p < .05000								
	{1} M=12.620	{2} M=10.193	{3} M=11.700	{4} M=10.221	{5} M=12.744	{6} M=9.6443	{7} M=11.213	{8} M=9.6504	{9} M=10.436
Control {1}		0.000000	0.004186	0.000000	0.713199	0.000000	0.000026	0.000000	0.000000
AZP10/14 {2}	0.000000		0.000038	0.945488	0.000000	0.186824	0.006320	0.199318	0.501406
AZP10/28 {3}	0.004186	0.000038		0.000053	0.001497	0.000000	0.127202	0.000000	0.000049
AZP50/14 {4}	0.000000	0.945488	0.000053		0.000000	0.165412	0.007892	0.177192	0.551782
AZP50/28 {5}	0.713199	0.000000	0.001497	0.000000		0.000000	0.000008	0.000000	0.000000
CPF10/14 {6}	0.000000	0.186824	0.000000	0.165412	0.000000		0.000042	0.988564	0.032394
CPF10/28 {7}	0.000026	0.006320	0.127202	0.007892	0.000008	0.000042		0.000064	0.015367
CPF50/14 {8}	0.000000	0.199318	0.000000	0.177192	0.000000	0.988564	0.000064		0.037652
CPF50/28 {9}	0.000000	0.501406	0.000049	0.551782	0.000000	0.032394	0.015367	0.037652	

Table 10 (c): Post-hoc statistical analysis (Fisher's LSD Test) of end length data of *O. mossambicus* at end of intermittent exposure experiment
Data for female fish only

Treatment	LSD Test; Variable: End length Marked effects are significant at p < .05000 {1} M=10.936	{2} M=8.9306	{3} M=10.292	{4} M=8.5214	{5} M=10.644	{6} M=8.8314	{7} M=9.9402	{8} M=8.1825	{9} M=8.5220
Control {1}		0.000006	0.071992	0.000000	0.444199	0.000000	0.009379	0.000000	0.000000
AZP10/14 {2}	0.000006		0.001238	0.390960	0.000113	0.823126	0.022025	0.124585	0.323131
AZP10/28 {3}	0.071992	0.001238		0.000018	0.331446	0.000087	0.332212	0.000001	0.000000
AZP50/14 {4}	0.000000	0.390690	0.000018		0.000001	0.474671	0.001024	0.477521	0.998651
AZP50/28 {5}	0.444199	0.000113	0.331446	0.000001		0.000005	0.069124	0.000000	0.000000
CPF10/14 {6}	0.000000	0.823126	0.000087	0.474671	0.000005		0.004893	0.144433	0.393594
CPF10/28 {7}	0.009379	0.022025	0.332212	0.001024	0.069124	0.004893		0.000076	0.000084
CPF50/14 {8}	0.000000	0.124585	0.000001	0.477521	0.000000	0.144433	0.000076		0.411580
CPF50/28 {9}	0.000000	0.323131	0.000000	0.998651	0.000000	0.393594	0.000084	0.411580	

Table 11 (a): Statistical analysis (Anova) of end weight data of *O. mossambicus* at end of intermittent exposure experiment

Variable	Analysis of Variance Marked effects are significant at p < .05000 SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
End weight	34.3054	17	2.017964	50.54787	348	0.145253	13.8928	0.00000

Table 11 (b): Post-hoc statistical analysis (Fisher's LSD Test) of end weight data of *O. mossambicus* at end of intermittent exposure experiment. Data for male fish only

Treatment	LSD Test; Variable: End weight Marked effects are significant at p < .05000								
	{1} M=3.4293	{2} M=2.8382	{3} M=3.1889	{4} M=2.5481	{5} M=3.3701	{6} M=2.7971	{7} M=3.1503	{8} M=2.8802	{9} M=3.1693
Control {1}		0.000001	0.019122	0.000000	0.583460	0.000000	0.008684	0.000012	0.011322
AZP10/14 {2}	0.000001		0.002617	0.027136	0.000014	0.757067	0.009024	0.755811	0.004467
AZP10/28 {3}	0.019122	0.002617		0.000000	0.083456	0.000992	0.705883	0.010847	0.842152
AZP50/14 {4}	0.000000	0.027136	0.000000		0.000000	0.061564	0.000001	0.014391	0.000000
AZP50/28 {5}	0.583460	0.000014	0.083456	0.000000		0.000005	0.042405	0.000113	0.055189
CPF10/14 {6}	0.000000	0.757067	0.000992	0.061564	0.000005		0.003764	0.544378	0.001746
CPF10/28 {7}	0.008684	0.009024	0.705883	0.000001	0.042405	0.003764		0.029482	0.852819
CPF50/14 {8}	0.000012	0.755811	0.010847	0.014391	0.000113	0.544378	0.029482		0.016981
CPF50/28 {9}	0.011322	0.004467	0.842152	0.000000	0.055189	0.001746	0.852819	0.016981	

Table 11 (c): Post-hoc statistical analysis (Fisher's LSD Test) of end weight data of *O. mossambicus* at end of intermittent exposure experiment. Data for female fish only.

Treatment	LSD Test; Variable: End weight Marked effects are significant at p < .05000								
	{1} M=2.9932	{2} M=2.5003	{3} M=2.8097	{4} M=2.3989	{5} M=2.8253	{6} M=2.5573	{7} M=2.7302	{8} M=2.4453	{9} M=2.6088
Control {1}		0.000451	0.109495	0.000016	0.169996	0.000488	0.031944	0.000100	0.000705
AZP10/14 {2}	0.000451		0.021354	0.507069	0.021331	0.688317	0.102671	0.724006	0.412112
AZP10/28 {3}	0.109495	0.021354		0.001762	0.893299	0.032825	0.493524	0.006796	0.058384
AZP50/14 {4}	0.000016	0.507069	0.001762		0.002038	0.254455	0.016253	0.761537	0.103696
AZP50/28 {5}	0.169996	0.021311	0.893299	0.002038		0.033213	0.442464	0.007184	0.058702
CPF10/14 {6}	0.000488	0.688317	0.032825	0.254455	0.033213		0.168614	0.430882	0.656905
CPF10/28 {7}	0.031944	0.102671	0.493524	0.016253	0.442464	0.168614		0.043362	0.288271
CPF50/14 {8}	0.000100	0.724006	0.006796	0.761537	0.007184	0.430882	0.043362		0.216918
CPF50/28 {9}	0.000705	0.412111	0.058384	0.103696	0.058702	0.656905	0.288271	0.216918	

Table 12 (a): Statistical analysis (Kruskal-Wallis Anova) of condition factor data of *O. mossambicus* at end of intermittent exposure experiment

Depend.: CF	Code	Valid N	Sum of Ranks
	Kruskal-Wallis ANOVA by Ranks: CF Independent (grouping) variable: Treatment Kruskal-Wallis test: H (17, N=364) = 102.9628 p = .000		
Control - F	110	25	5547.000
AZP10/14 - F	111	17	1381.000
AZP10/28 - F	112	30	4787.000
AZP50/14 - F	113	16	1150.000
AZP50/28 - F	114	24	4746.000
CPF10/14 - F	115	16	3711.000
CPF10/28 - F	116	26	3256.000
CPF50/14 - F	117	15	2285.500
CPF50/28 - F	118	30	6139.000
Control - M	101	20	6398.000
AZP10/14 - M	102	12	1672.000
AZP10/28 - M	103	25	4861.500
AZP50/14 - M	104	13	1047.000
AZP50/28 - M	105	19	5536.500
CPF10/14 - M	106	18	2681.000
CPF10/28 - M	107	19	3840.000
CPF50/14 - M	108	12	1815.000
CPF50/28 - M	109	27	5575.000

Table 12 (b): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of condition factor (CF) data of *O. mossambicus* at end of intermittent exposure experiment Data for male fish only

Depend.: CF	Multiple Comparisons p values (2-tailed); CF Independent (grouping) variable: Treatment Kruskal-Wallis test: H (17, N=364) = 102.9628 p = .000								
	Control R:147.27	AZP10/14 R:61.353	AZP10/28 R:98.950	AZP50/14 R:44.765	AZP50/28 R:134.67	CPF10/14 R:100.25	CPF10/28 R:91.404	CPF50/14 R:77.567	CPF50/28 R:110.78
Control		0.000079	0.069620	0.000001	1.000000	0.394545	0.019243	0.007892	0.692420
AZP10/14	0.000079		1.000000	1.000000	0.002524	1.000000	1.000000	1.000000	0.184377
AZP10/28	0.069620	1.000000		0.077434	0.898350	1.000000	1.000000	1.000000	1.000000
AZP50/14	0.000001	1.000000	0.077434		0.000039	0.222152	0.365420	1.000000	0.006659
AZP50/28	1.000000	0.002524	0.898350	0.000039		1.000000	0.309674	0.010295	1.000000
CPF10/14	0.394545	1.000000	1.000000	0.222152	1.000000		1.000000	1.000000	1.000000
CPF10/28	0.019243	1.000000	1.000000	0.365420	0.309674	1.000000		1.000000	1.000000
CPF50/14	0.007892	1.000000	1.000000	1.000000	0.102945	1.000000	1.000000		1.000000
CPF50/28	0.692420	0.184377	1.000000	0.006659	1.000000	1.000000	1.000000	1.000000	

Table 12 (c): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of condition factor (CF) data of *O. mossambicus* at end of intermittent exposure experiment Data for female fish only

Depend.: CF	Multiple Comparisons p values (2-tailed); CF Independent (grouping) variable: Treatment Kruskal-Wallis test: H (17, N=364) = 102.9628 p = .000								
	Control R:121.20	AZP10/14 R:39.708	AZP10/28 R:77.440	AZP50/14 R:29.846	AZP50/28 R:107.47	CPF10/14 R:89.194	CPF10/28 R:68.526	CPF50/14 R:79.625	CPF50/28 R:95.019
Control		0.000108	0.081518	0.000003	1.000000	1.000000	0.020826	0.617891	1.000000
AZP10/14	0.000108		0.882795	1.000000	0.004310	0.196080	1.000000	1.000000	0.030501
AZP10/28	0.081518	0.882795		0.128719	1.000000	1.000000	1.000000	1.000000	1.000000
AZP50/14	0.000003	1.000000	0.128719		0.000229	0.023135	0.881656	0.332930	0.001917
AZP50/28	1.000000	0.004310	1.000000	0.000229		1.000000	0.431363	1.000000	1.000000
CPF10/14	1.000000	0.196080	1.000000	0.023135	1.000000		1.000000	1.000000	1.000000
CPF10/28	0.020826	1.000000	1.000000	0.881656	0.431363	1.000000		1.000000	1.000000
CPF50/14	0.617891	1.000000	1.000000	0.332930	1.000000	1.000000	1.000000		1.000000
CPF50/28	1.000000	0.030501	1.000000	0.001917	1.000000	1.000000	1.000000	1.000000	

Table 13 (a): Statistical analysis (Anova) of liver somatic index (LSI) data of *O. mossambicus* at end of intermittent exposure experiment

Variable	Analysis of variance Marked effects are significant at p < .05000							
	SS Effects	df Effects	MS Effect	SS Error	df Error	MS Error	F	p
LSI	76.1464	17	4.4792	80.30126	348	0.230751	19.41142	0.00000

Table 13 (b): Post-hoc statistical analysis (Fisher's LSD Test) of liver somatic index (LSI) data of *O. mossambicus* at end of intermittent exposure experiment Data for male fish only

Treatment	LSD Test; Variable: LSI Marked effects are significant at p < .05000								
	{1} M=2.8552	{2} M=1.5459	{3} M=2.6758	{4} M=1.8563	{5} M=2.5203	{6} M=2.8433	{7} M=1.7267	{8} M=2.8958	{9} M=1.9584
Control {1}		0.000000	0.164049	0.000000	0.014237	0.937519	0.000000	0.794688	0.000000
AZP10/14 {2}	0.000000		0.000000	0.060414	0.000000	0.000000	0.228363	0.000000	0.004945
AZP10/28 {3}	0.164049	0.000000		0.000000	0.237985	0.260729	0.000000	0.148342	0.000000
AZP50/14 {4}	0.000000	0.060414	0.000000		0.000017	0.000000	0.387653	0.000000	0.484294
AZP50/28 {5}	0.014237	0.000000	0.237985	0.000017		0.037933	0.000000	0.018074	0.000025
CPF10/14 {6}	0.937519	0.000000	0.260729	0.000000	0.037933		0.000000	0.761080	0.000000
CPF10/28 {7}	0.000000	0.228363	0.000000	0.387653	0.000000	0.000000		0.000000	0.072705
CPF50/14 {8}	0.794688	0.000000	0.148342	0.000000	0.018074	0.761080	0.000000		0.000000
CPF50/28 {9}	0.000000	0.004945	0.000000	0.484294	0.000025	0.000000	0.072705	0.000000	

Table 13 (c): Post-hoc statistical analysis (Fisher's LSD Test) of liver somatic index (LSI) data of *O. mossambicus* at end of intermittent exposure experiment Data for female fish only

	LSD Test; Variable: LSI Marked effects are significant at $p < .05000$								
Treatment	{1} M=2.9674	{2} M=1.8837	{3} M=2.8238	{4} M=2.2490	{5} M=2.6359	{6} 2.9977	{7} M=2.1132	{8} M=2.8996	{9} M=2.3290
Control {1}		0.000000	0.328997	0.000037	0.033368	0.832732	0.000000	0.710710	0.000010
AZP10/14 {2}	0.000000		0.000000	0.058297	0.000028	0.000000	0.195975	0.000000	0.007899
AZP10/28 {3}	0.328997	0.000000		0.000527	0.199670	0.242477	0.000002	0.653663	0.000240
AZP50/14 {4}	0.000037	0.058297	0.000527		0.025855	0.000024	0.432636	0.000798	0.622220
AZP50/28 {5}	0.033337	0.000028	0.199670	0.025855		0.022659	0.000883	0.137580	0.035330
CPF10/14 {6}	0.832723	0.000000	0.242477	0.000024	0.022659		0.000000	0.584069	0.000007
CPF10/28 {7}	0.000000	0.195975	0.000002	0.432636	0.000883	0.000000		0.000012	0.134458
CPF50/14 {8}	0.710710	0.000000	0.653663	0.000080	0.137580	0.584069	0.000012		0.000691
CPF50/28 {9}	0.000010	0.007899	0.000240	0.622220	0.033533	0.000007	0.134458	0.000691	

Table 14 (a): Statistical analysis (Kruskal-Wallis Anova) of gonadosomatic index (GSI) data of male *O. mossambicus* at end of intermittent exposure experiment

Depend.: GSI	Code	Valid N	Sum of Ranks
	Kruskal-Wallis ANOVA by Ranks: GSI Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=201) = 14.16992 p = .0774		
Control	101	26	2889.000
AZP10/14	102	17	1550.000
AZP10/28	103	30	3233.000
AZP50/14	104	17	1702.000
AZP50/28	105	24	2019.000
CPF10/14	106	16	1155.000
CPF10/28	107	26	3374.000
CPF50/14	108	15	1451.000
CPF50/28	109	30	2928.000

Table 14 (b) Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of GSI data of male *O. mossambicus* at end of intermittent exposure experiment.

Depend.: GSI	Control R:111.12	AZP10/14 R:91.176	AZP10/28 R:107.77	AZP50/14 R:100.12	AZP50/28 R:84.125	CPF10/14 R:72.188	CPF10/28 R:129.77	CPF50/14 R:96.733	CPF50/28 R:97.600
	Multiple Comparisons p values (2-tailed); GSI Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=201) = 14.16992 p = .0774								
Control		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP10/14	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP10/28	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
AZP50/14	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000
AZP50/28	1.000000	1.000000	1.000000	1.000000		1.000000	0.200501	1.000000	1.000000
CPF10/14	1.000000	1.000000	1.000000	1.000000	1.000000		0.066115	1.000000	1.000000
CPF10/28	1.000000	1.000000	1.000000	1.000000	0.200501	0.066115		1.000000	1.000000
CPF50/14	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000
CPF50/28	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	

Table 14 (c): Statistical analysis (Kruskal-Wallis Anova) of gonadosomatic index (GSI) data of female *O. mossambicus* at end of intermittent exposure experiment

Depend.: GSI	Kruskal-Wallis ANOVA by Ranks: GSI Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=165) = 16.41278 p = .0368		
	Code	Valid N	Sum of Ranks
Control	101	20	1900.000
AZP10/14	102	12	1055.000
AZP10/28	103	25	2022.000
AZP50/14	104	13	1255.000
AZP50/28	105	19	920.000
CPF10/14	106	18	1649.000
CPF10/28	107	19	1670.000
CPF50/14	108	12	1238.000
CPF50/28	109	27	1986.000

Table 14 (b) Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of GSI data of female *O. mossambicus* at end of intermittent exposure experiment.

Depend.: GSI	Multiple Comparisons p values (2-tailed); GSI Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=165) = 16.41278 p = .0368								
	Control R:95.000	AZP10/14 R:87.917	AZP10/28 R:80.880	AZP50/14 R:96.538	AZP50/28 R:48.421	CPF10/14 R:91.611	CPF10/28 R:87.895	CPF50/14 R:103.17	CPF50/28 R:73.556
Control		1.000000	1.000000	1.000000	0.084242	1.000000	1.000000	1.000000	1.000000
AZP10/14	1.000000		1.000000	1.000000	0.898676	1.000000	1.000000	1.000000	1.000000
AZP10/28	1.000000	1.000000		1.000000	0.921462	1.000000	1.000000	1.000000	1.000000
AZP50/14	1.000000	1.000000	1.000000		0.185029	1.000000	1.000000	1.000000	1.000000
AZP50/28	0.084242	0.898676	0.921462	0.185029		0.215549	0.391579	0.067892	1.000000
CPF10/14	1.000000	1.000000	1.000000	1.000000	0.215549		1.000000	1.000000	1.000000
CPF10/28	1.000000	1.000000	1.000000	1.000000	0.391579	1.000000		1.000000	1.000000
CPF50/14	1.000000	1.000000	1.000000	1.000000	0.067892	1.000000	1.000000		1.000000
CPF50/28	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	

Table 15 (a): Statistical analysis (Kruskal-Wallis Anova) of data for AChE activity of *O. mossambicus* at end of intermittent exposure experiment

Depend.: AChE	Code	Valid N	Sum of Ranks
	Kruskal-Wallis ANOVA by Ranks: AChE Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=137) = 64.58161 p = 0.000		
Control	101	17	2145.000
AZP10/14	102	15	1149.000
AZP50/14	103	15	339.000
AZP10/28	104	15	1007.000
AZP50/28	105	15	1000.000
CPF10/14	106	15	631.000
CPF50/14	107	15	938.000
CPF10/28	108	15	1174.000
CPF50/28	109	15	1070.000

Table 15 (b): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of AChE activity data of *O. mossambicus* at end of intermittent exposure experiment.

Depend.: AChE	Control R:126.18	AZP10/14 R:76.600	AZP50/14 R:22.600	AZP10/28 R:67.133	AZP50/28 R:66.667	CPF10/14 R:42.067	CPF50/14 R:62.533	CPF10/28 R:78.267	CPF50/28 R:71.333
	Multiple Comparisons p values (2-tailed); AChE Independent (grouping) variable: Treatment Kruskal-Wallis test: H (8, N=137) = 64.58161 p = 0.000								
Control		0.015198	0.000000	0.000965	0.000833	0.000000	0.000216	0.023619	0.003457
AZP10/14	0.015198		0.007010	1.000000	1.000000	0.618790	1.000000	1.000000	1.000000
AZP50/14	0.000000	0.007010		0.076392	0.085053	1.000000	0.211147	0.004416	0.027818
AZP10/28	0.000965	1.000000	0.076392		1.000000	1.000000	1.000000	1.000000	1.000000
AZP50/28	0.000833	1.000000	0.085053	1.000000		1.000000	1.000000	1.000000	1.000000
CPF10/14	0.000000	0.618790	1.000000	1.000000	1.000000		1.000000	0.450082	1.000000
CPF50/14	0.000216	1.000000	0.211147	1.000000	1.000000	1.000000		1.000000	1.000000
CPF10/28	0.023619	1.000000	0.004416	1.000000	1.000000	0.450082	1.000000		1.000000
CPF50/28	0.003457	1.000000	0.027818	1.000000	1.000000	1.000000	1.000000	1.000000	

Table 16 (a): Statistical analysis (Kruskal-Wallis Anova) of feeding response time of *O. mossambicus* following pesticide exposure

Depend.: Response time	Kruskal-Wallis ANOVA by Ranks: Response time Independent (grouping) variable: Treatment Kruskal-Wallis test: H (4, N=90) = 79.71992 p = 0.000		
	Code	Valid N	Sum of Ranks
Control	101	18	296.500
AZP50	105	18	1467.000
AZP10	106	18	859.500
CPF50	107	18	1102.500
CPF10	108	18	369.500

Table 16(b): Post-hoc statistical analysis (Multiple comparison of mean ranks) of feeding response time of *O. mossambicus* following pesticide exposure

Depend.: Response time	Multiple Comparisons p values (2-tailed); Response time Independent (grouping) variable: Treatment Kruskal-Wallis test: H (4, N=90) = 79.71992 p = 0.000				
	Control R:16.472	AZP10/14 R:81.5	AZP10/28 R:74.750	AZP50/14 R:61.250	AZP50/28 R:20.528
Control		0.000000	0.003285	0.000003	1.000000
AZP50	0.000000		0.001063	0.200514	0.000000
AZP10	0.003285	0.001063		1.000000	0.017718
CPF50	0.000003	0.200514	1.000000		0.000290
CPF10	1.000000	0.000000	0.017718	0.000029	

Table 17 (a): Statistical analysis (Kruskal-Wallis Anova) of food consumption of O. mossambicus following pesticide exposure

Depend.: Consumption	Kruskal-Wallis ANOVA by Ranks: Consumption Independent (grouping) variable: Treatment Kruskal-Wallis test: H (4, N=90) = 58.26628 p = 0.000		
	Code	Valid N	Sum of Ranks
Control	101	18	1258.000
AZP50	105	18	311.500
AZP10	106	18	567.000
CPF50	107	18	714.000
CPF10	108	18	1217.000

Table 17(b): Post-hoc statistical analysis (Multiple Comparison of Mean Ranks) of food consumption of *O. mossambicus* following pesticide exposure

Depend.: Consumption	Multiple Comparisons p values (2-tailed); Consumption Independent (grouping) variable: Treatment Kruskal-Wallis test: H (4, N=90) = 58.26628 p = 0.000				
	Control R:71.389	AZP10/14 R:17.306	AZP10/28 R:31.500	AZP50/14 R:39.694	AZP50/28 R:67.611
Control		0.000000	0.000046	0.002731	1.000000
AZP50	0.000000		1.000000	0.101405	0.000000
AZP10	0.000046	1.000000		1.000000	0.000337
CPF50	0.002731	0.101405	1.000000		0.013470
CPF10	1.000000	0.000000	0.003370	0.013470	

APPENDIX 3: DATA SUMMARY

Table 1: Mean start weights of *E. fetida* used for intermittent exposure to azinphos-methyl and chlorpyrifos.

No of animals (n)	Start weight of group (g)	Mean weight per worm (g)
10	0.9224	0.0922
10	0.9322	0.0932
10	0.8918	0.0892
10	0.8484	0.0848
10	0.9821	0.0982
10	1.0352	0.1035
10	0.9020	0.0902
8	0.7810	0.0976
8	0.7296	0.0912
10	0.8697	0.0870
10	0.8301	0.0830
10	0.8532	0.0853
10	0.7985	0.0799
10	0.8664	0.0866
10	0.9525	0.0953
10	1.0559	0.1056
10	1.0617	0.1062
10	0.9507	0.0951
10	1.0111	0.1011
10	1.0181	0.1018
10	0.8909	0.0891
10	0.8626	0.0863
10	0.8901	0.0890
10	0.8481	0.0848
10	0.8421	0.0842
10	0.8111	0.0811
8	0.7715	0.0964
8	0.7406	0.0926
8	0.8240	0.1030
8	0.7613	0.0952
8	0.7929	0.0991
8	0.7783	0.0973
Mean	0.872	0.096
Std Dev	0.093	0.007

Table 2: Summary of growth data of *E. fetida* during an intermittent 12-week exposure to chlorpyrifos (14-day treatment interval)

		Control						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.8301	1.1615	1.8218	2.4204	2.8704	3.4818	4.5191
n=10	Replicate 2	1.0617	1.1056	1.7396	2.4377	3.2237	4.1897	4.9870
n=10	Replicate 3	0.9525	1.2249	1.7439	2.1498	2.8555	3.9971	4.6002
	Mean	0.948	1.164	1.768	2.336	2.983	3.890	4.702
	Std Dev	0.116	0.060	0.046	0.161	0.208	0.366	0.250

		Treatment: 5% of LC_{50} value (4.64 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.8532	1.1579	1.8796	2.2801	2.4211	3.2784	4.0191
n=10	Replicate 2	0.7985	1.0477	1.7744	2.0671	2.5667	3.2578	4.0582
n=10	Replicate 3	1.0559	1.1557	1.5371	1.9414	2.5482	3.6611	4.1805
	Mean	0.9025	1.1204	1.7304	2.0962	2.5120	3.3991	4.0859
	Std Dev	0.136	0.063	0.175	0.171	0.079	0.227	0.084

		Treatment 25% of LC_{50} value (23.20 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=8	Replicate 1	0.7783	1.0746	1.6554	1.9441	2.1115	2.3225	2.9671
n=10	Replicate 2	0.8664	1.1858	1.7909	2.0401	2.4691	2.5295	3.3761
n=10	Replicate 3	0.9761	1.0845	1.7955	2.1261	2.4018	3.0126	3.7111
	Mean	0.8736	1.1150	1.7473	2.0368	2.3275	2.6215	3.3514
	Std Dev	0.099	0.062	0.080	0.091	0.190	0.354	0.373

Table 3: Summary of growth data of *E. fetida* during an intermittent 12-week exposure to chlorpyrifos (28-day treatment interval)

		Control						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.9224	1.1861	1.6707	2.3924	3.2641	4.6036	4.8605
n=10	Replicate 2	0.8484	1.0402	1.4981	2.1903	3.0588	4.1696	4.4979
n=8	Replicate 3	0.7810	1.1699	1.7156	2.1612	2.5725	3.1628	4.3046
	Mean	0.851	1.132	1.628	2.248	2.965	3.979	4.554
	Std Dev	0.071	0.080	0.115	0.126	0.355	0.739	0.282

		Treatment: 5% of LC_{50} value (4.64 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.9322	1.1822	1.6966	2.2353	3.0284	3.6022	3.9197
n=10	Replicate 2	0.9821	1.1804	1.6458	2.1883	2.8502	3.5874	4.1327
n=8	Replicate 3	0.7269	1.2410	1.7979	2.3001	2.6338	3.0641	3.4152
	Mean	0.8804	1.2012	1.7134	2.2412	2.8375	3.4179	3.8225
	Std Dev	0.135	0.034	0.077	0.056	0.198	0.306	0.368

		Treatment 25% of LC_{50} value (23.20 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.8918	1.1109	1.6401	2.0863	2.8724	3.4860	4.0220
n=10	Replicate 2	0.9020	1.1367	1.5488	1.9769	2.5773	3.1142	3.4417
n=10	Replicate 3	0.8697	1.2669	1.7901	2.2939	2.8701	2.6050	3.3968
	Mean	0.8878	1.1715	1.6597	2.1190	2.7733	3.0684	3.6202
	Std Dev	0.017	0.084	0.122	0.161	0.170	0.442	0.349

Table 4: Summary of growth of *E. fetida* during an intermittent 12-week exposure to azinphos-methyl (14-day treatment interval)

		Control						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.9507	1.1932	1.7701	2.4373	3.0388	4.1021	5.6912
n=8	Replicate 2	0.7715	0.8619	1.2401	1.9097	2.4686	3.5197	4.3989
n=10	Replicate 3	0.8901	0.9573	1.3459	2.3936	2.9728	3.7398	4.2111
	Replicate 4	0.8111	0.9411	1.3568	2.4668	3.2459	4.2469	5.1272
	Mean	0.8559	0.9884	1.4282	2.3019	2.9315	3.9021	4.8571
	Std Dev	0.0802	0.1428	0.2339	0.2632	0.3298	0.3324	0.6821

		Treatment: 5% of LC_{50} value (1.27 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	1.0111	1.2241	1.7184	1.7913	1.8640	2.0534	1.0803
n=10	Replicate 2	0.8909	1.1151	1.7630	2.1248	1.9570	1.2792	1.1510
n=8	Replicate 3	0.8240	0.9494	1.4509	1.8864	2.0541	1.8062	1.1657
	Mean	0.9087	1.0962	1.6441	1.9342	1.9584	1.7129	1.1323
	Std Dev	0.095	0.138	0.169	0.172	0.095	0.395	0.046

		Treatment: 25% of LC_{50} value (6.35 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	1.0181	1.1532	0.4460	0.2105	Terminated - 100% mortality		
n=10	Replicate 2	1.0352	1.2921	0.8921	0.0345	Terminated - 100% mortality		
n=10	Replicate 3	0.8626	1.0096	1.1270	0.3252	Terminated - 100% mortality		
	Mean	0.9720	1.1516	0.8217	0.1901	Terminated - 100% mortality		
	Std Dev	0.095	0.141	0.346	0.146	Terminated - 100% mortality		

Table 5: Summary of growth data of *E. fetida* during an intermittent 12-week exposure to azinphos-methyl (28-day treatment interval)

		Control						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.9507	1.1932	1.7701	2.4373	3.0388	4.1021	5.6912
n=8	Replicate 2	0.7715	0.8619	1.2401	1.9097	2.4686	3.5197	4.3989
n=10	Replicate 3	0.8901	0.9573	1.3459	2.3936	2.9728	3.7398	4.2111
n=10	Replicate 4	0.8111	0.9411	1.3568	2.4668	3.2459	4.2469	5.1272
	Mean	0.8356	0.9884	1.4282	2.3019	2.9315	3.9021	4.8571
	Std Dev	0.0789	0.1428	0.2339	0.2632	0.3298	0.3324	0.6821

		Treatment: 5% of LC_{50} value (1.27 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=8	Replicate 1	0.7406	0.9072	1.4911	1.8734	2.0581	3.5136	4.5947
n=10	Replicate 2	0.8481	0.9437	1.3130	1.6102	2.3181	3.1740	4.0198
n=8	Replicate 3	0.7613	1.0602	1.4718	2.4370	2.8429	3.8325	4.5929
	Mean	0.7833	0.9704	1.4253	1.9735	2.4064	3.5067	4.4025
	Std Dev	0.057037	0.07991	0.09773	0.4224	0.39978	0.3293	0.3314

		Treatment: 25% of LC_{50} value (6.35 mg/kg)						
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	0.8585	0.9541	1.3992	1.0692	1.2545	1.3116	2.8165
n=10	Replicate 2	0.8421	1.0705	1.0428	1.0901	1.2220	1.3846	1.8293
n=8	Replicate 3	0.7929	0.8676	1.1141	1.0041	1.0525	1.1265	1.7659
	Mean	0.8312	0.9641	1.1854	1.0545	1.1763	1.2742	2.1372
	Std Dev	0.034139	0.101817	0.18859	0.04485	0.10847	0.13305	0.589116

Table 6: Data for ChE activity of *E. fetida* following a 12-week intermittent exposure to azinphos-methyl and chlorpyrifos using various exposure regimes

Treatment	ΔA/min	Protein content	Calculations	Enzyme activity
Control 1-1	0.2510	73.523	0.2320	232.0
Control1-2	0.4020	92.066	0.4653	465.3
Control 1-3	0.4640	58.430	0.3409	340.9
Control1-4	0.4540	78.698	0.4492	449.2
Control 2-1	0.2786	101.55	0.3557	355.7
Control 2-2	0.165	101.12	0.2098	209.8
Control 2-3	0.1878	168.71	0.3984	398.4
Control 2-4	0.224	72.66	0.2046	204.6
Control 2-5	0.2554	110.39	0.3545	354.5
Control 3-1	0.3332	134.023	0.5615	561.5
Control 3 -2	0.1996	109.961	0.2760	276.0
Control 3-2	0.2268	139.974	0.3992	399.2
Control 3 -3	0.2736	135.317	0.4655	465.5
Control 4-1	0.2774	135.446	0.4724	472.4
Control 4-2	0.2392	117.982	0.3548	354.8
Control 4-3	0.2736	110.737	0.3809	380.9
Control 5-1	0.1652	156.92	0.3259	325.9
Control 5-2	0.1844	113.71	0.2636	263.6
Control 5-3	0.1376	141.01	0.2440	244.0
Control 5-4	0.3096	158.34	0.6164	616.4
Control 6-1	0.2144	162.61	0.4384	438.4
Control 6-2	0.1582	172.32	0.3428	342.8
Control 6-3	0.1784	164.04	0.3679	367.9
Mean	---	---	---	370.4
Std Dev	---	---	---	104.5
A5/14R1-1	0.004	116.69	0.0059	5.9
A5/14R1-2	0.004	131.31	0.0064	6.4
A5/14R1-3	0.005	112.98	0.0070	7.0
A5/14R1-4	0.004	130.14	0.0065	6.5
A5/14R1-5	0.006	138.81	0.0097	9.7
Mean	---	---	---	7.1
Std Dev	---	---	---	1.5
A5/28R2-1	0.007	82.10	0.0075	7.5
A5/28R2-2	0.014	158.34	0.0272	27.2
A5/28R2-3	0.006	104.10	0.0083	8.3
A5/28R2-4	0.011	104.01	0.0138	13.8
A5/28R2-5	0.011	97.28	0.0130	13.0
A5/28R2-6	0.007	113.45	0.0101	10.1
A5/28R2-7	0.006	115.74	0.0086	8.6
A5/28R2-8	0.012	109.57	0.0159	15.9
A5/28R2-9	0.015	142.95	0.0272	27.2
A5/28-1R1	0.0000	23.286	0.0000	0.0

A5/28-2R1	0.0000	43.553	0.0000	0.0
A5/28-3R1	0.0410	105.649	0.0545	54.5
A5/28-4R1	0.0530	46.787	0.0312	31.2
A5/28-5R1	0.0490	56.059	0.0345	34.5
A5/28-6R1	0.0890	41.828	0.0468	46.8
A5/28-7R1	0.0390	46.356	0.0227	22.7
A5/28-3R3	0.0470	56.705	0.0335	33.5
A5/28-4R3	0.0000	27.382	0.0000	0.0
A5/28-6R3	0.0090	50.668	0.0057	5.7
A5/28-7R3	0.0000	71.367	0.0000	0.0
Mean	---	---	---	18.0
Std Dev	---	---	---	16.0
A25/28-1R2	0.0000	15.524	0.0000	0.0
A25/28-2R2	0.0000	61.449	0.0000	0.0
A25/28-3R2	0.0000	55.627	0.0000	0.0
A25/28-4R2	0.0000	34.713	0.0000	0.0
A25/28-5R2	0.0000	30.617	0.0000	0.0
A25/28-6R2	0.0000	31.695	0.0000	0.0
A25/28R1-1	0.000	118.59	0.0000	0.0
A25/28R1-2	0.003	97.97	0.0036	3.6
A25/28R1-4	0.001	120.66	0.0012	1.2
A25/28R1-5	0.002	105.05	0.0023	2.3
A25/28R1-6	0.000	96.42	0.0000	0.0
A25/28R1-7	0.001	128.07	0.0021	2.1
A25/28R1-8	0.000	117.46	0.0000	0.0
A25/28R1-9	0.000	93.23	0.0000	0.0
A25/28R1-10	0.002	88.66	0.0019	1.9
Mean	---	---	---	0.7
Std Dev	---	---	---	1.2
C5/14R1-2	0.0564	95.41	0.0677	67.7
C5/14R1-3	0.0704	124.08	0.1098	109.8
C5/14R1-5	0.033	119.99	0.0498	49.8
C5/14R2-1	0.0738	116.75	0.1083	108.3
C5/14R2-2	0.1342	131.20	0.2214	221.4
C5/14R2-3	0.0496	142.95	0.0891	89.1
C5/14R2-4	0.0398	149.85	0.0750	75.0
C5/14R2-5	0.0682	143.92	0.1234	123.4
C5/14R3-1	0.0542	179.30	0.1222	122.2
C5/14R3-2	0.0782	151.62	0.1491	149.1
C5/14R3-3	0.0798	128.59	0.1290	129.0
C5/14R3-4	0.1062	165.72	0.2213	221.3
C5/14R3-5	0.1038	142.56	0.1861	186.1
C5/14R3-6	0.0542	119.15	0.0812	81.2
Mean	---	---	---	123.8
Std Dev	---	---	---	54.1

C25/14R1-1	0.0432	95.19	0.0517	51.7
C25/14R1-2	0.0396	130.34	0.0649	64.9
C25/14R1-3	0.032	155.35	0.0625	62.5
C25/14R1-4	0.0412	103.71	0.0537	53.7
C25/14R2-1	0.0286	154.59	0.0556	55.6
C25/14R2-2	0.0396	126.67	0.0631	63.1
C25/14R2-3	0.0554	157.83	0.1099	109.9
C25/14R2-4	0.034	134.00	0.0573	57.3
C25/14R2-5	0.0176	88.62	0.0196	19.6
C25/14R2-6	0.0352	131.52	0.0582	58.2
C25/14R3-1	0.0266	127.94	0.0428	42.8
C25/14R3-2	0.0444	140.75	0.0786	78.6
C25/14R3-3	0.0334	132.34	0.0556	55.6
C25/14R3-4	0.0428	141.01	0.0759	75.9
C25/14R3-5	0.0288	120.57	0.0437	43.7
C25/14R3-6	0.0182	162.48	0.0372	37.2
Mean	---	---	---	58.1
Std Dev	---	---	---	19.9
C5/28R3-1	0.048	165.071	0.1002	100.2
C5/28R3-2	0.024	193.661	0.0589	58.9
C5/28R3-3	0.023	186.417	0.0547	54.7
C5/28R3-4	0.026	155.757	0.0508	50.8
C5/28R3-5	0.056	194.049	0.1371	137.1
C5/28R3-6	0.049	181.759	0.1118	111.8
C5/28R1-1	0.035	188.875	0.0822	82.2
C5/28R1-2	0.025	176.714	0.0557	55.7
C5/28R1-3	0.040	187.451	0.0951	95.1
C5/28R1-4	0.036	215.265	0.0974	97.4
C5/28R1-5	0.043	221.087	0.1193	119.3
C5/28R1-6	0.043	258.085	0.1389	138.9
C5/28R2-1	0.1350	116.559	0.1978	197.8
C5/28R2-2	0.1020	169.599	0.2175	217.5
C5/28R2-3	0.1638	129.884	0.2675	267.5
C5/28R2-4	0.1592	132.083	0.2644	264.4
C5/28R2-5	0.1626	125.873	0.2573	257.3
Mean	---	---	---	135.7
Std Dev	---	---	---	76.4
C25/28R2-1	0.016	203.881	0.0419	41.9
C25/28R2-2	0.025	185.640	0.0585	58.5
C25/28R2-3	0.010	188.228	0.0246	24.6
C25/28R2-5	0.013	189.004	0.0311	31.1
C25/28R2-6	0.020	201.811	0.0509	50.9
C25/28R3-1	0.011	177.749	0.0255	25.5
C25/28R3-2	0.013	154.722	0.0258	25.8
C25/28R3-4	0.011	171.022	0.0232	23.2
C25/28R3-5	0.030	144.502	0.0554	55.4

C25/28R3-6	0.023	195.084	0.0569	56.9
C25/28R1-1	0.0852	111.643	0.1196	119.6
C25/28R1-2	0.0570	164.166	0.1177	117.7
C25/28R1-3	0.0782	178.655	0.1757	175.7
C25/28R1-5	0.0592	139.457	0.1038	103.8
C25/28R1-6	0.0752	167.270	0.1582	158.2
Mean	---	---	---	71.2
Std Dev	---	---	---	50.8

Δ A/min = change in absorption/minute of tissue samples (calculated from spectrophotometer readings); P.C. = protein content of samples; Calculations = calculation of enzyme activity after Fisher (2000); Sample volume = 200 µl; Path length = 0.5848 cm; Molar Extinction Coefficient of DTNB reagent = 79.5328

Table 7: Summary of ChE activity of *E. fetida* following intermittent exposure to azinphos-methyl and chlorpyrifos

Azinphos-methyl treatments					
	Mean	**SD**	**% activity**	**SD [%]**	**CV (%)**
Control	370.1	101.9	100.00	27.54	27.5
AZP5/14	7.1	1.5	1.92	0.40	21.0
AZP5/28	18.0	16.04	4.87	4.33	89.0
AZP25/28	0.7	1.18	0.20	0.32	159.5

Chlorpyrifos treatments					
	Mean	**SD**	**% activity**	**SD [%]**	**CV (%)**
Control	370.1	101.9	100.00	27.54	27.54
CPF5/14	123.8	54.09	33.46	14.62	43.69
CPF25/14	60.7	17.66	16.40	4.77	29.09
CPF5/28	135.7	76.42	36.67	20.65	56.32
CPF25/28	71.2	50.82	19.25	13.73	71.34

Table 8a: Summary of reproductive endpoints of control *E. fetida* following a 12-week experimental period. (HPC = number of hatchlings produced per cocoon)

		No of cocoons	No of cocoons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=10	Replicate 1	18	1.8	2.3	2	89
n=10	Replicate 2	50	5.0	2.8	2	96
n=10	Replicate 3	33	3.7	2.8	1	97
n=10	Replicate 4	21	2.1	2.7	1	95
n=10	Replicate 5	45	4.5	2.8	0	100
n=8	Replicate 6	25	3.2	2.7	0	100
n=10	Replicate 7	24	2.4	2.7	0	100
n=8	Replicate 8	29	3.6	2.7	1	97
n=10	Replicate 9	34	3.8	2.6	0	100
n=10	Replicate 10	29	2.9	2.4	1	97
	Total	308.0	---	---	8	---
	Mean	30.8	3.2	2.6	0.8	97.1
	Std Dev	3.2	1.0	0.2	0.7	3.4

Table 8b: Summary of reproductive endpoints of *E. fetida* following a 12-week exposure to chlorpyrifos at 5% (4.64 mg/kg) of the LC_{50} value (Treatment group: CPF5/28)

		No of cocoons	No of cocoons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=10	Replicate 1	19	1.9	2.1	3	84
n=10	Replicate 2	13	1.3	2.2	1	92
n=8	Replicate 3	16	2	2.6	0	100
	Total	48	---	---	4	---
	Mean	16	1.6	2.3	1.3	92.0
	Std Dev	2.4	0.3	0.2	1.5	8.0

Table 8c: Summary of reproductive endpoints of *E. fetida* following a 12-week exposure to chlorpyrifos at 25% (23.2 mg/kg) of the LC_{50} value (Treatment group: CPF25/28)

		No of cocoons	No of coons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=10	Replicate 1	1	0.1	2.0	0	100
n=10	Replicate 2	10	1	2.5	2	80
n=10	Replicate 3	8	0.8	2.4	1	88
	Total	19	---	---	3.0	---
	Mean	6.3	0.6	2.3	1.0	89.3
	Std Dev	4.7	0.5	0.3	1.0	10.1

Table 8d: Summary of reproductive endpoints of *E. fetida* following a 12-week exposure to chlorpyrifos at 5% (4.64 mg/kg) of the LC_{50} value (Treatment group: CPF5/14)

		No of cocoons	No of coons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=10	Replicate 1	11	1.1	2.5	1	91
n=10	Replicate 2	13	1.3	2.1	0	100
n=10	Replicate 3	20	2	2.5	3	85
	Total	44	---	---	---	---
	Mean	14.7	1.5	2.4	1.33	92.0
	Std Dev	4.7	0.5	0.3	1.53	7.5

Table 8e: Summary of reproductive endpoints of *E. fetida* following a 12-week exposure to chlorpyrifos at 25% (23.2 mg/kg) of the LC_{50} value (Treatment group: CPF25/14)

		No of cocoons	No of coons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=8	Replicate 1	2	0.2	2.0	0	100
n=10	Replicate 2	4	0.4	2.0	1	75
n=10	Replicate 3	11	1.1	2.3	1	91
	Total	17	---	---	2	---
	Mean	5.67	0.6	2.1	0.67	88.7
	Std Dev	4.73	0.5	0.2	0.58	12.7

Table 8f: Summary of reproductive endpoints of *E. fetida* following a 12-week exposure to azinphos-methyl at 5% (1.27 mg/kg) of the LC_{50} value (Treatment group: AZP5/28)

		No of cocoons	No of coons/worm	HPC (mean)	Unviable cocoons	Cocoon viability (%)
n=8	Replicate 1	3	0.4	3.7	0	100
n=10	Replicate 2	7	0.7	1.8	1	86
n=8	Replicate 3	5	0.6	3.7	1	80
	Total	15	---	---	2	--
	Mean	5.0	0.5	3.1	0.67	88.7
	Std Dev	2.0	0.2	1.1	0.58	10.3

No cocoons were produced in the two groups exposed to azinphos-methyl at a 14-day interval (AZP5/14 and AZP25/14) and the group exposed to azinphos-methyl at 25% of the LC50 value for a 28 day interval (AZP25/28).

Table 9: Neutral red retention times (in minutes) of earthworms exposed to azinphos-methyl and chlorpyrifos using various exposure regimes. Two slides were counted per worm.

Treatment	Ind. No	Slide A (min)	Slide B (min)	Mean (min)
Control	1	23	29	26
Control	2	21	37	29
Control	3	29	27	28
Control	4	21	25	23
Control	5	27	27	27
Control	6	33	33	33
Control	7	37	31	34
Control	8	37	37	37
Control	9	31	29	30
Control	10	33	29	31
Control	11	35	33	34
Control	12	33	35	34
Control	13	39	39	39
Control	14	39	35	37
Mean	---	31	32	32
Std Dev	---	6.27	4.35	4.65
CPF5/14	1	11	9	10
CPF5/14	2	13	11	12
CPF5/14	3	17	15	16
CPF5/14	4	13	11	12
CPF5/14	5	9	19	14
CPF5/14	6	17	11	14
CPF5/14	7	11	11	11
CPF5/14	8	15	13	14
CPF5/14	9	13	13	13
CPF5/14	10	11	11	11
CPF5/14	11	15	9	12
CPF5/14	12	17	11	14
CPF5/14	13	11	13	12
CPF5/14	14	15	21	18
CPF5/14	15	19	17	18
CPF5/14	16	21	13	17
CPF5/14	17	15	17	16
CPF5/14	18	15	17	16
Mean	---	14	13	14
Std Dev	---	3.14	3.47	2.47
CPF25/14	1	11	11	11
CPF25/14	2	17	11	14
CPF25/14	3	11	15	13
CPF25/14	4	9	11	10
CPF25/14	5	13	13	13
CPF25/14	6	15	13	14

CPF25/14	7	17	9	13
CPF25/14	8	13	7	10
CPF25/14	9	9	9	9
CPF25/14	10	13	11	12
CPF25/14	11	9	13	11
CPF25/14	12	15	13	14
CPF25/14	13	9	15	12
CPF25/14	14	9	9	9
CPF25/14	15	11	11	11
CPF25/14	16	15	11	13
CPF25/14	17	15	9	12
CPF25/14	18	15	9	12
Mean	---	13	11	12
Std Dev	---	2.87	2.22	1.62
CPF5/28	1	13	17	15
CPF5/28	2	15	21	18
CPF5/28	3	23	25	24
CPF5/28	4	15	13	14
CPF5/28	5	19	11	15
CPF5/28	6	15	19	17
CPF5/28	7	23	15	19
CPF5/28	8	23	17	20
CPF5/28	9	15	17	16
CPF5/28	10	15	11	13
CPF5/28	11	17	17	17
CPF5/28	12	17	13	15
CPF5/28	13	23	19	21
CPF5/28	14	21	21	21
CPF5/28	15	19	21	20
CPF5/28	16	23	19	21
CPF5/28	17	25	19	22
CPF5/28	18	21	21	21
Mean	---	19	18	18
Std Dev	---	3.82	3.81	3.16
CPF25/28	1	15	13	14
CPF25/28	2	19	9	14
CPF25/28	3	11	11	11
CPF25/28	4	11	11	11
CPF25/28	5	17	19	18
CPF25/28	6	15	11	13
CPF25/28	7	11	7	9
CPF25/28	8	13	13	13
CPF25/28	9	11	9	10
CPF25/28	10	13	17	15
CPF25/28	11	19	15	17
CPF25/28	12	21	17	19

CPF25/28	13	9	9	9
CPF25/28	14	13	13	13
CPF25/28	15	9	9	9
CPF25/28	16	13	15	14
CPF25/28	17	13	15	14
CPF25/28	18	3	15	9
Mean	---	13	13	13
Std Dev	---	4.25	3.38	3.12
AZP5/14	1	9	11	10
AZP5/14	2	9	7	8
AZP5/14	3	9	7	8
AZP5/14	4	7	9	8
AZP5/14	5	13	13	13
AZP5/14	6	9	11	10
AZP5/14	7	11	9	10
AZP5/14	8	11	9	10
AZP5/14	9	9	9	9
AZP5/14	10	11	9	10
Mean	---	10	9	10
	---	1.69	1.84	1.51
AZP5/28	1	15	9	12
AZP5/28	2	19	13	16
AZP5/28	3	11	15	13
AZP5/28	4	13	13	13
AZP5/28	5	11	15	13
AZP5/28	6	11	13	12
AZP5/28	7	15	13	14
AZP5/28	8	11	11	11
AZP5/28	9	13	11	12
AZP5/28	10	11	15	13
AZP5/28	11	13	11	12
AZP5/28	12	11	15	13
AZP5/28	13	9	13	11
AZP5/28	14	11	11	11
AZP5/28	15	13	11	12
AZP5/28	16	9	13	11
AZP5/28	17	9	13	11
AZP5/28	18	11	11	11
Mean	---	12	13	12
Std Dev	---	2.50	1.76	1.32
AZP25/28	1	7	9	8
AZP25/28	2	7	7	7
AZP25/28	3	9	11	10
AZP25/28	4	7	11	9
AZP25/28	5	11	9	10
AZP25/28	6	7	11	9

AZP25/28	7	9	11	10
AZP25/28	8	7	7	7
AZP25/28	9	7	11	9
AZP25/28	10	11	9	10
AZP25/28	11	9	7	8
AZP25/28	12	9	11	10
Mean	---	8	10	9
	---	1.56	1.73	1.16

Table 10a: Burrowing times (in seconds) for control *E. fetida* individuals at 2-week intervals during a 12-week exposure period.

Control								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	68	75	54	73	58	62	68
n=10	Replicate 2	58	62	70	60	68	62	78
n=10	Replicate 3	62	65	70	76	70	68	77
n=10	Replicate 4	56	64	72	62	70	83	115
n=10	Replicate 5	72	64	66	68	78	70	105
n=8	Replicate 6	54	64	62	84	72	71	62
n=10	Replicate 7	66	76	69	75	67	70	77
n=8	Replicate 8	61	64	64	61	73	79	88
n=10	Replicate 9	58	58	59	63	77	67	62
n=10	Replicate 10	62	66	76	81	91	98	82
	Mean	62	66	66	70	72	73	81
	Std Dev	5.6	5.6	6.6	8.7	8.6	11.0	17.4

Table 10b: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to chlorpyrifos (Treatment group: CPF5/14)

Treatment: 5% of LC_{50} value (4.64 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	62	57	84	92	96	115	100
n=10	Replicate 2	58	66	70	76	102	132	95
n=10	Replicate 3	68	76	77	87	118	105	98
	Mean	63	66	77	85	105	117	98
	Std Dev	5.0	9.5	7.0	8.2	11.4	13.7	2.5

Table 10c: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to chlorpyrifos (Treatment group: CPF25/14)

Treatment: 25% of LC_{50} value (23.2 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=8	Replicate 1	70	70	83	93	135	130	140
n=10	Replicate 2	64	98	71	91	144	150	160
n=10	Replicate 3	73	72	78	108	153	151	170
	Mean	69	80	77	97	144	144	157
	Std Dev	4.6	15.6	6.0	9.3	9.0	11.8	15.3

Table 10d: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to chlorpyrifos (Treatment group: CPF5/28)

Treatment: 5% of LC_{50} value (4.64 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	54	67	80	102	92	103	136
n=10	Replicate 2	67	62	77	97	90	100	102
n=10	Replicate 3	70	68	75	78	86	74	110
	Mean	64	66	77	92	89	92	116
	Std Dev	9	3	3	13	3	16	18

Table 10e: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to chlorpyrifos (Treatment group: CPF25/28)

Treatment: 25% of LC_{50} value (23.2 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	62	73	86	115	130	140	131
n=10	Replicate 2	58	63	96	140	136	130	138
n=10	Replicate 3	68	73	86	98	100	121	161
	Mean	63	70	89	118	122	130	143
	Std Dev	5	6	6	21	19	10	16

Table 10f: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to azinphos-methyl (Treatment group: AZP5/14)

Treatment: 5% of LC_{50} value (1.27 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	62	62	71	162	205	298	516
n=10	Replicate 2	70	66	85	98	307	271	498
n=8	Replicate 3	68	64	73	124	266	261	520
	Mean	67	64	76	128	259	277	511
	Std Dev	4.2	2.0	7.6	32.2	51.3	19.1	11.7

Table 10g: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to azinphos-methyl (Treatment group: AZP25/14)

Treatment: 25% of LC_{50} value (6.35 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	68	70	350	600			
n=10	Replicate 2	58	74	443	600			
n=10	Replicate 3	72	110	220	600	No data 100% mortality		
	Mean	66	85	338	600			
	Std Dev	7	22	112	0			

Table 10h: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to azinphos-methyl (Treatment group: AZP5/28)

Treatment: 5% of LC_{50} value (1.27 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	74	78	110	121	121	86	181
n=10	Replicate 2	67	61	90	90	112	90	210
n=10	Replicate 3	66	64	95	150	93	85	224
	Mean	69	68	98	120	109	87	205
	Std Dev	4	9	10	30	14	3	22

Table 10i: Burrowing times (in seconds) for *E. fetida* individuals at 2-week intervals during a 12-week exposure to azinphos-methyl (Treatment group: AZP25/28)

Treatment: 25% of LC_{50} value (6.35 mg/kg)								
		Week 0	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**
n=10	Replicate 1	62	95	94	298	250	275	290
n=10	Replicate 2	78	116	102	355	220	270	283
n=10	Replicate 3	69	80	78	281	296	242	320
	Mean	70	97	91	311	255	262	298
	Std Dev	8	18	12	39	38	18	20

Table 11: Data for number of worms in clean and contaminated soils following a two-chamber avoidance experiment using two concentrations of azinphos-methyl and chlorpyrifos.

Treatment	Replicate no	Contaminated soil	Clean soil
AZP 25% of LC_{50} value (6.35 mg/kg)	1	4	6
	2	4	6
	3	7	3
	4	6	4
	5	7	3
Total		28	22
AZP 50% of LC_{50} value (12.70 mg/kg)	1	2	8
	2	5	5
	3	5	5
	4	6	4
	5	3	7
Total		21	29
CPF 25% of LC_{50} value (23.20 mg/kg)	1	3	7
	2	4	6
	3	6	4
	4	6	4
	5	3	7
Total		22	28
CPF 50% of LC_{50} value (46.6 mg/kg)	1	5	5
	2	6	4
	3	4	6
	4	5	5
	5	4	6
Total		24	26

Table 12: Start weight and length of *O. mossambicus* groups at start of intermittent exposure experiment

Number of animals (n)	Mean start weight (g)	Mean start length (mm)
20	1.192	320
20	1.090	310
20	0.789	280
20	0.649	260
20	0.812	300
20	0.877	300
20	1.071	310
20	1.048	310
20	1.060	310
20	0.733	280
20	0.809	290
20	0.878	300
20	0.811	290
20	0.688	270
20	0.613	260
20	0.755	280
20	0.866	290
20	0.711	270
20	0.646	270
20	1.192	310
20	1.028	310
20	0.738	280
20	0.968	300
20	0.647	280
20	0.648	270
20	0.685	280
20	0.673	270
20	0.781	280
20	0.723	280
20	0.890	300
20	0.690	270
Mean (all groups)	0.831	288
Std. Dev.	0.170	17.103

Table 12: Data for growth and morphological parameters of *O. mossambicus* following a 12-week intermittent exposure to azinphos-methyl and chlorpyrifos. CF = condition factor; LSI = liver somatic index; GSI = gonadosomatic index. (Data presented for female fish only.)

DATA FOR FEMALES ONLY						
Gender	**Treatment**	**End weight (g)**	**End length (cm)**	**CF**	**LSI**	**GSI**
F	Control	19.71	10.9	3.14	3.48	5.38
F	Control	19.00	10.7	3.15	3.14	4.25
F	Control	17.86	10.3	3.35	3.30	0.42
F	Control	36.01	13.0	3.34	3.15	5.03
F	Control	22.00	10.6	3.80	3.16	0.20
F	Control	19.14	10.7	3.18	2.81	4.24
F	Control	15.15	9.7	3.58	2.64	0.85
F	Control	19.71	11.2	3.05	2.76	4.87
F	Control	17.35	10.7	3.03	3.01	1.51
F	Control	18.97	11.2	2.94	2.88	1.87
F	Control	17.65	10.6	3.22	2.91	6.88
F	Control	20.44	11.3	3.04	2.33	2.87
F	Control	22.06	11.5	3.16	3.37	5.28
F	Control	22.35	11.5	3.20	3.36	5.18
F	Control	18.82	11.2	2.92	2.33	2.82
F	Control	23.97	11.6	3.31	3.19	4.99
F	Control	20.71	11.0	3.18	2.68	2.16
F	Control	15.71	9.6	3.66	3.33	1.68
F	Control	20.00	10.9	3.19	2.62	2.22
F	Control	19.14	10.7	3.18	2.84	4.13
Mean		20.29	10.9	3.23	2.96	3.34
Std Dev		4.29	0.7	0.22	0.33	1.87
F	AZP10/14	12.73	9.2	2.74	2.05	1.84
F	AZP10/14	10.91	8.6	2.92	1.58	0.46
F	AZP10/14	13.77	9.5	2.72	2.12	4.51
F	AZP10/14	12.41	9.0	2.74	1.71	4.92
F	AZP10/14	10.25	8.4	2.82	1.97	0.90
F	AZP10/14	15.06	9.4	2.94	1.74	7.01
F	AZP10/14	18.23	10.3	2.71	1.68	4.92
F	AZP10/14	9.87	8.2	2.84	1.19	2.25
F	AZP10/14	12.91	8.9	2.97	2.23	1.81
F	AZP10/14	11.17	8.8	2.74	1.79	0.34
F	AZP10/14	9.87	8.4	2.77	2.38	5.64
F	AZP10/14	11.43	8.6	3.06	2.16	2.87
Mean		12.38	8.9	2.83	1.88	3.12
Std Dev		2.44	0.6	0.12	0.33	2.21
F	AZP10/28	13.94	9.9	2.89	3.07	0.79
F	AZP10/28	16.34	10.3	2.98	2.41	3.24
F	AZP10/28	14.93	10.1	2.84	3.55	2.50
F	AZP10/28	24.51	11.4	3.27	3.71	4.47

F	AZP10/28	14.65	9.9	3.03	3.12	2.12
F	AZP10/28	20.70	11.1	2.98	3.18	1.16
F	AZP10/28	22.68	12.0	2.62	2.96	2.48
F	AZP10/28	19.72	10.8	3.07	3.29	1.22
F	AZP10/28	17.08	10.1	3.16	3.09	2.64
F	AZP10/28	13.47	9.9	2.71	2.97	5.30
F	AZP10/28	20.97	11.1	2.95	2.48	4.29
F	AZP10/28	22.78	11.3	3.09	3.59	3.98
F	AZP10/28	18.75	10.6	3.08	2.71	2.97
F	AZP10/28	14.17	9.6	3.10	1.97	1.28
F	AZP10/28	14.03	9.7	2.94	1.79	1.53
F	AZP10/28	21.25	10.8	3.22	3.22	2.64
F	AZP10/28	17.08	10.0	3.30	2.37	5.86
F	AZP10/28	20.28	10.8	3.08	2.53	4.22
F	AZP10/28	19.44	11.0	2.84	2.40	3.67
F	AZP10/28	16.39	10.1	3.03	2.66	3.89
F	AZP10/28	9.03	8.3	3.01	2.67	0.50
F	AZP10/28	15.14	9.6	3.32	3.17	1.19
F	AZP10/28	13.47	9.9	2.71	2.72	1.36
F	AZP10/28	13.75	9.9	2.77	2.54	1.42
F	AZP10/28	11.67	9.2	2.92	2.41	1.54
Mean		17.05	10.3	3.00	2.82	2.65
Std Dev		3.90	0.8	0.18	0.49	1.49
F	AZP50/14	8.85	4.6	2.76	2.72	4.47
F	AZP50/14	16.92	10.0	2.78	2.76	6.63
F	AZP50/14	6.54	7.6	2.48	2.48	2.73
F	AZP50/14	7.95	7.9	2.60	2.49	3.54
F	AZP50/14	17.14	10.3	2.68	1.84	4.97
F	AZP50/14	16.62	9.7	3.03	1.71	4.90
F	AZP50/14	10.00	8.3	2.94	2.10	0.74
F	AZP50/14	11.43	8.8	2.80	1.92	4.89
F	AZP50/14	8.83	8.1	2.85	2.53	0.37
F	AZP50/14	11.56	9.1	2.59	2.48	3.18
F	AZP50/14	11.30	9.1	2.54	2.47	1.77
F	AZP50/14	12.99	9.1	2.92	1.68	1.95
F	AZP50/14	9.22	8.2	2.84	2.07	4.47
Mean		11.49	8.5	2.75	2.25	3.43
Std Dev		3.52	1.4	0.17	0.38	1.85
F	AZP50/28	15.82	10.4	3.09	2.64	0.93
F	AZP50/28	17.01	10.4	3.32	3.05	0.41
F	AZP50/28	8.66	8.7	2.97	2.66	1.30
F	AZP50/28	12.09	9.6	3.09	2.93	0.54
F	AZP50/28	7.76	8.2	3.13	1.84	0.66
F	AZP50/28	13.28	9.9	3.10	3.57	0.47
F	AZP50/28	20.87	11.6	2.81	2.63	1.21
F	AZP50/28	26.38	11.9	3.30	2.65	5.44

F	AZP50/28	17.83	10.4	3.30	2.88	3.51
F	AZP50/28	20.72	11.0	3.26	2.38	1.14
F	AZP50/28	13.58	10.0	3.03	2.54	2.98
F	AZP50/28	19.70	10.9	3.39	3.52	0.84
F	AZP50/28	16.87	10.6	3.16	1.82	0.40
F	AZP50/28	19.70	11.3	3.01	2.85	1.62
F	AZP50/28	29.40	12.7	3.21	2.96	1.72
F	AZP50/28	14.03	10.4	2.74	1.78	2.36
F	AZP50/28	20.00	11.0	3.31	2.74	0.33
F	AZP50/28	31.49	12.7	3.44	2.96	1.42
F	AZP50/28	15.07	10.4	2.94	1.69	0.61
Mean		17.91	10.6	3.14	2.64	1.47
Std Dev		6.27	1.2	0.19	0.54	1.31
F	CPF10/14	13.38	9.1	2.75	2.86	1.64
F	CPF10/14	10.38	8.1	3.02	4.34	5.10
F	CPF10/14	9.00	8.4	2.39	2.50	3.07
F	CPF10/14	6.38	7.3	2.61	4.44	2.52
F	CPF10/14	11.50	8.9	2.57	3.43	1.03
F	CPF10/14	16.71	8.9	3.85	3.01	4.85
F	CPF10/14	15.19	9.0	3.35	3.19	4.63
F	CPF10/14	25.06	10.8	3.22	3.22	6.68
F	CPF10/14	20.63	10.1	3.18	2.83	6.03
F	CPF10/14	13.54	9.1	2.87	2.39	4.21
F	CPF10/14	17.47	10.0	2.80	2.60	1.88
F	CPF10/14	9.87	7.8	3.27	2.66	0.58
F	CPF10/14	9.11	7.7	3.17	2.78	0.45
F	CPF10/14	14.05	9.1	2.97	3.10	2.12
F	CPF10/14	12.15	8.5	3.19	2.64	6.30
F	CPF10/14	19.37	9.7	3.35	2.86	7.08
F	CPF10/14	12.53	8.7	3.01	2.30	0.64
F	CPF10/14	9.24	7.7	3.22	2.81	0.62
Mean		13.64	8.8	3.05	3.00	3.30
Std Dev		4.76	0.9	0.35	0.58	2.33
F	CPF10/28	13.15	9.3	3.05	1.72	0.46
F	CPF10/28	12.47	9.0	3.17	2.46	0.94
F	CPF10/28	11.78	9.0	2.99	2.46	0.43
F	CPF10/28	17.81	10.1	3.21	2.31	4.62
F	CPF10/28	16.16	10.4	2.69	1.99	0.21
F	CPF10/28	10.27	9.0	2.61	1.30	10.04
F	CPF10/28	10.82	8.8	3.01	2.34	1.00
F	CPF10/28	20.41	11.2	2.64	2.04	2.11
F	CPF10/28	15.14	9.9	2.88	2.24	6.01
F	CPF10/28	15.81	9.7	3.13	2.74	3.99
F	CPF10/28	18.38	10.5	2.87	2.36	5.25
F	CPF10/28	25.95	11.6	3.02	2.39	4.07
F	CPF10/28	24.38	11.5	3.00	2.02	5.70

F	CPF10/28	18.22	10.1	3.28	2.17	2.45
F	CPF10/28	14.52	10.1	2.62	1.79	2.44
F	CPF10/28	17.95	10.4	2.98	2.20	5.72
F	CPF10/28	13.15	9.6	2.80	2.08	1.22
F	CPF10/28	13.56	9.6	2.89	2.08	2.05
F	CPF10/28	11.37	8.8	3.17	1.46	4.08
Mean		15.86	9.9	2.95	2.11	3.30
Std Dev		4.36	0.9	0.21	0.35	2.55
F	CPF50/14	8.81	7.7	2.69	3.42	6.06
F	CPF50/14	15.12	9.0	2.89	3.61	1.49
F	CPF50/14	23.53	9.8	3.50	2.31	7.06
F	CPF50/14	19.41	9.4	3.22	3.43	4.06
F	CPF50/14	13.18	8.4	3.13	2.74	5.12
F	CPF50/14	15.88	9.1	2.96	2.99	7.80
F	CPF50/14	9.53	7.9	2.69	2.87	3.30
F	CPF50/14	10.12	7.5	3.28	2.66	2.94
F	CPF50/14	9.17	7.7	2.80	2.46	1.71
F	CPF50/14	9.64	7.7	2.95	2.86	0.31
F	CPF50/14	6.31	6.9	2.72	2.88	3.26
F	CPF50/14	8.10	7.0	3.31	2.56	2.85
Mean		12.40	8.2	3.01	2.90	3.83
Std Dev		5.16	0.9	0.27	0.41	2.29
F	CPF50/28	14.30	8.6	3.04	2.14	5.00
F	CPF50/28	15.23	8.7	3.11	3.25	2.42
F	CPF50/28	9.65	7.6	3.02	2.54	2.35
F	CPF50/28	15.23	8.5	3.37	2.42	0.24
F	CPF50/28	17.79	9.2	3.10	3.02	1.61
F	CPF50/28	16.86	9.1	3.06	1.79	6.60
F	CPF50/28	10.47	7.7	3.13	1.46	0.66
F	CPF50/28	13.02	8.3	3.13	2.45	5.27
F	CPF50/28	12.91	8.5	2.85	2.32	2.20
F	CPF50/28	13.95	8.7	2.84	1.41	6.65
F	CPF50/28	16.86	8.7	3.44	1.98	5.75
F	CPF50/28	20.00	9.6	3.16	3.66	4.53
F	CPF50/28	27.62	10.6	3.29	3.54	3.46
F	CPF50/28	20.00	9.6	3.16	2.17	1.28
F	CPF50/28	16.19	9.2	2.98	2.03	2.20
F	CPF50/28	10.83	7.9	3.17	2.33	0.61
F	CPF50/28	14.88	8.8	3.08	2.03	0.39
F	CPF50/28	12.62	8.3	3.09	2.39	0.34
F	CPF50/28	9.05	7.5	3.04	1.96	0.48
F	CPF50/28	14.47	8.7	3.04	2.55	4.73
F	CPF50/28	11.88	8.2	2.94	2.54	4.59
F	CPF50/28	7.41	6.9	3.07	2.55	2.07
F	CPF50/28	14.71	8.5	3.35	2.73	1.05
F	CPF50/28	12.12	8.5	2.76	1.75	2.13

F	CPF50/28	16.12	9.2	2.89	2.12	1.47
F	CPF50/28	6.24	6.7	2.86	1.77	0.30
F	CPF50/28	13.06	8.4	3.10	1.99	0.45
Mean		14.20	8.5	3.08	2.33	2.55
Std Dev		4.31	0.8	0.16	0.56	2.09

Table 13: Data for growth and morphological parameters of *O. mossambicus* following a 12-week intermittent exposure to azinphos-methyl and chlorpyrifos. CF = condition factor; LSI = liver somatic index; GSI = gonadosomatic index. (Data presented for male fish only.)

		Data for males only				
Gender	**Treatment**	**End weight (g)**	**End length (cm)**	**CF**	**LSI**	**GSI**
Male	Control	24.85	9.7	5.88	1.61	0.52
Male	Control	26.18	12.6	2.80	3.00	0.75
Male	Control	26.62	12.1	3.28	2.48	0.42
Male	Control	29.12	12.8	3.01	3.18	0.65
Male	Control	13.82	10.1	2.86	2.66	0.37
Male	Control	24.71	12.5	2.74	0.31	1.02
Male	Control	31.03	12.9	3.10	3.19	0.61
Male	Control	31.76	13.4	2.87	2.48	0.84
Male	Control	28.24	12.5	3.13	3.22	1.00
Male	Control	38.09	14.4	2.75	3.29	0.97
Male	Control	29.85	12.9	2.98	3.12	0.47
Male	Control	27.50	12.2	3.27	2.73	0.45
Male	Control	49.57	14.1	3.58	3.42	0.38
Male	Control	48.29	14.1	3.48	3.24	0.53
Male	Control	49.29	14.3	3.45	3.40	0.47
Male	Control	28.14	11.6	3.71	3.83	0.28
Male	Control	56.71	14.9	3.53	2.86	0.75
Male	Control	19.14	10.0	3.91	3.55	0.13
Male	Control	31.14	12.0	3.68	3.48	0.20
Male	Control	40.57	13.7	3.21	2.26	0.54
Male	Control	33.00	13.4	2.78	3.03	0.81
Male	Control	37.71	12.9	3.62	3.33	0.31
Male	Control	25.29	11.4	3.46	3.06	0.22
Male	Control	36.00	13.1	3.24	2.43	0.72
Male	Control	37.14	13.7	2.94	2.68	1.53
Mean		32.95	12.7	3.33	2.87	0.60
Std Dev		10.04	1.4	0.63	0.72	0.32
Male	AZP10/14	16.71	9.6	3.01	1.54	0.32
Male	AZP10/14	8.99	8.0	2.84	1.43	0.07
Male	AZP10/14	11.65	8.9	2.68	1.15	0.49
Male	AZP10/14	9.49	7.5	3.65	1.52	0.17
Male	AZP10/14	19.22	10.6	2.68	1.36	0.97
Male	AZP10/14	14.42	9.7	2.63	1.49	0.41
Male	AZP10/14	20.65	11.0	2.59	1.60	0.48
Male	AZP10/14	6.88	7.4	2.86	1.33	0.15
Male	AZP10/14	16.75	9.4	3.46	2.21	0.42
Male	AZP10/14	21.82	11.2	2.64	1.32	0.57
Male	AZP10/14	20.39	11.3	2.38	1.52	0.61
Male	AZP10/14	24.94	11.6	2.72	1.41	0.34
Male	AZP10/14	13.38	9.5	2.65	1.61	0.35

Male	AZP10/14	24.03	11.4	2.71	1.73	0.66
Male	AZP10/14	22.73	11.3	2.66	1.55	0.63
Male	AZP10/14	33.38	12.7	2.73	1.70	0.85
Male	AZP10/14	30.39	12.2	2.82	1.82	0.99
Mean		18.58	10.2	2.81	1.55	0.50
Std Dev		7.39	1.6	0.31	0.24	0.27
Male	AZP10/28	10.83	8.8	3.12	2.82	0.02
Male	AZP10/28	16.94	10.6	2.78	2.37	0.33
Male	AZP10/28	28.89	12.5	2.85	2.69	0.69
Male	AZP10/28	34.58	12.9	3.10	3.56	0.74
Male	AZP10/28	26.67	12.2	2.82	2.09	0.67
Male	AZP10/28	30.83	12.4	3.15	3.15	0.27
Male	AZP10/28	22.08	12.2	2.33	2.76	0.44
Male	AZP10/28	24.72	11.9	2.80	2.07	0.64
Male	AZP10/28	26.11	11.7	3.17	2.14	0.70
Male	AZP10/28	31.39	12.9	2.81	3.01	0.55
Male	AZP10/28	22.78	11.1	3.20	2.17	0.18
Male	AZP10/28	27.36	12.1	2.99	3.11	0.32
Male	AZP10/28	35.42	13.5	2.79	2.14	0.72
Male	AZP10/28	31.81	12.2	3.36	2.44	0.40
Male	AZP10/28	21.94	11.3	2.97	3.10	1.02
Male	AZP10/28	25.56	12.2	2.70	2.83	0.29
Male	AZP10/28	28.75	12.4	2.94	2.40	1.02
Male	AZP10/28	20.28	11.1	2.85	2.17	0.63
Male	AZP10/28	25.14	8.1	9.28	2.21	0.96
Male	AZP10/28	35.56	13.3	2.89	3.30	0.76
Male	AZP10/28	33.33	13.3	2.71	3.46	0.79
Male	AZP10/28	24.23	11.4	3.24	2.59	0.17
Male	AZP10/28	25.63	12.3	2.76	2.68	1.42
Male	AZP10/28	19.44	11.0	2.91	2.09	0.67
Male	AZP10/28	29.44	12.1	3.29	2.97	0.54
Male	AZP10/28	9.58	8.9	2.72	2.93	0.02
Male	AZP10/28	14.51	10.0	2.88	2.92	0.77
Male	AZP10/28	29.30	13.1	2.59	2.95	0.52
Male	AZP10/28	18.59	10.7	3.01	2.18	0.67
Male	AZP10/28	28.45	13.0	2.59	2.98	0.50
Mean		25.34	11.7	3.12	2.68	0.58
Std Dev		6.75	1.4	1.18	0.45	0.31
Male	AZP50/14	16.10	9.7	2.94	2.20	0.24
Male	AZP50/14	9.74	8.4	2.73	2.23	0.26
Male	AZP50/14	11.69	9.0	2.74	2.34	0.49
Male	AZP50/14	25.06	11.4	2.83	1.33	0.73
Male	AZP50/14	14.94	9.7	2.73	1.48	0.48
Male	AZP50/14	20.39	10.8	2.75	1.19	0.56
Male	AZP50/14	13.90	9.7	2.54	1.22	0.57
Male	AZP50/14	21.56	10.9	2.80	1.70	0.55

Male	AZP50/14	17.66	10.4	2.66	1.98	1.25
Male	AZP50/14	16.75	10.1	2.72	0.78	1.31
Male	AZP50/14	25.77	11.4	2.85	2.17	0.39
Male	AZP50/14	30.64	12.2	2.79	2.57	0.59
Male	AZP50/14	23.72	11.7	2.45	1.91	0.49
Male	AZP50/14	8.59	8.2	2.56	2.59	0.31
Male	AZP50/14	14.49	10.0	2.38	2.80	0.28
Male	AZP50/14	7.69	7.8	2.64	1.52	0.21
Mean		17.42	10.1	2.69	1.88	0.54
Std Dev		6.65	1.3	0.15	0.58	0.32
Male	AZP50/28	57.97	15.9	3.01	2.74	0.79
Male	AZP50/28	47.68	14.6	3.19	3.82	0.51
Male	AZP50/28	43.62	14.1	3.30	3.40	0.43
Male	AZP50/28	50.00	14.9	3.16	2.84	0.43
Male	AZP50/28	28.84	10.3	5.56	1.71	0.07
Male	AZP50/28	16.96	10.7	2.89	1.63	0.39
Male	AZP50/28	34.64	13.6	2.88	2.80	0.81
Male	AZP50/28	16.12	10.9	2.78	3.02	0.12
Male	AZP50/28	42.24	14.0	3.41	2.79	0.46
Male	AZP50/28	22.69	11.5	3.33	2.30	0.24
Male	AZP50/28	32.54	13.7	2.80	2.26	0.29
Male	AZP50/28	38.51	14.0	3.11	2.66	0.59
Male	AZP50/28	26.12	12.1	3.29	2.01	0.34
Male	AZP50/28	34.93	13.9	2.91	1.94	0.72
Male	AZP50/28	24.33	11.9	3.18	2.13	0.19
Male	AZP50/28	28.21	13.0	2.87	2.82	0.52
Male	AZP50/28	30.75	13.6	2.73	2.73	0.79
Male	AZP50/28	26.87	12.5	3.04	2.50	0.52
Male	AZP50/28	35.67	14.0	2.88	2.14	0.65
Male	AZP50/28	13.43	10.0	2.99	2.91	0.44
Male	AZP50/28	14.48	9.9	3.37	2.17	0.61
Male	AZP50/28	28.51	12.7	3.11	1.68	0.37
Male	AZP50/28	27.91	12.5	3.16	2.37	0.13
Male	AZP50/28	23.73	11.3	3.62	3.13	0.50
Mean		31.11	12.7	3.19	2.52	0.46
Std Dev		11.42	1.7	0.55	0.55	0.21
Male	CPF10/14	21.77	10.8	2.80	2.64	0.54
Male	CPF10/14	21.27	10.0	3.41	3.20	0.16
Male	CPF10/14	17.22	9.1	3.64	2.75	0.33
Male	CPF10/14	22.66	10.6	3.02	2.47	0.51
Male	CPF10/14	19.49	10.4	2.79	2.08	0.27
Male	CPF10/14	16.58	9.0	3.66	2.83	0.33
Male	CPF10/14	17.09	9.9	2.84	2.73	0.46
Male	CPF10/14	8.86	7.8	2.94	3.25	0.40
Male	CPF10/14	15.32	9.0	3.38	3.06	0.48
Male	CPF10/14	15.82	9.9	2.63	3.59	0.69

Male	CPF10/14	11.27	8.5	2.96	2.16	0.26
Male	CPF10/14	16.84	10.3	2.50	2.67	0.71
Male	CPF10/14	9.87	8.2	2.84	3.17	0.24
Male	CPF10/14	22.78	10.6	3.04	3.09	0.36
Male	CPF10/14	19.75	10.6	2.63	3.29	0.32
Male	CPF10/14	15.13	9.6	2.65	2.52	0.46
Mean		16.98	9.6	2.98	2.84	0.41
Std Dev		4.31	0.9	0.36	0.42	0.16
Male	CPF10/28	26.49	11.6	3.08	1.56	0.33
Male	CPF10/28	23.92	11.9	2.60	1.42	1.20
Male	CPF10/28	24.05	11.5	2.90	1.87	0.70
Male	CPF10/28	27.70	12.0	2.91	1.43	0.40
Male	CPF10/28	22.84	7.8	2.75	1.61	0.88
Male	CPF10/28	21.76	10.8	3.14	1.65	0.18
Male	CPF10/28	11.49	8.8	3.10	1.96	0.07
Male	CPF10/28	28.11	12.0	2.95	1.96	1.33
Male	CPF10/28	22.43	11.1	3.01	1.26	0.83
Male	CPF10/28	18.65	10.9	2.60	3.71	0.94
Male	CPF10/28	32.05	12.6	3.01	2.44	0.76
Male	CPF10/28	26.58	12.5	2.57	1.54	0.78
Male	CPF10/28	24.38	11.8	2.80	1.65	0.92
Male	CPF10/28	33.56	12.7	3.05	2.54	0.60
Male	CPF10/28	24.38	11.9	2.70	1.71	0.97
Male	CPF10/28	24.93	11.9	2.76	1.67	0.98
Male	CPF10/28	29.59	11.9	3.28	1.53	0.40
Male	CPF10/28	23.42	7.9	2.78	0.75	1.31
Male	CPF10/28	29.18	12.2	3.02	1.88	0.40
Male	CPF10/28	17.81	10.5	2.85	1.72	0.75
Male	CPF10/28	25.89	11.6	3.08	1.88	0.72
Male	CPF10/28	33.01	12.6	3.09	1.70	0.94
Male	CPF10/28	24.66	12.1	2.64	1.03	1.08
Male	CPF10/28	24.38	11.9	2.70	1.47	0.74
Male	CPF10/28	11.92	9.3	2.77	1.66	0.15
Male	CPF10/28	14.11	9.5	3.14	1.28	0.46
Mean		24.13	11.2	2.90	1.73	0.72
Std Dev		5.73	1.4	0.20	0.55	0.35
Male	CPF50/14	24.24	10.7	2.73	2.89	0.92
Male	CPF50/14	23.29	10.4	2.91	2.39	0.40
Male	CPF50/14	12.24	8.4	2.91	2.55	0.36
Male	CPF50/14	26.47	11.1	2.71	2.56	0.43
Male	CPF50/14	20.36	10.2	2.69	3.15	0.80
Male	CPF50/14	22.86	10.7	2.63	2.17	0.63
Male	CPF50/14	17.62	9.5	2.89	3.06	0.50
Male	CPF50/14	18.33	9.8	2.79	2.80	0.66
Male	CPF50/14	24.29	10.6	2.89	3.08	0.44
Male	CPF50/14	19.52	9.4	3.33	2.84	0.14

Male	CPF50/14	18.57	9.9	2.73	3.12	1.44
Male	CPF50/14	8.45	7.5	2.84	3.34	0.17
Male	CPF50/14	12.14	8.5	2.85	2.93	0.22
Male	CPF50/14	14.40	8.7	3.11	3.00	0.29
Male	CPF50/14	16.19	9.5	2.66	3.54	1.02
Mean		18.60	9.7	2.84	2.90	0.56
Std Dev		5.21	1.0	0.18	0.36	0.36
Male	CPF50/28	51.90	13.3	3.10	2.42	0.78
Male	CPF50/28	33.93	11.4	3.22	1.65	0.37
Male	CPF50/28	19.40	9.3	3.43	2.14	0.20
Male	CPF50/28	35.36	12.3	2.72	1.79	1.41
Male	CPF50/28	41.79	13.0	2.71	2.42	0.68
Male	CPF50/28	13.10	8.3	3.21	2.07	0.24
Male	CPF50/28	23.10	10.2	3.05	2.21	0.23
Male	CPF50/28	32.74	11.4	3.11	2.03	0.23
Male	CPF50/28	15.48	8.7	3.34	1.84	0.04
Male	CPF50/28	42.14	12.5	3.06	1.80	0.57
Male	CPF50/28	23.69	10.2	3.13	1.46	0.18
Male	CPF50/28	20.00	9.5	3.28	1.55	0.21
Male	CPF50/28	21.29	10.2	2.75	1.62	0.72
Male	CPF50/28	28.12	11.3	2.70	2.14	0.64
Male	CPF50/28	19.76	10.2	2.55	1.92	0.52
Male	CPF50/28	24.82	10.7	2.80	1.69	0.49
Male	CPF50/28	19.29	9.5	3.09	2.01	0.18
Male	CPF50/28	21.53	10.5	2.60	1.77	0.81
Male	CPF50/28	23.06	10.8	2.52	1.74	0.73
Male	CPF50/28	18.35	9.4	3.05	2.54	0.29
Male	CPF50/28	26.12	10.6	3.05	1.81	0.23
Male	CPF50/28	13.65	8.7	2.86	1.90	0.17
Male	CPF50/28	26.71	10.8	2.92	2.03	1.21
Male	CPF50/28	29.41	11.1	3.01	2.65	1.03
Male	CPF50/28	20.00	9.8	2.90	1.88	0.55
Male	CPF50/28	18.84	9.5	2.94	2.44	0.88
Male	CPF50/28	19.07	9.3	3.20	1.94	0.27
Male	CPF50/28	18.60	9.3	3.13	1.64	0.91
Male	CPF50/28	22.67	10.0	3.07	1.57	0.91
Male	CPF50/28	28.95	11.0	2.90	2.07	0.94
Mean		25.10	10.4	2.98	1.96	0.55
Std Dev		8.90	1.2	0.23	0.31	0.35

Table 14: Data for AChE activity of *O. mossambicus* following a 12-week intermittent exposure to azinphos-methyl and chlorpyrifos using different exposure regimes.

Treatment group	Δ A/min	P.C.	Calculations	AChE activity
ControlR1-1	0.086	22.80	0.024783469	24.78
ControlR1-2	0.112	23.84	0.033428596	33.43
ControlR1-3	0.097	19.76	0.024118153	24.12
ControlR1-4	0.086	23.35	0.025318455	25.32
ControlR1-5	0.101	23.39	0.029779422	29.78
ControlR2-1	0.098	20.45	0.025201246	25.20
ControlR2-2	0.102	22.07	0.028200572	28.20
ControlR2-3	0.069	19.45	0.016885034	16.89
ControlR2-4	0.089	20.24	0.022558856	22.56
ControlR2-5	0.088	18.10	0.020000019	20.00
ControlR3-1	0.113	22.38	0.031858329	31.86
ControlR3-2	0.100	21.72	0.02725846	27.26
ControlR3-3	0.103	20.54	0.026504162	26.50
ControlR3-4	0.114	20.70	0.029539234	29.54
ControlR3-5	0.055	19.14	0.013191181	13.19
ControlR4-1	0.107	24.46	0.032970756	32.97
ControlR4-2	0.152	25.34	0.04851899	48.52
ControlR4-3	0.064	18.76	0.015082314	15.08
ControlR4-4	0.111	20.76	0.028957268	28.96
ControlR4-5	0.147	28.00	0.051884368	51.88
Mean	---	---	---	30.05
Std. Dev.	---	---	---	8.41
AZP50/14R1-1	0.061	15.733	0.012040301	12.04
AZP50/14R1-2	0.056	15.116	0.010567301	10.57
AZP50/14R1-3	0.065	17.213	0.014110998	14.11
AZP50/14R1-4	0.059	13.586	0.010135735	10.14
AZP50/14R1-5	0.042	15.585	0.008321478	8.32
AZP50/14R2-1	0.035	11.613	0.005047114	5.05
AZP50/14R2-2	0.035	11.662	0.005122324	5.12
AZP50/14R2-3	0.035	10.774	0.004723186	4.72
AZP50/14R2-4	0.052	14.746	0.009696753	9.70
AZP50/14R2-5	0.029	9.318	0.003401616	3.40
AZP50/14R3-1	0.021	13.438	0.003517305	3.52
AZP50/14R3-2	0.024	10.922	0.003263767	3.26
AZP50/14R3-3	0.030	13.734	0.005105814	5.11
AZP50/14R3-4	0.028	17.608	0.00613249	6.13
AZP50/14R3-5	0.026	12.822	0.004159239	4.16
Mean	---	---	---	7.02
Std. Dev	---	---	---	3.50
CPF10/14R1-1	0.053	9.664	0.006435727	6.44
CPF10/14R1-2	0.066	10.922	0.009017702	9.02
CPF10/14R1-3	0.040	14.549	0.007274309	7.27

CPF10/14R1-4	0.037	10.502	0.004824301	4.82
CPF10/14R1-5	0.058	14.104	0.010327168	10.33
CPF10/14R2-1	0.044	15.215	0.008423584	8.42
CPF10/14R2-2	0.046	6.333	0.003646975	3.65
CPF10/14R2-3	0.058	14.771	0.010684894	10.68
CPF10/14R2-4	0.071	12.303	0.011024711	11.02
CPF10/14R2-5	0.054	13.882	0.009501305	9.50
CPF10/14R3-1	0.045	12.377	0.00703435	7.03
CPF10/14R3-2	0.098	18.669	0.02291729	22.92
CPF10/14R3-3	0.059	12.427	0.00924465	9.24
CPF10/14R3-4	0.079	17.780	0.01760915	17.61
CPF10/14R3-5	0.087	16.127	0.017604472	17.60
Mean	---	---	---	10.37
Std. Dev.	---	---	---	5.23
AZP50/28R1-1	0.062	15.481	0.011996948	12.00
AZP50/28R1-2	0.082	19.527	0.020214734	20.21
AZP50/28R1-3	0.094	18.849	0.022316802	22.32
AZP50/28R1-4	0.083	17.196	0.017938157	17.94
AZP50/28R1-5	0.070	15.629	0.013775458	13.78
AZP50/28R2-1	0.058	12.607	0.009262388	9.26
AZP50/28R2-2	0.074	14.507	0.01355213	13.55
AZP50/28R2-3	0.044	9.363	0.005148323	5.15
AZP50/28R2-4	0.056	11.830	0.008344357	8.34
AZP50/28R2-5	0.070	11.028	0.009641442	9.64
AZP50/28R2-6	0.069	14.692	0.012770581	12.77
AZP50/28R2-7	0.085	16.764	0.01799716	18.00
AZP50/28R3-1	0.072	14.223	0.012947302	12.95
AZP50/28R3-2	0.097	20.711	0.02528173	25.28
AZP50/28R3-3	0.054	11.225	0.007640426	7.64
AZP50/28R3-4	0.053	12.743	0.008432816	8.43
AZP50/28R3-5	0.070	14.605	0.012817949	12.82
Mean	---	---	---	13.92
Std. Dev.	---	---	---	5.77
AZP10/28R1-1	0.063	11.978	0.009492944	9.49
AZP10/28R1-2	0.049	10.979	0.006717892	6.72
AZP10/28R1-3	0.071	14.852	0.013283455	13.28
AZP10/28R2-1	0.081	11.225	0.011455934	11.46
AZP10/28R2-2	0.112	10.831	0.015195159	15.20
AZP10/28R2-3	0.078	11.793	0.011511119	11.51
AZP10/28R2-4	0.088	15.444	0.016994426	16.99
AZP10/28R2-5	0.062	11.645	0.009053282	9.05
AZP10/28R3-1	0.085	15.617	0.016768839	16.77
AZP10/28R3-2	0.080	19.860	0.020035128	20.04
AZP10/28R3-3	0.073	19.367	0.017654242	17.65
AZP10/28R3-4	0.092	17.862	0.020661783	20.66
AZP10/28R3-5	0.077	16.875	0.016302236	16.30

Mean	---	---	---	13.76
Std Dev	---	---	---	4.31
CPF50/14R1-1	0.073	17.44	0.015907373	15.91
CPF50/14R1-2	0.080	16.75	0.016807924	16.81
CPF50/14R1-3	0.072	15.54	0.014077174	14.08
CPF50/14R1-4	0.077	16.36	0.01581544	15.82
CPF50/14R1-5	0.066	13.99	0.011561392	11.56
CPF50/14R2-1	0.059	16.70	0.012432309	12.43
CPF50/14R2-2	0.062	18.70	0.014515445	14.52
CPF50/14R2-3	0.063	15.86	0.012466151	12.47
CPF50/14R2-4	0.057	15.86	0.011269401	11.27
CPF50/14R2-5	0.089	20.65	0.022986544	22.99
CPF50/14R3-1	0.054	15.05	0.010205364	10.21
CPF50/14R3-2	0.050	11.89	0.007460862	7.46
CPF50/14R3-3	0.055	14.75	0.010208652	10.21
CPF50/14R3-4	0.051	14.31	0.009139725	9.14
CPF50/14R3-5	0.055	15.64	0.010901887	10.90
Mean	---	---	---	13.05
Std. Dev.	---	---	---	3.83
CPF10/28R1-1	0.078	14.41	0.014154372	14.15
CPF10/28R1-2	0.091	15.64	0.017876996	17.88
CPF10/28R1-3	0.090	16.60	0.018781838	18.78
CPF10/28R1-4	0.113	21.51	0.030547807	30.55
CPF10/28R1-5	0.108	15.27	0.020782275	20.78
CPF10/28R2-1	0.083	13.96	0.014595972	14.60
CPF10/28R2-2	0.060	8.96	0.006737383	6.74
CPF10/28R2-3	0.058	11.45	0.008300117	8.30
CPF10/28R2-4	0.068	14.16	0.012167067	12.17
CPF10/28R2-5	0.072	13.62	0.012305747	12.31
CPF10/28R3-1	0.125	16.53	0.025920341	25.92
CPF10/28R3-2	0.110	17.20	0.023815444	23.82
CPF10/28R3-3	0.085	13.77	0.014718543	14.72
CPF10/28R3-4	0.089	14.83	0.016505272	16.51
CPF10/28R3-5	0.055	9.23	0.006326668	6.33
CPF10/28R1-1	0.067	15.93	0.013429022	13.43
CPF10/28R1-2	0.052	14.28	0.00932752	9.33
CPF10/28R1-3	0.074	16.70	0.015477384	15.48
CPF10/28R1-4	0.073	16.18	0.014854844	14.85
CPF10/28R1-5	0.048	13.06	0.007938786	7.94
Mean	---	---	---	16.24
Std. Dev.	---	---	---	6.96
AZP10/14R2-1	0.125	17.28	0.027082189	27.08
AZP10/14R2-2	0.097	15.17	0.018479528	18.48
AZP10/14R2-3	0.092	15.05	0.017471482	17.47
AZP10/14R2-4	0.113	16.37	0.023216216	23.22
AZP10/14R2-5	0.072	12.71	0.01144363	11.44

AZP10/14R3-1	0.095	18.36	0.021947995	21.95
AZP10/14R3-2	0.071	13.58	0.01209585	12.10
AZP10/14R3-3	0.072	14.67	0.013191729	13.19
AZP10/14R3-4	0.083	15.64	0.016270885	16.27
AZP10/14R3-5	0.067	13.11	0.011095829	11.10
Mean	---	---	---	15.55
Std. Dev.	---	---	---	5.35
CPF50/28R1-1	0.097	16.23	0.019860041	19.86
CPF50/28R1-2	0.061	14.98	0.011567358	11.57
CPF50/28R1-3	0.081	16.33	0.016606149	16.61
CPF50/28R1-4	0.119	16.21	0.024184411	24.18
CPF50/28R1-5	0.085	15.20	0.01621021	16.21
CPF50/28R2-1	0.059	13.17	0.009795239	9.80
CPF50/28R2-2	0.088	14.78	0.016307892	16.31
CPF50/28R2-3	0.084	17.28	0.018303474	18.30
CPF50/28R2-4	0.108	15.36	0.020925531	20.93
CPF50/28R2-5	0.079	17.94	0.017770598	17.77
CPF50/28R3-1	0.058	12.35	0.00906689	9.07
CPF50/28R3-2	0.077	13.21	0.012851528	12.85
CPF50/28R3-3	0.057	12.19	0.008724388	8.72
CPF50/28R3-4	0.066	12.04	0.009990913	9.99
CPF50/28R3-5	0.045	12.13	0.006840514	6.84
Mean	---	---	---	14.6
Std. Dev.	---	---	---	5.17

Δ A/min = change in absorption/minute of tissue samples (calculated from spectrophotometer readings); P.C. = protein content of samples; Calculations = calculation of enzyme activity after Fisher (2000) Sample volume = 200 µl; Path length = 0.5848 cm; Molar Extinction Coefficient of DTNB reagent = 79.5328.

Table 15: Data for feeding experiment monitoring feeding response time and food consumption as endpoints. The experiment was conducted using 2 fish per tank and offering 20 pellets of food in total. The time to first feeding attempt was measured in seconds. (CPF10% = 0.005 mg/l; CPF50% = 0.025 mg/l; AZP10% = 0.0007mg/l; AZP 50% = 0.0035 mg/l)

Treatment	Replicate no	Time to first feeding attempt (s)	No of pellets consumed
Control	1	3	20
	1	5	20
	1	3	20
	1	6	18
	1	4	14
	1	4	16
	2	4	17
	2	6	20
	2	3	19
	2	5	18
	2	5	20
	2	7	20
	3	8	20
	3	5	20
	3	5	15
	3	9	15
	3	3	20
	3	10	16
Mean		5.41	18.12
Std Dev		2.06	2.18
CPF10%	1	5	20
	1	6	20
	1	6	20
	1	8	14
	1	5	18
	1	3	16
	2	4	16
	2	7	17
	2	4	20
	2	5	19
	2	8	19
	2	4	18
	3	7	20
	3	7	18
	3	8	15
	3	6	16

	3	5	15
	3	6	17
Mean		5.63	17.79
Std Dev		1.61	2.02
	1	24	14
	1	30	14
	1	31	18
	1	25	15
	1	20	13
	1	26	11
	2	18	13
	2	22	17
CPF50%	2	24	14
	2	27	14
	2	19	11
	2	32	10
	3	30	11
	3	24	14
	3	23	16
	3	27	16
	3	22	17
	3	22	11
Mean		24.78	13.83
Std Dev		3.99	2.32
	1	12	10
	1	20	16
	1	21	13
	1	14	13
	1	18	12
	1	16	9
	2	24	16
	2	16	11
AZP10%	2	21	14
	2	24	18
	2	19	14
	2	17	14
	3	24	6
	3	22	11
	3	16	14
	3	11	13
	3	13	11
	3	16	12
Mean		18.00	12.61

Std Dev		4.13	2.79
	1	48	10
	1	65	14
	1	42	14
	1	40	9
	1	47	6
	1	48	11
	2	52	10
	2	60	10
AZP50%	2	53	13
	2	50	11
	2	51	9
	2	57	6
	3	48	11
	3	51	9
	3	61	6
	3	63	9
	3	58	13
	3	55	14
Mean		52.72	10.28
Std Dev		6.94	2.65