Rapid Enzymatic Detection of Organophosphorous and Carbamate Pesticides in Water

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By

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Abstract

The increased use of pesticides has resulted in a corresponding increase in concern for the effect they may have on the health of humans and other non-target organisms. The two main areas of concern are the toxicological effects that mixtures of pesticides may have as well as the endocrine disrupting effects. Although the individual pesticides may be present at concentrations below the levels deemed to be detrimental to health, it has been argued that their combined effect may still result in elevated health risks. Another important aspect of pesticide risk assessment requires a consideration of the breakdown products of pesticides and their effect on human health. There has been very little research into the effects of degradation products and this issue should be addressed as these could potentially pose a higher risk than their parent compounds.

One of the most important bio-markers available for use is the ubiquitous enzyme acetylcholinesterase (AChE). This enzyme is responsible for one of the most important functions in the body; namely nerve impulse transmission, upon which all life depends. The inhibition of this enzyme indicates toxicity and as a subsequence, a threat to the organism's well-being. Bioassays have also recently been developed to test chemicals for endocrine disrupting effects. These tests rely on a dose response equivalent to that of the most potent well known estrogen 17-β estradiol. Any chemical that has a measurable response is deemed to display endocrine disrupting effects.

This first aim of this study was to investigate the toxicological and endocrine disrupting effects of three organophosphorus pesticides; aldicarb, parathion and demeton-S-methyl, in addition to two breakdown products; aminophenol and *p*-nitrophenol. Two carbamate pesticides; carbaryl and carbofuran were also analysed. The toxicological effects of mixtures of the parent pesticide compounds were tested to assess if any antagonistic, additive or synergistic effects were observed. This data was then used in conjunction with an artificial neural network to assess if individual pesticides could be distinguished from mixtures of pesticides. A final objective was to sample various Eastern Cape water sources, utilising the enzymatic assay to determine the presence of any of these pesticides in these samples.

There were several conclusions drawn from this study. AChE was successfully used as an assay to test the toxicity of the pesticides under investigation, based on their inhibition of this enzyme. An important factor for consideration throughout the study was the need to establish basal and monitor AChE activity (i.e. the need to monitor AChE activity in the absence of any pesticide). This ensured accurate comparison of the results obtained. It was found that demeton-S-methyl was the most potent of these pesticides followed by carbaryl, parathion, aldicarb and finally carbofuran, and that carbofuran could potentiate AChE. The results indicated that pesticide mixtures generally exhibited an additive inhibitory effect on AChE, although at some concentrations of pesticides, synergistic and antagonistic effects were noted. From the data using mixtures of pesticides, a feed forward neural network was created that was successfully able to distinguish individual pesticides from mixtures within its training parameters.

None of the pesticides tested displayed endocrine disrupting properties in the Yeast Estrogen Screen (YES), T47D-KBluc and MDA-kb2 bio-assays. Other studies reported mixed results in this regard and thus no final conclusions could be drawn. The Blaauwkrantz River, Kariega River, Sundays River, Swartkops River and Kowie River were all tested for pesticides and although positive results were recorded, conventional methods indicated that there were no pesticides in the rivers. There were, however, trace metals present which are known to inhibit AChE, thus causing a false positive result. These results indicated that AChE can be used as a high throughput initial pre-screening tool, but that it cannot serve as a substitute for more accurate conventional testing methods.

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ABBREVIATIONS

AChE	Acetylcholinesterase
AcSChI	Acetylthiocholine iodide
ANNs	Artificial neural networks
CPs	Carbamate pesticides
CPRG	Chlorophenol red-β-D-galactopyranoside
DDT	Dichloro-diphenyl-trichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
DNTB	5'5'-dithio-bis-2-nitrobenzoic acid
EDCs	Endocrine disrupting compounds
EPA	Environmental Protection Agency
ER-α	Estrogen receptor α
ERE	Estrogen responsive elements
EROD	Ethoxyresorufin-O-deethylase
EU	European Union
GC	Gas chromatography
GHT	Grahamstown
Hsp 70	Heat shock protein
HPLC	High performance liquid chromatography
IC_{20}	Concentration giving 20% inhibition
LDH	Lactate dehydrogenase
LOD	Limit of detection
OPs	Organophosphorus pesticides
PAN	Pesticide Action Network
PCR	Principal component regression
PLS	Partial least squares
POPs	Persistent organic pollutants

RBF-ANN	Radial basis function neural network
UV	Ultra violet
YES assay	Yeast estrogen screen assay

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CHAPTER 1: Literature Review and Introduction to the Present Study

1. Pesticides

Pesticides are chemicals that have been developed and produced in order to control agricultural pests on food crops as well as crops grown for their commercial value. These are used to increase yields and therefore maximize economic returns on the crops. Food crops that need protection include staple foods such as rice, wheat, barley, maize/corn and potatoes, as well as non-staples such as sugar cane, citrus fruits, tea, coffee and vegetables. Commercial crops of value include tobacco, cotton, flowers and trees of ornamental value. Pesticides can be divided into various categories, i.e. herbicides, fungicides, avicides, rodenticides, insecticides, nematicides and molluscides (Johnston, 2000). Some pesticides may fall into more than one category as they may target different pests.

Pesticides may be known by various names, such as common names, brand names or chemical names. Standardisation of names is done through the International Union of Pure and Applied Chemistry (IUPAC) and Chemical Abstracts. Each pesticide must have a IUPAC name as well as a chemical abstract name and chemical abstract registry number (CAS-RN) (Tomlin, 2006). Pesticide regulation further requires that several details on the pesticide be supplied before it is listed as an approved pesticide. These details include:

- Chemical composition indicating what elements are present besides hydrogen and carbon;
- Physical properties such as appearance, melting and boiling points, density, solubility;
- Classification and mode of action;
- Stability with regards to oxidation and UV light;
- Methods of degradation and half-life values;
- Toxicity towards plants, humans and birds;
- Lethal Dose (LD) levels, i.e. the concentrations at which the pesticides are toxic;
- Carcinogenic properties;

- Environmental fate;
- Method of application, i.e. direct application as a liquid or powder and aerial spraying.

As part of the information available on pesticides, the chemical formula indicates the elements that are present as well as functional groups. These functional groups also determine the classification of the pesticide. Pesticides fall into several classes of which the most common ones are organochlorines, organophosphates, carbamates, pyrethroids and other urea based pesticides.

1.1. Organochlorines (OCs)

Most of the older pesticides fall into this category. OCs do not have a general structure but as the name implies they are chlorinated hydrocarbons, the common examples being dichloro-diphenyl-trichloroethane (DDT), dichloro-diphenyl-dichloroethylene (DDE), aldrin, dicofol, heptachlor, endosulfan, chlordane, mirex and pentachlorophenol (Telang et al., 1982; Wolff et al., 1993). DDT use was promoted during World War II to protect soldiers and civilians from typhus and malaria. After the war, due to its cost effectiveness, it was used for agricultural purposes in addition to combating malaria. Its structure is shown in Figure 1.

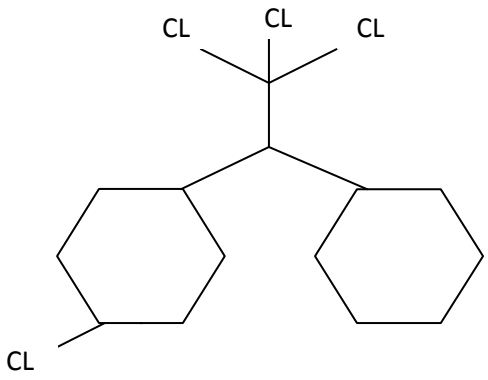


Figure 1: Chemical structure of dichloro-diphenyl-trichloroethane (DDT)

In her book, Silent Spring, Rachel Carson pointed out that the excessive use of pesticides was harming not only the wildlife, but humans as well (Carson, 1962). This is due to the

fact that most OCs degrade at an extremely slow rate. For example, DDT has a half-life of over 10 years, and as a result, persists in the environment and accumulates in plant and animal tissue over time.

Another additional factor that has raised concerns is the stability of OCs. With time, these stable OCs can be transported to regions where they are not produced or used (Burger, 2005). Therefore, these compounds have been banned internationally and measures put in place to remove any residues resulting from these compounds. These decisions were made during the Stockholm Convention on persistent organic pollutants (POPs) in 2001 (Lallas, 2001). The main aim of the conference was to identify and eliminate the use of the most dangerous POPs, as well as regulating the use of any POPs that were not banned (Lallas, 2001). However, the use of DDT is still permitted in certain countries, under strict regulation, as it remains the cheapest and most effective pesticide against malaria mosquitoes. At the same time, alternative pesticides have been used as substitutes for OCs. These fall into several different categories, two important ones being organophosphorus and carbamate pesticides. These classes of pesticides have a shorter half-life than organochlorines and therefore a lower persistence.

1.2. Carbamate Pesticides (CPs)

Figure 2 illustrates the general structure of CPs. Carbamates are esters of carbamic acid where the "R" represents any atom or group of atoms that will give each carbamate a distinct identity.

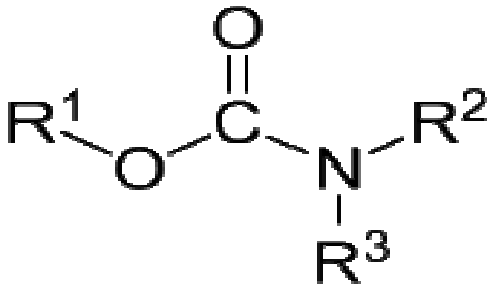


Figure 2: Chemical structure of CPs (Adapted from Dorofeeva and Tarakanov, 1987)

Some of the commonly used CPs in South Africa are carbofuran, carbaryl and aldicarb (Figure 3) (Slabbert et al., 2004; Burger, 2005).

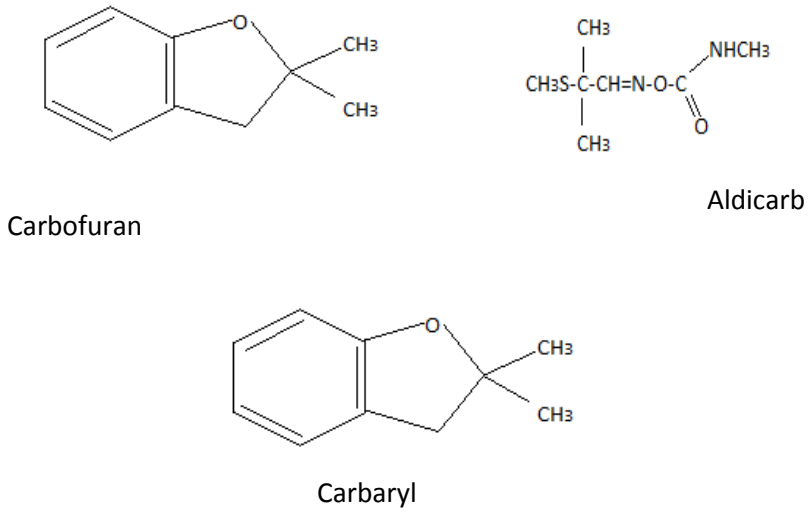


Figure 3: Diagram of structures of Carbofuran, Carbaryl and Aldicarb

Aldicarb or 2-methyl-2-(methylthio)propionaldehyde O-methylcarbamoyloxime is an insecticide with CAS registry number 116-06-3. It's a white crystalline solid with a sulphorous ordour. It is rapidly oxidized to its sulphoxide. Aldicarb is applied directly to the soil to control chewing and sucking insects. Examples of these are aphids, whiteflies, leaf miners, spider mites and nematodes. It is used for both outdoor and indoor protection of ornamental plants, sugar beet, potatoes, onions, citrus fruits, cotton, sugar cane, vine nurseries, coffee, bananas and soya beans to mention a few. Aldicarb is absorbed rapidly through the roots and translocated throughout the plant (Tomlin, 2006).

Carbaryl or 1-naphthyl methylcarbamate, commonly known according to its trade name Sevin, has a CAS registry number 63-25-2 and is usually found in a tan crystalline form. It is also an insecticide used for dealing with chewing and sucking insects on many different crops such as vegetables, cotton, rice, tobacco, cereals, beet, maize, sorghum, potatoes and tree fruits including citrus. It has also been used as a growth regulator, for the control of earthworms and as an animal ectoparasiticide.

Carbofuran, also known as 2, 3-dihydro-2, 2-dimethylbenzofuran-7-yl methylcarbamate or trade names Furadan or Curater with CAS RN 1563-66-2, is in the form of white crystals. It is used for the control of soil dwelling as well as foliage feeding insects, examples of which are white grubs, millipedes, weevils and nematodes. It's used to protect the same range of crops as carbaryl. All CPs are known for their ability to inhibit the enzyme AChE.

1.3. Organophosphorus Pesticides (OPs)

OPs is the term used to describe organic phosphorus (V)-containing compounds and these compounds are known for their neurotoxic properties. Figure 4 illustrates the general structure of OPs. OPs are referred to as nerve agents due to the fact that they act on the enzyme AChE and are more toxic than CPs as they inhibit AChE irreversibly **(**Dekundy et al., 2007)**.**

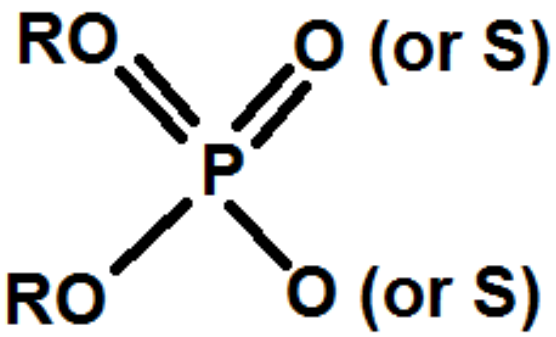
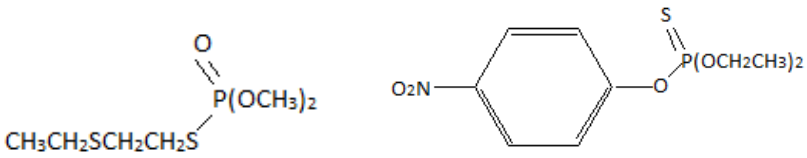


Figure 4: Structure of an OP. R represents either a methyl or ethyl group, OR represents an alkyl, alkoxy, alkylthio, aryl, or heterocyclic aryloxy, arylthio or a heterocyclic analog.

In South Africa the most common OPs in use are parathion and demeton-S-methyl (Figure 5) (Slabbert et al., 2004; Burger, 2005).



Demeton-S-Methyl

Parathion

Figure 5: Structures of demeton-S-methyl and parathion

Parathion, known as O, O-diethyl O-4-nitrophenyl phosphorothioate, is a pale yellow liquid with a phenol odour. It is also an insecticide and is used for the same purposes as carbaryl and carbofuran for the control of sucking and chewing insects. It is for the most part also used for the same crops. Parathion is slowly being phased out of use as it has been found to be extremely hazardous to health and has thus been placed in group 1A in the list of potentially dangerous pesticides by the World Health Organization (WHO) (WHO Report, 2009).

Demeton–S-methyl, known as 2-ethylthioethyl O,O-dimethyl phosphorothioate, is a pale yellow oil that is commonly used in conjunction with demeton-O-methyl. It is used as an insecticide and acaricide. It controls aphids which include virus vectors, sucking insects, saw flies and spider mites in fruits and vegetables, potatoes, cereals and ornamental plants. It is, however, being phased out of use by the WHO and declared obsolete (WHO Report, 2009).

There are many more examples of OPs and CPs - some of these are displayed in Table 1.

Table 1: List of OPs and CPs used worldwide

Carbamate Pesticides		Organophosphorus Pesticides	
Alanycarb	BMPC	Methyl parathion	Chlorpyrifos
Alcamate	Bufencarb	Dichlorvos	Diazinon
Aldicarb sulfoxide	Butacarb	Phosmet	Tetrachlorvinphos
Aldicarb	Carbaryl	Acetoxon	Azinphos methyl
Aldoxycarb	Carbonolate	Acethion	Acephate
Allyxycarb	Isoprocarb	Akton	Azinphos-methyl
Aminocarb	Mexacarbate	Amiton	Bomyl
Asulam	Promacyl	Bromophos	Bensulide
Barban	Proxpur	Butamifos	Chlorphoxim
Bendiocarb	Oxamyl	Carbophenothion	Chlormephos
Benfuracarb	Methomyl	Chlorpyrifos	Chlorthion
Benomyl	Promacyl	Cyanthoate	DDVP
Benthiavalicarb	Thiofanox	Demeton-s-sulfone	Cyanfenphos
Betamix	Trimethcarb	Demeton	Diazoxon
Diethofencarb	Hoppcide	dichlofenthion	Dimethoate
Bufencarb	Methiocarb	Dioxathion	Dialfor
Butacarb	Ethiofencarb	DMCP	Endothion
Butocarboxim	Xylylcarb	EPN	Ethion
Butoxycarboxim	Karbutilate	Fenthion	Famphur
Carbanolate	Terbucarb	Fonofos	Iprobenfos
Dimetan	Furadan	Malathion	Malaxon
Dichlormate	Pyrolan	Phorate	Propaphos
Decarbofuran	Propham	Propoxon	Pyraclofos
Chlorprocarb	Nitilacarb	Ronnel	Quinothion
Potassium asulam	Fenoxycarb	Trizophos	Trichloronat
Biscarbofuran N,N'-disulfide	Thiophanate-methyl	Isazophos-methyl	Methyl carbofenthion

1.4. Pesticide Distribution in South Africa

Much research has been performed to determine the presence of OPs and CPs in water courses in various countries, particularly focusing on studying pesticides commonly implemented in that area for agricultural use (Kuchler et al., 1997; Van der Hoek, 1998; Vanclooster et al., 2000; Maumbe and Swinton, 2003; Lopez-Espinosa et al., 2008; Haylamicheal and Dalvie, 2009; Landau-Ossondo et al., 2009). Studies have also been performed on the effect of pesticides on the health of both humans and animals in a given location. Table 2 shows a summary of pesticides that have been found in water sources in South Africa.

Table 2: Summary of studies performed to determine pesticide distribution in water sources in South Africa.

Reference	**Pesticides Identified**	**Area Tested**
Grange et al. (2003)	1,2 dichlorobenzene, 1,4 dichlorobenzene, 2-methylnaphtalene, 4-chloro-3,5-dimethylphenol, Atrazine, Anthracene, Camphor, Diazinon, Dibutylphthalate, Dimethylphthalate, Dimethylpyridine, Isobutylphthalate, o-hydroxybiphenyl	Johannesburg surface water samples
Fatoki and Awofulu (2003)	Aldrin, ß - BHC	Marine water and fresh water sediments in the Eastern Cape
London and Meyers (1995a,b)	Aldicarb, amitrole, Azinphos-methyl, Azocyclotin, Bromoxynil, Captab, Chlorpyrifos, Citrus bands, Copper oxychloride, Dimethoate, Dinitro-ortho-cresol(DNOC), Dithiocarbamate, EDB, Endosulfan, Ethylene dibromide, Glyphosate, Mancozeb, MCPA, Methidathion, Metiram, Mineral oil, Parathion, Paraquat, Prochloraz, Simazine, Sulphur, Tartar emetic, Vanmidothion	Investigation of most used pesticides in southern Africa
Bouwman et al. (2003)	Atrazine, DDT (use restricted to malaria mosquito control)	KwaZulu Natal Region
Du Preez et al. (2003)	Atrazine	Pesticide levels in different seasons in the northern and central parts of South Africa
Bennett et al. (2003)	Azinphos-methyl, Chlorpyrifos, Endosulfan, Prothiofos	Constructed wetlands of Lourens River, Cape Town
Dabrowski and Schulz (2003a +b)	Azinphos-methyl	Western Cape region
Dabrowski et al. (2002 a+b, 2003)	Azinphos-methyl, Chlorpyrifos, Deltamethrin, Endosulfan, Procymidone	Western Cape region
Schulz and Dabrowski (2003)	Azinphos-methyl	Western Cape region
Solomons et al. (2003)	Chlorpyrifos, Deltamethrin, Endosulfan, Fenarimol, Iprodione, Penconazole, Prothiofos	Western Cape region
London et al. (2000)	Azinphos-methyl, Chlorpyrifos, Copper oxychloride, Endosulfan, Fenarimol, Iprodione, Penconazole, Prothiofos, Simazine	Western Cape region
Davies (1997)	Azinphos-methyl, Chlorpyrifos, Endosulfan, Penconazole	Western Cape region
Weaver (1993)	Vinclozin, Triadimefon, simazine, prothios, proxpur, propineb, propetamphos,profenofos, procymidone, pirifenox, penconazole, paraquat, oryzalen, omethoate, nuarimol, mevinphos, methiocarb, methidathion, metaldehyde, MCPA, mancozeb, iprodione, hexaconazole, formothion, folpet, fenvalerate, fenthion, fenamiphos, EPTC, endosulfan, diquat,dinocap, dimethoate, dichlorovos, deltamethrin, cypermethrin, cyhalothrin, cyfluthrin, copper	Usage of pesticides in Hex River Valley in the Western Cape

	oxychloride, chlorpyrifos, carbofuran, carbaryl, captan, bromopropylate, benomyl, atrazine, alphamethrin, aldicarb, Mancozeb	
Heath et al. (2003)	BHC, DDE, Heptachlor, Lindane	Crocodile River, Mpumalanga
Snyman et al. (2005)	Copper oxychloride	Areas with vineyards and orchards
Leslie (2003)	α – cypermethrin	Western Cape Region
Sereda and Meinhardt (2003)	DDT (use restricted to malaria mosquito control), Deltamethrin	KwaZulu Natal Region
McGregor (1999)	Chlorpyrifos, Endosulfan	Western Cape region
Schulz et al. (2001)	Chlorpyrifos, Endosulfan	Western Cape region
Naidoo and Buckley (2003)	Simazine	South Africa

As can be observed from Table 2, most studies have been carried out in the Western Cape region where most vineyards and orchards are located (see Figure 6). Very little data is available on pesticide use and distribution for the rest of the country, but is most likely associated with the growth of crops.

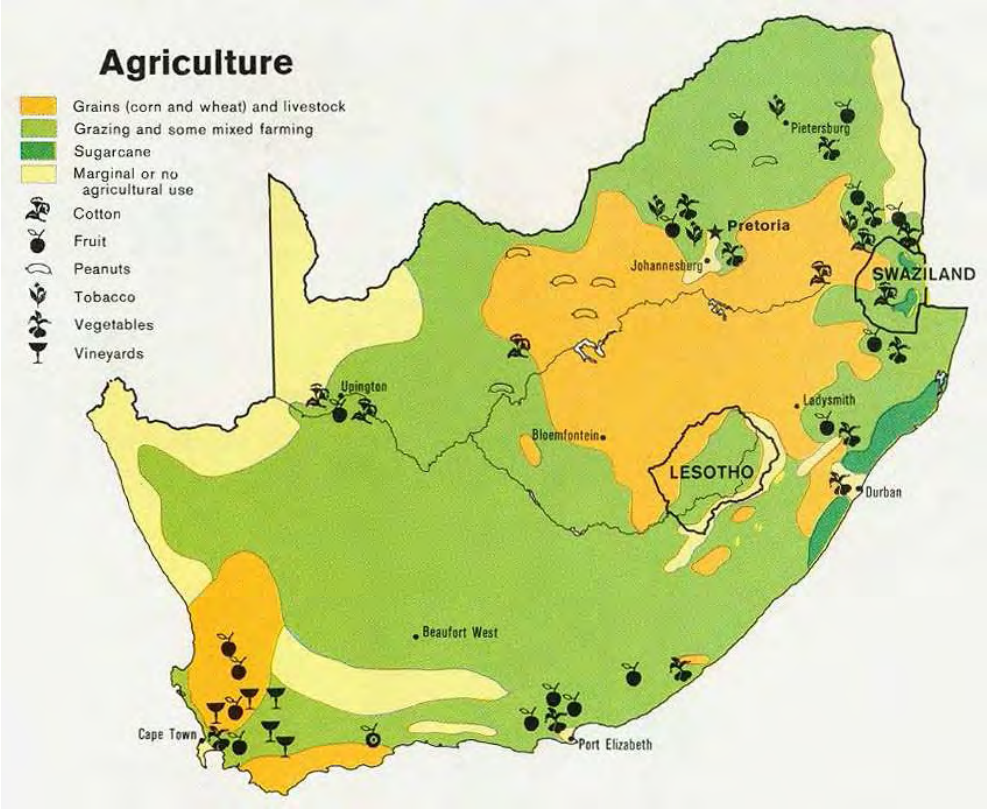


Figure 6: Map of South Africa indicating the various regions in which crops are grown. Agroinformatics (2010)

The studies that have been carried out on the presence of pesticides that could potentially be endocrine disrupting compounds (EDCs) in South African water sources include Aneck-Hahn et al. (2007), Awofolu and Fatoki (2003) and Barnhoorn et al. (2003). Only Awofolu and Fatoki (2003) based their research on water sources in the Eastern Cape and analysed the presence of organochlorine pesticides (OCs), most of which have been banned in terms of the Stockholm Convention due to their endocrine disrupting properties.

The limited data on pesticide use in and around South Africa is due to the abolition of laws which previously required agrochemical manufacturers to provide information on the quantities of pesticides sold and their uses (PAN, 1995). However, Maharaji (2005) was able to ascertain some of the commonly used groups of pesticides and it was evident that CPs and OPs played a

major role in the protection of crops in South Africa and this trend was predicted to continue. As hundreds of different pesticides exist, analysis and identification of potential contamination of water is more complex without information on the use of pesticides in a particular area.

1.5. Problems Associated with Pesticides

Through anthropogenic activities such as aerial spraying of pesticides and natural causes such as run-off, pesticides often end up contaminating water sources. As a result, pesticides have come under scrutiny due to the various health problems associated with their use (Maroni et al., 1986; Van Der Hoek et al., 1998; Amr, 1999; Ecobichon, 2001; Wesseling etal., 2005; Aqiel et al., 2006, Aneck Hahn et al., 2007). It has been found that the pesticides are not only toxic to the intended targets, but also to humans and animals drinking contaminated water. Indeed, most pesticides are suspected to have endocrine disrupting properties. For this reason, the detection of pesticide contamination in water is essential to protect the health and well-being of animals and humans exposed to such contamination. As many different pesticides may be used in the same area, the effect of pesticide mixtures requires investigation and the cumulative toxicity of risk should be considered.

Two important problems that require assessment are the toxicity and endocrine disrupting properties of pesticides.

1.5.1. Toxicity

According to Webster's New World Medical Dictionary (2008), "toxicity" is defined as the degree to which a substance can harm an organism. Acute toxicity refers to the damage done when exposed to harmful substances over a short period of time, whereas chronic toxicity refers to accumulated exposure over time. There are, however, many factors that have an impact on the degree of toxicity of a substance. Therefore, toxicity of pesticides will depend on the chemical structure and dosage of the pesticide, as well as the susceptibility of the organism exposed to the pesticide. Species, age, physical and nutritional state of the target will all be relevant factors (Hayes, 2008). Toxicity is indicated based on the lethal dose (LD) that will kill 50% of the sample population of test animals (LD_{50}). Toxicity for humans may result from oral

ingestion, as well as through skin, eyes and lungs. Therefore, pesticides present a serious health hazard and their presence in water sources needs to be constantly monitored.

1.5.2. Endocrine Disrupting Effects of Pesticides

A problem that has recently arisen is the potential adverse effects that pesticides may manifest on animals and humans as a result of the disruption of the endocrine system. This can take place at concentrations far below those identified as toxic and these represent long-term effects that may not be detected immediately. Pesticides that have a detrimental effect on the endocrine systems of organisms are termed endocrine disrupting compounds (EDCs) and are defined as exogenous agents that interfere with natural hormones in the body that are responsible for maintenance, reproductive development and/or behaviour of organisms by interrupting synthesis, secretion, transport or binding of hormones (USEPA, 2010). A simpler definition has been coined as chemical substances that have a detrimental effect on the endocrine system.

The endocrine system consists of the pituitary, thyroid, pineal, thymus, ovary and testis, salivary, sweat and adrenal glands. These glands and the hormones they excrete regulate various bodily functions (Bertók, 1998). Together they make up the reproductive system, the neurological system, and control thyroid function as well as the immune system (Aoki, 2001). The regulation of normal body functions by these systems include maintaining blood glucose levels, food utilisation, fluid balance and reproductive cycles in animals and humans (Vogel, 2004). Severe physiological problems, birth defects, developmental problems, infertility and other reproductive ailments are some of the manifestations of EDCs. EDCs can potentially result in these disruptions at exposure levels lower than carcinogenic exposure level. This highlights the importance of detecting pesticide contamination in water sources at low concentrations as legal limits do not necessarily indicate safe levels of contamination. One of the main methods of testing for endocrine disrupting properties is to use a bioassay such as the Yeast Estrogen Screen assay (YES Assay).

1.5.2.1. YES Bioassay

The YES assay involves the use of yeast cells which do not have any natural estrogen receptors. Yeast cells are transfected with the human estrogen receptor alpha (ER-α) gene together with an

expression plasmid that contain the estrogen-responsive elements (ERE) and *lac-z* reporter gene which encode for the enzyme β-galactosidase (Routledge and Sumpter, 1996). There is a strong promoter region on the expression plasmid that has ERE sequences that the ER-α, which is expressed, can bind to. Figure 7 below illustrates the structure of the plasmid.

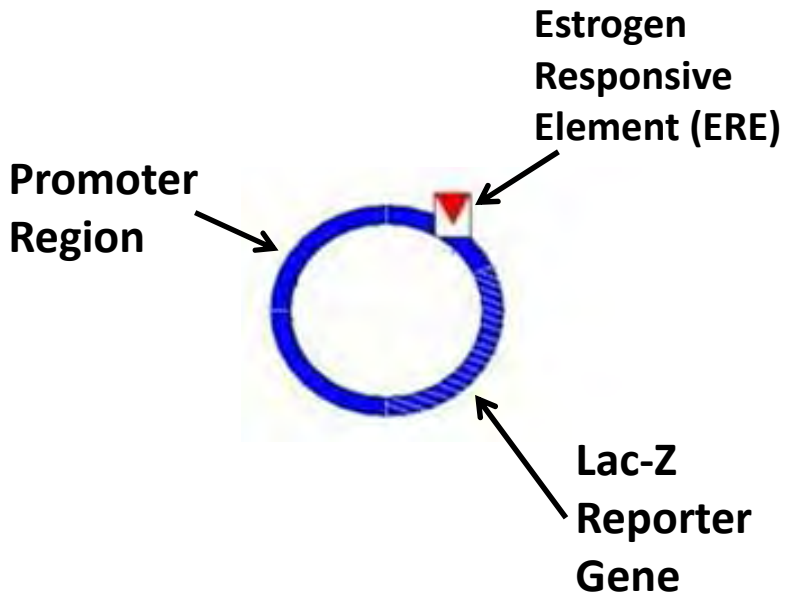


Figure 7: Diagrammatic representation of the plasmid inserted into the yeast cell with a strong reporter sequence, estrogen responsive elements (ERE) and the *lac-z* gene. (Adapted from University of Auckland, 2011).

When an active ligand binds, the estrogen-occupied receptor interacts with transcriptional factors and other components to modulate gene transcription. Figures 7 and 8 depict this as the red triangle that fits into a receptor. The end result is the expression of the reporter gene *lac-z* and production of the enzyme β-galactosidase, shown by the purple square in Figure 8. The enzyme (β-galactosidase) is secreted into the medium which contains a chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), which is metabolized, resulting in a colour change from yellow to red. This colour change can be measured using a spectrophotometer at an absorbance of 540 nm, hence allowing for the evaluation of estrogenic activity of the substance being tested (Routledge and Sumpter, 1996).

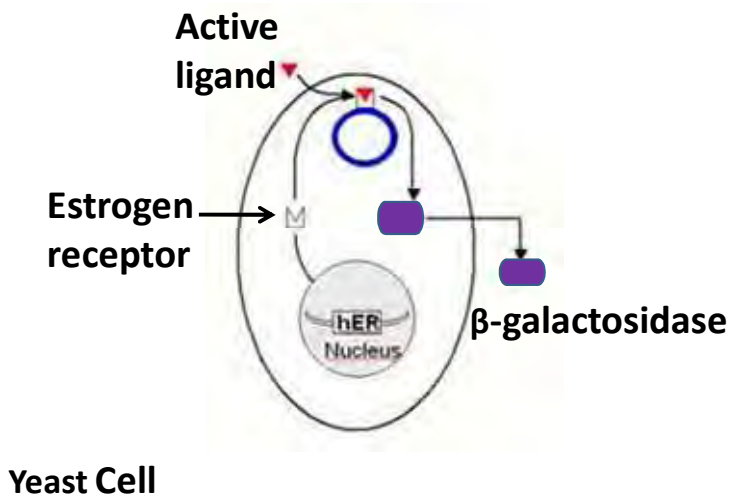


Figure 8: Diagrammatic representation of the effect of an estrogenic compound on the yeast cell in the YES assay which results in the production of the enzyme β-galactosidase and assessment of endocrine activity. (Adapted from University of Auckland, 2011).

The estrogenic effect is measured relative to that of 17β-estradiol (E_2). A dose response curve for E_2 and the test chemicals are constructed. Based on the dose response curves the Relative Potency (RP) of each test chemical is calculated using the EC_{50} value of the test chemical relative to E_2. Figure 9 below is an example of a possible estrogenic response in the YES assay.

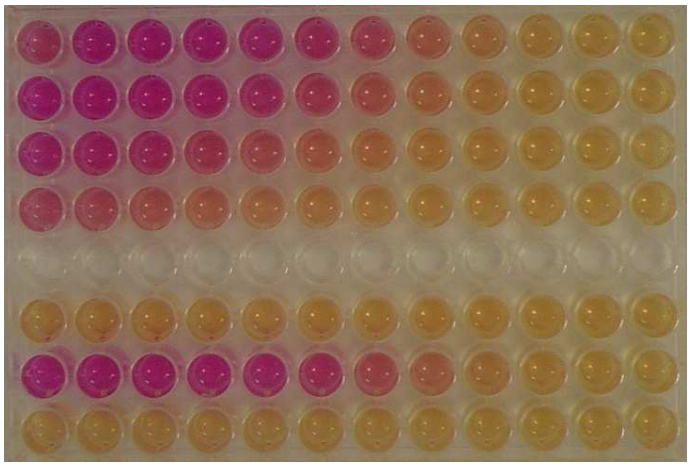


Figure 9: Picture of a microtiterplate showing a positive response for an estrogenic sample.

There have been many studies conducted in various parts of the world that assessed the estrogenic effects of various pesticides (Kuchler et al., 1997; Van der Hoek, 1998; Vanclooster et al., 2000; Maumbe and Swinton, 2003; Lopez-Espinosa et al., 2008; Haylamicheal and Dalvie, 2009; Landau-Ossondo et al., 2009). These studies have concentrated on those pesticides most often used and their effect on the environment. Limited studies have been undertaken in South Africa to determine the presence of pesticides in water sources as well as to assess the estrogenic effects of these compounds (Awofolu and Fatoki, 2003; Barnhoorn et al., 2003 and Aneck-Hahn et al., 2007).

1.5.2.2. Other Bioassays

The YES assay is not the only bio-assay available for detection of endocrine disrupting properties. The T47D-KBluc reporter gene assay is another assay available to test for estrogenicity in samples. It is based on the same principle as the YES assay in that it has cells that are transfected with the human estrogen receptor alpha (ER-α) gene, but the T47D cells also have the human estrogen receptor beta (ER-β) incorporated. This is in addition to the expression plasmid with estrogen-responsive elements (ERE) and *lac-z* reporter. This assay is therefore much more responsive to some estrogenic substances that the YES assay may not detect.

Endocrine disruption can also be androgen based, and the aforementioned assays cannot detect this type of endocrine disruption (Tamura et al., 2001). The MDA-kb2 reporter gene assay has been used to detect this type of endocrine disruption. The MDA-MB-453 human breast cancer cells were selected for this assay as they have a high level of endogenous androgen receptors (AR) as well as glucocorticoid receptors (GR) and can thus detect any substances that elicit an AR or GR response (Wilson et al., 2002).

1.6. Detection of Pesticides

In order to detect the pesticides at extremely low levels, several conventional analytical techniques have been established. Most of these involve sample preparation, extraction, isolation, separation and detection. All these processes are used to ensure minimal loss of sample and removal of interfering substances. Among the methods employed for pesticide

detection are gas chromatography (GC), high performance liquid chromatography (HPLC), UV spectroscopy, fluorescence spectroscopy and mass spectroscopy (Pogačnik and Franko, 2001; Chen et al., 2006; Muir and Sverko, 2006). These methods are extremely sensitive with regards to pesticide detection, but have several shortcomings. The most important factor is the high cost involved. Many countries, including South Africa, do not have the economic resources required to purchase the equipment or materials required to use the equipment, nor are they able to successfully maintain the equipment in the long run (Ni et al., 2007). This renders these methods unfeasible for use by most organisations.

The need for highly trained and skilled personnel is another challenge faced by most countries. There is insufficient training of personnel to be able to carry out the testing of samples. A third and very important shortcoming is the amount of time required for sample preparation and the actual running of the tests for pesticide detection (Dabrowski et al., 2002a). The harmful nature of pesticides requires that detection methods be as rapid as possible and this puts all the above mentioned methods at a disadvantage. Having considered these issues, the use of enzymatic assays for pesticide detection promises to be an attractive alternative option.

1.7. Enzymatic Methods of Detection

Many enzymes have been suggested for the detection of pesticides. Evtugyn et al. (1998) discussed several enzymes that could be used. Most of these were based on the inhibition of an enzyme, the degree of which would correlate to the concentration of pesticide present. Other enzymatic methods make use of the production of hydrolysis products which are used as a positive signal. Enzymes suggested for use were cholinesterases, ureases, glucose oxidase, tyrosinase, alcohol dehydrogenase, amino oxidase, aldehyde dehydrogenase, cytochrome c, catalase and peroxidase. The choice of enzyme would be based on the application for which it is required as well as its relevant properties, i.e. stability, sensitivity or availability (Evtugyn et al., 1998). This study was based on the use of one of the enzymes that fall in the group of cholinesterases; namely acetylcholinesterase.

1.7.1. Acetylcholinesterase (AChE)

AChE (E.C 3.1.1.7) is a ubiquitous neuro-regulatory enzyme that forms part of the cholinesterase family of enzymes. AChE is responsible for the catalytic hydrolysis of acetylcholine into choline and acetic acid as illustrated in Figure 10.

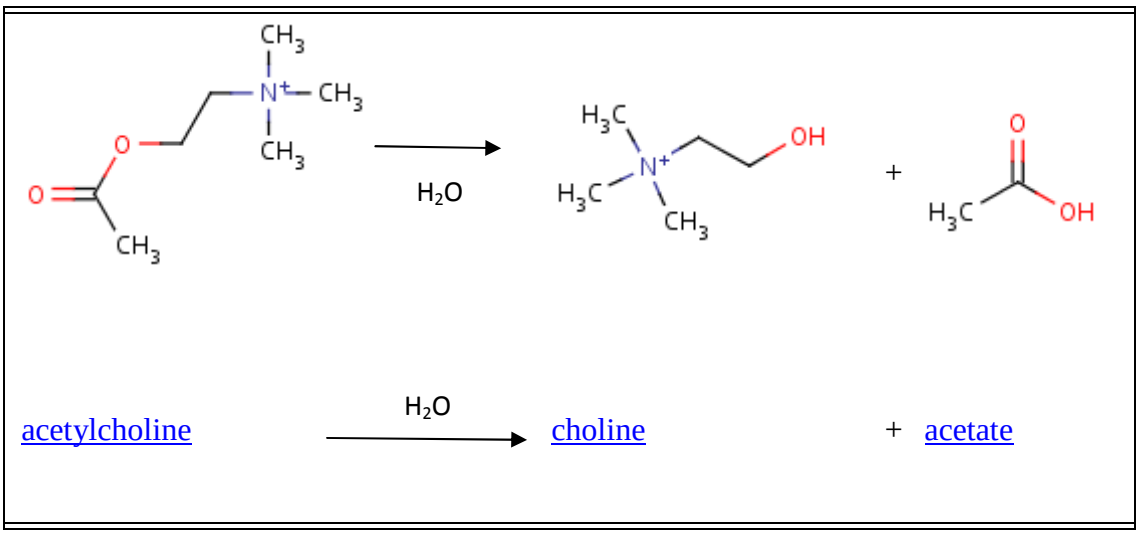


Figure 10: Reaction catalyzed by AChE that results in the breakdown of acetylcholine to acetate and choline.

The relevance of this reaction can be highlighted in synaptic transmission in neuromuscular junctions and cholinergic nervous system where it is required for termination. Upon transmission of a nerve impulse it results in the release of acetylcholine from the pre-synaptic neuron which travels across the synaptic cleft and activates receptors on the post synaptic neuron to continue impulse transmission. AChE (as is shown in Figure 10) is responsible for the breakdown of acetylcholine which stops the activation of the receptors on the post-synaptic neuron and as a subsequence stops the nerve impulse transmission. This breakdown of AChE allows the body to return to resting potential (Voet and Voet, 2004). Figure 11 below illustrates this process.

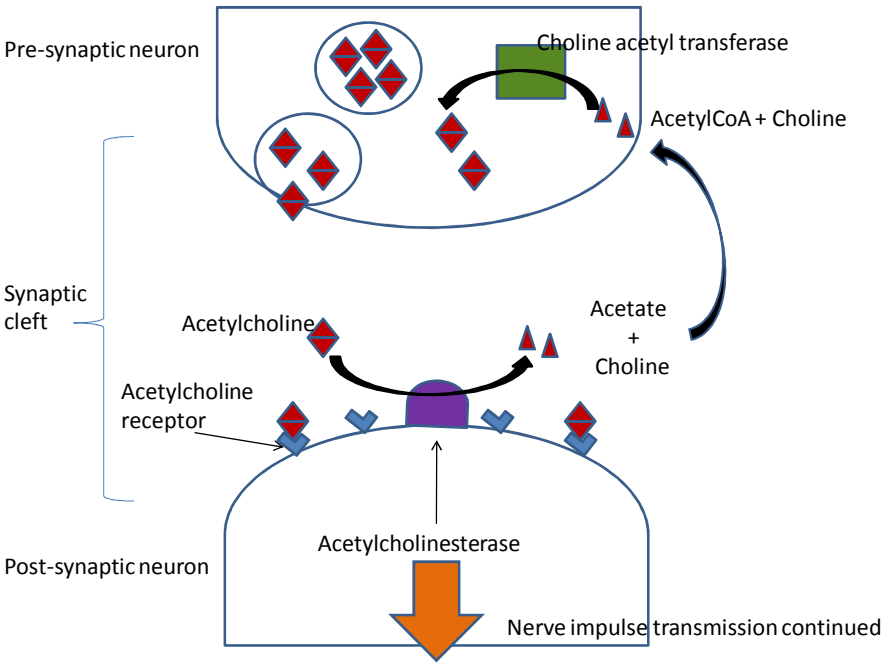


Figure 11: Schematic illustration of nerve transmission during which acetylcholine diffuses across the synaptic cleft and continues transmission of a nerve impulse and the manner in which it is broken down by AChE and the nerve impulse transmission is terminated. (Adapted from Voet and Voet, 2004)

This allows for the choline and acetic acid produced to be recycled and returned to the pre-synaptic neuron. Acetylcholine is then resynthesized in readiness for the next nerve impulse transmission. Acetylthiocholine iodide, acetylcholine chloride or fluorescein diacetate are also AChE substrates. The important role of AChE in the neural systems of organisms makes it necessary to distinguish which chemicals/compounds affect the enzyme. Any compound that has an adverse effect on the enzyme is referred to as an inhibitor. Several compounds have been found to adversely affect AChE, and in extreme cases (with total inhibition of AChE), can result in what is referred to as a cholinergic crisis. Prolonged seizure of limbs, convulsions and even central respiratory failure, which can eventually lead to death, are all indicative of a cholinergic crisis (Lallement et al., 1992 and Tonduli et al., 1999). Research by Fukuto (1990) indicated that AChE is inhibited by CPs and OPs and this inhibition can be used to test for the presence of these pesticides in water. The degree of inhibition of AChE would correspond to the concentration of pesticide present in the water sample (Andreescu et al., 2002).

CPs can reversibly inhibit the enzyme AChE by the carbamylation of the serine residue in the active site of AChE. This results in most carbamates being classed as true competitive

inhibitors (Wilson et al., 1960). Given sufficient recovery time, the carbamylation of the active site can be reversed and the inhibitory effects of the CP will disappear. OPs, unlike carbamates, are regarded as irreversible inhibitors and act as "suicide substrates" (Walker, 2003). Upon acceptance of an OP molecule into its active site, the serine moiety of the active site of the enzyme is phosphorylated. Fukuto (1990) reported that the dephosphorylation of the active site is so slow such that it can take up to several days so that the reaction is generally termed irreversible. The presence of pesticides can thus be determined using the inhibitory effect of both CPs and OPs on AChE.

1.7.2. Effects of Pesticide Mixtures

Several studies have investigated the effect of individual pesticides on AChE inhibition, but very few studies have investigated the effects of pesticide mixtures on this enzyme. According to Richardson et al. (2001), who assessed the effect of two pesticides on AChE activity, lower concentrations of the pesticides had a dose additive effect with regards to the inhibition of AChE. For the experiments in this study, "*dose additive"* was defined as the theoretically calculated inhibition which accounts for the number of AChE molecules that would be available for inhibition by a second compound following inhibition by a first compound. Therefore, this referred to more than just the simple mathematical summation of percentage inhibition, which is also called "*response additive"* (Richardson et al., 2001).

Further investigations by Tahara et al. (2005) also demonstrated the effect of two pesticides on AChE by keeping the concentration of one pesticide constant and varying the concentration of the second pesticide. Despite not explicitly defining the term "*additive effect"*, a similar conclusion was reached (i.e. additive inhibition of AChE) and additional tests were carried out to assess the effects of three pesticides. Their study noted that there was an additional inhibitory effect, but they could not conclusively state that the inhibitory effect on AChE was additive (Tahara et al., 2005). Their results seem to correlate to that of *dose additivity* that was described by Richardson et al. (2001).

Kok and Hasirci (2004), using *in vitro* studies, determined that binary combinations of aldicarb and carbaryl, as well as aldicarb and carbofuran, resulted in a less than additive effect. Karanth

et al. (2001, 2004) carried out *in vivo* tests in rats with chlorpyrifos and parathion and found that sequential exposure resulted in cumulative toxicity.

1.7.3. Chemometrics

Chemometrics is a scientific method used in statistics, mathematics and computer science to obtain information from systems through data driven analysis. It can be used to solve both predictive and descriptive problems and falls across many disciplines (Beatle and Jackson, 1990). Models of systems are made with the aim of identifying any structures and relationships within the system that could predict behaviour or be used to solve problems. This requires that sample data sets be provided from which this information can be obtained. The sample data is used for calibration which involves the development of a statistical model that fits the data set provided. Artificial neural networks (ANNs) are one of the applications of chemometrics, other examples include genetic algorithms, expert systems and machine logic to name a few (Massart et al., 1988).

1.7.3.1. Neural Networks

ANNs are mathematical models that model the functionality of the brain. ANNs try to emulate the brain's ability to process non-linear data and high parallelism as well as its robustness and ability to handle faults and failures. This is achieved by adaptive processing elements which are analogous to neurons in the brain (Veelenturf, 1995). As in the brain, they are interconnected and carry out complex computations and data processing. ANNs are designed to learn through updating the internal representation of the system in response to external stimuli provided by the sample data so it can perform the specific tasks assigned to it. This involves adjustments of weights, links, creating or deleting connections and or changing firing rules similar to the way the brain does with individual neurons (Veelenturf, 1995).

The brain is capable of learning, can generalize and also handle imprecise data, and these are all properties that ANNs try to incorporate in their functionality. Each ANN has the following features, given some input, each has a weight (w) and bias that are applied to it and the summation ($\sum$) of these are used to form an activation function that is used to calculate the final output as shown below (Figure 12).

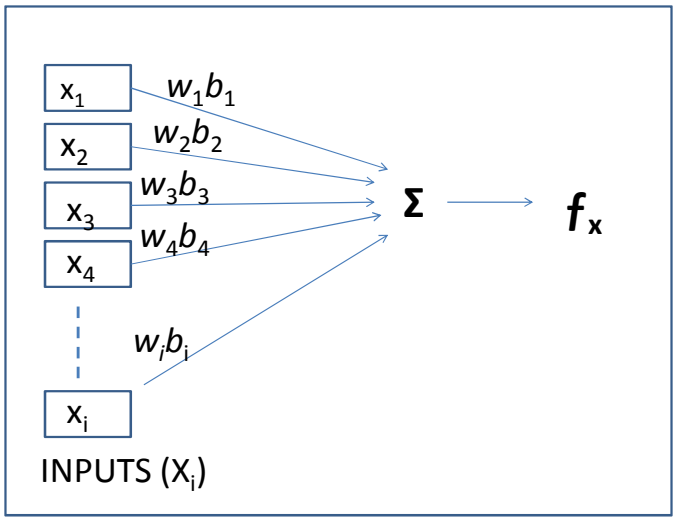


Figure 12: Architecture of a neural network. X indicates the initial inputs into the neural network; w represents the weights that are applied to each input; b represents the bias that is allotted to each weighting. Σ refers to the summation of the weightings and bias that result in the function (f_x) that can be used to predict outputs.

As with the human brain, learning is performed reiteratively with the aid of several examples. Continuously, each input has the weight and bias adjusted until the final output is as close to the desired result as possible. There is a degree of error that is incorporated into each activation function. The error function is from all the weights and forms an irregular multidimensional, complex hyperplane with many peaks and saddle points. The learning aims to obtain the set of weights that will correspond to a global minimum error. Artificial neural networks are therefore said to "learn" with regard to pattern recognition, mapping and completing and can thus be used to predict outcomes as opposed to creating whole new biological systems and this is one of their main advantages.

Chemometrics for data analysis by Ni et al. (2007; 2009) was undertaken in several studies that dealt with pesticide mixtures. From these studies it was indicated that individual pesticides could be identified from mixtures. A study was based on the different oxidation rates of the pesticides aminocarb and carbaryl when reacted in an alkaline medium with potassium ferricyanide (Ni et al., 2009). Partial least squares (PLS) and principal component regression (PCR), both factor analysis based multivariate tools, as well as the radial basis function neural network (RBF-ANN), were used for chemometric calibration in the studies. Application of

these tools was then performed for pesticide simultaneous prediction during the study involving two pesticides (Ni et al., 2009). According to Ni et al. (2009), RBF-ANN was used due to its versatility and ability to deal well with non-linear data. From this ANN they were able to successfully determine the presence of aminocarb and carbaryl from a mixture. Another study, also conducted by Ni et al. (2007), successfully identified the presence of four pesticides, namely carbaryl, carbofuran, isoprocarb and propoxur. After determination of the voltammetric behaviour of each pesticide after alkaline hydrolysis with sodium perchlorate and sodium hydroxide, repeats were done with laboratory samples containing varying pesticide concentrations. The calibration methods mentioned in the previous study were applied and a modified version of the RBF-ANN method was once again selected for the chemometric analysis as it provided the most satisfactory results. Similar success was recorded for the identification of individual pesticides, using chemometrics, by Danzer and Schwedt (1996), Dock et al. (2005), Guiberteau et al. (1995) as well as Ni et al. (2004a; 2004b; 2005 and 2007). In some cases more than one enzyme was used, for example, Danzer and Schwedt (1996) managed to differentiate eleven pesticides and two metals using three enzymes. Bachmann et al. (2000) and Bachmann and Schmid (1999) managed to use feed-foward artificial neural networks (FF-ANNs) as opposed to RBF ANNs to differentiate pesticides.

Previous research in our laboratory could not distinguish between pesticides or pesticide mixtures using AChE (Mwila, 2009). Chemometrics has been suggested as a means of accomplishing this based on the success of Ni et al. (2009). Using their method and making use of differences in reaction rates, it has been hypothesized that individual pesticides could be identified in mixtures.

1.7.4. Disadvantages of Enzymatic Methods

Enzyme assays have been criticised for several reasons. The main disadvantages have been described as lack of specificity, interference, loss of activity and problems with solubility. Luque de Castro and Herrera (2003) pointed out that the enzyme can be inhibited by various other compounds such as heavy metals, inorganic and organic compounds similar in structure to pesticides, hence the challenge in using enzymes in environmental testing. Simonian et al. (2005) pointed out further problems such as false positives and loss of enzyme activity during storage when biosensors are used. In order to overcome this problem, baseline testing would be

required at all times which would lengthen the process of testing, as well as the regeneration of the biosensors (Flounders et al., 1999; Mulchandani et al., 2001).

The issue of poor specificity and inability to distinguish pesticides is also another disadvantage of enzymatic assays. However, this is one of the problems addressed in our study. Solvent use was another major factor with regard to the use of enzymes. Most pesticide solubilities vary depending on the solvent used. The degree of solubility would therefore have an effect on the enzyme. According to Everett and Rechnitz (1998), a compound near its solubility limit may result in a suspension that would affect the enzyme's ability to interact with the compound. Further studies carried out by Mionetto et al. (1994) found that water soluble solvents could reduce the activity of AChE as they stripped the enzyme of the water of solvation, thus inactivating it, whereas non-polar solvents were found to activate AChE activity. Similar conclusions were reached by other researchers with other solvents (Fennouh et al., 1997; Campanella et al 1999; Andreescu et al., 2002). Therefore, in conclusion, it can be stated that most solvents can be used but these must be at concentrations that do not have a major effect on the enzyme activity.

1.8. Problem Statement

The adverse health effects of pesticides require that a rapid detection mechanism be developed that will assist in determining their presence in water sources. Humans and animals are being exposed to an ever increasing number of pesticides simultaneously, and this necessitates the assessment of the effects of pesticide mixtures. The endocrine disrupting effects of pesticides also needs to be determined as very little information is available for most OPs and CPs. More data is required on the presence of OPs and CPs in water sources in South Africa, especially in the Eastern Cape region.

1.9. Hypothesis

The hypothesis can be separated in to three parts and these are as follows;

1. Mixtures of OPs and CPs and their degradation products can be rapidly detected in water using an AChE-enzyme assay and their estrogenic properties assessed using the YES assay.

2. The AChE assay can also be used to test environmental water samples in the Eastern Cape.
3. Neural networks can be used to identify individual pesticides within mixtures.

1.10. Aims and Objectives

The main aim of this study was to use AChE to detect sub-lethal levels of pesticides, determine the endocrine disrupting properties of these pesticides as well as assess pesticide distribution in the Eastern Cape. From this aim the following were the objectives set:

- To optimize the AChE assay and determine its limit of detection (LOD),
- To determine the effects of mixtures,
- To identify individual pesticides in pesticide mixtures using chemometrics,
- To establish the endocrine disrupting effects of a number of CPs and OPs, and their degradation products, using the YES assay,
- To use the AChE assay to investigate the relative distribution of the above pesticides (and breakdown products) in surface water fractions in the Eastern Cape.

CHAPTER 2 -Methods and Materials

2.1 *Detection of Individual Pesticides using AChE*

The micro-Ellman assay makes use of AChE and acetylthiocholine iodide (AcSChI) as a substrate. Upon addition of 5'5'-dithio-bis-2-nitrobenzoic acid (DNTB: Ellman's reagent) to the enzyme and substrate, a yellow colour is formed that can be monitored using spectroscopy (Ellman et al., 1961).

Optimization of the micro-Ellman Assay was carried out with several sources of enzyme being considered in order to increase the sensitivity of the assay. According to Villatte et al. (1998), the best sensitivity for pesticides was exhibited by AChE from purified *Drosophilla melanogaster* as compared to *Electrophorus electricus*, bovine erythrocyte, *Torpedo californica* and *Caenorhabditis elegans*. However, due to lack of availability of AChE from *Drosophilla melanogaster*, AChE from *Electrophorus electricus* was selected. Optimal concentrations of DNTB and AcSChI were determined experimentally and found to be 0.01 M and 0.075 M, respectively. This was done to ensure that neither the substrate nor DNTB was limiting the reaction rate and reduced possible false positives. Sodium phosphate buffer (0.1 M) was used at pH 7.5 for the assay. The advantage of this assay is that it can also be used to assess aqueous samples.

The first set of experiments was carried out for different concentrations of individual OPs to assess the inhibitory effect of each pesticide. In addition, the effect of varying pre-incubation times of AChE with pesticides was also investigated.

Basal AChE activity (i.e. AChE activity in the absence of any pesticide) was always kept constant by testing enzyme activity levels prior to inhibition studies and ensuring similar activity levels were always obtained. For each experiment, a 160 µl aliquot of buffer was added to 10 µl of DNTB and 20 µl of AChE. These were placed in a microtiterplate well and 10 µl of acetylthiocholine was added in order to start the reaction. A Powerwave$_X$ microtiterplate reader was used to record the absorbance at 412 nm every 30 seconds for 10 minutes. All assays were performed in quadruplicate. In order to ensure standardization between different batches of enzyme, the AChE concentration was selected to ensure similar activity levels for all experiments for the "No pesticide" control.

2.2 Analysis of the effect of Pesticide Mixtures on AChE

2.2.1 Mixtures

In the experiments that required analysis of mixtures, the total pesticide volume was kept constant at 120 µl per sample. This required that volumes for individual pesticides in each reaction be reduced as follows: for two pesticides, 60 µl of each was used, for three pesticides, 40 µl of each, for four pesticides, 30 µl of each and for five pesticides, 24 µl of each were incubated with 120 µl of enzyme. This ensured that the total pesticide concentration was kept the same as in the individual pesticide assay. The enzyme and pesticides were pre-incubated for 15 minutes prior to addition of substrate and measurement of AChE activity. Assays of mixtures were performed in quadruplicate.

Tables 3 and 4 display the pesticide mixtures that were tested for four and three pesticides, respectively. The pesticides were tested at three concentrations described as low ($1x10^{-7}$µg/L), medium ($1x10^{-3}$µg/L) and high (0.1 µg/L).

Table 3: Summary of the pesticide combinations tested for four pesticides

	Parathion	Demeton	Carbaryl	Carbofuran	Aldicarb
Parathion		√	√	√	√
Demeton	√		√	√	√
Carbaryl	√	√		√	√
Carbofuran	√	√	√		√
Aldicarb	√	√	√	√	

Table 4: Summary of combinations of pesticides tested for three pesticides.

	Parathion	Aldicarb	Demeton	Carbofuran	Carbaryl
Parathion		√	√		
		√		√	
		√			√
Aldicarb	√		√		
	√			√	
	√				√
Demeton		√		√	
		√			√
	√			√	
	√				√
Carbofuran	√				√
	√	√			
Carbaryl	√			√	
	√	√			
		√		√	

2.2.2 Effect of Increased Pesticide Concentration on Activity and Variability in Results

The sensitivity of the assay also required that the effect of varying the enzyme concentrations be investigated. Therefore, the enzyme concentrations were increased 2 fold, 5 fold and 10 fold to 0.2 units, 0.5 units and 1 unit, respectively. All other components were kept constant, as described in Sections 2.1 and 2.2, with pesticide concentration being the only variable tested.

2.3 Chemometrics

There were 5 pesticides that were investigated in this study and each was present at one of 8 concentrations. Each pesticide was assigned an identification term, T representing toxin, to distinguish it from the other pesticides i.e. T1 represent aldicarb. This process was repeated for each of the concentrations as well i.e. C1 represents 0.1 µg/L. The full list for all the pesticides and concentrations is shown below:

T1	Aldicarb
T2	Carbaryl
T3	Carbofuran
T4	Demeton
T5	Parathion

	µg/L
C1	1×10^{-1}
C2	1×10^{-2}
C3	1×10^{-3}
C4	1×10^{-4}
C5	1×10^{-5}
C6	1×10^{-6}
C7	1×10^{-7}
C8	1×10^{-8}

Pesticides were therefore denoted as follows: (T*i*,C*i*) identifies a pesticide and its concentration. Each pesticide mixture was identified by a set of ordered pairs of the form:

$\{(T1, C1), \cdots, (Tk, Ck)\}$ of k ordered pairs

The total number of possible pesticide mixtures if M_i denotes the number of i-pesticide mixtures can be determined as shown below:

$$M_1 = 5 \cdot 8$$

$$M_2 = \frac{5 \cdot 4 \cdot 8^2}{2!}$$

$$M_3 = \frac{5 \cdot 4 \cdot 3 \cdot 8^3}{3!}$$

$$M_4 = \frac{5 \cdot 4 \cdot 3 \cdot 2 \cdot 8^4}{4!}$$

$$M_5 = \frac{5 \cdot 4 \cdot 3 \cdot 2 \cdot 1 \cdot 8^5}{5!}$$

The total number of statistically possible pesticide mixtures is therefore:

5*8+5*4*8^2/2+5*4*3*8^3/6+5*4*3*2*8^4/24+5*4*3*2*1*8^5/120 = 59048

Carrying out the testing of all possible combinations of mixtures would be an extremely difficult and time consuming task and therefore a small number of representative assays were selected. Assays of the change in reaction rates for individual and pesticide mixtures were obtained from the first and second objectives using the micro-Ellman assay. A FF-ANN was constructed in MATLAB and trained on all of the available data. The trained neural network could then identify mixtures close to those on which it was trained but naturally, it could not identify mixtures which varied greatly from those in the training set.

2.3.1 Preparation of Input Patterns

It was originally intended that the results for each pesticide or pesticide mixture would be the input patterns. However, the variability in the enzyme activity for which account had to be made. Therefore, the no pesticide reaction for each batch was added to the reaction profiles for each pesticide. For example, the no pesticide reaction profile for batch 1 as well as the reaction profile for T1C1 (Aldicarb, 0.1 µg/L) are shown in Table 5. The final output would be a 1x 42 matrix with the first set of data values being those seen in the column labelled batch 1 and the next set of values those labelled T1C1.

Table 5: Tabulated data indicating the no pesticide absorbance values as batch 1 in the first column and T1C1 represents the absorbance values for the inhibited reaction by aldicarb at 0.1 µg/L in the second column.

Batch 1	T1C1
0.36	0.50
0.47	0.50
0.51	0.53
0.53	0.55

0.55	0.57
0.56	0.59
0.57	0.61
0.59	0.63
0.62	0.64
0.63	0.66
0.66	0.67
0.67	0.68
0.69	0.70
0.71	0.71
0.72	0.71
0.75	0.72
0.77	0.73
0.78	0.74
0.80	0.75
0.82	0.77
0.85	0.78

For each mixture, a 5×8 zero matrix was created and if pesticide i at concentration j is present in a mixture then a 1 was inserted in position (i, j) in the matrix, creating a $0,1$-matrix, T. From this, a 40×1 column vector is created from the columns of T using the MATLAB colon operator. The function, *target.m* (which can be found in Appendix 3) was created for this purpose.

For example, if the mixture is $\{(2,4),(5,3)\}$ then

```
>> [t, T]=target(c)
```

T =

0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	0

2.3.2 Training

The neural network, called toxinsnet, was created and trained using the script: *toxins_train.m*. Pseudo code for this script is as follows:

- load the data file
- create the net using the MATLAB function, newff :

```
net=newff (p, t, [150,150], {'tansig','tansig','tansig'},'trainscg');
```

This created a neural network with 150 neurons in layers 1 and 2 and the number of neurons in layer 3 was determined by the dimension of the target vectors, which was 40. The number, s_1 and s_2 of neurons in layers 1 and 2 were determined by means of a supervising script which formed neural networks with s_1 and s_2 ranging from 50 to 200 and identifying that combination which produced the best fit to the targets.

The transfer function in each layer is the tansig function. Since this is a pattern-recognition problem, the last layer also has the tansig transfer function instead of the usual purelin function.

The net was trained using the scaled conjugate gradient training function, trainscg, which yielded better results than the resilient back propagation training function: trainrp.

Training of the net was done over 3000 epochs. The output during training can be seen in Figures 13 and 14. These data indicate how successfully the neural network was trained.

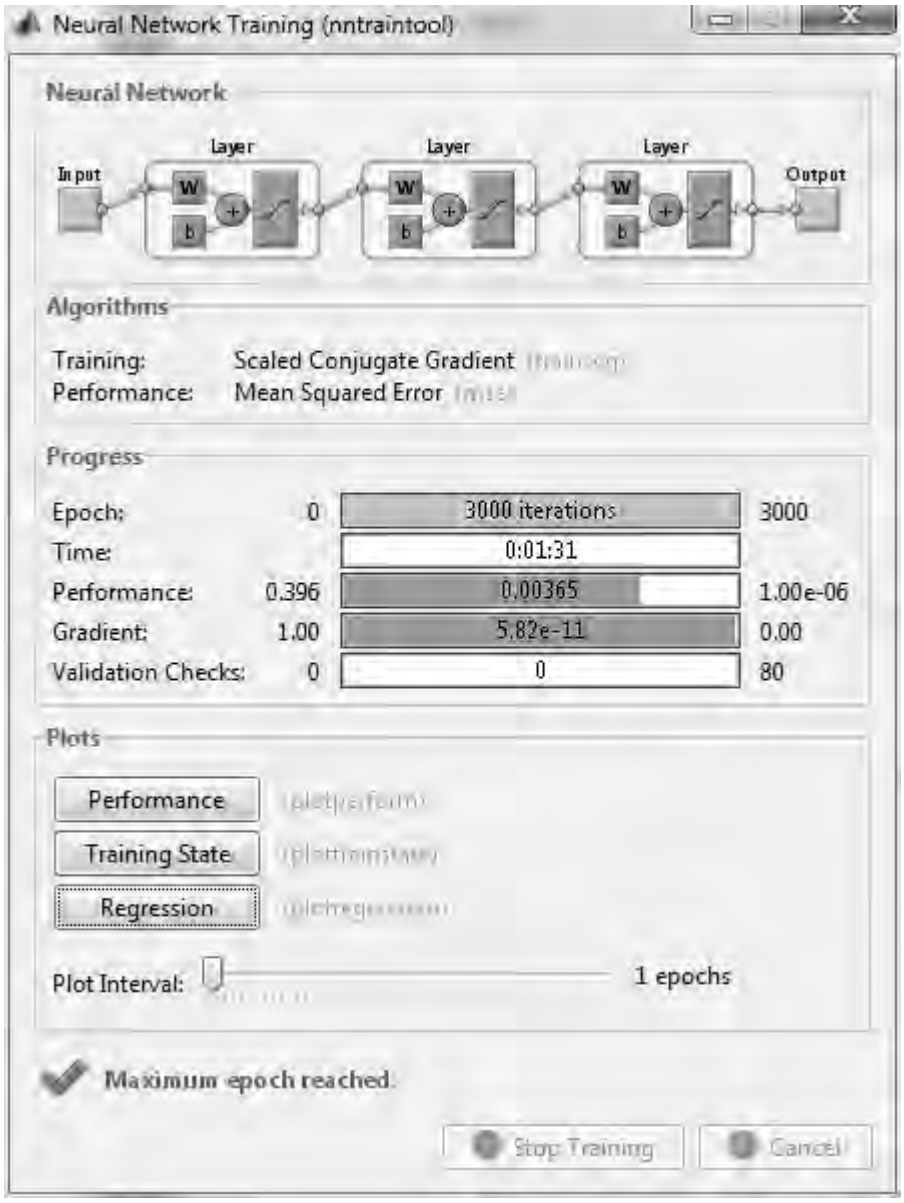
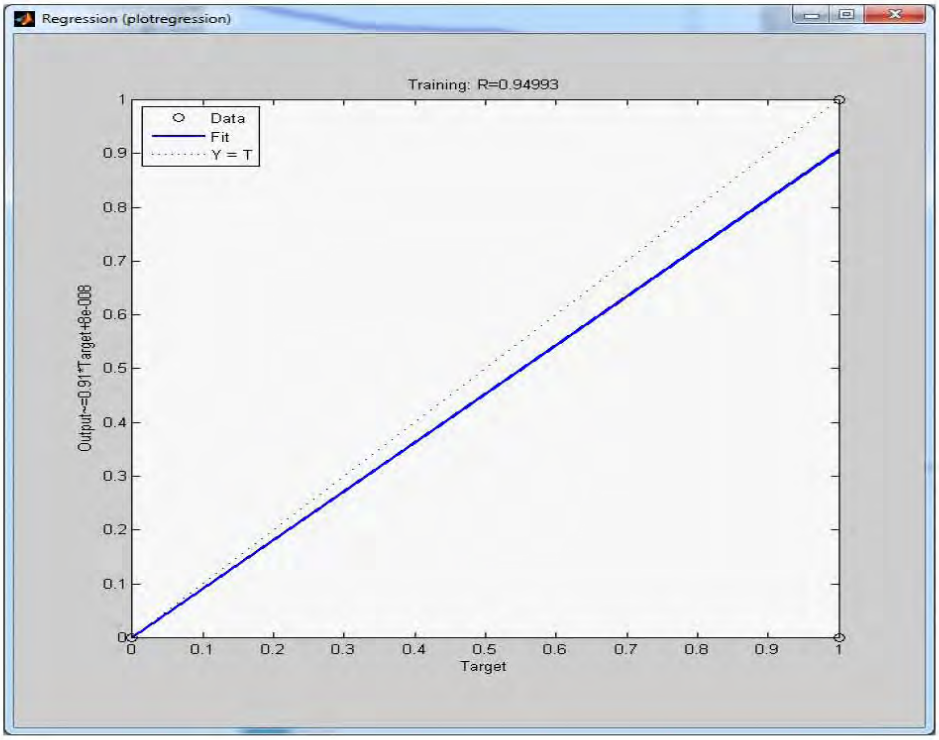
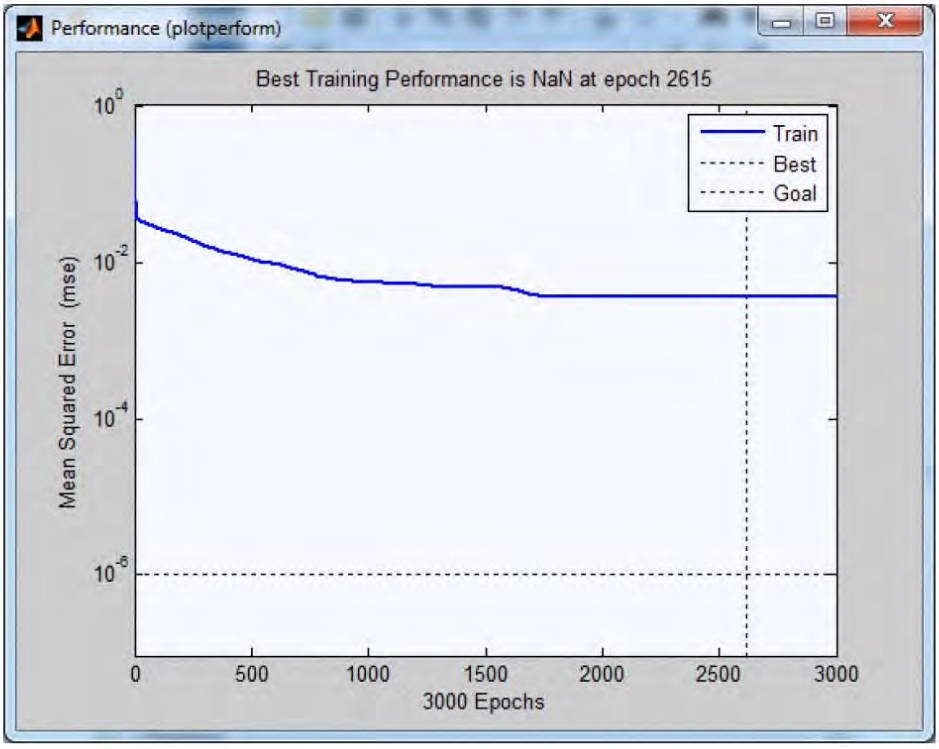


Figure 13: Output from training of the neural network over 3000 epochs using the trainscg function.



A



B

Figure 14A and B: Diagrams showing training and optimisation of artificial neural network parameters to ensure minimum global errors were achieved.

2.4 Bio-Assays

2.4.1 Yeast Estrogen Screen Assay (YES Assay)

2.4.1.1 Sample Preparation

Stock concentrations of the test chemicals (1 mg/ml) were prepared in ethanol, prior to dosing of plates. Powdered samples were immediately dissolved in ethanol, but because the yeast cells are adversely affected by organic solvents, in the case of the liquid samples pre-evaporation had to be performed. Once the organic solvents were removed, the dried sample was then diluted into 1 ml of ethanol to maintain the same stock concentration of 1 mg/ml.

2.4.1.2 YES Assay

The YES assay was performed according to the method of Routledge and Sumpter (1996) with minor adjustments (Aneck-Hahn et al, 2005; Bornman et al. 2007). Serial dilutions of test chemicals and controls were made in ethanol solvent in 96 well optically flat bottom microplates. Ten (10) µl aliquots were transferred to a second sterile 96 well microtiter plate and allowed to evaporate to dryness. Two hundred (200) µl of the assay medium that contained yeast and chromogenic CPRG was placed into each well. Each plate contained a row with ethanol solvent (blank) as well as an E_2 standard curve (17β-estradiol) with concentrations ranging between $1x10^{-8}$ M and $4.8x\ 10^{-12}$ M. The plates were sealed with parafilm and placed in an incubator at 32^oC for 3-5 days. After 3 days incubation, the plates were read using a Titertek Multiskan MCC/340 plate reader at absorbances of 540 nm and 620 nm (Figure 15). The plates were resealed and returned to the incubator and readings taken (as before) for days 4 and 5. All experiments were performed in triplicate. The readings at 540 nm and 620 nm were used to correct the readings obtained for turbidity of the yeast cells (Aneck-Hahn et al 2005; Bornman et al., 2007).

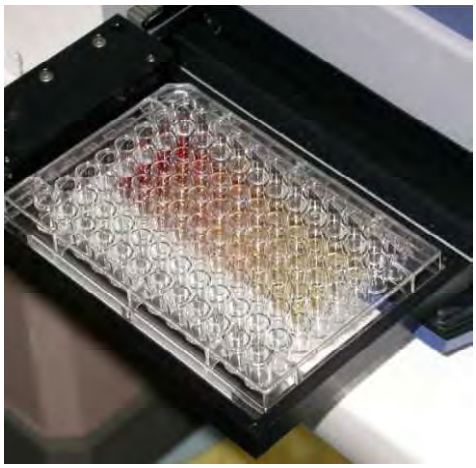


Figure 15: An example of a YES plate after 3 days incubation, prior to reading in the spectrophotometer.

2.4.2 T47D-KBluc Reporter Gene Assay

2.4.2.1 Sample Preparation

All test chemicals were processed according to the protocol described in Wilson et al. (2004).

2.4.2.2 Maintenance of Cell Cultures

T47D-KBluc cells were maintained in RPMI growth media supplemented with 2.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), 100 µg/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B. One week prior to the assay, cells were placed in growth media modified by replacement of 10% FBS with 10% (w/v) dextran-charcoal treated FBS excluding antibiotic supplements (Wilson et al., 2004).

2.4.2.3 Experimental procedure

Cells were seeded at 5 x 10^4 cells per well in 96-well luminometer plates and allowed to attach overnight. Dosing dilutions were prepared in growth media containing 5% (w/v) dextran-charcoal treated FBS and vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and

background control (vehicle plus ICI). Each sample was tested alone as well as in the presence of 0.1 nM E_2 or ICI. Cells were incubated 24 h with 0.1 ml per well dosing solution at 37°C, 5% CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 0.025 ml lysis buffer. Luciferase activity was determined using a LUMIstar OPTIMA luminometer and quantified as relative light units (RLU). Each well received 0.025 ml reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/ml BSA, pH 7.8), followed by 0.025 ml D-luciferin 5 s later. Relative light units were converted to a fold induction above the vehicle control value.

2.4.3 MDA-kb2 Reporter Gene Assay

2.4.3.1 Sample Preparation

The test chemicals were processed according to the protocol described in Wilson et al. (2002).

2.4.3.2 Maintenance of Cell Cultures

MDA-kb2 cells were maintained in Lebovitz's L-15 growth media supplemented with 10% FBS, 100 µg/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B.

2.4.3.3 Experimental Procedure

Cells were seeded at $5x10^4$ cells per well in 96-well luminometer plates and allowed to attach overnight. Dosing solutions were prepared in growth media and vehicle (ethanol) did not exceed 0.2%.

Each plate contained agonist positive control (dihydrotestosterone, DHT), negative control (vehicle only), antagonist control (DHT plus flutamide) and background control (vehicle plus flutamide). Each sample was tested alone as well as in the presence of 0.1 nM DHT or flutamide. Cells were incubated for 24 h with 0.1 ml/well dosing solution at 37°C, without supplemental CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 0.025 ml lysis buffer. Luciferase activity was determined using a LUMIstar OPTIMA luminometer and quantified as Relative Light Units (RLU). Relative light units were converted to a fold induction above the vehicle control value.

2.5 Environmental Sampling

Environmental samples were collected from various surface water sources in the Eastern Cape. In order to assess the distribution of pesticides in water samples in the Eastern Cape, it needs to be borne in mind that more than one pesticide may be present at any given time in a water sample. For water sample analysis, all experiments were performed as previously explained, except that the buffer volume was reduced to 140 µl and 20 µl of the water sample was added. All assays were carried out in quadruplicate.

The photos in Figure 16 were taken during water sampling.

Figure 16: Sampling photographs taken at the Kariega River sampling site by the N2 Bridge in Kenton-on-Sea

Two sets of water sampling were carried out. The first water sampling was performed in October, 2010 at the locations indicated below. The second set of water sampling was performed in March, 2011. These samples were sent to the South African Bureau of Standards (SABS) laboratory in Cape Town to be analysed using conventional testing methods.

2.5.1 Sampling Sites

The following paragraphs indicate the sampling sites which were selected for analysis of their pesticide content; the locations are indicated on the maps in Figures 17-21. Sites were selected

on the basis that they represented some of the main water sources in the Eastern Cape and are represented by a circle with a filled in centre (Θ).

2.5.1.1 Blaauwkrantz River, Grahamstown

The area around the Blaauwkrantz River is used mainly for agricultural purposes, but also passes through an informal settlement in Grahamstown. Pollution in the river is very visible, and although the water quality appears to be poor, very little information is available on the river and its condition (see location of the river in Figure 17).

Figure 17: Map of Grahamstown indicating the location of the sampling site at the Blaauwkrantz River.

2.5.1.2 Sundays River, Colchester

The Sundays River (Figure 18) has a catchment area that is mainly used for agricultural activities. Emmerson (1989) as well as Scharle and Baid (2005) established that agricultural activities contribute to the input of organophosphate fertilisers and pesticides into nearby rivers and ultimately estuaries. It was noted that pollution was relatively mild compared to other estuaries such as Swartkops, as the urban settlement, because of its small size, does not contribute much in the way of pollution with regards to the river.

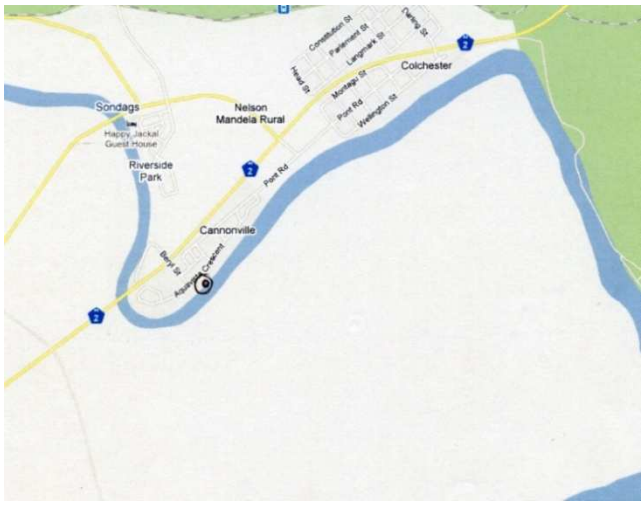


Figure 18: Map of Colchester indicating the location of the sampling site at the Sundays River just after the N2 Bridge. Tap water samples from the urban settlement in Colchester were also collected.

2.5.1.3 Kowie River, Port Alfred

The Kowie Estuary in Port Alfred (Figure 19) is an open estuary that flows through an urbanized area and is subject to substantial environmental degradation. This is due to the construction of the marina along the length of the river before it empties into the ocean. Recent issues have arisen as a result of the persistent dredging in the river mouth region as well as the land and sewage run-off into the river which is affecting the fauna and flora of the ecosystem.

Figure 19: Map of Port Alfred indicating the location of the sampling sites at the Kowie River just before and after the urban settlement, near the river mouth.

2.5.1.4 Kariega River, Kenton

The Kariega Estuary has a small urban settlement that produces minimal liquid effluent that is disposed of in the river and its catchment area is characterised by minimal industrial activities (Figure 20). Orr et al. (2008) demonstrated that sediments in the Kariega Estuary were enriched in cadmium (Cd), copper (Co), nickel (Ni) and lead (Pd), but the Kariega River is considered relatively pristine. However, the study also observed that there were significant seasonal variations in the sediment enrichment by Co, Ni and Pb as well as in the Cd and Pb concentrations in the water column.

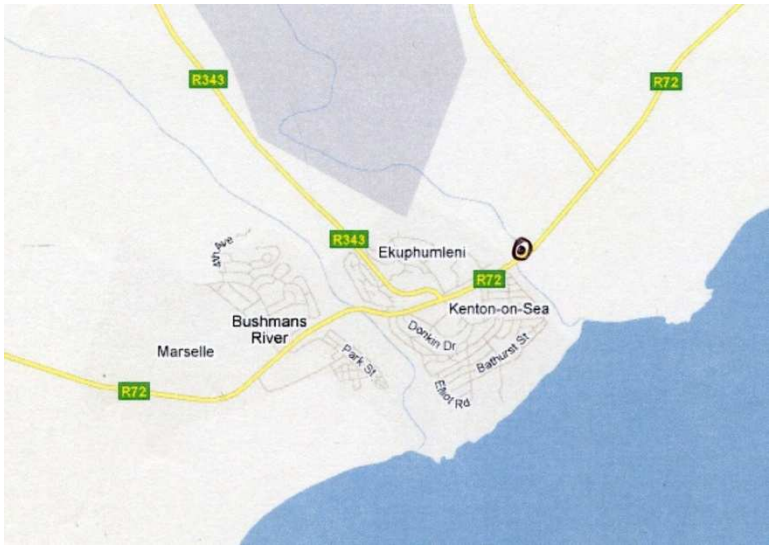


Figure 20: Map of Kenton-on-Sea indicating the location of the sampling site at the Kariega River just before the R72 Bridge.

2.5.1.5 Swartkops River, Port Elizabeth

Previous studies carried out by Binning and Baird (2001) demonstrated that heavy metal concentrations in the sediments of Swartkops Estuary have increased remarkably over the 1980s and 1990s. The extent of pollution has been so significant that the annual Redhouse river mile race was moved due to the health risk that the river's pollution posed for the swimmers in 2010 (Herald, 2010).There are numerous industrial activities such as clay mining, salt works, sewerage treatment works, wool washeries and tanneries situated in the region as well as a dense population in the lower catchment area around the Swartkops River estuary (Figure 21).

Motherwell and Markman Canals are sources of pollutants which include the under-serviced, heavily populated residential townships and industrial developments, respectively (Fatoki and Mathabatha, 2001). The Chatty River also exits into the Swartkops Estuary in the lower reaches and flows through an informal settlement.

Figure 21: Map of Port Elizabeth indicating the location of the sampling sites at the Swartkops River at the Grahamstown Road Bridge, Amsterdam Way and at Bluewater Bay.

CHAPTER 3 - RESULTS

3.1Individual Pesticides

The results from the individual pesticides are displayed in Figures 22 to 25 and summarized in Table 3. Several different types of graphical plots were assessed in an attempt to clearly illustrate the results from the individual pesticides. XY plots were ideal as they gave an accurate representation of the differences in the selected pesticide concentrations. However, these plots failed to accurately show the linear trends observed as data points were all clustered together near the Y axis and thus the line graph was selected as a better alternative for the purposes of illustration.

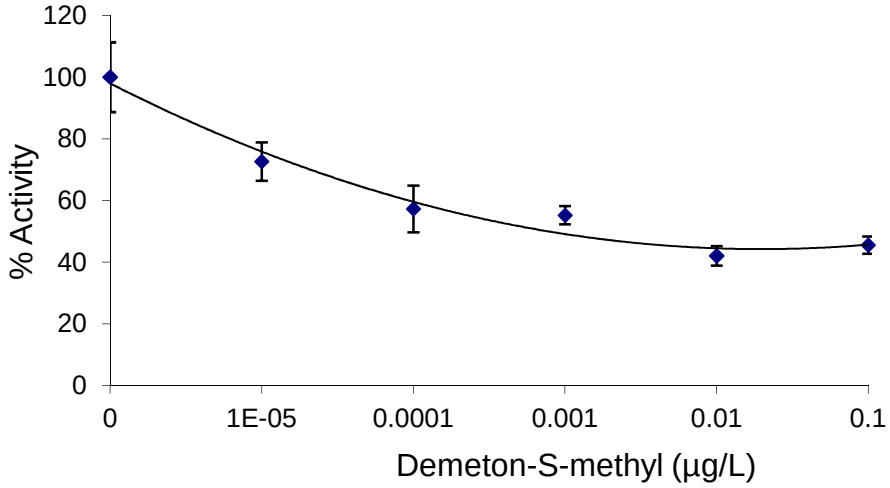


Figure 22: Residual AChE activity after 15 minutes incubation with demeton-S-methyl at concentrations ranging from 0 to 0.1 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means ± SD (n=4).

Figure 22 shows that an increased concentration of demeton-S-methyl resulted in an increased inhibition of AChE. The maximum degree of inhibition was approximately 55%, which reached a plateau at concentrations greater than 1 x 10^{-3} µg/L. This indicated that the inhibition of AChE was proportional to pesticide only up to a certain threshold concentration.

Demeton-S-methyl and parathion were found to inhibit AChE quite significantly as can be observed in Figures 22 and 23. Demeton-S-methyl resulted in a residual activity of 45% whilst parathion resulted in a residual activity of 50%. Aldicarb inhibited the enzyme to a lesser degree and led to a residual AChE activity of 70% (see Figure 24).

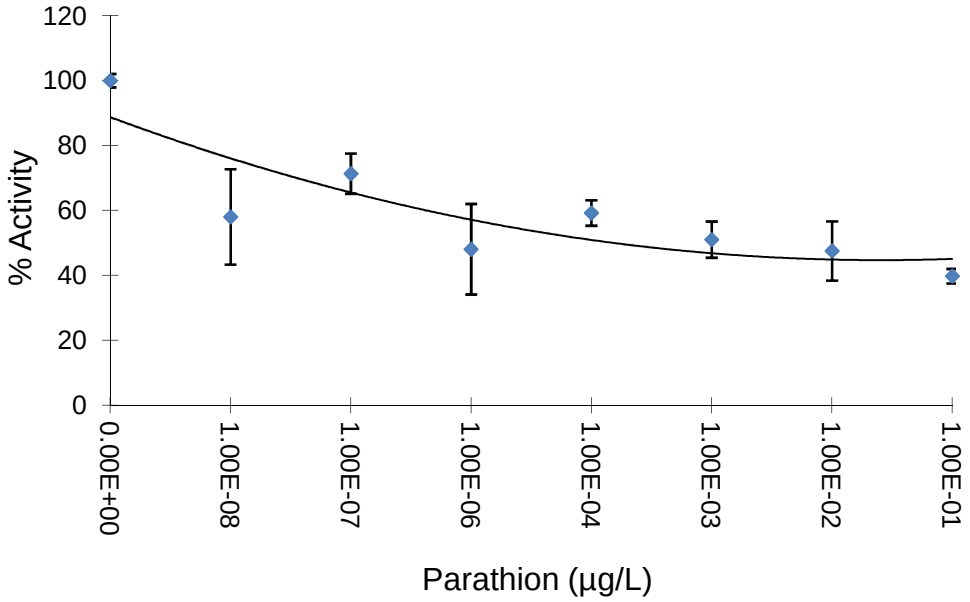


Figure 23: Residual AChE activity after 15 minutes incubation with parathion at concentrations ranging from 0 to 0.1 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means ± SD (n=4).

Parathion displayed a lesser degree of inhibition than demeton-S-methyl with the greatest inhibition being approximately 50% at all concentrations higher than 1×10^{-4} µg/L.

Aldicarb exhibited the lowest degree of inhibition with respect to AChE -a residual activity of approximately 70% at 1×10^{-2} µg/L. Although there was a gradual inhibition, there was no asymptotic behaviour as observed with the other pesticides tested.

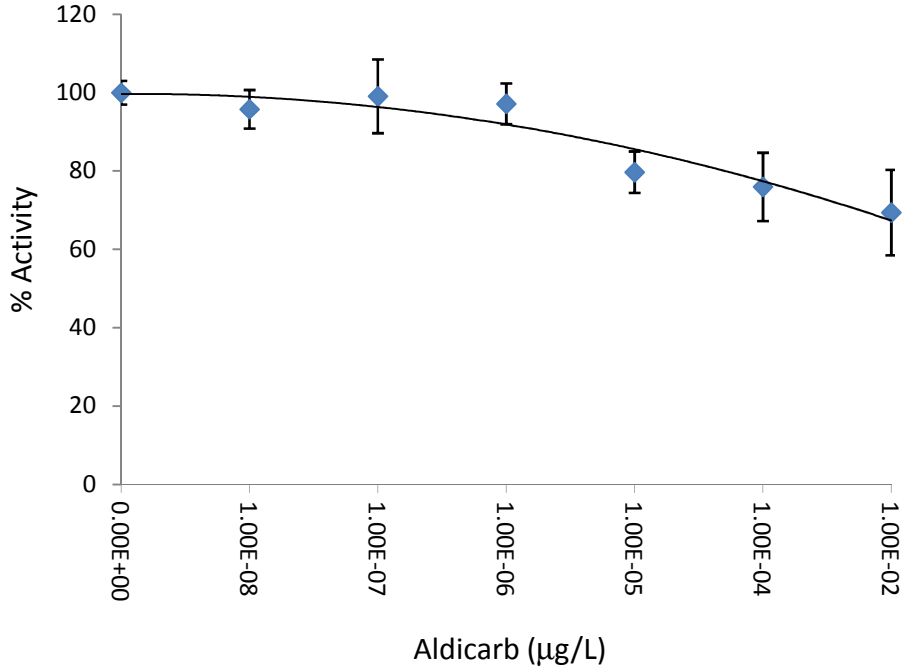


Figure 24: Residual AChE activity after 15 minutes incubation with aldicarb at concentrations ranging from 0 to 0.01 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means ± SD (n=4).

Carbofuran, as can be observed in Figure 25, did not display a gradual change in inhibition as would be expected. No general trend was noted and AChE displayed stimulation at concentrations of $1x10^{-5}$µg/L, slight inhibition and then stimulation at 0.1 µg/L.

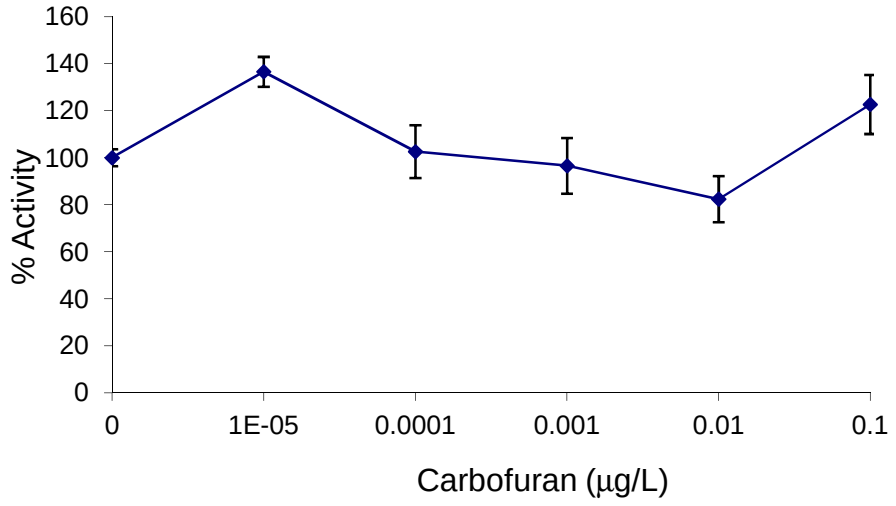


Figure 25: Residual AChE activity after 15 minutes incubation with carbofuran at concentrations ranging from 0 to 0.1 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means ± SD (n=4).

Table 6 displays the IC_{20} levels for each pesticide. IC_{20} refers to the concentration of pesticide that will result in a 20% inhibition of the enzyme activity. This value was selected as it was considered a substantial inhibition and would allow for easier monitoring of the effects of pesticide mixtures. Of the five pesticides tested, demeton-S-methyl displayed the greatest inhibition of AChE. This was followed by carbaryl, parathion, aldicarb and carbofuran; which displayed the least inhibition. As can be observed; detection was achieved at concentration levels far below the required legal limits. Comparison with literature limits of detection also display marked improvement, with the exception of carbofuran (Table 6).

Table 6: Summarised data for IC_{20} levels for individual pesticides tested over the range of 0 µg/L to 0.1 µg/L, compared to LODs in literature.

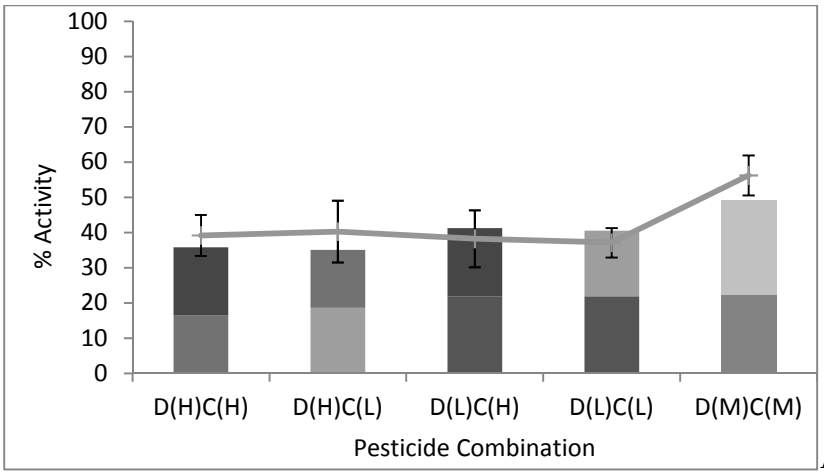
Pesticide	**IC_{20} (µg/L) (This study)**	**Max allowable limits in water (EU) (µg/L)**	**LODs reported in Literature**
Carbofuran	0.01	0.1	0.001 µg/L (No et al., 2007) 2.2 µg/L (Martorell et al., 1997) 20 µg/L (Bachmann and Schmid, 1999)
Aldicarb	$1x10^{-5}$	0.1	0.2 µg/L (Nunes et al., 2004) 24 µg/L (Arduini et al., 2006) 4.8 µg/L (Campanella et al., 1999)
Parathion	$5x10^{-6}$	0.1	0.001 µg/L (No et al., 2007) 10 ng/ml (Del Carlo et al., 2004)
Carbaryl	$1x10^{-7}$	0.1	0.001 µg/L (No et al., 2007) 25 µg/L (Arduini et al., 2006) 108 µg/L (Andreou and Clonis, 2002)
Demeton-S-Methyl	$5x10^{-9}$	EPA limit 0.1 µg/L	Not reported

After analysis of individual pesticides was performed, the effect of mixtures on AChE activity was also considered.

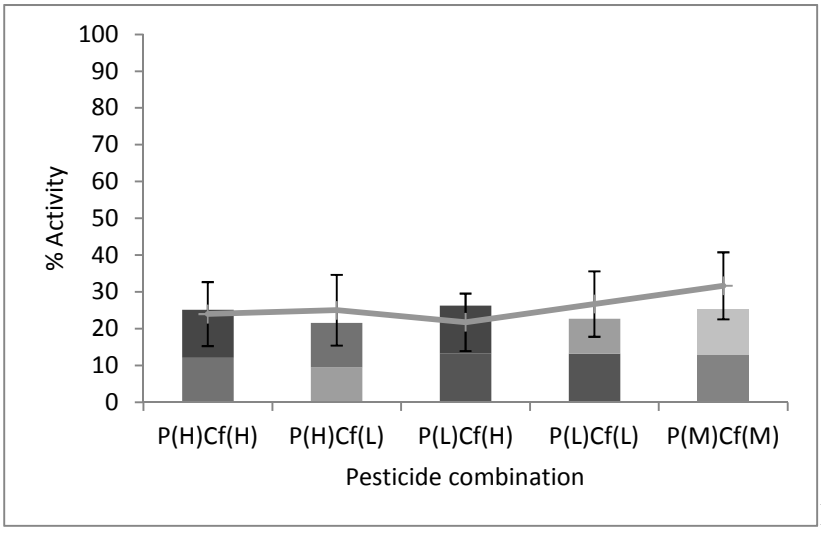
3.2 Effect of mixtures of pesticides

3.2.1 Mixtures

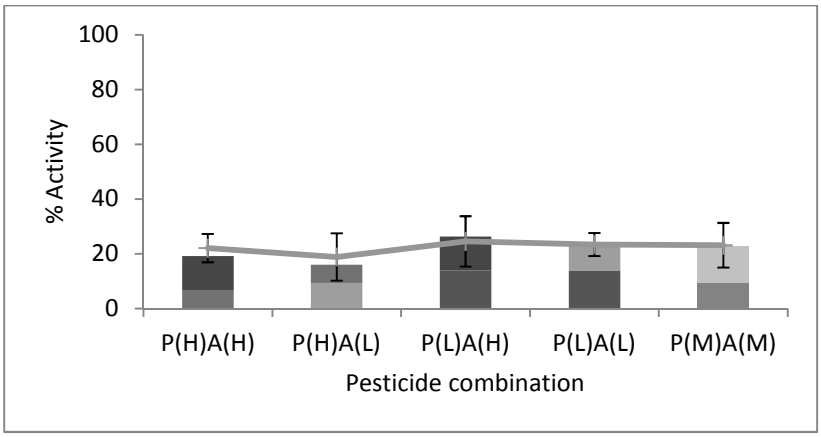
Figures 26 to 28 illustrate the effects of mixtures of pesticides on AChE. The graphs display the theoretical sum of inhibition by the individual pesticides (shown by the bar graph) as well as the actual observed inhibition (shown by the line graph).



A



B



C

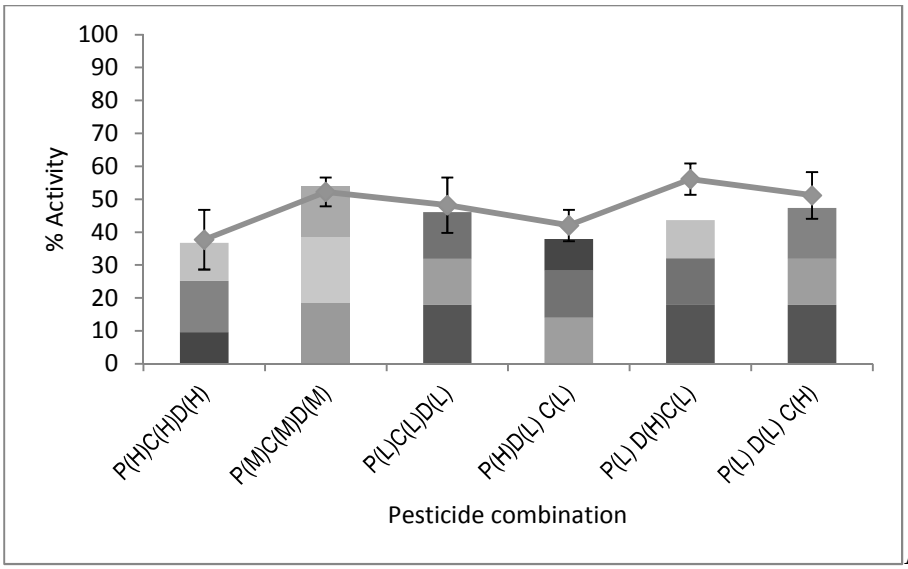
Figure 26 (A-C). Graphs showing residual AChE activity after inhibition by 2 pesticides at low (L), medium (M) and high (H) with total concentration below 0.1 µg/L at all times. Pesticides are denoted P = parathion, D = demeton-S-methyl, A = aldicarb, C = carbaryl, Cf = carbofuran. The bar graph indicates the predicted theoretical summation of the individual inhibitions and the line graph represents the actual inhibition in the presence of the pesticide mixture. Values represent the mean ±SD (n=3).

The binary mixtures in Figure 26A all displayed a combined inhibition of between 50% and 65%. Mixtures denoted by D(H)C(H), D(M)C(M),D(H)C(L) all demonstrate that the predicted values were slightly less than the actual observed values. For the binary mixtures D(L)C(H), D(L)C(L), the observed inhibition was slightly less than predicted. It can be observed that all these differences were within the standard deviation range.

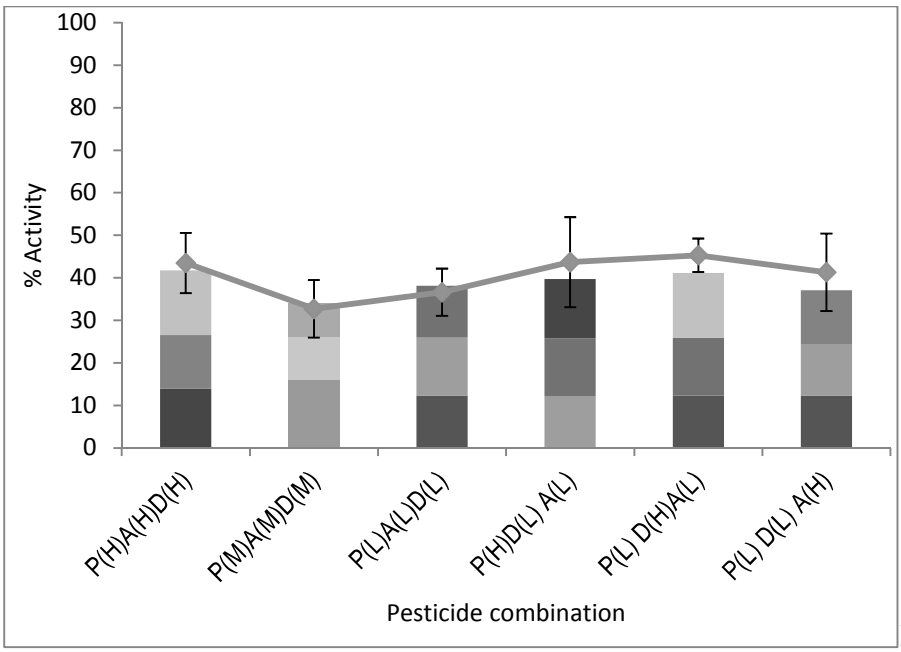
In Figure 26B the mixture P(H)Cf(H) was the only sample where the observed inhibition was equal to the predicted inhibition. For P(L)Cf(H) the predicted inhibition was greater than the observed inhibition whilst the remainder all displayed lower predicted inhibition than the observed. All the differences were within the standard deviation range and therefore not considered significant enough. Residual activity levels were between 20% and 30%.

In Figure 26C the predicted and observed inhibitory effects were equal with the exception of P(H)A(H) and P(H)A(L). Residual AChE activity was between 15% and 25%.

In Figures 27A and 27B, when all pesticide concentration levels were equal, the predicted and observed inhibition levels were also equal. When differing concentrations were used it was noted that the observed inhibition was greater than the predicted inhibition. Some of the results did not fall within the deviation range, but inhibition was within 35% and 55%.



A

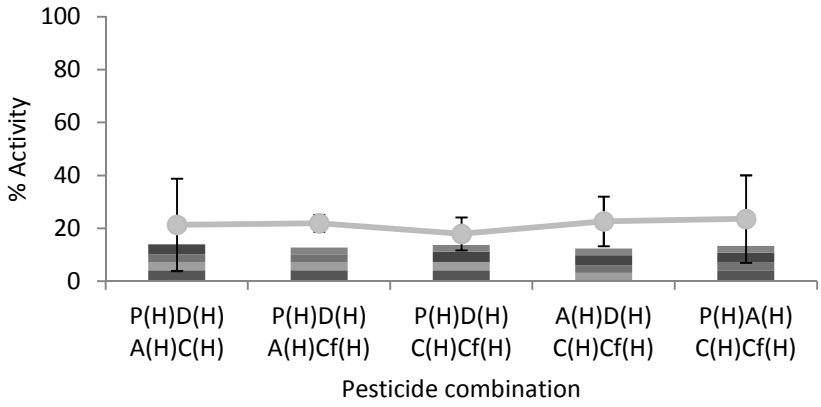


B

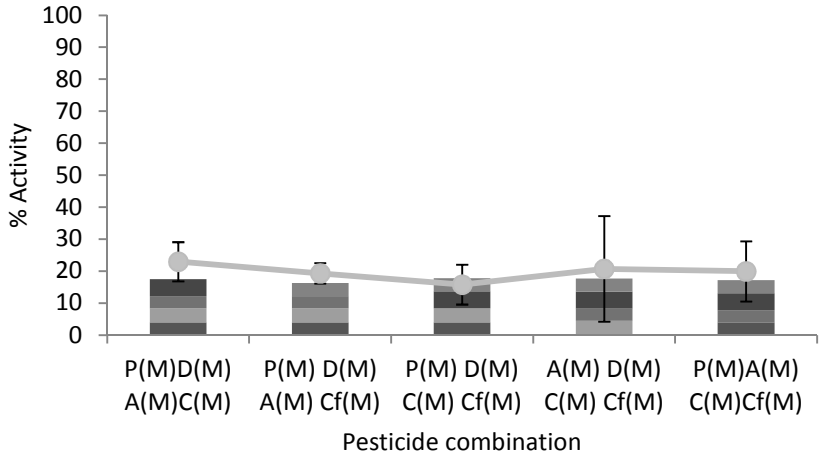
Figure 276(A-B): Graphs showing residual AChE activity after inhibition by combinations of three pesticides at varying concentrations of 0.1 µg/L (H), $1x10^{-3}$ µg/L (M), $1x10^{-7}$ µg/L (L). Pesticides are denoted P = parathion, D = demeton-S-methyl, A = aldicarb, C = carbaryl, Cf = carbofuran. The bar graph indicates the predicted theoretical summation of the individual inhibitions and the line graph represents the actual inhibition in the presence of the pesticide mixture. Values represent the mean ±SD (n=3).

Figure 28A results indicated that all the pesticide mixtures (combinations of four), resulted in approximately 15-20% residual AChE activity. The observed inhibition levels were greater than those predicted for all samples. All results did fall within the deviation range with the exception of one mixture P(H)D(H)A(H)Cf(H). In Figures 28B and 28C activity levels were also noted to be around 15-

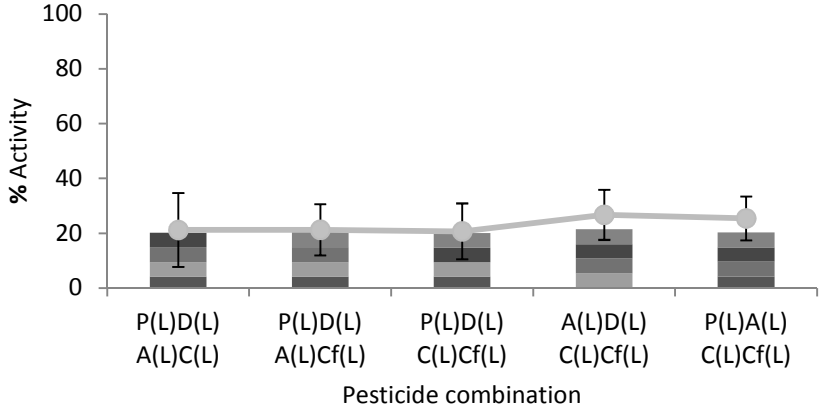
20% but the predicted and observed inhibition levels were more or less equal for the majority of the mixtures or within standard deviation for the remaining samples.



A



B



C

Figure 28: Graphs showing residual AChE activity after inhibition by combinations of four pesticides at concentrations of 0.1 µg/L (A), $1x10^{-3}$ µg/L (B), $1x10^{-7}$ µg/L (C). Pesticides are denoted P = parathion, D = demeton-S-methyl, A = aldicarb, C = carbaryl, Cf = carbofuran. The bar graph indicates the predicted theoretical summation of the individual inhibitions and the line graph represents the actual inhibition in the presence of the pesticide mixture. Values represent the mean ±SD (n=3).

The general trend noted for pesticide mixtures was an additive effect. There were some deviations from this result (examples being Figures 26-28). These deviations did, however, fall within the standard deviation for the data and were therefore not statistically significant. Figure 28A displayed a less than additive inhibitory effect. According to Kok and Hasirci (2004), competition may take place between pesticides at higher concentrations and this would lower the inhibitory effect. This is termed antagonistic interaction and may account for the results observed in Figure 28A.

3.2.2 Effect of Increased Pesticide Concentration on Activity and Variability in Results

The effect of varying the enzyme concentration on assay reproducibility and sensitivity was also investigated. Figures 29 to 31 display the results for aldicarb, demeton-S-methyl as well three pesticide mixtures at 0.2 units, 0.5 units and 1 unit of enzyme.

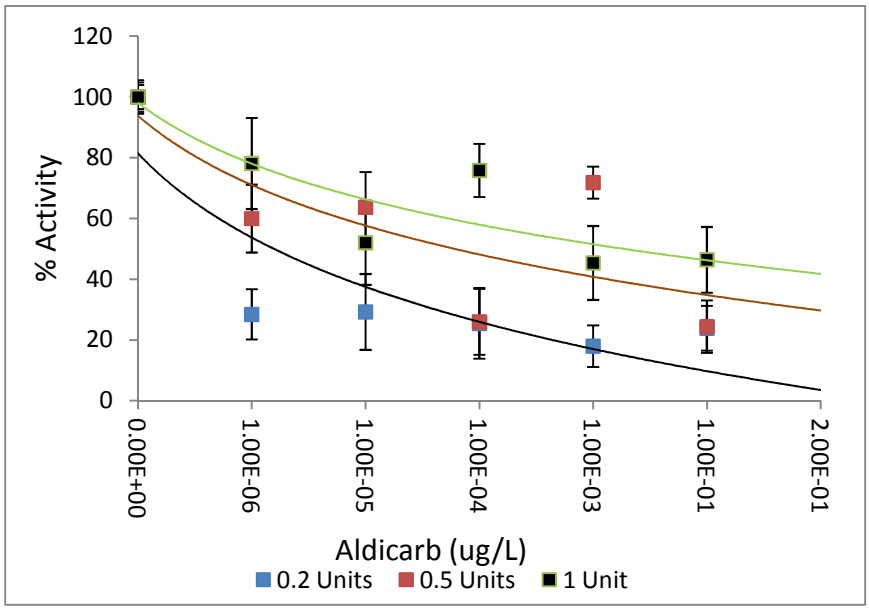


Figure 29: Graph indicating the effect of increased enzyme concentration to 0.2 Units, 0.5 Units and 1 Unit at varying concentrations of aldicarb.

In the case of aldicarb, Figure 29 indicated that an increase in enzyme concentration reduced the degree of inhibition observed, although this was not the case with demeton-S-methyl (Figure 30). At all enzyme concentrations tested, there was an decrease in enzyme activity up to concentrations of 1 x 10^{-4} µg/L. Thereafter, activity levels began to increase up to concentrations of 0.1 µg/L. These results indicated that for demeton-S-methyl, the enzyme appeared to recover from the inhibition by the pesticides at all units of enzyme activity tested. In addition, the standard deviations did not decrease

with increasing enzyme concentration in the case of aldicarb, but some decrease was noted for demeton-S-methyl.

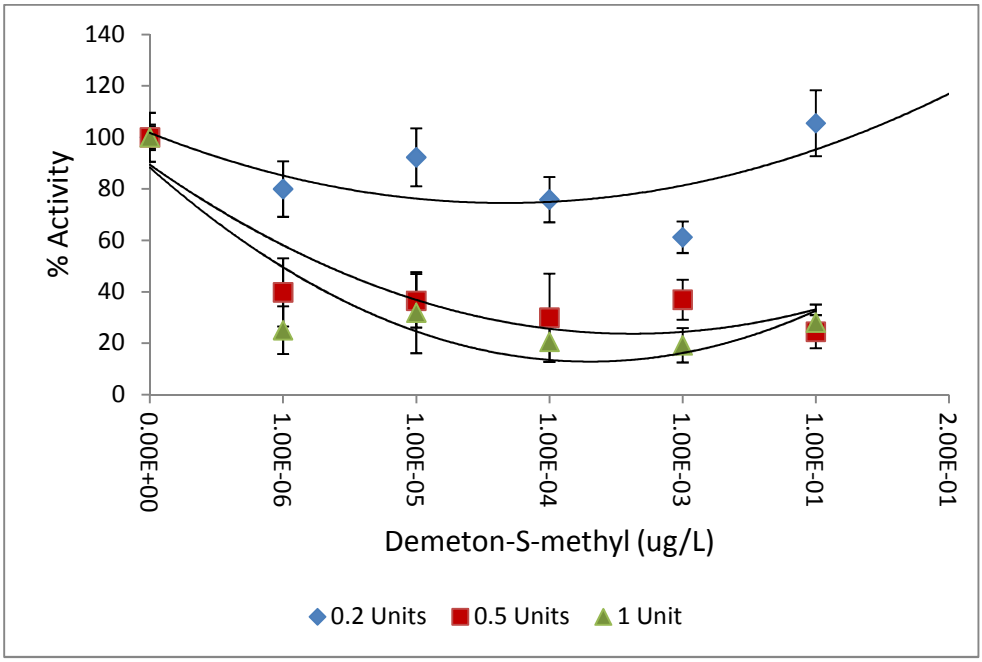


Figure 300: Graph indicating the effect of increased enzyme concentration to 0.2 Units, 0.5 Units and 1 Unit at varying concentrations of demeton-S-methyl.

Analysis of the results from Figure 31 indicated that for different pesticide combinations, an increase in pesticide concentration resulted in a corresponding decrease in AChE activity at 0.2 units as well as at 0.5 units of enzyme. However, this was not the observed response for 1 unit of enzyme. For 1 unit of enzyme, the largest degree of inhibition was observed at medium concentrations (D(M)A(M)Cf(M)), and the least inhibition at high pesticide concentration ((D(H)A(H)Cf(H)), this result was unexpected. The highest degree of inhibition was expected to occur at the highest concentrations of each pesticide.

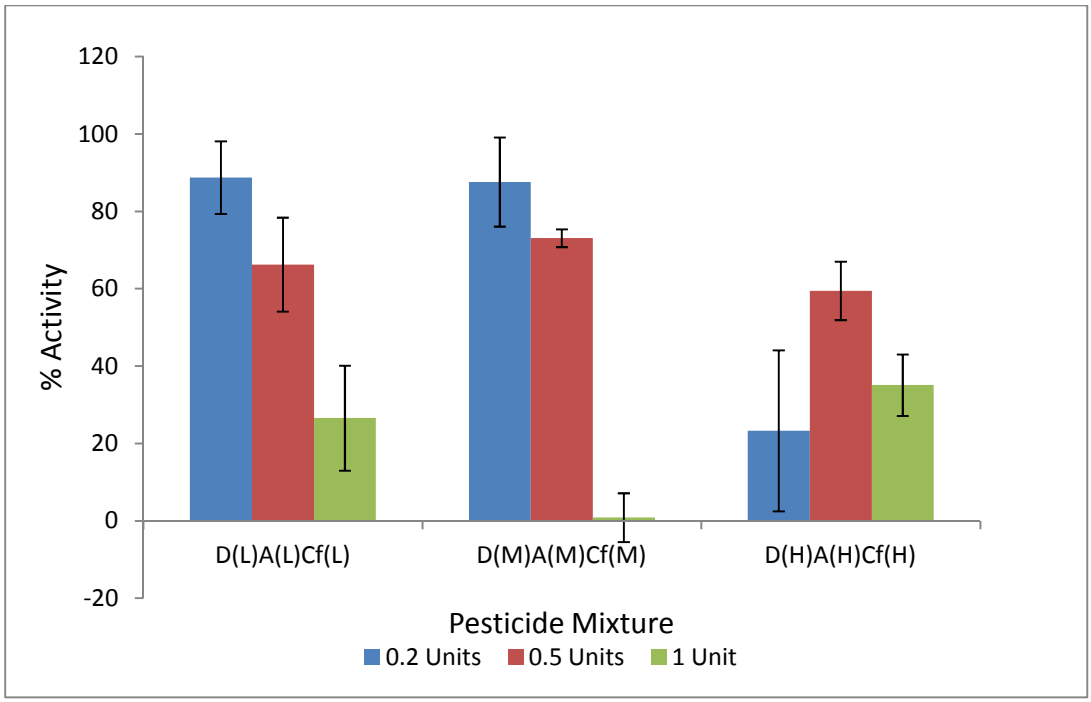


Figure 31: Bar graph indicating the effects of varying enzyme concentration (0.2 Units, 0.5 Units and 1 Unit) after inhibition by combinations of three pesticides at concentrations of 0.1 µg/L (H), $1x10^{-3}$ µg/L (M), $1x10^{-7}$ µg/L (L). Pesticides are denoted D = demeton-S-methyl, A = aldicarb, and Cf = carbofuran. Values represent the mean ±SD (n=3).

3.3 Chemometric analysis

Chemometric analysis was carried out using a neural network. Activation using toxinsnet on an input pattern, p, was obtained by simulating input patterns using the MATLAB function *sim*. A program called *toxins_test* was written which performed this task for all input patterns. Some output from this script was as follows (Figure 32).

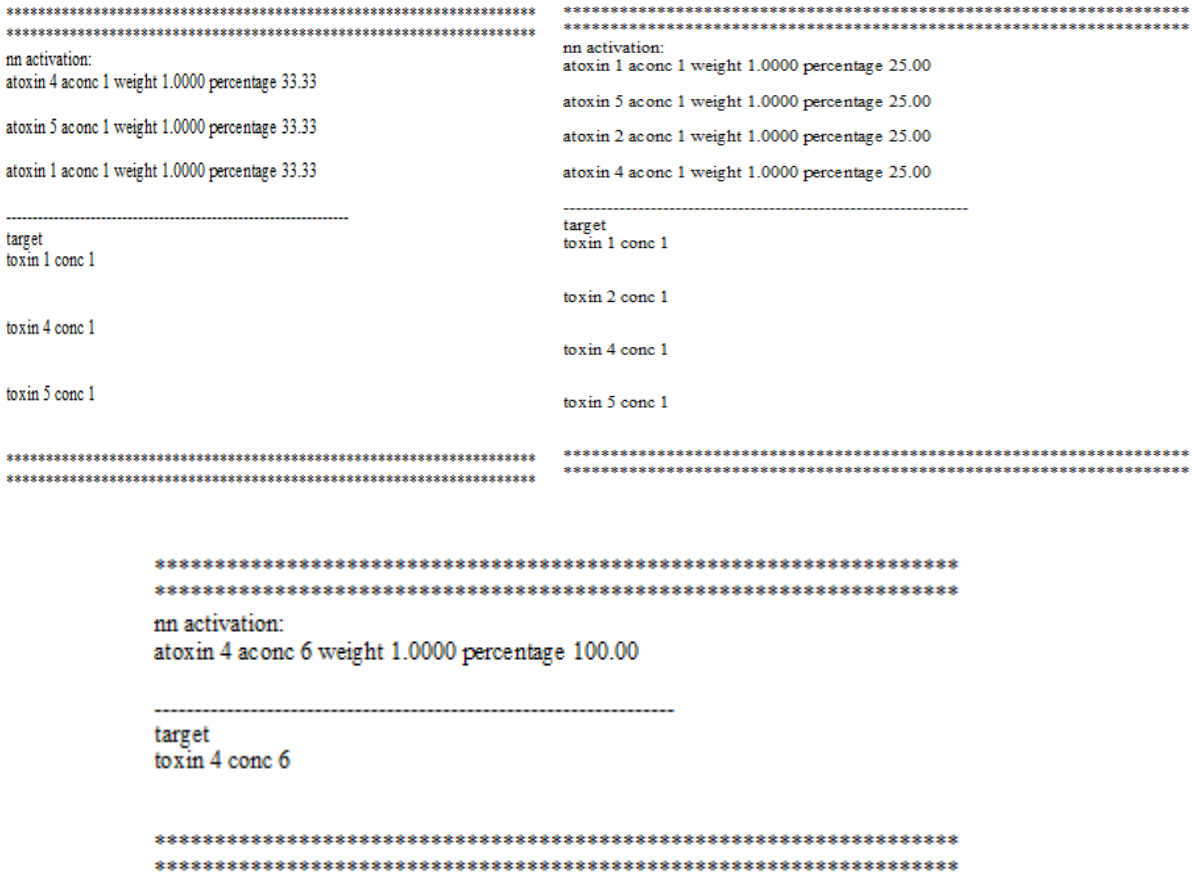


Figure 32: Three examples of correct sample output from the neural network after simulation of input data. It indicates the samples predicted as likely being present (labelled as activation) as well as the actual pesticides that were in the sample data (labelled as target).

The neural network was able to correctly identify mixtures of pesticides. Figure 32 demonstrates the ANN correctly identifying mixtures of one, three and four pesticides after being trained. The data was also perturbed and neural network tested to assess how well it dealt with data that deviated from the norm. The deviation was input as a percentage and the neural network was able to successfully determine the pesticides present up to 5% deviations. Figure 33 demonstrates some of the errors made by the neural network.

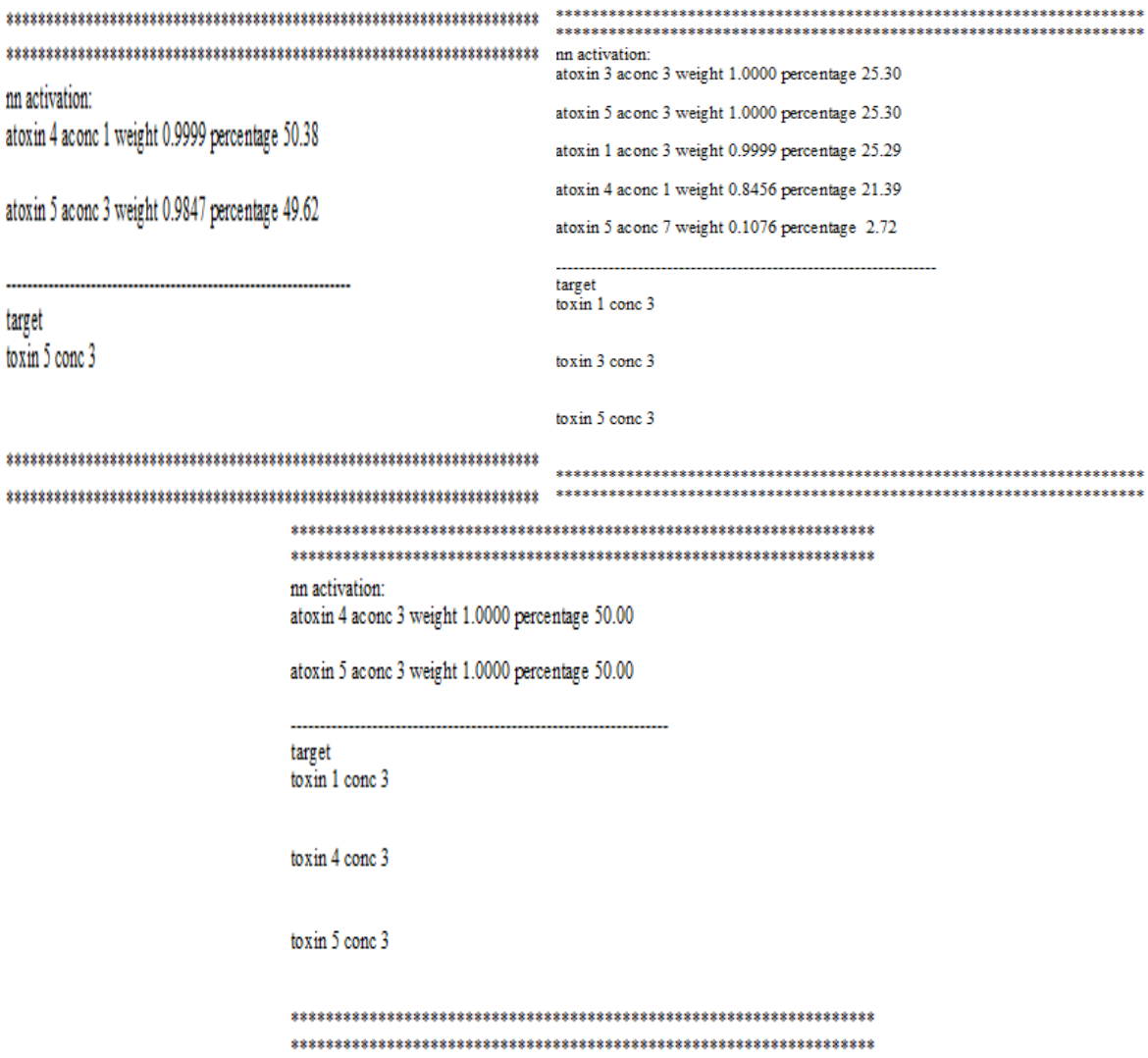


Figure 33: Three examples of incorrect sample output from the neural network after simulation of input data. It indicates the samples predicted as likely being present (labelled as activation) as well as the actual pesticides that were in the sample data (labelled as target).

On the top left hand side of Figure 33, the ANN identified pesticide four at concentration one as being present in the mixture which was incorrect. A similar error was made as can be seen in the output located at the top right hand corner where pesticides four at concentration one and pesticide five at concentration seven were also indicated as present in the mixture which was incorrect. An omission was also made of pesticide one at concentration three in the output at the bottom of Figure 33.

3.4 Bio-Assays

3.4.1 YES Assay

The results from the YES assay are recorded in Figure 34. Shown below are the absorbance readings obtained from each pesticide and this has been compared relative to the standard 17ß-estradiol (E_2). As can be observed, all the pesticides appeared to have a constant adjusted absorbance of approximately 1.4. This indicated that none of the pesticides tested compared significantly at any concentration to the standard.

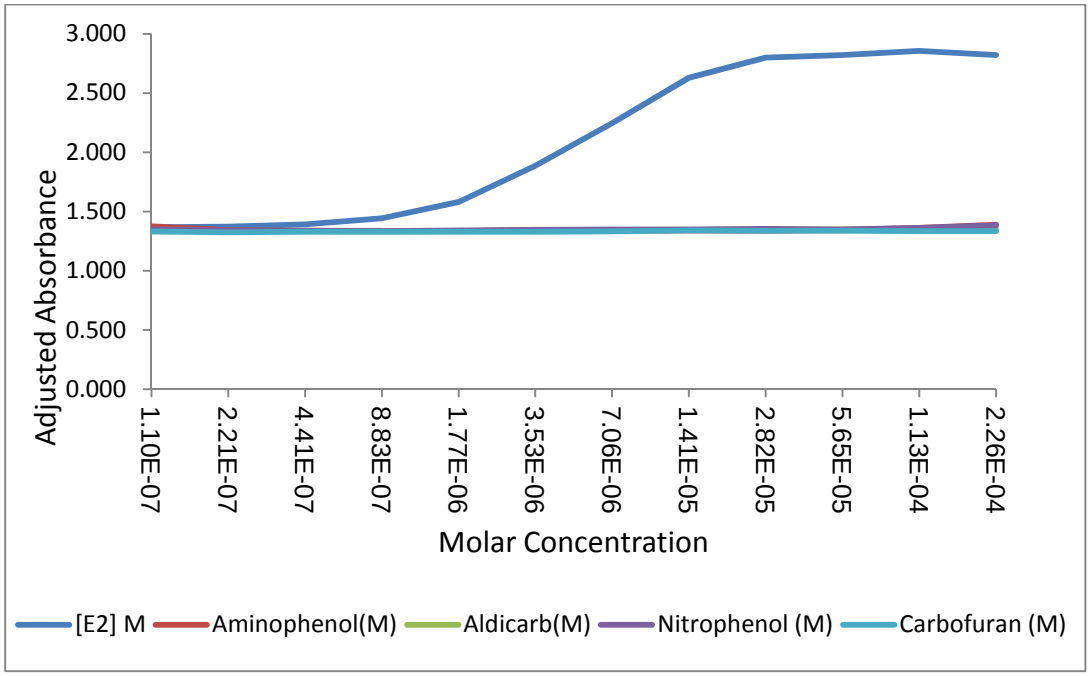


Figure 34: Graph showing estrogenic response of aminophenol, aldicarb, nitrophenol and carbofuran pesticides relative to the standard 17ß-estradiol (E_2) at varying concentrations.

3.4.2 Other Bioassays

The results from the other two assays are shown in the Figures 35 and 36. The T47D-KBluc assay confirmed that none of the samples tested had an estrogenic response. The MDA-kb2 assay also indicated that none of the samples elicited an androgenic or glucocorticoid response.

3.4.2.1 MDA-kb2 Assay

All the pesticides tested exhibited fold inductions of approximately one (1). This comparison was made with the standard dihydrotestosterone (DHT). A sample is considered to have a response if the fold induction reaches a minimum of 2 and this was not observed in any of the samples.

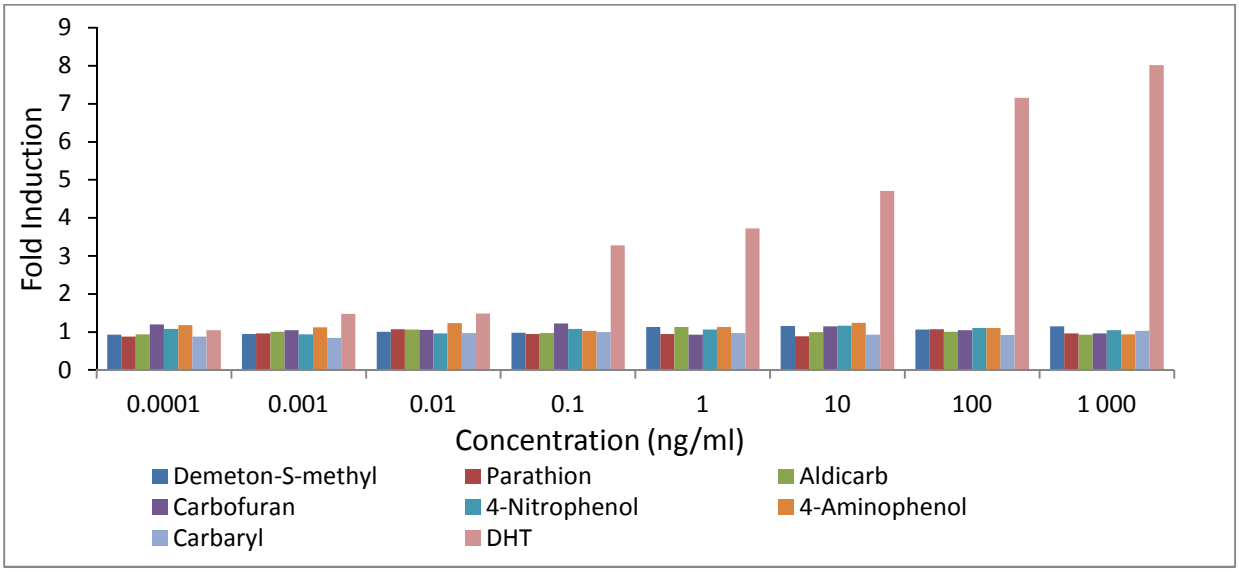


Figure 35: Graph showing fold induction response of aminophenol, aldicarb, 4-nitrophenol, carbaryl, parathion, demeton-S-methyl and carbofuran pesticides relative to the standard dihydrotestosterone (DHT) at varying concentrations.

3.4.2.2 T47D-KBluc Assay

Shown in Figure 36 are the fold induction values for all the pesticides tested as compared to the standard 17-β-estradiol (E_2). As can be observed at very low concentrations, E_2 exhibited an induction fold of 5.9. The induction values for the remaining pesticides were all in the range of 1 ± 0.3, which are not considered significant enough to have produced a dose response.

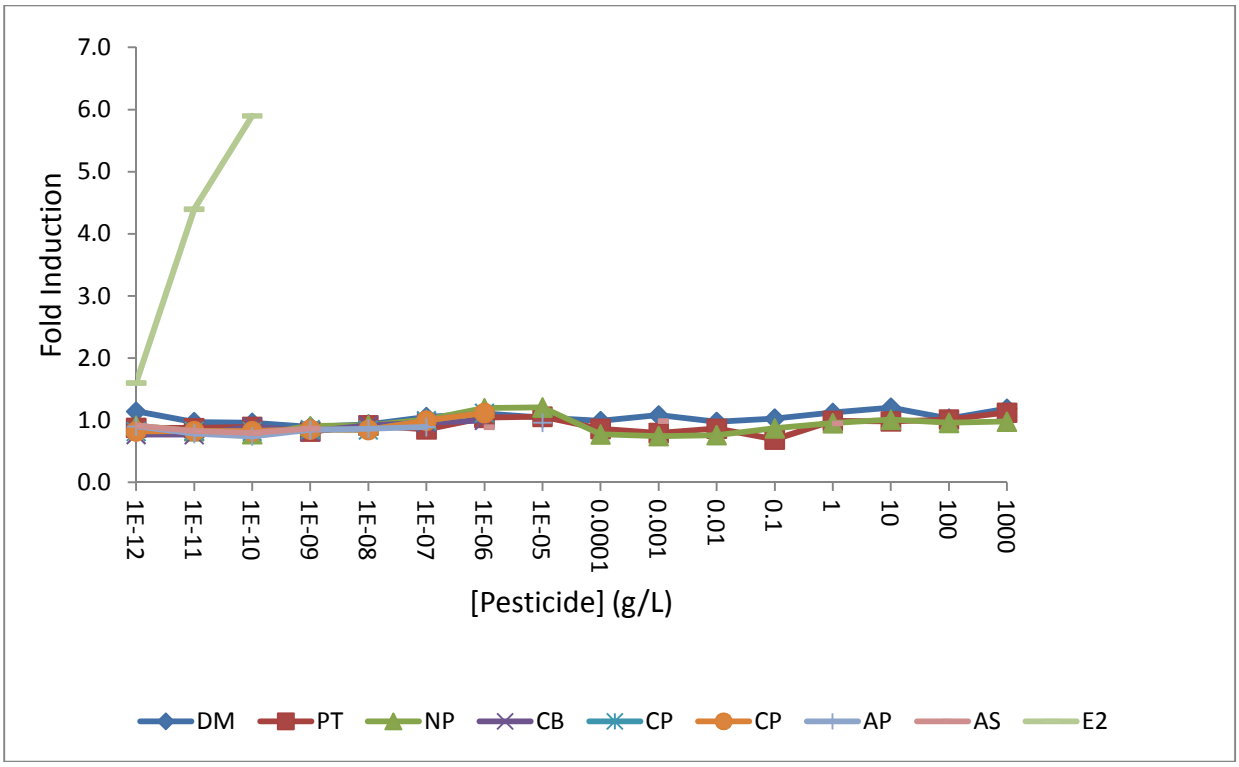


Figure 36: Graph showing the fold induction response of 17-β estradiol denoted as E2, aminophenol denoted as AP, aldicarb denoted as AS, nitrophenol denoted as NP, carbaryl denoted as CB, parathion denoted as PT, demeton-S-methyl denoted as DM and carbofuran denoted as CP. The effect of the pesticides and E2 were investigated at varying concentrations.

3.5 Environmental Sampling

Table 7 represents a tabulated summary of the data obtained from the first set of environmental samples from water sources around the Eastern Cape. Figure 37 displays this data in graphical form. Each of the samples is presented relative to the uninhibited reaction, shown as "no pesticide" in Figure 37. Inhibition was indicated to have occurred in instances where AChE activity was lower in the water sample than the uninhibited reaction.

Table 7: Tabulated data of gradients indicating inhibition of AChE by each water sample, used to calculate total activity relative to the reaction without any inhibition. %SD represents percentage standard deviation (n=4). *Represents points before and after the disposal of sewerage into the water sources.

Water Sample	Gradient	% Activity	%SD
No Pesticide	0.1549	100.0	9.4
Port Elizabeth Tap Water	0.122	78.7	25.6
Colchester Tap Water	0.0449	28.9	16.9
Port Alfred Marina	0.1145	73.9	13.9
Swartkops Bluewater Bay	0.1981	127.8	23.3
Sundays N2 Bridge	0.1346	86.8	18.0
Port Alfred Centenary Way before Sewerage*	0.1015	65.5	22.7
Port Alfred Centenary Way After Sewerage *	0.1019	65.7	21.8
Kariega Before Bridge	0.1782	115.0	24.1
Swartkops Grahamstown Bridge	0.0804	0.5	14.2
Blaauwkrantz River Grahamstown	0.0529	34.1	16.2
Grahamstown Tap Water	0.0798	51.5	11.8

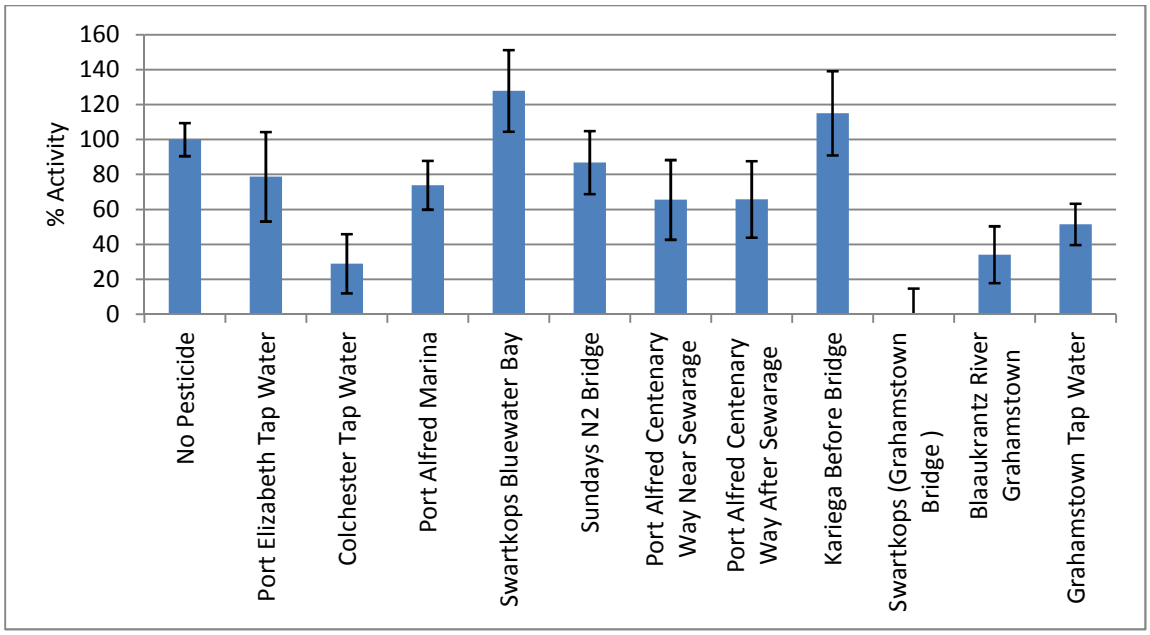


Figure 37: Graphical representation of results from first set of water samples taken from various locations in the Eastern Cape, displaying the degree of AChE inhibition. The % activity is based on the reaction rate (gradients) for each sample relative to the uninhibited reaction (No Pesticide). Data points represent the mean ± SD (n=3).

A second set of water sampling results is shown summarised in Table 8 and Figure 38. These samples were also sent for analysis to the South African Bureau of Standards (SABS) laboratories and the results are attached as Appendix 2. The results indicated that there were residual AChE activity of 17.96%, 36.53%, 32.77%, 33.25%, 25.61% and 27.18% for Kariega River, Sundays River, Colchester tap water, Swartkops River sampled at Amsterdam Way and Grahamstown Bridge as well as Blaaukrantz River in Grahamstown, respectively. Results from the SABS laboratories stated that there were no pesticides present. Enzyme inhibition could have been as a result of the presence of metals or the high sensitivity of the AChE assay.

Table 8: Tabulated data of gradients indicating inhibition of AChE by each water sample, used to calculate total activity relative to the reaction without any inhibition for the second set of water samples.

	Gradient	% Activity	%SD
No Pesticide	0.0824	100	0.0
KariegaBefore Bridge	0.0148	17.96	2.79
Sundays N2 Bridge	0.0301	36.53	2.26
Colchester Tap Water	0.027	32.77	1.49
Swartkops (Amsterdam Way)	0.0274	33.25	1.38
Swartkops (Grahamstown Bridge)	0.0211	25.61	1.84
Blaauwkrantz River Grahamstown	0.0224	27.18	0.45

The results obtained varied substantially from those obtained in the initial testing of the water samples as can be observed in Figures 37 and 38, as well as having reduced standard deviations.

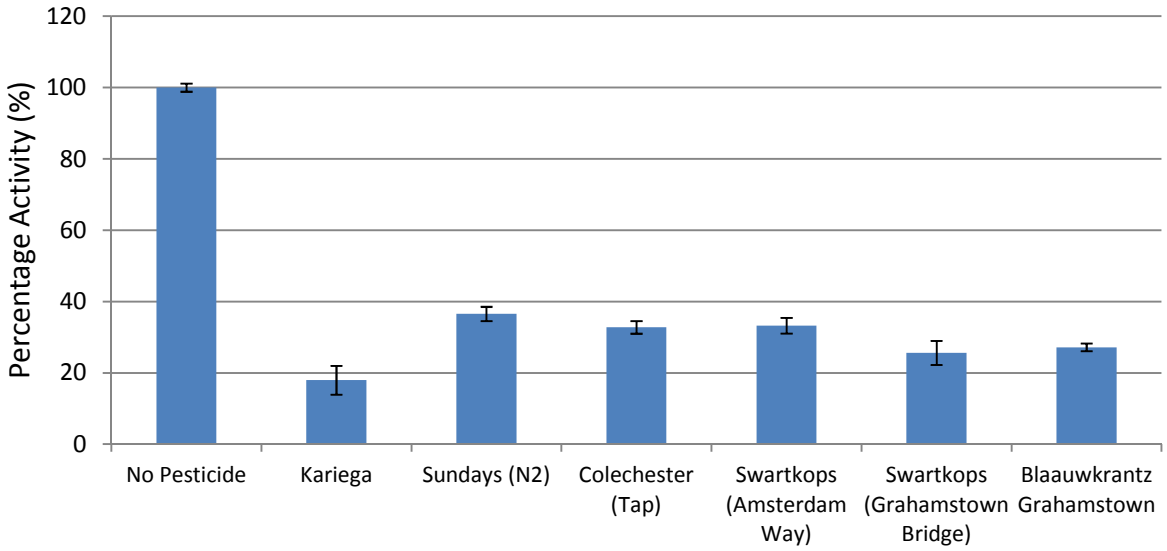


Figure 38: Graphical representation of results from second set of water samples taken from various locations in the Eastern Cape, displaying the degree of AChE inhibition. The % activity is based on the reaction rate (gradients) for each sample relative to the uninhibited reaction (No Pesticide). Data points represent the ± SD (n=4).

CHAPTER 4 - Discussion

4. Discussion

4.1. Individual Pesticides

Optimization of the micro-Ellman assay was undertaken in this study. High sensitivity levels were ensured by the selection of AChE from electric eel, low concentrations of enzyme and a pre-incubation time of enzyme with pesticide of fifteen minutes. Free enzyme was utilised as this allowed for lower detection limits as compared to immobilized enzymes, such as those used in biosensors (Van Dyk and Pletschke, 2011).

The effect of individual pesticides on AChE was tested in this study and the majority of the results indicated that the pesticides inhibited AChE in a linear manner until certain threshold concentrations were reached. Table 6 displays the inhibition of AChE, and therefore the potency of each pesticide towards the enzyme. The potency varied between the different pesticides with demeton-S-methyl being the most potent followed by carbaryl, parathion, aldicarb and carbofuran being the least potent. It is generally known that pesticides containing a P=O moiety (OPs such as demeton) are far more inhibitory than OPs containing a P=S moiety (such as parathion) (Fukuto, 1990; Marty et al., 1995). Demeton-S-methyl would therefore be expected to be more inhibitory than parathion which is confirmed by our results (see structures in Figure 5). The CPs tested were found to have a low to intermediate inhibitory effect on AChE. This can be explained as a result of CPs displaying a lower degree of irreversible inhibition of AChE than OPs. Fukuto (1990) explained that cholinesterases, which have been inhibited by CPs through carbamylation, are spontaneously regenerated and thus the inhibition can be reversed within relatively short periods of time (Fukuto, 1990). The chemical structure of the specific pesticide dictates the degree of inhibitory effect noted (Fukuto, 1990). As indicated by Fukuto (1990), the affinity of OPs for the AChE is dictated by the reactivity of the phosphorus, while the affinity of CPs is dictated by their steric fit into the active site. Enzyme sources, as well as the specific enzyme, have all demonstrated an effect on the variation in inhibitor response (Marques et al., 2004). According to studies by Marques et al. (2004), AChE purified *Drosophila melanogaster* had a much higher sensitivity as compared to that purified from *Electrophorus electricus* and bovine erythrocytes. Several mutations were also performed

that were able to increase the sensitivity of AChE from *Drosophila melanogaster*. This study made use of AChE from *Electrophorus electricus* due to its commercial availability.

Current data on individual pesticides indicates that there may be variation in the toxicity to different animals such as rats, mice, chickens, fish and various insect such as bees. LD_{50} is defined as the dose that is required to kill 50% of the given population and is used as a means of comparison across studies. These studies; however, displayed great variations with regard to the results observed. For example, some studies indicated that the LD_{50} for rats, given carbaryl orally, ranged between 500 mg/kg and 600 mg/kg by weight (WHO Report, 1986), 250 mg/kg (Budavari, 1996) and 230 to 850 mg/kg (Hayes and Laws, 1991). Results from these *in vivo* studies show considerable variations and this could lead to questionable conclusions. *In vitro* tests could therefore be considered an alternative method of comparison. Standardization of *in vitro* tests is much more simplified and data collection would be much simpler for a larger number of pesticides.

A study by Iyaniwura (1989) presented *in vitro* data for aldicarb, carbofuran and oxamyl using the inhibition of rat plasma cholinesterase. Iyaniwura (1989) found that the concentrations that produced 25% and 50% inhibition of cholinesterase (IC_{25} and IC_{50}) were, respectively, 1 x 10^{-8}M and 5 x 10^{-7}M for aldicarb, 1 x10^{-9}M and 1.5 x 10^{-7}M for carbofuran, 5 x 10^{-11}M and 5 x 10^{-8}M for oxamyl. The study also demonstrated that, for a mixture consisting of equal proportions of all three carbamates, the IC_{25} and IC_{50} values were 4 x 10^{-11}M and 6.5 x 10^{-8}M, respectively. Results from this study (data not shown) indicated that the IC_{25} for aldicarb was 5.25 x 10^{-11}M. This result may not be identical to that reported by Iyaniwura (1989); however, comparison would be simpler as there is no need to account for other factors such as the weight of the organism and any other *in vivo* processes that may affect inhibition. Details of enzyme source and activity levels would also be required for meaningful comparison. Due to the fact that the test concentrations were below those required by regulation and that the study conducted by Iyaniwura (1989) included only three pesticides, no further comparison can be made. However, these results indicated that the use of an *in vitro* IC value should be considered for initial research purposes before further investigations can be carried out on the effect of these pesticides *in vivo*.

Carbofuran was the exception to the general trend of inhibition as was observed in Figure 24. AChE activity appeared to be stimulated at certain concentrations of carbofuran, a phenomenon referred to by scientists as "hormesis". Hormesis has been defined as a dose response occurrence characterized by stimulation of an enzyme at low concentrations of a toxin (i.e. pesticide) and inhibition at high concentrations (Calabrese and Baldwin, 2001). Although the mechanism is not well understood, it has been hypothesized that the stimulatory response arises as a result of overcompensation to an initial change or disruption in the natural equilibrium state or homeostasis (Calabrese and Baldwin, 2001). At higher concentrations of toxin, there is thought to be insufficient compensation and hence inhibition is observed. Calabrese and Blain (2004) indicated in their study that 7% of all the hormetic responses analysed were observed in the category of OPs and pesticides. It can therefore be hypothesized that carbofuran may have a similar effect on the AChE. This trend is still greatly disputed but is finding greater acceptance in the science field.

In addition, the study conducted by Calabrese and Blain (2004) on hormesis also stated that the stimulation range, which is typically quite narrow, falls within a factor of 10 from the no observed adverse effect level (NOAEL), but can be as large as 1000 fold, which accounts for the effects observed in the case of carbofuran (Calabrese and Blain, 2004). The effect of carbofuran at concentrations greater than the NOAEL would need to be studied further and the concentrations at which it can inhibit AChE determined. As yet, there are no strict rules to determine which chemicals will exhibit hormesis properties, but this may be an important consideration when identifying which pesticides are labelled as dangerous and also the effect this has in terms of mixtures of pesticides.

Another observed result that requires further investigation is the plateau effect observed with parathion and demeton-S-methyl (see Figures 22 and 23). Tahara et al. (2005) also observed similar behaviour with some of the pesticides they tested but did not discuss the possible causes for this effect. This phenomenon may have an important impact on the effect of pesticide mixtures on AChE inhibition. Consideration needs to be made of the non-linear behaviour of individual pesticides as this would have an impact on whether an additive effect would be observed at all inhibitor and enzyme concentrations.

Studies by Kok and Hasirci (2004) demonstrated that, at concentrations between 1 ppb and 50 ppb (or 1 x 10^{-6} g/L and 5 x 10^{-5} g/L), the effect of varying pre-incubation times did not have a significant effect on the degree of inhibition after ten minutes up to thirty minutes. Allowing for an increased incubation time would ensure that the pesticides had interacted sufficiently with AChE, and that this response could be noted by an increase in inhibition. However a balance needs to be maintained with regards to longer pre-incubation times. CPs are known to be reversible inhibitors and a reversal of inhibition may impact on the response of AChE. Therefore incubation time is an important consideration when trying to determine the degree to which a pesticide inhibits AChE irrespective of its concentration. Optimisation studies resulted in the selection of a pre-incubation time of fifteen minutes in this study.

However, the main problem associated with testing individual pesticides (as explained previously) is that generally humans and animals are exposed to multiple pesticides. This is as a result of mixing of pesticides in an attempt to protect crops, increase crop yields and therefore economic returns. As noted earlier, there has been very little research into exactly how these mixtures of pesticides affect the health of humans and animals. The next section of the study shows some of these effects and their health implications for humans and animals.

4.2. Pesticide Mixtures

Several studies have investigated the *in vivo* and *in vitro* effects of inhibition of pesticide mixtures. Rats, fish and other marine animals have been used for *in vivo* studies. Karanth et al. (2004) investigated sequential and simultaneous exposure to two OPs and were able to deduce that sequential exposure resulted in higher levels of inhibition. Gennings et al. (2004) observed in rats that with 5 OPs there was a synergistic effect. One of the five OPs under consideration was malathion; however, when it was removed from the mixture, the effect was found to be additive. Therefore, malathion was found to potentiate the inhibitory effects of other pesticides. *In vivo* studies are able to evaluate the total toxicological effect on the organism and not just the specific enzyme being targeted. This total evaluation is advantageous to assess how an organism responds as a whole; however, when trying to determine the individual interactions between pesticides, the inconsistencies in the results (as Richardson et al., 2001, has pointed out) become a cause for concern.

In vitro studies carried out by Richardson et al. (2001) made use of AChE extracted from the brain and serum. Using the definition of dose additive as an additive effect resulting from the same mode of action, as in the case of OPs, they were able to determine that binary mixtures resulted in an additive effect if added simultaneously and higher than additive effect if added sequentially. It was also pointed out that, even though exposure may be simultaneous, due to the varying affinity of AChE for individual pesticides, it would be expected that the pesticide with the greatest affinity would preferentially inhibit AChE. This could result in a less than additive response which could be amplified when exposure was sequential. Tahara et al. (2005) also investigated the effect of 15 different OPs in combinations of 2 and 3 using pseudocholinesterase. The results indicated that, in the majority of cases, the inhibitory effect was additive, although a few cases resulted in a less than additive effect. Bocquene et al. (1995) used the crude homogenates from marine animals to test binary mixtures of OPs. Results demonstrated a synergistic effect and they concluded that the synergistic effect was greater when a CP and OP where combined, as opposed to using combinations of only OPs or only CPs.

Consideration of these studies brings to the fore the inconsistencies in results. Richardson et al. (2001) and Tahara et al. (2005) found additive results whereas Kok and Hasirci (2004) found less than additive effect when competition was possible, and Bocquene et al. (1995) found a greater than additive effect. From these studies only Bocquene et al. (1995) used combinations of OPs and CPs, whilst other researchers looked at mixtures of only OPs or CPs. This indicates that studying the effects of pesticide mixtures is extremely complex and thus far there are no clear patterns which can be used to determine the effects of pesticide mixtures. In addition, several factors may affect the comparison of results.

The sources of enzymes present a difficulty when trying to compare results as these differ across experiments. It has been shown that different sources of enzymes have different sensitivity and as a result will have different activity levels (Marques et al., 2004). Some researchers even employed the use of genetically modified AChE, optimized for detection of pesticides (Villatte et al., 1998). Whilst researching the effects of pesticide mixtures it was noted that enzyme activity varied substantially per batch and methods of standardization had to be employed to account for this. **In this study the AChE enzyme activity levels were always tested prior to the inhibition studies to ensure that basal AChE activity (i.e. AChE activity**

in the absence of any pesticide) was consistently at the same level. Enzyme activity levels are a key factor for consideration in any inhibition studies.

The pesticides themselves, despite being classified in the same group, differ in structure and thus have an effect on function. This indicates the need for further research into the interactions between pesticides. In an interaction-based study by Iyaniwura (1989), it was found that for the mixture of all three carbamates, aldicarb was found to be potentiated by carbofuran and oxamyl. The effects of carbofuran were also potentiated by aldicarb and oxamyl, but the presence of carbofuran and aldicarb was found to reduce cholinesterase inhibition by oxamyl. The results observed in our study do not appear to support these findings with regard to aldicarb and carbofuran; however, the presence of the third pesticide oxamyl may explain the differences in results. This, however, brings to the fore the importance of an extensive assessment of pesticides for any interactions that may take place.

The results from this section indicated that enzyme concentration will play a role in the results obtained with regard to IC levels, as well as the effect that mixtures will have on the enzyme. In these tests, the pesticide concentrations and substrate concentrations were kept constant as enzyme concentration was varied. As was observed, the IC_{20} levels for the individual pesticides shifted to the right; this represented an increase in inhibitor concentration and therefore lower sensitivity. These results are in line with known enzyme kinetic data. As the enzyme concentration increased, the ratio of enzyme to pesticide changed accordingly. There would be fewer pesticide molecules and more enzyme molecules available to participate in the reaction and this would increase the reaction rate. This was observed in the case of using 0.5 units and 0.2 units of pesticide for the mixtures. However, in the case of 1 unit in the presence of individual pesticides, there was a deviation from the normal trend. This deviation cannot currently be explained on the basis of classical enzyme kinetics.

At low concentrations of enzyme, minor changes in absorbance became more significant than at higher concentrations of enzyme. With a decrease in sensitivity, results were expected to show a marked reduction in variability, which should be evident as a decrease in standard deviations. This, however, was not clearly evident in the results up to 1 unit of AChE. No further increase in enzyme concentration was considered as this would have impacted extremely negatively on

the sensitivity of the assay. These results highlight the fact that enzyme activity variability is an important contributing factor in any study of this kind.

4.3. Bioassays

A compound can be described as having endocrine disrupting effects if it produces a dose response above the detection limit of the assay. The results obtained indicated that none of the pesticides tested displayed any endocrine disrupting effects above the detection limits for the YES, T47D-KBluc and MDA-kb2 assays; which is evident in Figures 34-36. Current literature provides no data on the possible estrogenic and androgenic effects of demeton-S-methyl, despite its being labelled a possible EDC by the World Health Organization. Results from the bio-assay indicated that the pesticide demeton-S-methyl, as well as the two breakdown products tested (*p*-nitrophenol and aminophenol), have no androgenic or estrogenic effects. However, further research is necessary to substantiate this claim. Further research could include testing of these pesticides in more sensitive assays, at higher concentrations than those included in this study, as well as testing for other types of endocrine disrupting effects apart from androgenic and estrogenic effects.

The results of this study are in agreement with those of Kojima et al. (2004), Oh et al. (2007) and Nishihara et al. (2000). Nishihara et al. (2000) concluded that carbaryl, carbofuran and aldicarb displayed no estrogenic effects. Their study made use of a yeast hybrid assay with human estrogen receptor α (hERα) and a TIF2 promoter. Kojima et al. (2004), in a different study, tested a group of 200 chemicals using Chinese hamster ovary (CHO) cells that had been modified to incorporate hER receptors (α and β) and androgen receptors (AR) to test for both estrogenic and androgenic activity. Their study concluded that carbaryl and carbofuran elicited no estrogenic effects. Oh et al. (2007) reported that carbaryl had no estrogenic effects using the E-screen bio-assay with MCF-7 breast cancer cell line. The E-screen bio-assay has both hER and human progesterone receptor (hPR). These tests all achieved the same results with the use of hER (α and β) and hAR, however the tests differed in the type of cells and cell lines that were used. Our study made use of yeast cells (as did Nishihara et al., 2000), however their study used a different set of promoters and lacked the ERE sequence present in this study. The other studies used CHO cells and MCF-7 breast cancer cells in which proliferation of the cells

would indicate an estrogenic or androgenic response. As indicated earlier, none of the pesticides showed any positive response in any of these studies.

There are also some contradictory reports in literature. In the same battery of tests carried out by Kojima et al. (2004) with CHO cells, it was found that parathion exhibited anti-androgenic effects. Research by Sohoni et al. (2001) and Tamura et al. (2001) indicated that parathion should have exhibited anti-androgenic effects in the MDA-kb2 assay. This, however, was not observed in our study. The current study achieved only initial screening of pesticides for androgenic activity and full dose response curves would be required for further investigation before more conclusive results can be obtained.

Klotz et al. (1997) and Oh et al. (2007) used MCF-7 breast cancer cell lines transfected with hER gene as well and this allowed for a valid comparison of results. Klotz et al. (1997)'s results indicated that parathion, carbaryl and aldicarb could all be hER and hPR agonists. Once again, this is in disagreement with the results observed in our study. Therefore, studies have still not been able to conclusively determine whether or not carbaryl, carbofuran and aldicarb possess the ability to affect estrogenic or androgenic receptors.

A very important issue with regards to determination of ER and AR activity was raised by Klotz and co-workers (1997). Despite the fact that results may indicate a sample does not have any endocrine disrupting effects; there may still be reason to believe that the sample could behave as an EDC. This theory was based on the mechanism of action of OPs and CPs. Fukuto (1990) described the basis of inhibition as the phosphorylation or carbamylation of the serine residue in the active site. Klotz et al. (1997) theorized that the carbamate may not necessarily carbamylate the serine residue in the active site, but may act by carbamylation of residues near the hER or hPR. This would, in turn, affect the receptors, albeit indirectly. According to Fukuto (1990), this should be accounted for in testing for estrogenic and androgenic properties of samples.

As the concern over EDCs has increased, further steps have been taken to assess the extent to which pesticides affect the health of organisms. The Global Water Research Coalition (GWRC) commissioned the investigation into several bio-assays with regard to determining the feasibility of use in identifying EDCs. Among the assays considered were: the YES, T47D-KBluc, E-Screen, Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER-Calux) and MELN assays. One important point highlighted was the difficulty of standardization of tests and the difficulties that arise when making comparisons. Results from the study indicated that the ER-Calux assay was one of the best assays for testing water samples, but that the high cost, time required for processing samples and the requirement for highly trained personnel were major drawbacks. The E-Screen and YES assays were identified as the next best assays in the battery of tests. These were found to have good sensitivity and reproducibility and were deemed to be adequate alternatives to the ER-Calux assay. The T47D-KBluc was said to possibly be a good assay but required more testing to verify its feasibility. Taking into consideration these submissions, in conjunction with the results obtained, it can be concluded that the data obtained from Oh et al. (2007) and Nishara et al. (2000) may be considered the most accurate. This would imply that carbaryl, carbofuran and aldicarb do not have estrogenic effects, which is in agreement with the results obtained in our study.

4.4. Chemometrics

The identification of individual pesticides from mixtures with the aid of chemometrics has been undertaken by several researchers. Methods used for data collection included voltammetry, spectrophotometric kinetic analysis and enzymatic biosensors. Analytical methods assessed in these studies included classical least squares (CLS), principal component regression (PCR), partial least squares (PLS), Kalman filter, radial basis function artificial neural networks (RBF-ANN), back propagation artificial neural networks (BP-ANN), cluster analysis, factor analysis, multidimensional variance and discriminatory analysis (MVDA) and feed forward artificial neural networks (FF-ANN). Bachmann & Schmid (1999), as well as Bachmann et al. (2000), were able to successfully use a FF-ANN to detect individual pesticides. Different results indicated that the use of an RBF-ANN, based on the lowest prediction error and percentage recovery, was best according to Ni et al. (2004a; 2004b; 2005; 2007 and 2009). Of the studies considered, Danzer and Schwedt (1996) were able to determine the largest number of pesticides from mixtures using chemometrics. Using eleven pesticides, two metals and three different

enzymes (AChE, an acid phosphatase and an alkaline phosphatase), as well as chemometrics, Danzer and Schwedt (1996) were able to differentiate all compounds. Ni et al. (2007) were also able to distinguish two CPs from mixtures using only AChE. This study has shown the first example of data collection using only one enzyme; AChE, together with an ANN for analysis, which was able to successfully identify five pesticides (two OPs and three CPs) from mixtures.

The results showed that the FF-ANN could distinguish these pesticides quite successfully within the parameters of its training. This necessitates that, in order to use ANNs, the possible combinations of pesticides present must be known before the system can be successfully used for identification of pesticides.

There are some other considerations that could be included in future studies. The neural network was made to assist with regard to the identification of individual pesticides in a mixture, but its success depends solely on how well the system was trained. One of the main short comings associated with enzymatic assays is the interference from other compounds such as metals. Therefore in order to successfully identify pesticides, even in the presence of interfering compounds, the interfering compounds could also be incorporated into the ANN as an input.

The incorporation of interfering compounds would also aid the use of ANNs in environmental sampling. Further research would involve training of the neural network with data that included the effects of interfering compounds. Once this has been successfully achieved, environmental testing could be achieved using the ANN.

4.5. Environmental Sampling

Two sets of water sampling were carried out on the water sources as described in Section 2.5. Currently there is no data available on the presence of pesticides in water samples in the Eastern Cape for comparison. The only study that is available on the Eastern Cape was carried out by Awofolu and Fatoki (2003) and they assessed the presence of OCs only. Analysis of the first data set indicated that most of the water samples, with the exception of Swartkops

(Bluewater Bay) and Kariega (before bridge), could have pesticides present, as these samples were clearly inhibitory to AChE activity. Some of the other samples appeared to have no pesticides present as no inhibition was detected. Retesting was done for the samples that displayed minimal or excessive inhibition of AChE. The samples that displayed a moderate degree of inhibition were not retested due to cost constraints. The second data set indicated high inhibition in all of the retested samples.

The sample from Swartkops River Bluewater Bay had a percentage residual activity of 127.8 % ± 23.3%, which was higher than the assay containing no pesticide (referred to as No Pesticide in Figure 37). The reason for the increased enzyme activity of the sample was not clear. However, due to the large standard deviations obtained for this sample, an error may have occurred in the readings observed. The results obtained from the first sampling of Swartkops River Bluewater Bay sample contradicted the expected results. Due to the industrial effluent and sewerage waste that enters the river upstream, it was expected that much greater inhibition of AChE would be observed. This was not the case, however, as the enzyme appeared to have been activated rather than inhibited by contaminants in the water. The samples were collected after the incoming tide and this may have resulted in the dilution of any pesticides that were present in the river water.

The Kariega River samples displayed residual AChE activity of 115.0% ± 24.1%, which was in agreement with known data which indicated that the estuary was relatively pristine, and therefore would display minimal inhibition of AChE. The relatively high standard deviation (24.1%) may also account for the higher than expected readings. The second data set indicated AChE activity of 17.96% ±2.79% for this sample. This result was unexpected; however, the fact that the tide was outgoing at the time of sample collection may have accounted for the difference observed. Reports from a SABS certified laboratory (see Appendix 2), indicated that there were no pesticides present; however, some trace metals were found.

Results from the water samples taken from Swartkops Grahamstown Road and Colchester Tap Water displayed excessively high levels of AChE inhibition. The excessive inhibition of AChE (99.5%) in the Swartkops Grahamstown Road sample could be attributed to the extreme pollution evident in the river. It has been reported that heavy metals such as lead and cadmium inhibit the activity of AChE after incubation of AChE and the heavy metal under investigation

for several days. This was done *in vivo* by dosing fish with known concentration of metals and homogenizing the fish after 2, 7 and13 days and assessing the AChE activity in the tissue (Jebali et al., 2006). Several studies also looked at shorter incubation periods of 30 minutes (Abdollahi et al., 1998; Richardson et al., 2001) and reached similar conclusions. Binning and Baird (2001) also pointed out that the degree of metal pollution had increased over the years and also concluded that this may account for the levels of inhibition observed. The results from the certified SABS laboratory also indicated trace metals in this sample. As was previously stated, the polluted state of the river resulted in the change of venue for the Redhouse Mile race which is held annually in the Swartkops River, giving further evidence of pollution although exact details were never published.

The first data set indicated that the Colchester Tap water had 28.9% ± 16.9% residual activity and 32.775% ± 1.49% residual activity in the second round of sampling. Although all the samples had trace metals present, these water samples exhibited stronger inhibition of AChE compared to the remaining water samples. Further research into activities in the area has shown that there may be other inhibitors present in the water source as construction of a bridge over the river could have resulted in other chemicals being washed into the river.

Port Elizabeth Tap water recorded residual AChE activity of 78.7%, which indicated relatively mild inhibition. Port Alfred Centenary way samples both before and after the inflow of sewerage into the water source, were relatively similar at 65.5% and 65.7%. There may be inadequate treatment of sewerage in this region, which could account for the high inhibition of AChE. Insufficient treatment of the water may have resulted in many possible contaminants that would affect AChE negatively. Further chemical analysis of the water samples is necessary to ascertain the exact composition of the sewerage effluent. The Port Alfred Marina water sample had residual AChE activity of 73.9% ± 13.9%, which is in contrast with the results above just discussed. The Marina, however, is also subject to tidal variations (low and high tide) which would dilute most of the contaminants upstream that may enter the river. Due to this tidal variation it would be expected that there would be minimal inhibition of AChE.

The Blaauwkrantz River located in Grahamstown had residual AChE activity of 34.1% and Grahamstown tap water had 51.5%. Grahamstown has been experiencing water shortages due to the severe decrease in rainfall in the area with heavy metals being reported in the water by

the media. This has resulted in severe problems with water quality and hence would have an impact on AChE activity, as was observed.

Upon further analysis of the sampling sites, it was observed that the samples were predominantly salt water samples. Therefore, testing of the effect of salt on AChE activity was undertaken at three different salt concentrations. The final concentrations of salt tested per assay were equivalent to those found in fresh water, brackish and salt water which were 0.5%, 1.7% and 3.5%, respectively. Results from this test indicated that the salinity does not have a major effect on AChE, as was previously thought within the parameters tested. A study by Scaps and Borot (2000) indicated that increased salinity (sodium chloride concentration) had a minimal effect on the activity of AChE. However, they did note that, in conjunction with an increase in temperature, there was a noted effect on the activity of AChE (Scaps and Borot, 2000). Therefore this indicates that the water source's salt concentration was not a major factor when testing for pesticides in water sources.

Several other studies have been conducted that investigated the use of AChE in environmental testing. Among these are Bernabei et al. (1993), Wang et al. (1996), Andreou and Clonis (2002), Suwansa-ard et al. (2005), Arduini et al. (2006),Vamvakaki and Chaniotakis (2007), Hildebrandt et al. (2008), Viswanathan et al. (2009) and Wu et al. (2009). These studies tested environmental samples that had been spiked with known concentrations of pesticide and were successfully able to detect these pesticides. However, the main aim of this study has been the use of AChE to test un-spiked environmental samples. The optimization of this assay indicated that this should be feasible as very low concentrations of pesticide were detectable. It must be noted that, due to the potential for false positives that may arise from the presence of other inhibitors, AChE testing must be validated using standard methods such as HPLC, GC and MS. It can be concluded that, with the aid of an ANN and suitable data on interfering factors, AChE can be successfully used as a pre-screening tool for environmental samples.

With increased sensitivity of the assay, results from this study also showed marked increase in the percentage deviations. Due to the fact that very small quantities of samples were being analysed, any minor variations would have an impact on the absorbance levels. This was clearly evident in Figures 23, 26, 27, 31 and 37, despite ensuring that basal AChE levels were kept

constant. There are several possible factors that could account for these variations. Enzymes are known to show variations in activity despite all attempts at maintaining standard conditions. It must also be borne in mind that some of the pesticides tested were dissolved in organic solutions to maintain pesticide stability. These could have had an impact, as the micro-Ellman AChE assay is designed to allow for testing aqueous media and organic solvents could potentially have caused interference in the test. In addition, environmental samples may also have contained other substances that could cause interference within the tests. All of these may have accounted for the larger variations noted in the results. From this study it can be observed that with increased sensitivity there is a unavoidable resultant increase in variation.

CHAPTER 5– Conclusion

The growing increase in concern over the effect of exposure to multiple pesticides on the health of humans and other non-target organisms has prompted investigation into their *in vivo* effects on different animals and *in vitro* effects on different bio-markers. The main areas of concern under investigation are acute toxicity of pesticide mixtures that may not be at individual toxic levels, as well as the endocrine disrupting effects of selected pesticides. The aim of this study was to achieve several important objectives with regard to the detection and identification of pesticides, the effects that mixtures would have on AChE, their potential endocrine disrupting effects and the distribution of pesticide contamination within water sources in the Eastern Cape of South Africa.

The first objective was to optimise the micro-Ellman assay; particularly with regard to increasing sensitivity and determining the limits of detection. This was achieved via the selection of AChE from electric eel which has a high sensitivity and the testing of individual pesticides to determine the concentrations of pesticides that could be detected. The results obtained demonstrated that pesticide detection well below the standards set by water regulation authorities was possible using this enzymatic assay. This study also indicated that different pesticides inhibit AChE to varying degrees.

A second objective was to determine the effects of pesticide mixtures on AChE. The study was performed in order to determine whether pesticide mixtures had an additive or synergistic effect on AChE. Several pesticide combinations were selected and results indicated that, generally, pesticide mixtures resulted in an additive effect. There were, however, a few instances in which the result was less than additive, also called antagonistic, and some situations in which the observed result was slightly more than additive, also called synergistic. The results raised an important point, that although pesticides may have the same mechanisms of action, this will not necessarily result in a mixture having the sum of inhibition equal to that of the individual pesticides. Another important point that arose during this study was the need to assess the interactions which may take place in mixtures of pesticides. Pesticides have the ability to

potentiate the effect of some pesticides and inhibit the effects of others. This factor is especially important in the context of environmental monitoring of pesticides, and involves a consideration of pesticides known to display this effect and the circumstances under which this warrants consideration.

The third objective of this study was to use chemometrics and the differences in inhibition rates from individual pesticides to determine the identity of pesticides in a mixture. This concept has been shown to a viable option by earlier studies that used several different enzymes to distinguish between different pesticides and metals. Our study has demonstrated that chemometrics can be used effectively to distinguish up to five pesticides in a mixture with one enzyme (i.e. AChE). In addition, it has been found that a basic FF-ANN can be used to achieve this objective. The results showed that careful consideration has to be made with regards to the way in which the input data is prepared in order for the system to be trained and to be able to successfully identify individual pesticides in a mixture.

The endocrine disrupting effects of aldicarb, carbaryl, carbofuran, demeton-S-methyl and parathion were also analysed to achieve the 4th objective of this study. These pesticides did not exhibit any positive response in the three bio-assays used, namely the YES, T47D-KBluc and MDA-kb2 assays. Two breakdown products (*p*-nitrophenol and aminophenol) were also tested and the results indicated no endocrine disrupting effects. These results were not in agreement with literature and therefore no conclusive statements could be made regarding the endocrine disrupting effects of the pesticides. Another aspect of endocrine disruption that should not be overlooked is the aspect of receptor versus non-receptor mediated responses. There are many different types of receptors in the human body and it is not feasible to test all of these in the context of endocrine disruption, as many of the receptors must be tested before reaching a conclusion on whether a pesticide is an endocrine disruptor or not.

The final objective was the analysis of water samples in the Eastern Cape taken from various water sources. Analysis of the results indicated that the water samples exhibited an inhibitory effect on AChE activity which should indicate the presence of pesticides. However, further analysis using traditional testing methods indicated that no pesticides were present in the water

sources and only trace metals were found. This result emphasizes one of the main disadvantages of enzyme assays, interference of enzymes by other compounds. While this is an important factor, it should be kept in mind that enzymatic assays were not designed to replace traditional testing methods. They are rather to be used as an initial pre-screening tool for large numbers of samples, after which subsequent tests should be performed for verification.

The common theme running through each of the sections in this study was the integrated approach that is required for pesticide detection, monitoring and management. This study showed that each of the individual research components can be built upon and used to further improve our knowledge on pesticides and the effects that they have on the health of organisms. The first and second objectives of the study involved the testing of individual pesticides and of pesticide mixtures and assessing the effects of these mixtures. These results were then used as input data into the ANN and were successfully used to identify individual pesticides in a mixture of up to five pesticides. Although not implemented in this study, the ANN could then be assessed for its ability to determine which OPs and CPs are present in an environmental sample.

The debate on the usefulness of AChE as a bio-marker for monitoring of toxicity levels in water still continues. The fact that it is able to detect pesticides at very low concentrations is of great value; however, the interference by other compounds is a major disadvantage. Among those compounds that have been investigated are various metals and other compounds found in water, such as the detergent sodium dodecyl sulphate (SDS). Our research suggests that if these inhibitors could be accounted for when creating a test system, the disadvantages of using AChE may be minimized.

It has been found that some pesticides exhibit greater potency upon oxidation, for example parathion has a stronger potency in its oxon-form. This study did not account for such information; further studies could include this information and increase the accuracy of the testing system.

The results obtained indicated that, in the case of the tested pesticides, the general trend for pesticides was an additive effect, but that this conclusion cannot be applied to all pesticides. Some pesticides resulted in less than additive effects and others in more than additive effects. All pesticide mixtures need to be assessed based on their own merits such as mechanisms of action, the degree of inhibition as well as any interactions that may arise with other pesticides. Therefore, with the five pesticides that were tested, further analysis must be carried out with regards to the potentiating effects of the pesticides and the total effect this has on the mixture. It is acknowledged that such a study is not always feasible, however all effort must be made to ensure that any conclusions be thoroughly substantiated by as much evidence as is possible.

The ANN was successfully used to distinguish pesticides within the parameters of its training. Further research in this regard could include the addition of interfering factors that were discussed in the previous section. In addition, with these improvements, the ANN could be customized for use in effective environmental sampling. The feasibility of this would require that some information be available on the pesticides that may be encountered in the water sources.

The inconclusive results from the endocrine disruption assays warrant the need for further research. More sensitive assays, such as the AR-Calux and ER-Calux assays, could be used to verify androgenic and estrogenic effects of the pesticides. This conclusion is based on the considerations of the GWRC which indicated that these two tests are the most sensitive and reliable assays of the five methods that were evaluated. Although the results indicated that none of the pesticides possessed endocrine disrupting properties, it should be born in mind that they were tested at very low concentrations. This study can dispel concerns of low concentrations of pesticide resulting in endocrine disrupting (ED) effects. This may imply that ED effects could still be manifested at higher concentrations of the pesticides. This could therefore be another issue considered with regards to pesticides. In addition, the ED effects of pesticide mixtures could also be assessed in a similar manner to that in which toxicity was tested using AChE.

Future studies could also incorporate the effect of not only pesticides on AChE, but also other inhibitors such as metals likely to be present in water samples being tested as well as any interactions that these may have. In this manner, ANNs could be much more useful with regard to environmental sampling as interfering factors would be accounted for.

A method for standardising the bioassays is also required in order to compare analyses and data between various research groups. In addition, the use of *in vivo* studies may be considered in conjunction with the bioassays, in order to confirm estrogenic and androgenic characteristics of samples. Another factor that should be considered is non-receptor mediated EDC effects.

The testing of water samples by the SABS laboratory indicated that no pesticides were present in the water samples. These results could have been influenced by the tidal variations associated with most of the water samples that were tested. This implies that the effect of tidal variations as well as seasonal variations on the results needs to be considered. Additionally, the majority of the water samples taken were from downstream of the rivers. For more accurate comparisons to be made, samples could be taken at different intervals starting closer to the source of the river and at various points before and after industrial/urban activity. This had been previously considered but due to access problems this could not be investigated. Another possible reason for the differences may be that the optimized assay may have been much more sensitive than the conventional testing methods used by the SABS. However time constraints did not allow for further investigation of this aspect of this study.

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Appendix 1

List of Chemicals and Suppliers

Adenosine triphosphate	Sigma Aldrich (Cat. No. A7699)
Agar	Sigma (Cat. No.A9915)
Acetylcholinesterase (Electric Eel)	Sigma Aldrich (Cat. No.C2888-1KU)
Acetylthiocholine iodide (AcSChI)	Sigma Aldrich (Cat. No.A5751-59)
Adenine	Merck (Cat. No.1.00838)
Aldicarb	Sigma Aldrich (Cat. No.33386)
Aminophenol	Sigma Aldrich (Cat. No.35837)
Ammonium sulphate ($(NH_4)_2SO_4$)	Laboratory supplies (Cat. No.R0350/500 g)
Anhydrous magnesium sulphate ($MgSO_4$)	BDH (Cat. No.291184P)
Antibiotic/antimycotic solution	Scientific Group (Cat. No. 15240-062)
Beetle Luciferin, potassium salt	Whitehead Scientific (Cat. No.E1603)
Biotin	BDH (Cat. No. 44011 4H)
Bovine Serum Albumin (BSA)	Sigma Aldrich (Cat. No.A7906)
Carbaryl	Sigma Aldrich (Cat. No.36856)
Carbofuran	Sigma Aldrich (Cat. No.32056)
Cell culture freezing media	Scientific Group (Cat. No. 11101-011)
Chlorophenol red-β-D-galactopyranoside(CPRG)	Roche Diagnostics(Cat. No.10884308001)
Copper (II) sulphate anhydrous ($CuSO_4$)	Roche Diagnostics (Cat. No.278504G)
D(+)-glucose	Merck (Cat. No. 8337)
D-glucose	Merck (Cat. No.8337)
Demeton-S-methyl	Sigma Aldrich (Cat. No.34234)
Ethanol HPLC Grade	Sigma Aldrich (Cat. No. 34870)

Ferric Sulphate ($Fe_2(SO_4)_3$)	Sigma (Cat. No.F-1135)
Fetal Bovine Serum	Separations (Cat. No.SH30071.03)
Fetal Bovine Serum, charcoal/dextran treated	Separations (Cat. No. SH30068.03)
Glycerol	Sigma (Cat. No.G2025)
Glycylglycine	Sigma Aldrich (Cat. No.G7278)
Hanks' balanced salt solution (HBSS) 10x	Scientific Group (Cat. No.14185-045)
ICI 182 780	Tocris (Cat. No. 1047)
Inositol	BDH (Cat. No.380443)
L-arginine-HCl	Merck (Cat. No.1.01543)
L-aspartic acid	BDH (Cat. No.370225W)
L-glutamic acid	BDH (Cat. No.371024T)
L-histidine	BDH (Cat. No.372214E)
L-isoleucine	BDH (Cat. No.371236G)
L-leucine	BDH (Cat. No.371213)
L-lysine –HCl	BDH (Cat. No.371293P)
L-methionine	BDH (Cat. No.371315E)
L-phenyalanine	Merck (Cat. No.1.07256)
L-serine	BDH (Cat. No.371465R)
L-threonine	BDH (Cat. No.371505Y
L-tyrosine	BDH (Cat. No.371562R)
L-valine	BDH (Cat. No.37160)
Magnesium Chloride ($MgCl_2$) 1 M Solution	Sigma Aldrich (Cat. No.M1028)
Parathion	Sigma Aldrich (Cat. No.45607**)**

Pantothenic acid	Merck (Cat. No.111 993)
Phosphate buffered solution (PBS) 10x	Scientific Group (Cat. No.14080-048)
p-Nitrophenol	Sigma Aldrich (Cat. No.35836)
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma (Cat. No.P-0662)
Potassium hydroxide (KOH) pellets	Saarchem (Cat. No.504 44 00)
Pyridoxine	BDH (Cat. No.449865Q)
Reporter lysis buffer	Whitehead Scientific (Cat. No.E3971)
RPMI 1640 medium (with glutamine, without phenol red)	Sigma Aldrich (Cat. No.R3)8755)
Sodium bicarbonate ($NaHCO_3$)	Sigma Aldrich (Cat. No.S5761)
Thiamine	BDH (Cat. No.440055N)
Trypsin EDTA (10x)	Scientific Group (Cat. No. 15400-054)
1 M HEPES buffer	Scientific Group (Cat. No.15630-056)
5'5'-dithio-bis-2-nitrobenzoic acid (DNTB)	Sigma Aldrich (Cat. No. D21820-0)
17-β Estradiol (E_2)	Sigma Aldrich (Cat. No. E8875)
100 mM Sodium pyruvate	Scientific Group (Cat. No.11360-039)

TEST REPORT

SABS

Your ref: BC 013023
Our ref: 2819
Enquiries: L MAGQI
Date: 2011-04-29
Report No: 2819/M4987
Page: 1 of 3

Rhodes University
ATTENTION: Katayi Mwila
P O Box 94
GRAHAMSTOWN
6140

WATER SAMPLES

1 DESCRIPTION OF SAMPLES

The following samples were submitted by Katayi Mwila to our Cape Town laboratory on 30 March 2011.

SABS CODE	SAMPLE DESCRIPTION	
CT 0408	Sample No. 1	(11-03-30)
CT 0409	Sample No. 2	(11-03-30)
CT 0410	Sample No. 3	(11-03-30)
CT 0411	Sample No. 4	(11-03-30)
CT 0412	Sample No. 5	(11-03-30)
CT 0413	Sample No. 6	(11-03-30)
CT 0414	Sample No. 7	(11-03-30)
CT 0415	Sample No. 8	(11-03-30)
CT 0416	Sample No. 9	(11-03-30)
CT 0417	Sample No. 10	(11-03-30)
CT 0418	Sample No. 11	(11-03-30)
CT 0419	Sample No. 12	(11-03-30)

2 ANALYTICAL DURATION

The analysis commenced on 31 March 2011 and was completed on 03 May 2011.

3 IMPORTANT NOTES

Results marked * are not SANAS accredited and are not included in the SANAS Schedule of accreditation for this laboratory.

Results pertain to samples as supplied. The estimated uncertainty of measurements for all results is obtainable from the laboratory at request. Original report from subcontractors is available on request.

Preservation techniques used, where required, were based on the recommendations supplied in ISO 5667/3 'Guidelines on the preservation and handling of samples' and Method 1060/C 'Sample preservation' from 'Standard Methods for the Examination of Water and Wastewater', APHA-AWWA-WPCF, 1995 19th Edition.

SABS COMMERCIAL (PTY) LTD - WATER CHEMISTRY LABORATORY
Reg No T0090, Liesbeek Park Way, Rosebank

HEARSHAW AND KINNES ANALYTICAL LABORATORY
Reg No T0232, 9 Regent Park, Bell Crescent, Westlake Business Park, Tokai.

Liesbeek Parkway Rosebank Cape Town, PO Box 615 Rondebosch 7701
Tel: +27 (021) 681-6700, Fax: +27 (021) 681-6701.

SOUTH AFRICAN BUREAU OF STANDARDS SUID-AFRIKAANSE BURO VIR STANDAARDE

TEST REPORT No. 2819/M4987
TOETSVERSLAGNO

4 RESULTS OF ANALYSIS

DETERMINANDS	METHOD USED	METHOD NUMBER	LAB Ref.	RESULTS			
				CT 0408	CT 0409	CT 0410	CT 0411
Pesticides in µg/L	GC-MS	DF01	T0232	ND	ND	ND	ND
*Arsenic as As in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Cadmium as Cd in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Chromium as Cr in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Copper as Cu in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Lead as Pb in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Mercury as Hg in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Selenium as Se in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10

DETERMINANDS	METHOD USED	METHOD NUMBER	LAB Ref.	RESULTS			
				CT 0412	CT 0413	CT 0414	CT 0415
Pesticides in µg/L	GC-MS	DF01	T0232	ND	ND	ND	ND
*Arsenic as As in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Cadmium as Cd in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Chromium as Cr in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Copper as Cu in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Lead as Pb in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Mercury as Hg in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Selenium as Se in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10

SOUTH AFRICAN BUREAU OF STANDARDS SUID-AFRIKAANSE BURO VIR STANDAARDE

TEST REPORT No.
TOETSVERSLAGNO 2819/M4987

5 RESULTS OF ANALYSIS (Continued)

DETERMINANDS	METHOD USED	METHOD NUMBER	LAB Ref.	RESULTS			
				CT 0416	CT 0417	CT 0418	CT 0419
Pesticides in µg/L	GC-MS	DF01	T0232	ND	ND	ND	NA
*Arsenic as As in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Cadmium as Cd in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Chromium as Cr in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Copper as Cu in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Lead as Pb in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10
*Mercury as Hg in µg/L	ICP-OES	SANS 11885:2008	T0090	<1	<1	<1	<1
*Selenium as Se in µg/L	ICP-OES	SANS 11885:2008	T0090	<10	<10	<10	<10

PLEASE NOTE

1. ND = Not Detected
2. NA = Not Applicable. No sample was supplied for the Pesticide analysis.

NM NDABENI
TECHNICAL SIGNATORY
Water/M4987/mcromey-hawke

L MAGQI
MANAGEMENT SIGNATORY