RAPID ENZYMATIC DETECTION OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES IN WATER

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

1. Background

Pesticides are important for growth of commercial crops, and this is especially noteworthy considering the number of crops of commercial value grown in South Africa. However, due to run-off they often contaminate water sources which may function as drinking water for humans and animals. Organophosphate and carbamate pesticides function by a similar mechanism, namely the inhibition of an enzyme, acetylcholinesterase (AChE). However, it is problematic that AChE occurs in all living organisms and thus pesticides also have an effect on unintended targets such as humans and animals. As a result of their toxic effects, contamination of water by these pesticides must be monitored to prevent adverse effects in humans and animals. Due to the cost and complexity of traditional analysis methods, alternative screening methods of water for pesticide contamination has to be identified. As AChE is the intended target for organophosphate and carbamate pesticides, it has been identified as a suitable method for detection of these pesticides with the concentration of the pesticide having a linear correlation to the inhibition of the enzyme. Using AChE as a detection mechanism has the advantages of being cheaper than most methods and allows for high initial throughput screening. In addition, its sensitivity allows for testing at toxicity levels below stipulated legal limits.

Determination of pesticide distribution in water sources around the world has received limited attention, and as a result, the Global Water Research Commission (GWRC) instituted programs that would rectify this omission. For this reason the WRC, a member of the GWRC, has initiated and funded various studies to identify and assess pesticide distribution in water sources around the country. This study was undertaken in line with this resolution to determine contamination of water sources in the Eastern Cape, South Africa.

In addition to toxic effects, pesticides have also been identified as having possible endocrine disrupting effects. Therefore this study also used the yeast estrogen screen (YES) bioassay to assess the estrogenic effects of selected pesticides. Endocrine disrupting compounds (EDCs) have been noted to be a major health hazard at exposure levels below those considered to be carcinogenic. These compounds can adversely affect the endocrine system at levels below the legal limit and adequate monitoring in water sources at such low levels can be problematic.

The main aims achieved during the study were the optimization of pesticide detection to achieve lower detection limits than the minima set by legislation; the use of this assay to determine pesticide contamination in Eastern Cape water sources; and the assessment of the estrogenic effects of these pesticides.

2. Aims

This study had the following aims:

- To optimize the use of the AChE assay in order to detect carbamate and organophosphorus based pesticides;
- To use the optimized AChE assay to detect pesticide mixtures and possibly identify individual pesticides in mixtures;
- To incorporate the use of a bioassay (YES Assay) into linking pesticides and their breakdown products to endocrine disrupting activity;
- To determine the relative distribution of organophosphorus and carbamate based pesticides in Eastern Cape water sources.

3. Methodology

Previous scoping studies have indicated that AChE is an enzyme that can be used successfully to detect the presence of carbamate pesticides (CPs) and organophosphorus pesticides (OPs) in water samples. The AChE assay was optimized and used to establish the detection of individual OPs and CPs and then used to test the effect of pesticide mixtures on the enzyme. The degree of enzyme inhibition displayed a correlation with the concentration of pesticide present. The assay was then used to detect the presence of pesticides in various Eastern Cape water sources, which were selected according to their location and accessibility. Chemometrics was also applied to the data obtained in order to ascertain if individual pesticides could be identified in mixtures.

The YES assay was used in order to determine the estrogenic effects of the pesticides under consideration. The modified yeast cells have receptors that are activated by any compound that has an estrogenic receptor mediated response. The estrogenic properties of all the compounds were measured relative to 17-β estradiol. The MDA-kb2 and T47D-KBluc assays were also used in conjunction with the YES assay. This enabled testing for androgenic activity as well.

4. Summary of results

It was concluded that the AChE assay could be used for the detection of pesticides at nanogram levels (ng/L). In addition, the assay could be used to detect pesticide mixtures, which were found to have an additive inhibitory effect on AChE. Whether or not individual pesticides can be differentiated from mixtures using the enzyme assay, together with analysis via chemometrics, is still being investigated. The results obtained from the samples that were tested in this assay did not appear to have any receptor mediated estrogenic or androgenic activity in the YES, T47D-KBluc or MDA-kb2 assays respectively. In addition, it has been found that some Eastern Cape water sources may be contaminated with pesticides as indicated by the AChE results and also contains heavy metals according to a South African Bureau of Standards report on the water samples.

5. Conclusions and Recommendations

The AChE assay has been optimized and found to be an effective and cost efficient method for determining the presence of pesticides. It may also be an effective tool in identifying individual pesticides within mixtures in conjunction with chemometric analysis. We are currently also immobilizing AChE onto electrospun nanofibers with the aim of increasing the stability and the feasibility of using this enzyme for pesticide detection. With regard to determining the estrogenic effect of pesticides, a wider range of pesticides needs to be tested and these tests should also include the various breakdown products that can be formed, regardless of the method of degradation. The YES assay proved to be an effective method of determining estrogenicity, but the fact that not all pesticides are receptor mediated needs to be considered.

6. Publications

The following review article has been published as part of this study:

Van Dyk, JS, Pletschke, BI (2011) “Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment”. Chemosphere. 82(3): 291-307.

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LIST OF 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

1. INTRODUCTION

1.1. Background information

Pesticides are compounds commonly used to assist in the growth of food and crops of commercial value. Pesticides can be divided into various categories, i.e. herbicides, fungicides, avicides, rodenticides and many more (Johnston, 2000). Pesticides have, however, become a worldwide problem 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 et al., 2005; Aqiel et al., 2006, Aneck-Hahn et al., 2007). Originally pesticides were organochlorine based, 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). However, these were found to persist in the environment and accumulate in plants and animals over time. In addition, because of their stability, organochlorines can be transported to regions where they are not produced or used (Burger, 2005). It is for this reason that 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). This conference aimed at identifying and eliminating the use of the most dangerous POPs and eliminating their use, as well as regulating the use of any POPs that were not banned (Lallas, 2001). Pesticides that have been used as suitable substitutes for organochlorines fall into several different categories, two important ones being organophosphorus and carbamate pesticides. Although these classes of pesticides have a shorter half-life than organochlorines and therefore a lower persistence, they still pose a problem in the environment as they are transported into water sources. The mechanism of these pesticides is the inhibition of a key neural enzyme, acetylcholinesterase, which is found in all living organisms, including animals and humans. Thus these pesticides are not only toxic to the intended targets, but also to humans and animals drinking contaminated water. 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.

A further problem with pesticides is that they can cause adverse health effects on animals and humans by acting on the endocrine systems of such organisms. This can take place at concentrations far below the concentrations identified as toxic and has long-term effects that may not be detected immediately. Pesticides that act on the endocrine systems of organisms are termed endocrine disrupting compounds (EDCs), which, according to the United States Environmental Protection Agency (USEPA), are defined as exogenous agents that interfere with synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for maintenance, reproduction development and/or behavior of organisms (USEPA, 2010), or more simply chemical substances that have a detrimental effect on the endocrine system. The endocrine system consists of all the glands and hormones that regulate various bodily functions. These include pituitary, thyroid, pineal, thymus, ovary and testis, salivary, sweat and adrenal glands (Bertók, 1998), which together make up the reproductive system, the neurological system, control thyroid function as well as the immune system (Aoki, 2001). All of these systems regulate normal body functions, which include blood glucose levels, food utilization, fluid balance and reproductive cycles in animals and humans (Vogel, 2004). The manifestations of EDCs include severe physiological problems, birth defects, developmental problems, infertility and other reproductive ailments. Endocrine disrupting compounds can cause these disruptions at exposure levels much lower than carcinogen exposure level. It is therefore important that detection of

pesticide contamination in water sources must take into account that pesticide concentrations at legal limits do not necessarily indicate that contamination levels are safe.

1.2. Acetylcholinesterase

Acetylcholinesterase (E.C. 3.1.1.7) is a neuroregulatory enzyme common to many species and is part of the cholinesterase family of enzymes. They are responsible for the catalytic hydrolysis of acetylcholine into choline and acetic acid as Figure 1 illustrates.

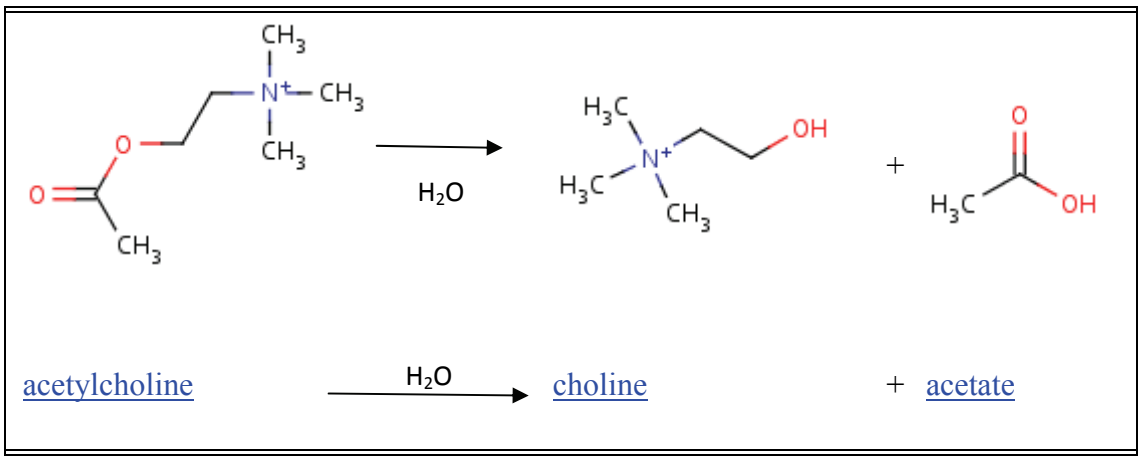

Figure 1: Reaction catalyzed by the enzyme acetylcholinesterase (EC 3.1.1.7).

This reaction is relevant for the termination of synaptic transmission in neuromuscular junctions and cholinergic nervous system. When a nerve impulse is transmitted 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. Acetylcholinesterase is responsible for the breakdown of acetylcholine which stops the activation of the receptors on the post-synaptic neuron and therefore stops the nerve impulse transmission. This allows the body to return to resting potential (Voet and Voet, 2004). Figure 2 below illustrates this process.

The choline and acetic acid are recycled and returned to the pre-synaptic neuron and acetylcholine is resynthesized in readiness for the next nerve impulse transmission.

Acetylcholinesterase can also use acetylthiocholine iodide, acetylcholine chloride or fluorescein diacetate as substrates. The vital function of AChE in the neural systems of organisms makes it very important to know what chemicals/compounds affect the enzyme. Any compound that negatively affects the enzyme is referred to as an inhibitor. There are various compounds that have been found to adversely affect AChE and in extreme cases (when AChE is totally inhibited) this is referred to as a cholinergic crisis. During a cholinergic crisis the organism can undergo prolonged seizure of limbs, convulsions and even central respiratory failure which can eventually lead to death (Lallement et al., 1992 and Tonduli et al., 1999). Fukuto (1990) indicates 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 corresponds to the concentration of pesticide present in the water sample (Andreescu et al., 2002).

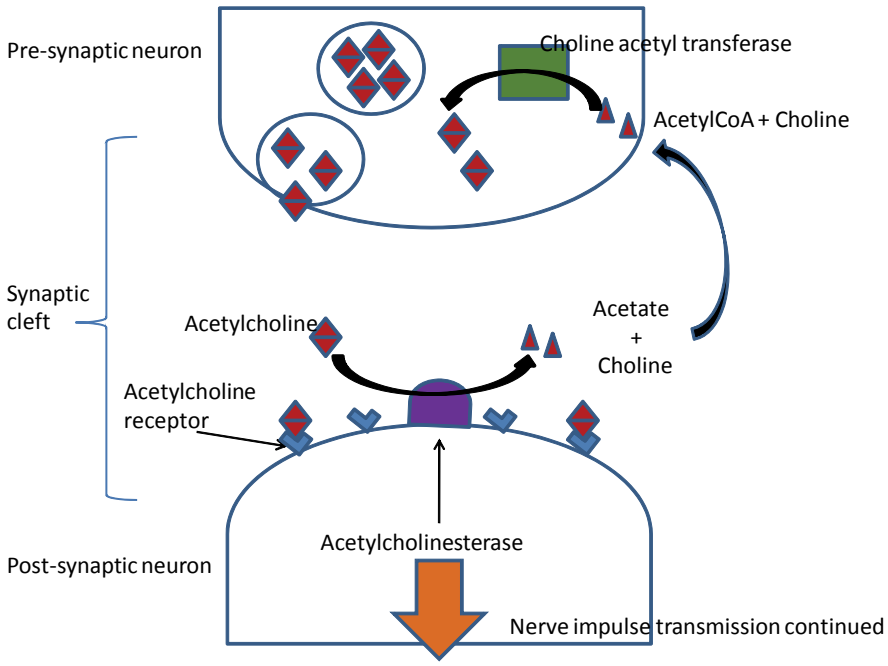

Figure 2: Schematic diagram of the synaptic cleft demonstrating the manner in which a nerve impulse is transmitted via activation of receptors on the post synaptic neuron by acetylcholine, as well as the manner in which it is broken down by AChE and nerve impulse transmission terminated. (Adapted from Voet and Voet, 2004)

1.3. Carbamate and organophosphate based pesticides – structure and function

Figure 3 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.

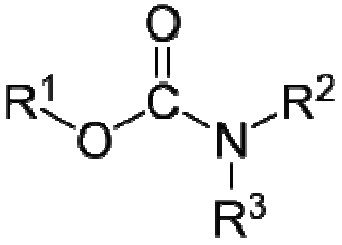

Figure 3: Chemical structure of carbamates (Adapted from Dorofeeva and Tarakanov, 1987)

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

Organophosphorus pesticides (OPs) is the term used to describe organic phosphorus (V)-containing compounds and these compounds are known for their neurotoxic capabilities. 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). The exact mechanism of inhibition is further described in this study.

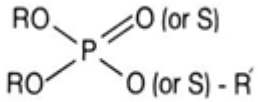

Figure 4: Structure of an organophosphorus compound. R represents either a methyl or ethyl group, OR represents an alkyl, alkoxy, alkylthio, aryl, or heterocyclic aryloxy, alrylthio or a heterocyclic analog.

There are various examples of available OPs and in South Africa; the most common OPs in use are parathion and demeton-S-methyl (Slabbert et al., 2004; Burger, 2005). Some of the CPs and OPs used worldwide 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

Previous research has been performed to determine the presence of OPs and CPs in water courses in various countries, 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 into the manner in which pesticides affect the health of both humans and animals in a given location.

1.4. Pesticide distribution in South Africa

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 analyzed the presence of organochlorine pesticides (OCs), most of which have been banned in terms of the Stockholm Convention due to their endocrine disrupting properties. This study aims to supplement the results reported by Awofolu and Fatoki (2003) which identified the presence of OCs in water samples in the Eastern Cape, but did not include OPs and CPs. Currently there are very limited data on pesticide use in and around South Africa, which 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). This would render a study of the presence of pesticides more difficult as no data exists which would give an indication as to the quantities of pesticides used in the country and the purpose for which it is used. 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. This study will therefore contribute to the available data on the presence of pesticides in water samples that are organophosphorus and carbamate based.

1.5. Available methods of detection

Several methods have been employed for pesticide detection such as 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, despite being extremely sensitive with regards to pesticide detection, have several shortcomings. Their greatest disadvantage 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 therefore renders these methods unfeasible for use by most organizations.

Another challenge is the need for highly trained and skilled personnel. 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., 2002). This poses a problem as the harmful nature of pesticides requires that detection methods be as rapid as possible. In light of all the observations above, the use of enzymatic assays for pesticide detection promises to be an attractive option.

1.6. Acetylcholinesterase enzymatic assay

Several bio-assays have been identified as possible methods of detection for OPs and CPs, including the use of the biomarkers ethoxyresorufin-O-deethylase (EROD), glucose, glycogen, acetylcholinesterase (AChE), heat shock protein (Hsp 70), pyruvate kinase and lactate dehydrogenase (LDH) (Slabbert et al., 2004). The AChE assay was selected for our study.

Carbamate pesticides reversibly inhibit the enzyme AChE. This is caused by the carbamylation of the serine residue in the active site of AChE, which results in most carbamates being classed as true competitive inhibitors (Wilson et al., 1960). With time the carbamylation is reversed and the inhibitory effects of the CP disappear. Organophosphorus pesticides, on the other hand, are regarded as irreversible inhibitors and act as "suicide substrates" (Walker, 2003). The serine moiety of the active site of the enzyme is phosphorylated upon acceptance of an OP molecule into its active site, and as Fukuto (1990) explains, the dephosphorylation of the active site is so slow (i.e. can take

up to several days) that the reaction is generally termed irreversible, explaining why OPs are referred to as "suicide substrates". Due to the inhibitory effect of both CPs and OPs on AChE, their presence can be detected using this enzyme.

There are many studies reporting data on the effect of individual pesticides on AChE inhibition, but very few studies have investigated the effects of pesticide mixtures on this enzyme. Richardson et al. (2001) assessed the effect of two pesticides on AChE activity and found that at lower concentrations the pesticides had a dose additive effect with regards to the inhibition of AChE. "Dose additive" in these experiments 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 and not just simple mathematical summation of percentage inhibition, also referred to as response additivity (Richardson et al., 2001). Tahara et al. (2005) also investigated the effect of two pesticides by keeping the concentration of one pesticide constant and varying the concentration of the second pesticide. Although they did not define 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. They noted that there was an additional inhibitory effect but could not conclusively state that the inhibitory effect on AChE was additive (Tahara et al., 2005).

Ni et al. (2007; 2009) have undertaken several studies that deal with pesticide mixtures, using chemometrics for data analysis. These studies indicated that individual pesticides could be identified from mixtures. One 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). Chemometric calibration was achieved with the use of 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). These tools were applied for pesticide simultaneous prediction during the study involving the two pesticides (Ni et al., 2009). Radial basis function neural network was used due to its versatility and ability to deal well with non-linear data. Ni et al. (2009) were able to successfully determine the presence of aminocarb and carbaryl from a mixture. The other study, also conducted by Ni et al. (2007), successfully identified the presence of four pesticides, namely carbaryl, carbofuran, isoprocarb and propoxur. The voltammetric behavior of each pesticide was determined after alkaline hydrolysis with sodium perchlorate and sodium hydroxide. This was repeated with laboratory samples containing varying pesticide concentrations and the calibration methods mentioned in the previous study applied. A modified version of the RBF-ANN method was once again selected for the chemometric analysis as it provided the most satisfactory results.

Research in our laboratory could not previously distinguish between pesticides or pesticide mixtures using AChE (Mwila, 2009) and, based on the research by Ni et al. (2007, 2009), chemometrics have been suggested as a means of accomplishing this. Using the method of Ni et al. (2009) and making use of the difference in reaction rates, it has been hypothesized that individual pesticides could be identified in mixtures.

1.7. Yeast Estrogen Screen assay

The YES assay has been used to assess the estrogenic activity of compounds. The YES assay involves the use of yeast cells which do not have any natural estrogen receptors. The 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 encoding for

the enzyme β-galactosidase (Routledge and Sumpter, 1996). The ER-α is expressed in a form that can bind to the ERE sequences that are located within a strong promoter region on the expression plasmid. The structure of the plasmid is illustrated in Figure 5 below.

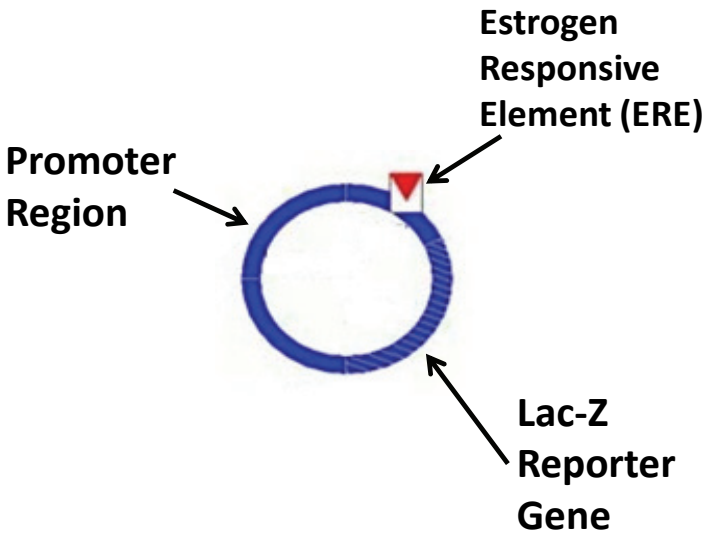

Figure 5: Diagram of the plasmid inserted into the yeast cell that contains a strong reporter sequence, estrogen responsive elements (ERE) and the lac-z gene. (Adapted from University of Auckland, 2011).

Therefore, upon binding of an active ligand, the estrogen-occupied receptor interacts with transcriptional factors and other components to modulate gene transcription. In Figure 5 and 6 this is depicted as the red triangle that fits into a receptor. This results in the expression of the reporter gene *lac-z* and production of the enzyme β-galactosidase, shown by the purple square in Figure 6. This enzyme is secreted into the medium containing a chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), which is metabolized and causes a color change from yellow to red. The color change can be measured using a spectrophotometer at an absorbance of 540 nm, hence allowing for the evaluation of estrogenic activity (Routledge and Sumpter, 1996). This process is depicted in Figure 6.

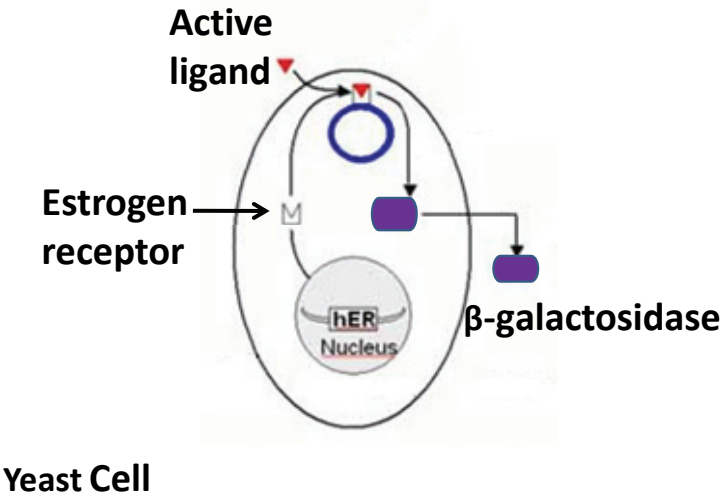

Figure 6: Diagrammatic representation of the effect of an estrogenic compound on the yeast cell in the YES assay which results in the production of the enzyme β-galactosidase. (Adapted from University of Auckland, 2011).

The estrogenic effect is measured relative to that of 17β-estradiol (E_2). A dose response curve for E_2 and the test chemical 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 7 below is an example of a possible estrogenic response in the YES assay.

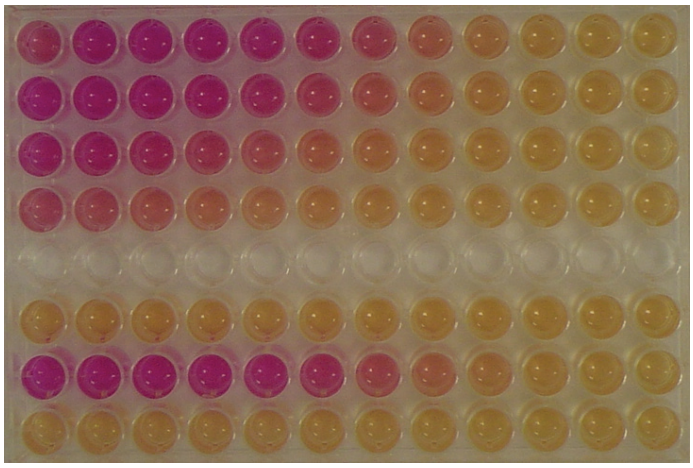

Figure 7: Picture of a microtiterplate showing a positive response for an estrogenic sample.

2. HYPOTHESIS

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

3. AIMS

- To optimize the AChE assay and determine its limit of detection (LOD) for various pesticides
- To identify individual pesticides in pesticide mixtures using chemometrics
- To establish the estrogenic activity 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 environmental samples in the Eastern Cape

4. METHODOLOGIES

The pesticides that were selected for analysis were aldicarb, carbofuran, carbaryl and parathion, as well as the breakdown products aminophenol and *p*-nitrophenol.

4.1. To optimize the AChE assay and determine its limit of detection (LOD)

The micro-Ellman assay was used in this study to determine AChE activity (Ellman et al., 1961). All reagents were purchased from Sigma Aldrich. The assay involved the use of AChE enzyme from electric eel (CAS number: 9000-81-1) and acetylthiocholine iodide (AcSChI) (CAS number: 1866-15-5) as substrate. Acetylthiocholine iodide was mixed with 5'5'-dithio-bis-2-nitrobenzoic acid (DNTB: Ellman's reagent) (CAS number: 69-78-3) which resulted in the formation of a color product that was monitored using UV spectroscopy (Ellman et al., 1961). Phosphate buffer was used at pH 7.5 and a concentration of 0.1 M for the assay. The AcSChI was made to 0.075 M and the DNTB was made up to 0.01 M (Ellman et al., 1961). This assay was carried out with AChE from electric eel and for different concentrations of OPs to assess the inhibitory effect of the various concentrations of the different pesticides (all HPLC grade). This assay was considered ideal because it can also be used to assess aqueous samples. In addition, the effect of varying pre-incubation times of AChE with pesticides was also investigated.

A 160 µl aliquot of buffer, 10 µl of DNTB and 20 µl of AChE were placed in a microtiterplate well and 10 µl of acetylthiocholine was added in order to start the reaction. The absorbance was measured at 412 nm every 30 seconds for 10 minutes using a Powerwave$_X$ microtiterplate reader. All assays were performed in quadruplicate. Standardization was achieved by ensuring that the AChE concentration used resulted in similar activity levels for all experiments for the no pesticide control.

4.2. To identify synergistic and/or additive effects in pesticide mixtures using chemometrics

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. 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.

After collecting data from the AChE assay using pesticide mixtures, chemometrics was applied to the data and information attained as to whether mathematical formulae can be used to determine whether any synergistic or additive effects could be established. There are two types of additive effect; dose additive and response additive (Richardson et al., 2001). Dose additive can be defined as the effect created when both chemicals act on the same site and each compound's contribution to the final toxicity is dependent on its potency. Response additive is the mathematical summation of the inhibitory effect of the compound (Richardson et al., 2001). The response additive effect was selected for this study as it is simpler to determine.

Generally an increase in the concentration of AChE at the concentrations of pesticide tested results in a decrease in the level of inhibition observed (as the ratio of enzyme to pesticide is reduced), but with a concomitant improvement in the variation (standard deviation) observed between replicates (data not shown). This would be due to the fact that sufficient enzyme would be present to allow the

reaction to proceed. This would; however, render the reaction less sensitive. Hence concentration levels of AChE were kept low to ensure high sensitivity.

MatLab programming software was chosen for this analysis. Chemometrics is based on the use of artificial neural networks (ANNs) and these are mathematical models that work on the same premise as the brain. The brain has the ability to deal with non linear data, high parallelism, is robust, has fault and failure tolerance, is capable of learning, can generalize and also handles imprecise data; ANNs try to model this functionality.

An ANN has the following architecture; each input has a weight (w) and bias that are applied to it and these are summed (∑) to give an activation function that is used to calculate the final output as shown below (Figure 8).

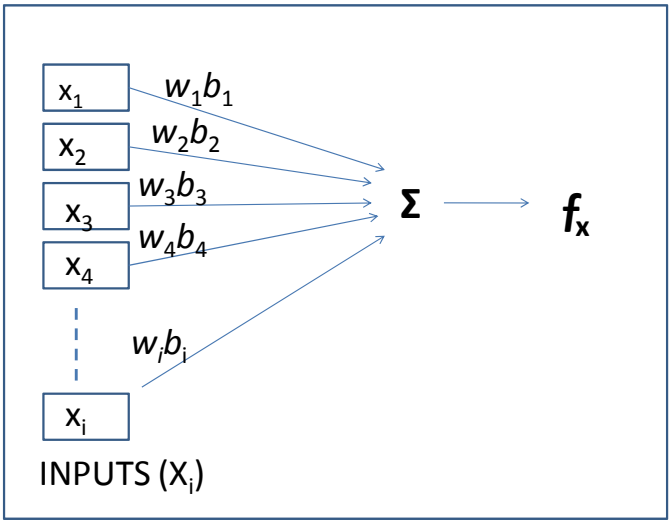

Figure 8: An example of a neural network. X represents the inputs into the neural network; w represents the weights that are applied to each input; b represents the bias that is allotted to each weighting. Σ is the summation of the weightings and bias that result in the function (f_x) that can be used to predict outputs.

The ANNs consist of adaptive processing elements which are analogous to neurons, and these are interconnected and can carry out complex computations and data processing. They are designed to learn through the process of updating their internal representation of the system in response to external stimuli so it can perform specific tasks. This involves adjustments of weights, links, creating or deleting connections and or changing firing rules of individual neurons. Learning is performed reiteratively with examples as with the human brain. Each input has the weight and bias adjusted continuously until the final output is as close to the desired result as possible. The learning strives to obtain the set of weights that will correspond to a global minimum error. The error is a function of all the weights and forms an irregular multidimensional complex hyperplane with many peaks and saddle points. Artificial neural networks therefore "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.

Four programs were written in MatLab for the purpose of applying chemometrics to the data that was attained. The first program involved preparing the data in a format that could be input into the neural network (i.e. creation of column vectors). These column vectors were then fed into a second program that would create the neural network and train it to recognize patterns within the data. This would mean that the neural network would recognize the various pesticides present in the sample.

The third program was written to test the neural network's ability to recognize different pesticides and the fourth program created simulated data to test the neural network that was created.

4.3. To establish the estrogenic activity of CPs and OPs, and their breakdown products, using the YES assay and other assays

The GWRC recommended the use of a suite of assays in order to assess the estrogenic activity of samples. In line with this the YES assay was used in conjunction with the T47D-KBluc (KBluc) assay. The KBluc assay involves the use of the human cancer cell line T47D, which has been transfected with a triplet ERE-promoter luciferase reporter gene construct. The assay assesses estrogenic and anti-estrogenic activity of samples (Wilson et al*.,* 2004).

The MDA-Kb2 assay (MDA) was used to screen the samples for androgenic activity. The MDA-Kb2 assay also makes use of a cancer cell line, MDA-MB-453. This has been stably transformed with a MMTV luciferase neo reporter gene construct. This cell line has both an AR (androgen receptor) and a GR (glucocorticoid receptor) which assess the androgenic and anti-androgenic properties of samples (Wilson et al*.,* 2002).

4.3.1. Preparation of test chemicals

Stock concentrations of 1 mg/ml of the test chemicals were prepared in ethanol, prior to dosing of plates. The powdered samples were dissolved in ethanol straight away, whilst the liquid samples, using other solvents, had to be evaporated as the yeast cells are adversely affected by organic solvents. Once evaporated, the dried sample was diluted into 1 ml of ethanol.

4.3.2. The Yeast Estrogen Screen assay

The YES assay was performed according to the method described by 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, microtiterplate 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 (17-β estradiol) with concentrations ranging between 1×10^{-8} M and 4.8×10^{-12} M. The plates were sealed with parafilm and placed in an incubator at 32°C for 3-5 days. After three days incubation, the plates were read using a Titertek Multiskan MCC/340 plate reader at absorbances of 540 nm and 620 nm. The plates were resealed and returned to the incubator and readings taken (as before) for days four and five. 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). A dose response curve for the positive control (E_2) and the test chemical were constructed. Based on the dose response curve the relative potency (RP) of each test chemical was calculated using the EC_{50} value of the test chemical relative to E_2.

4.3.3. The T47D-KBluc Reporter Gene assay

The test chemicals were processed according to the protocol described in Wilson et al. (2004). T47D-KBluc cells were maintained in RPMI growth media supplemented with 2.5 g/l glucose,

10 mM HEPES buffer, 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 medium modified by replacement of 10% FBS with 10% dextran-charcoal treated FBS excluding antibiotic supplements (Wilson et al., 2004).

Cells were seeded at 5×10^4 cells per well in 96-well luminometer plates and allowed to attach overnight. Dosing dilutions were prepared in growth media containing 5% dextran-charcoal treated FBS and vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (E_2), negative control (solvent only), antagonist control (E_2 plus ICI) and background control (solvent plus ICI). Each sample was tested alone as well as in the presence of 0.1 nM E_2 or ICI. Cells were incubated for 24 h with 100 µl/well dosing solution at 37^{o}C, with 5% CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 25 µl lysis buffer. Luciferase activity was determined using a microtiterplate luminometer and quantified as relative light units. Each well received 25 µl reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/ml BSA, pH 7.8), followed by 25 µl of 1 mM D-luciferin 5s later. Relative light units were converted to a fold induction above the vehicle control value.

A dose response curve for the positive control (E_2) and the test chemical were constructed. Based on the dose response curve the relative potency (RP) of each test chemical was calculated using the EC_{50} value of the test chemical relative to E_2.

4.3.4. The MDA-kb2 Reporter Gene assay

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

Cells were seeded at 5×10^4 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 100 µl /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 25 µl 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.

4.4. To use the AChE assay to investigate the relative distribution of various pesticides (and breakdown products) in environmental samples in the Eastern Cape

Environmental samples were collected from various surface water sources in the Eastern Cape and the AChE assay used in conjunction with chemometrics to determine the presence of CPs and OPs in water sources.

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.

4.4.1. Sampling sites

The following water sources were selected for analysis of pesticide content. These sites were selected on the basis that they represent some of the main water sources in the Eastern Cape and their locations are indicated on the maps in Figures 9-13. The area around Grahamstown is used mainly for agricultural purposes and the Blaauwkrantz River also passes through an informal settlement in Grahamstown. The river displays visible pollution 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 9).

Figure 9: Map of Grahamstown indicating the location of the sampling site at the Blaauwkrantz River.

The Sundays River (Figure 10) has a catchment area that is mainly used for agricultural activities. These agricultural activities contribute to the input of organophosphate fertilizers and pesticides into nearby rivers and ultimately estuaries as was established in studies by Emmerson (1989) and Scharle and Baid (2005). It was, however, established that pollution was relatively mild compared to other estuaries such as Swartkops. The urban settlement, because of its small size, does not contribute much in the way of pollution with regards to the river.

Figure 10: 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.

The lower catchment and the area around the Swartkops River estuary are densely populated and numerous industrial activities such as clay mining, salt works, sewerage treatment works, wool washeries and tanneries are situated in the region (Figure 11). Point sources of pollutants include the Motherwell and Markham Canals which drain under-serviced, heavily populated residential townships and industrial developments, respectively (Fatoki and Mathabatha, 2001). In addition to this, the Chatty River enters the Swartkops Estuary in the lower reaches and flows through an informal settlement. 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 degree of pollution has reached such significant levels 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 (The Herald, 2010).

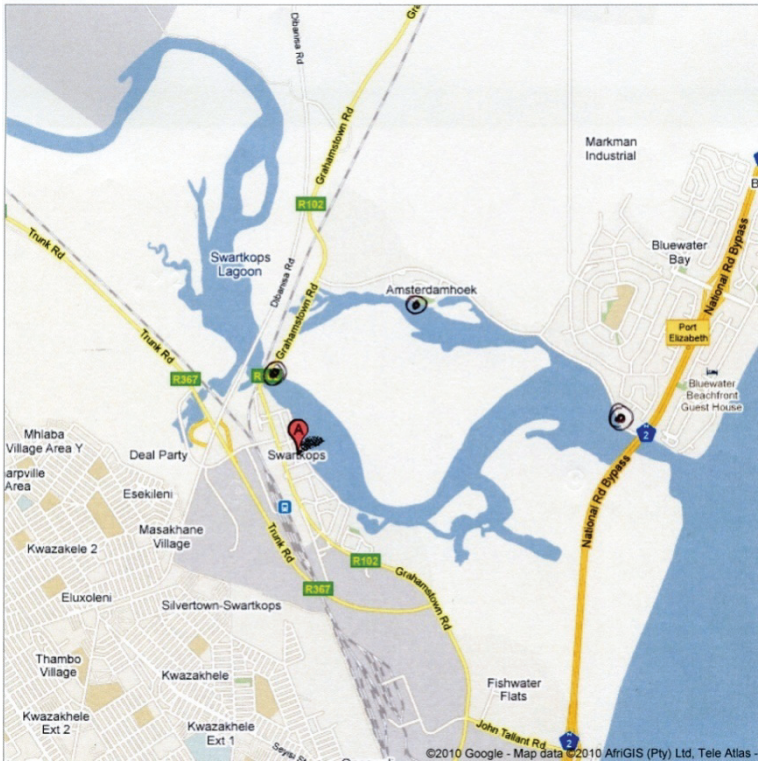

Figure 11: Map of Port Elizabeth indicating the location of the sampling sites at the Swartkops River at the Grahamstown Road Bridge, Amsterdam Way and at Bluewater Bay.

The Kowie Estuary in Port Alfred (Figure 12) is an open estuary that flows through an urbanized area and is subject to substantial environmental degradation due to the construction of the marina. Recent issues affecting the fauna and flora of the ecosystem have arisen as a result of the persistent dredging in the river mouth region and the land and sewage run-off into the river.

The Kariega Estuary catchment is characterized by minimal industrial activities and has a small urban settlement that produces minimal liquid effluent that is disposed of in the river (Figure 13). Thus, the Kariega River is considered relatively pristine, although Orr et al. (2008) demonstrated that sediments in the Kariega Estuary were enriched in cadmium (Cd), copper (Co), nickel (Ni) and lead (Pd). 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 12: Map of Port Alfred indicating the location of the sampling site at the Kowie River just before and after the urban settlement, near the river mouth

Figure 13: Map of Kenton-on-Sea indicating the location of the sampling site at the Kariega River just before the R72 Bridge.

The photos in Figure 14 were taken during water sampling.

Figure 14: The Kariega River sampling site by the N2 Bridge in Kenton-on-Sea

5. RESULTS

5.1. Individual pesticides

The first objective of this research project was to optimize the AChE assay and use it to assess the effect of individual pesticides on AChE activity. Optimization of the assay was completed and was able to detect pesticides at concentrations much lower than those reported in literature for similar assays except for carbofuran. The graphs in Figures 15 to 18 display the typical effect of individual pesticides on AChE activity in the assay. Figure 15 shows the inhibition of AChE by parathion.

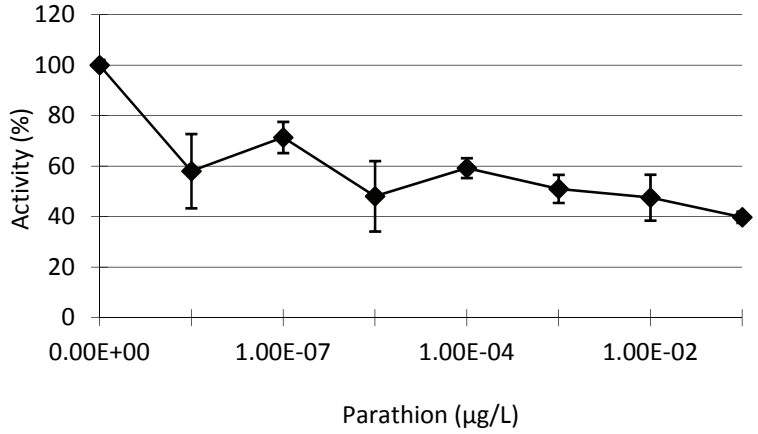

Figure 15: Results of inhibition of AChE by parathion after 15 minutes incubation period with concentration ranging from 0 to 0.1 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

Figures 16 and 17 represent the inhibition of AChE by demeton-S-methyl at low and high concentrations. The results show that demeton-S-methyl inhibits the enzyme up to 50% at concentrations of 0.1 µg/L.

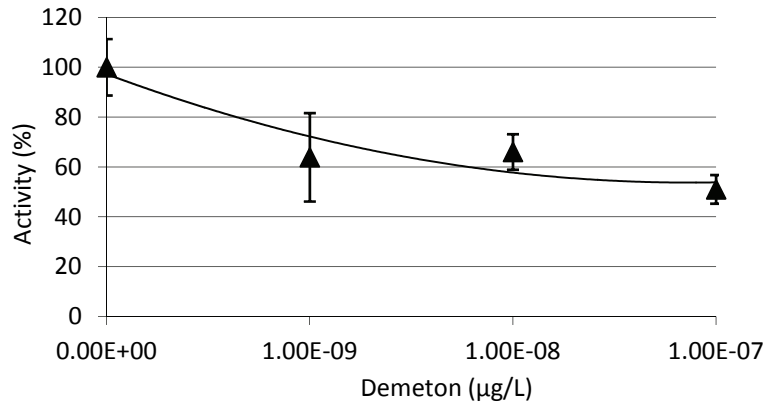

Figure 16: Graph showing AChE activity after 15 minutes incubation with demeton-S-methyl at very low concentrations. The results shown are relative to the uninhibited reaction. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

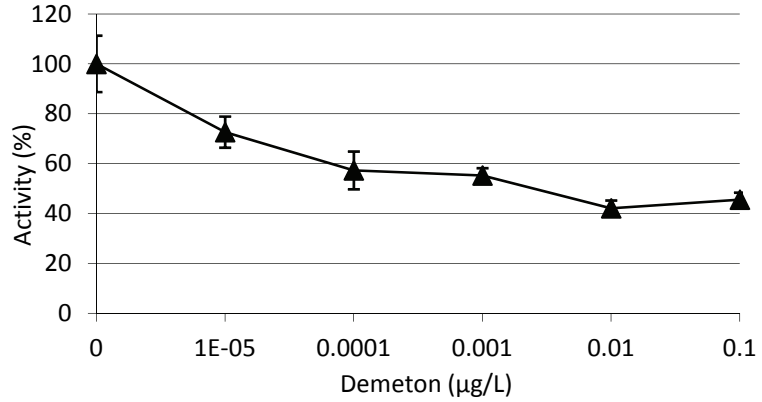

Figure 17: Graph showing AChE activity after 15 minutes incubation with demeton-S-methyl at moderately low concentrations. The results shown are relative to the uninhibited reaction. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

Figure 18 indicates that aldicarb did not significantly inhibit AChE as inhibition was only observed up to 30% at the concentrations of pesticide tested.

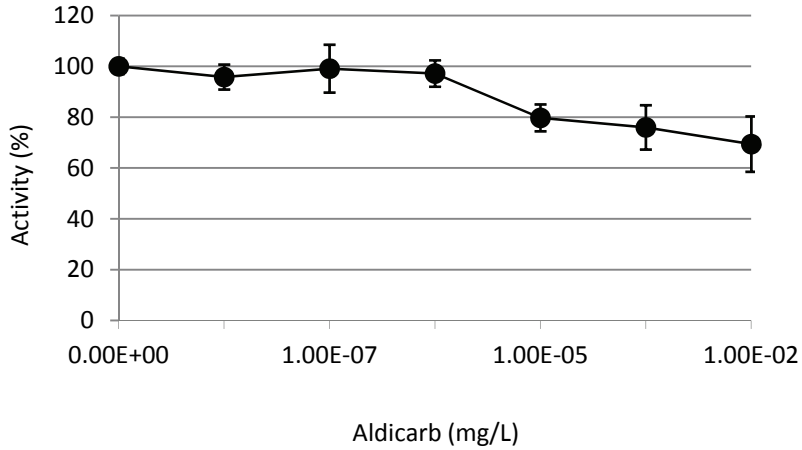

Figure 18: Graph showing AChE activity after 15 minutes incubation with aldicarb at concentrations of 0 to 0.1 µg/L. The results shown are relative to the uninhibited reaction. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

The IC_{20} value was used in order to compare limits of detection of the pesticides, as this represents a substantial inhibition. The IC_{20} is the concentration of pesticide that is required to inhibit the enzyme activity by 20%, i.e. the enzyme will only function at 80% of its full capacity. Table 2 gives IC_{20} values for the individual pesticides that were tested. In addition, details are also supplied on the required limit of detection according to the EU standards (Hamilton et al., 2003) which are the most stringent as well as some of the limits of detection (LODs) that have been achieved by other researchers. Table 2 shows that the optimized AChE assay is far more sensitive than any others reported thus far with the exception of carbofuran, and can be used successfully to test for pesticides at low concentrations.

Table 2: Summarized data for IC_{20} levels for individual pesticides tested over the range 0 µg/L-0.1 µg/L, compared to LODs in literature.

Pesticide	IC_{20} (µg/L) this study	Required LOD (EU) (µg/L)	LOD in Literature (µg/L)
Carbofuran	0.01	0.1	0.001 (No et al., 2007)
Carbaryl	1×10^{-7}	0.1	0.001 (No et al., 2007)
Aldicarb	1×10^{-5}	0.1	0.2 (Nunes et al., 2004)
Demeton-S-Methyl	5×10^{-9}	Not Stipulated	Not Known
Parathion	5×10^{-6}	0.1	0.001 (No et al., 2007)

5.2. Pesticide mixtures

A second objective of the project was to investigate whether pesticide mixtures had a synergistic or additive effect in their inhibition of AChE. Mixtures of pesticides and their effect on enzyme activity were examined under laboratory conditions prior to the analysis of environmental samples to allow us to interpret the data obtained from environmental samples. Pesticides were tested on their own and in combinations of two, three, four and five pesticides (see Table 3 and 4).

Table 3: Matrix summarizing the pesticide combinations tested for four pesticides

	Parathion	Demeton	Carbaryl	Carbofuran	Aldicarb
Parathion		✓	✓	✓	✓
Demeton	✓		✓	✓	✓
Carbaryl	✓	✓		✓	✓
Carbofuran	✓	✓	✓		✓
Aldicarb	✓	✓	✓	✓	

Table 4: Matrix summarizing combinations of pesticides tested for three pesticides.

	Parathion	Aldicarb	Demeton	Carbofuran	Carbaryl
Parathion		✓	✓		
		✓		✓	
		✓			✓
Aldicarb	✓		✓		
	✓			✓	
	✓				✓
Demeton		✓		✓	
		✓			✓
	✓			✓	
	✓				✓
Carbofuran	✓				✓
	✓	✓			
Carbaryl	✓			✓	
	✓			✓	
		✓		✓	

Their impact on enzyme activity was then measured and it was determined whether the mixture had an additive or synergistic effect on enzyme activity.

5.2.1. Carbamate pesticide only mixtures

A mixture of only carbamate pesticides was made and AChE inhibition measured. The graphs below show the results obtained.

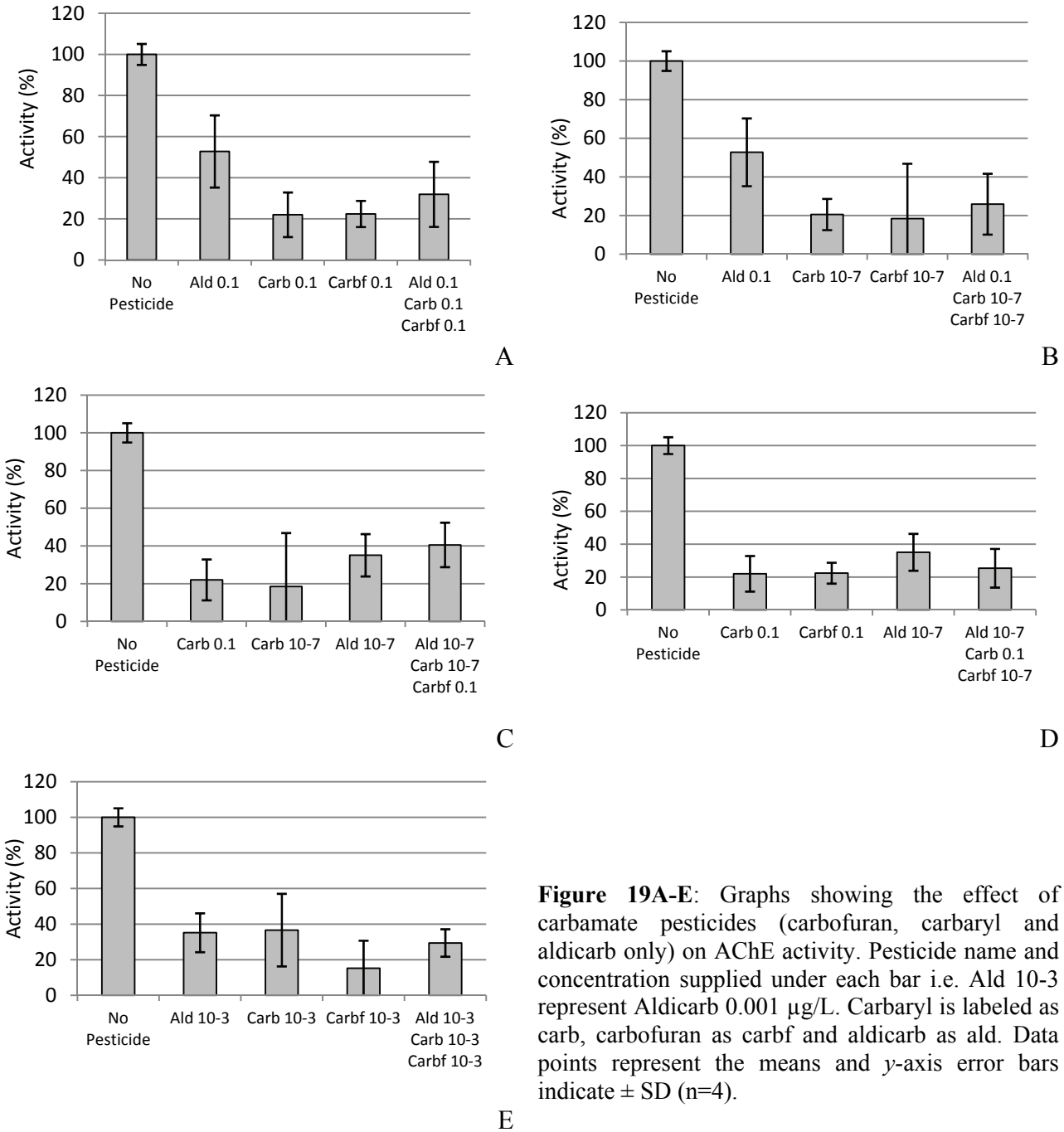

Figure 19A-E: Graphs showing the effect of carbamate pesticides (carbofuran, carbaryl and aldicarb only) on AChE activity. Pesticide name and concentration supplied under each bar i.e. Ald 10-3 represent Aldicarb 0.001 µg/L. Carbaryl is labeled as carb, carbofuran as carbf and aldicarb as ald. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

It must be noted that for each of the mixtures, the final concentration was the total indicated i.e. in the first graph, Figure 19A, the total concentration was 0.1 µg/L. This indicates that each pesticide contributed 0.033 µg/L. The same applies for all concentrations of pesticides. At all concentrations the carbamate pesticide mixtures were found to evince additive inhibition.

5.2.2. Organophosphate pesticide only mixtures

The same process was repeated with only OPs, which were also found to have an additive inhibition effect on AChE.

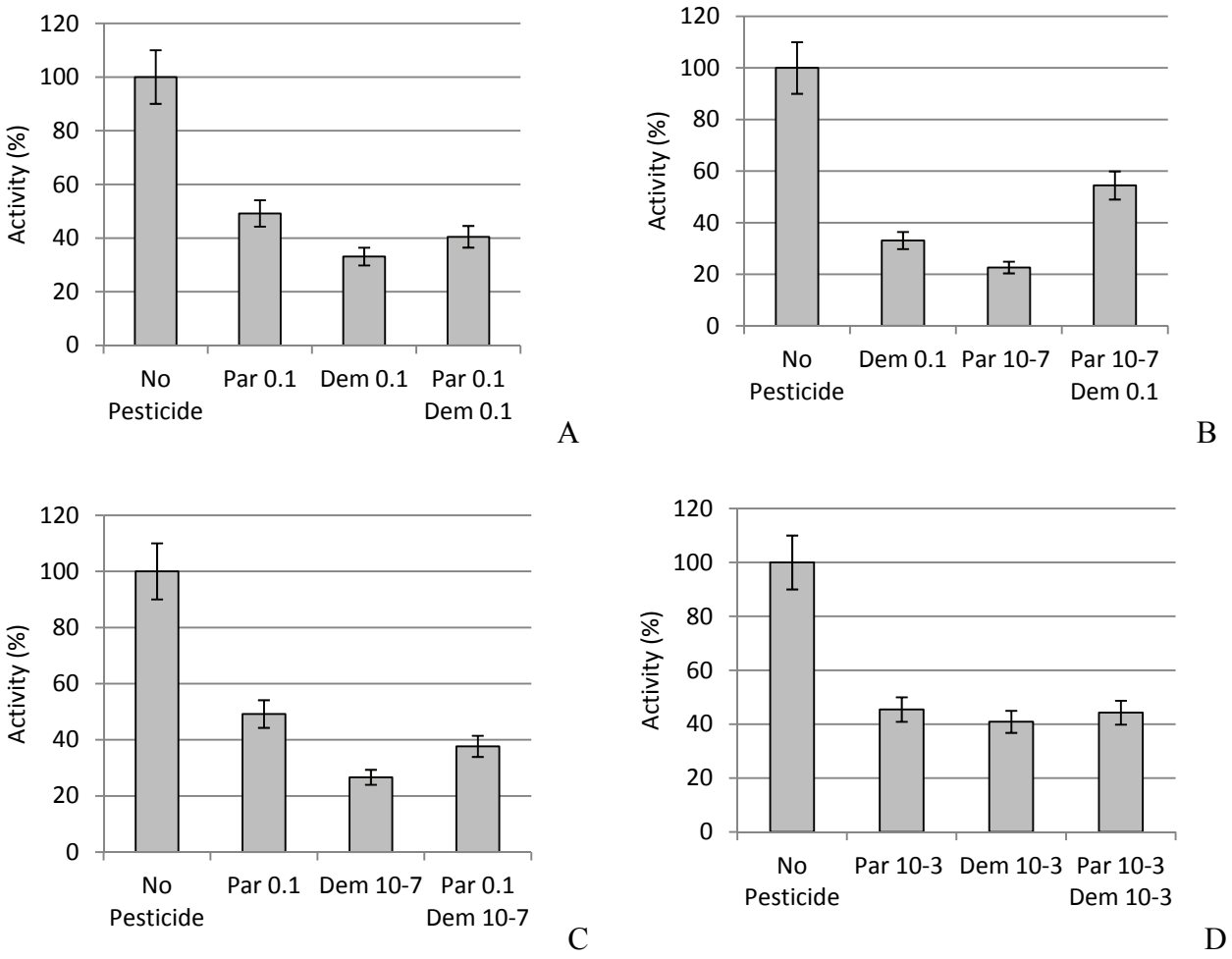

Figure 20A-D: Graphs showing the effect of organophosphorus pesticides (parathion and demeton-S-methyl only) on AChE activity. Pesticide name and concentration supplied under each bar i.e. Par 10-3 represents Parathion 0.001 µg/L. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

5.2.3. Carbamate and organophosphorus pesticide mixtures

Varying combinations of OPs and CPs were made and for each of these, AChE activity was measured after incubation of the pesticide mixture with the enzyme for 15 minutes. The combinations consisted of mixtures of two, three and four pesticides. These results can be observed in Figure 21. In addition, the effects of all five pesticides at low, medium and high concentrations were also investigated. Results can be observed in Figures 22 and 23.

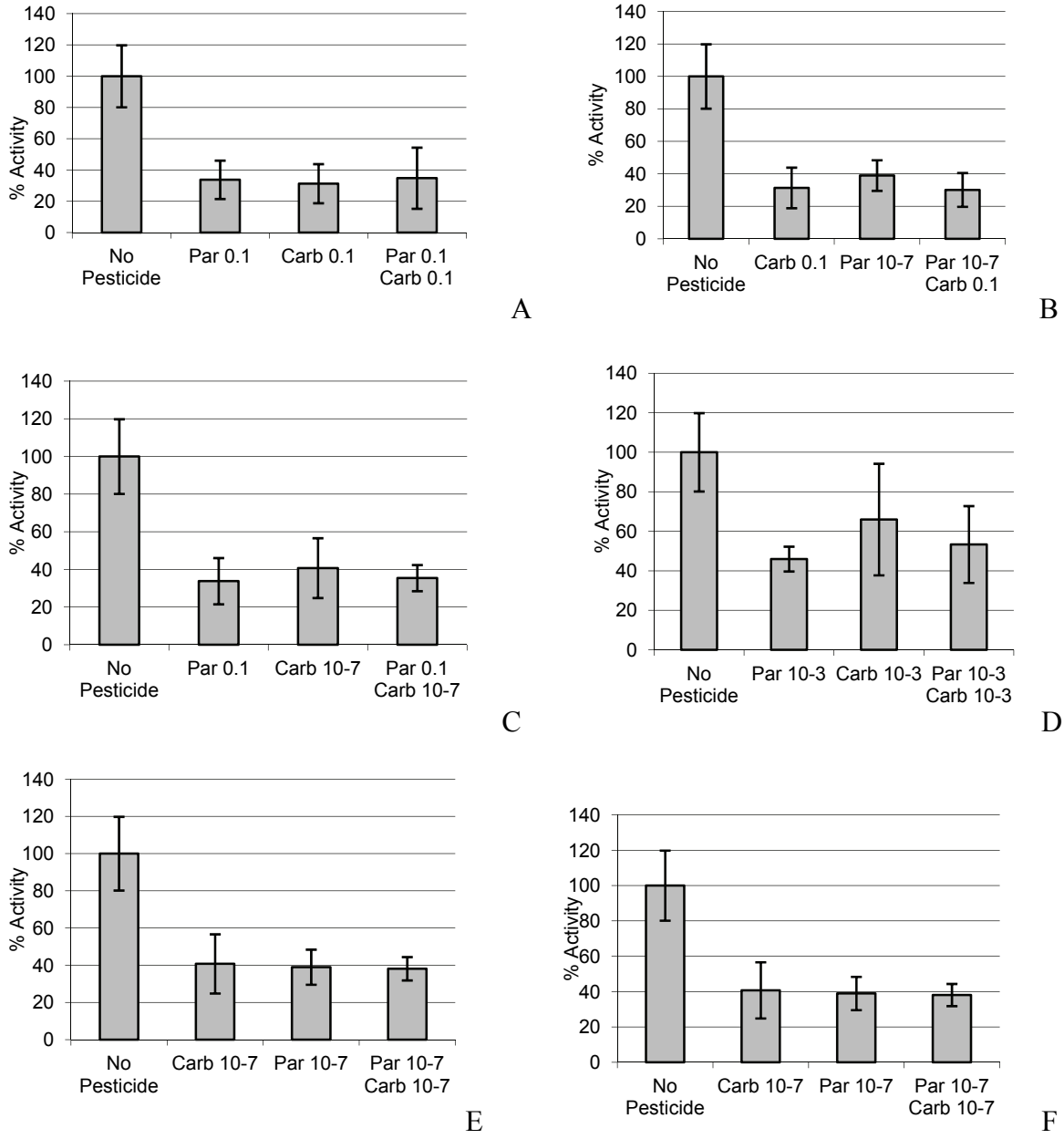

Figure 21A-F: Graphs showing mixtures of OPs and CPs showed an additive inhibition of AChE. Pesticide name and concentration supplied under each bar i.e. Par 10-3 represents Parathion 0.001 µg/L. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

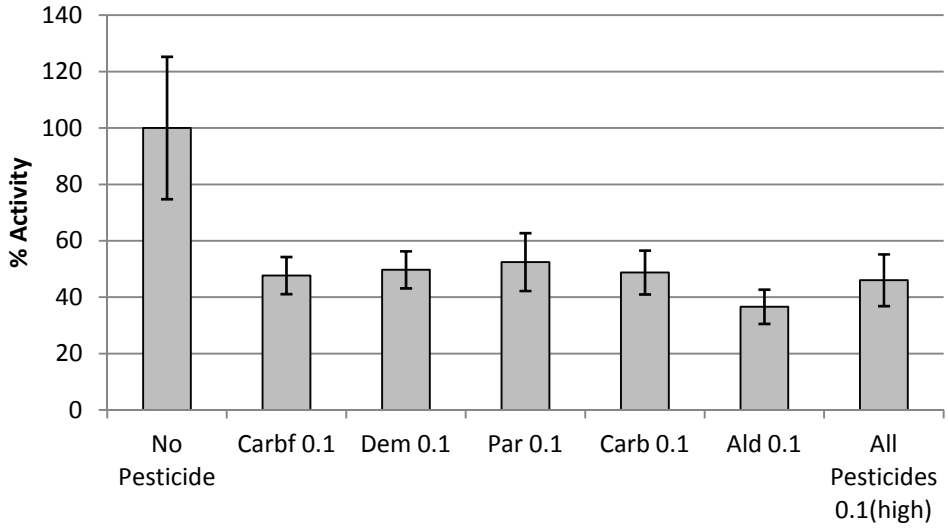

Figure 22: Graph showing AChE inhibition by individual pesticides, as well as a mixture of all 5 pesticides at the highest concentration tested. Pesticide name and concentration supplied under each bar i.e. Par 0.1 represents Parathion 0.1 µg/L. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

As was the case with individual mixtures of CPs and OPs, the pesticide mixtures of OPs and CPs were found to display an additive effect. Each of the pesticides was found to inhibit the enzyme by 75% to 80%. The same level of inhibition was observed when all the pesticides were mixed in one sample. The analysis of a mixture of five pesticides, OPs and CPs, at the highest concentration tested is shown in Figure 23.

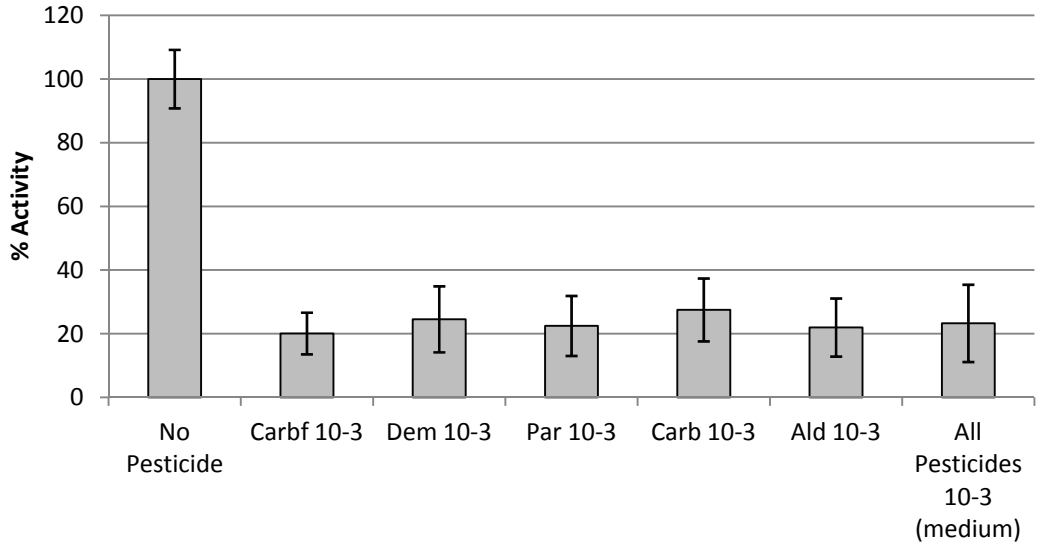

Figure 23: AChE inhibition by individual pesticides, as well as a mixture of all five pesticides. Pesticide name and concentration supplied under each bar i.e. Par 10-3 represents Parathion 0.001 µg/L. Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

From the results obtained, it was concluded that the pesticide concentration had an additive effect with regards to the inhibition of AChE at low concentrations.

5.3. Pesticide mixture detection using chemometrics

An ANN was created that focuses on identifying the pesticides that are present in a mixture. Up to now, the ANN has been trained to recognize individual pesticides given a specific data set. Thus far the ANN is able to correctly identify when single pesticides are present and it is now being "trained" in order to distinguish more than one pesticide. This process is reiterative and the ANN should progressively work more efficiently and correctly. As the "training" process has not been completed, the final results have not been included in this report.

5.4. Yeast Estrogen Screen assay

The results from the YES and the T47D-KBluc assays indicated that none of the samples tested had receptor mediated estrogenic activity above the detection limit of the assays.

The MDA assay also indicated that none of the samples had androgenic properties above the detection limit of the assay.

5.5. Environmental sampling

The environmental water samples were all collected on the same day and stored overnight at 4°C. Analysis of the samples was carried out the following day. The pesticides were extracted using solid phase extraction (SPE) and then analyzed using gas chromatography (GC). For metal analysis filtration was carried out using 0.45 µm membrane and samples preserved using nitric acid at pH 2. Appendix A indicates the results obtained using the AChE micro-Ellman assay as well as the water source from which it was obtained. The graphs represent the slope or rate of activity of the reaction relative to a reaction in the absence of any pesticide.

From the raw data as displayed in Appendix A, the gradient of the reaction was used as the average rate of reaction and these tabulated values are shown below for comparative purposes in Table 5 with the reaction containing no pesticide taken as having 100% activity.

Table 5: Tabulated data of gradients indicating inhibition of AChE by each water sample, used to calculate total activity relative to the reaction without any inhibition. RSD represents relative standard deviation.

Water Sample	Gradient	Activity (%)	RSD (%)
No Pesticide	0.1549	100.0	9.4
Port Elizabeth Tap Water	0.1220	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 Bridge GHT Road	0.0804	0.5	14.2
Blaauwkrantz Near EBRU	0.0529	34.1	16.2
Grahamstown Tap Water	0.0798	51.5	11.8

Figure 24 summarizes the data that was obtained from all the water samples that was calculated in Table 5. Each of the samples is presented relative to the uninhibited reaction, shown as "no pesticide" in Figure 24. Inhibition was said to have occurred if AChE activity was lower in the water sample than the uninhibited reaction.

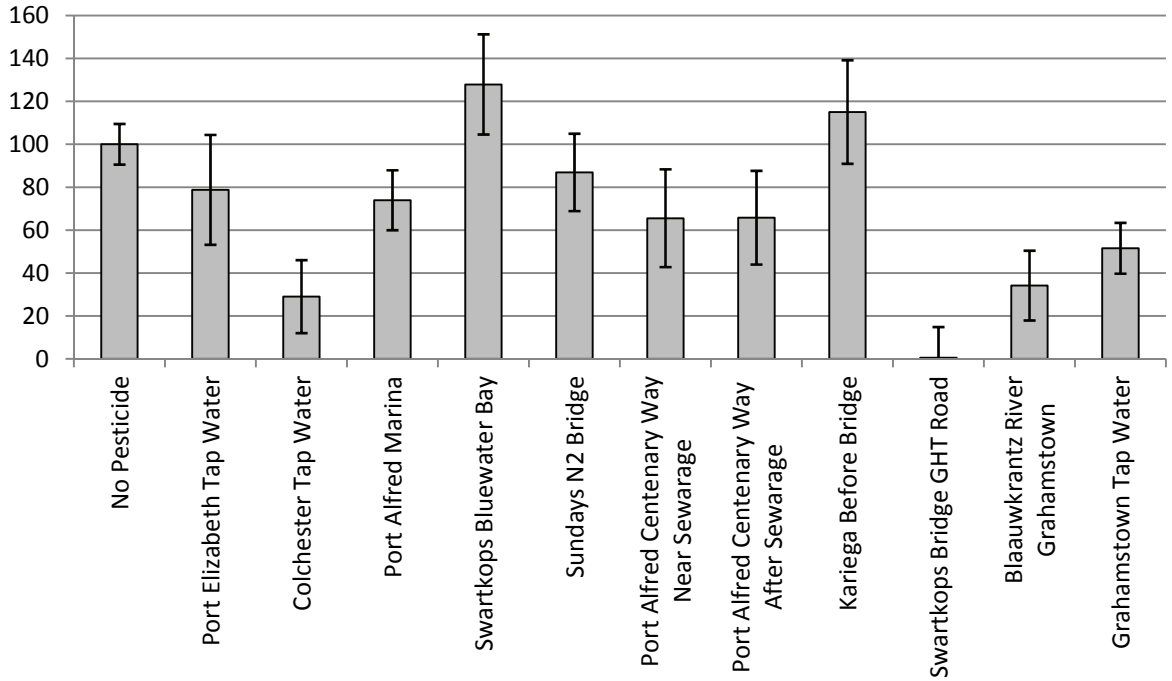

Figure 24: Results of AChE inhibition by water samples taken from various locations in the Eastern Cape. The activity (%) is based on the reaction rate (gradients) for each sample relative to the uninhibited reaction (No Pesticide). Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

A second round of sampling was undertaken in March 2011 and the assay carried out as before. Table 6 and Figure 25 show the tabulated and graphed data for water samples.

Table 6: 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 (%)	RSD (%)
No Pesticide	0.0824	100.00	0.00
Kariega	0.0148	17.96	2.79
Sundays (N2)	0.0301	36.53	2.26
Colchester (Tap)	0.0270	32.77	1.49
Swartkops (Amsterdam Way)	0.0274	33.25	1.38
Swartkops (Grahamstown Bridge)	0.0211	25.61	1.84
Blaauwkrantz Grahamstown	0.0224	27.18	0.45

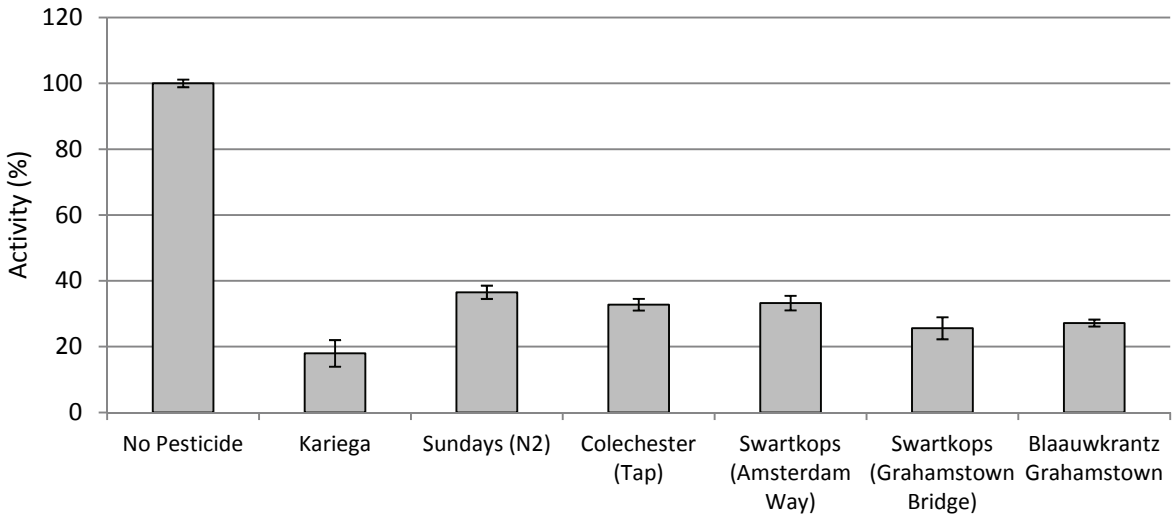

Figure 25: Results of AChE inhibition by the second set of water samples taken from various locations in the Eastern Cape. The value of activity (%) is based on the reaction rate (gradients) for each sample relative to the uninhibited reaction (No Pesticide). Data points represent the means and *y*-axis error bars indicate ± SD (n=4).

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

6. DISCUSSION

6.1. Detection of OPs and CPs using AChE

Acetylcholinesterase was found to be an effective tool in determining the presence of the selected CPs and OPs. It was used to detect the presence of these pesticides at nanogram levels and this indicated a high level of sensitivity. As Table 2 indicates, other researchers have not managed to achieve such sensitivity levels with the exception of carbofuran and this therefore renders this assay extremely useful in measuring CPs and OPs at very low concentrations. Due to the fact that sensitivity is an important consideration for toxicity testing below legal limits, this assay should remain an integral part of the biomarker assays established for this purpose. This increased sensitivity can be attributed to two main factors. As was discussed earlier, *Electrophorus electricus* (electric eel) AChE has been found to have the greater sensitivity comparable to genetically modified *Drosophila melanogaster (*common fruit fly) AChE hence its use (Marques et al*.,* 2004). The second factor, which increased the sensitivity of the assay, was the inclusion of a 15 minute pre-incubation period and the use of a microtiterplate assay.

The increased sensitivity allowed for the pesticide range tested to be adjusted and to ensure that the pesticides could be detected well within the limits stipulated by the relevant authorities. According to literature, the EU has the most stringent limits (Hamilton et al., 2003), most of which are 0.1 µg/L for individual pesticides. As Table 2 indicates, the assay is able to detect pesticides well below this range for the majority of pesticides tested.

In previous work (without a pre-incubation period and at higher pesticide concentrations) IC_{20} values of 1×10^{-5} µg/L, 2×10^{-3} µg/L and 5×10^{-5} µg/L were recorded for aldicarb, carbaryl and parathion, respectively (Mwila, 2009). As Table 2 demonstrates; a pre-incubation period in which the enzyme is allowed to interact with the pesticide prior to the substrate being added, plays a significant role on the sensitivity that is observed. In this study a constant period of 15 minutes was selected. The new IC_{20} values of 0.01 µg/L, 1×10^{-7} µg/L 1×10^{-5} µg/L, 5×10^{-9} µg/L and 5×10^{-6} µg/L were recorded for carbofuran, carbaryl, aldicarb, demeton-S-methyl and parathion, respectively.

The AChE assay was used to collect data on the effects of individual pesticides as well as pesticide mixtures on the activity of the enzyme. Data analysis suggests that mixtures of pesticides may have an additive inhibitory effect on AChE activity. This is assuming that additive effect is defined according to Richardson et al. (2001) which stipulates that additive effect means the summative inhibition of the enzyme. This method of assessment is appropriate as it takes into consideration the fact that both OPs and CPs act on AChE at the same site on the molecule (i.e. the active site) and this is a key feature in determining whether the effect is dose or response additive according to Richardson et al. (2001).

The conclusion that the effect of pesticide mixtures is additive is important in terms of the application of the assay in environmental sampling as it implies that the concentration of pesticide can be equated to the degree of inhibition, and this is important as identification of pesticides in pesticide mixtures can be simplified. In addition, this is also important in that it brings to the fore concerns over the fact that even though individual pesticides may be at no adverse effect level (NOAEL), their additive effect may still result in adverse effects as was suggested by Tahara et al. (2005). With this in mind, more stringent regulations can be put in place with regard to pesticide limits in water that account for the additive effect.

It should; however, be noted that at higher concentrations of pesticides, the additive effect observed may no longer take place. But this may be overcome if environmental samples are tested at different dilutions. This study however, is focused on testing water samples at levels lower than legal limits; therefore the total effect of these pesticides at low concentrations can be established as additive.

6.2. Estrogenic effects of OPs and CPs

The YES, KBluc and MDA assays all indicated that none of the pesticides tested displayed any estrogenic or androgenic effects above the detection limits of the assays. The results obtained are in accordance with those of Kojima et al. (2004), Oh et al. (2007) and Nishihara et al. (2000).

Kojima et al. (2004) used Chinese hamster ovary (CHO) cells which were modified to incorporate hER receptors (α and β) and androgen receptors (AR) to test for both estrogenic and androgenic activity. While testing a group of 200 chemicals, they found that carbaryl and carbofuran had no reported estrogenic effects. Oh et al. (2007), using the E-screen assay with MCF-7 breast cancer cell line that has both hER and human progesterone receptor (hPR), reported that carbaryl had no estrogenic effects. Nishihara et al. (2000) also concluded that carbaryl, carbofuran and aldicarb had no estrogenic effects using a yeast hybrid assay that had involved the use of hERα and TIF2 promoter.

The findings of this study appear to contradict some of the reports in literature. In the same tests carried out with CHO cells, Kojima et al. (2004) found that parathion exhibited anti-androgenic effects. According to Sohoni et al. (2001) and Tamura et al. (2001), parathion should have exhibited anti-androgenic effects in the MDA assay which was not observed in our study. However, the results of this study are inconclusive as full dose response curves for anti-androgenic activity were not included and should be investigated further. This study only included a screening plate for androgenic activity. In addition, Klotz et al. (1997)'s results for parathion corresponded to those of others in the field. Their study indicated that carbaryl and aldicarb could be hER and hPR agonists. The fact that Klotz et al. (1997) used MCF-7 breast cancer cell lines transfected with hER gene as well, allows for a valid comparison with the results of Oh et al. (2007). As previously indicated, it has not been conclusively determined whether or not carbaryl, carbofuran and aldicarb possess the ability to affect estrogenic or androgenic receptors.

Currently no data are available on the estrogenic or androgenic properties of demeton-S-methyl in literature, despite it having been declared a possible EDC. This study concluded that demeton-S-methyl appears to have no endocrine disrupting effects. However, more studies need to be performed to verify this result. The two breakdown products tested, *p*-nitrophenol and aminophenol reported no effects in all the assays. There are currently no reports in literature to compare with this result.

Klotz et al. (1997) raised a very important issue in discussing the results obtained. Their research indicated that, despite the fact that the 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. The mechanism of action of carbamates as described in Fukuto (1990) is based on carbamylation of the serine residue in the active site and the carbamate may still act by carbamylation of residues near the hER or hPR. This would, in turn, affect the receptors, albeit indirectly. Fukuto (1990) therefore suggested that this also be accounted for in testing for estrogenic and androgenic properties of samples.

The Global Water Research Coalition (GWRC) reported that several bioassays were tested with regard to their ability to detect water samples containing EDCs. This report analyzed the YES, T47D-KBluc, E-Screen, Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER-Calux) and MELN assays. The report highlighted the lack of standardization of these tests and the difficulties that arise when making comparisons. It did conclude that the ER-Calux was one of the best assays for testing water samples. However it was noted that this test is costly, time consuming and requires highly trained personnel. E-Screen and YES assays were identified to have good sensitivity and reproducibility and were adequate alternatives to the ER-Calux assay. T47D-KBluc was said to possibly be a good assay but required more testing to verify its feasibility. If these submissions are taken into consideration and used in conjunction with the results obtained, then 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 this study.

In order to obtain more conclusive results, the use of the AR-Calux and ER-Calux assays should be considered in order to verify androgenic and estrogenic effects of the pesticides, since it is considered to be the most sensitive and reliable assay of the five tested. A method for standardizing 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.

6.3. Relative distribution of OPs and CPs in water samples in the Eastern Cape

Water samples from different sources were taken on two occasions, as represented in Figures 24 and 25. The results from these samples varied quite substantially. Analysis of the first set of water samples indicated that most of the water sources, with the exception of Swartkops (Bluewater Bay) and Kariega (before bridge), could have pesticides present because all the samples were clearly inhibitory to AChE activity. The Swartkops (Bluewater Bay) sample had an activity of 127.8 % of that in the control, which was higher than the assay containing no pesticide (referred to as No Pesticide in Figure 24). The cause for the increased enzyme activity of the sample is not clear. However, one possible explanation is an error created as a result of the large standard deviations (23.3%) in the readings observed in this sample. The result obtained from the Swartkops River (Bluewater Bay) sample contradicted the expected results. Due to the industrial effluent and sewage that enters the river upstream, it would be expected that much greater inhibition of AChE would be observed. This was not the case as the enzyme appeared to have been activated rather than inhibited by contaminants in the water. With reference to the Kariega River samples, the result of 115.0% agrees with known data which indicate that the estuary is relatively pristine, and therefore would have minimal inhibition of AChE. The relatively high standard deviation (24.1%) may also account for the higher than expected readings.

Swartkops (Grahamstown Road) and Colchester (Tap Water) displayed excessively high levels of AChE inhibition not encountered during optimization of the assay as well as testing with laboratory grade pesticides. For the Swartkops (Grahamstown Road) sample, the excessive inhibition of AChE (99.5%) could be attributed to the extreme pollution evident in the river. There is currently no known reason as to why Colchester Tap water should inhibit AChE up to 28.9%, however further analysis of the water samples is being performed in order to ascertain possible explanations. From

these results it can be concluded that there are either excessively high pesticide levels or other inhibitors present in the water sources which also inhibit the AChE assay.

Port Elizabeth Tap water had a recorded activity of 78.7%, which is representative of mild inhibition. The results from Port Alfred Centenary way, before and after the sewerage pipe, were similar at 65.5% and 65.7% respectively. The high inhibition of AChE was expected as this water is believed to originate from an inadequate sewerage works treatment source. The lack of proper 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 presence of inhibitory compounds in the water samples.

The Port Alfred Marina water sample had a level of activity of 73.9%. The Marina, however, is subject to tidal variations (low and high tide) which would dilute most of the contaminants upstream that may flow in. Due to this tidal variation it would be expected that there would be minimal inhibition of AChE.

The Blaauwkrantz river sample had residual AChE activity of 34.1% and Grahamstown tap water had 51.5%. These sample results were also as expected as Grahamstown has been experiencing water shortages due to the decrease in rainfall in the area. This has resulted in severe problems with water quality and hence would have an impact on AChE activity, as was observed.

The second set of water samples analyzed (Figure 25) indicated higher levels of inhibition for all samples compared to the first set of samples taken. Most of the samples displayed almost 70% AChE inhibition with only 30% residual cholinesterase activity in most samples, with the exception of the Kariega River which only retained about 17% activity. The similarity of the results was unexpected, as some of the rivers are considered to be more pristine than others and should therefore not have resulted in equal inhibition of AChE. The statistical analysis of this data resulted in p-values less than 0.05 ($p < 0.05$) which indicates that the differences in sites were significant.

The second set of samples was sent to the South African Bureau of Standards (SABS) to validate the results found in the AChE assays. However, the results indicated that no pesticides were present in any of these samples. This was contrary to the expected results as AChE inhibition indicated that high concentrations of pesticides should be present. The SABS results did indicate that some heavy metals were present in these samples, which could explain the inhibition of AChE as it is known to be inhibited by metals (Appendix B). The report indicated that lead, arsenic, chromium, copper and selenium were found at concentrations less than 10 µg/L whilst cadmium and mercury were found at concentrations of less than 1 µg/L.

The presence of heavy metals has been reported in the some of the water sources that were tested (Orr et al., 2008). This may assist in verifying some of the results attained for the water samples as well as explain some of the unexpected results that were recorded. It has been reported that heavy metals such as lead and cadmium inhibit the activity of AChE after incubation. This was done *in vivo* by dosing fish with known concentration of metals and homogenizing the fish after two, seven and 13 days and assessing the AChE activity in the tissue (Jebali et al., 2006). Several studies also investigated using shorter incubation periods of 30 minutes (Abdollahi et al., 1998; Richardson et al., 2010) and reached similar conclusions. It therefore needs to be assessed whether the current pre-incubation time (15 minutes) allows for sufficient interaction with the metals that are present in the water samples and therefore allow for inhibition of the AChE.

The samples tested were predominantly salt water. The effect of salt on the enzyme AChE was analyzed. In order to achieve this, the AChE assay was carried out in the presence of salt (sodium chloride) concentrations equivalent to those found in sea water (3.5%), bracken (1.7%) as well as freshwater (0%). From this, the effect of salt on AChE activity was ascertained to be negligible as was concluded by Scaps and Borot (2000). Their study indicated that increased salinity (sodium chloride concentration) had minimal effect on the activity of AChE. However, they did note that, in conjunction with an increase in the temperature at which the test organisms were kept, there was a noted effect on the activity of AChE (Scaps and Borot, 2000). Re-testing of the above Eastern Cape samples is ongoing and will be continued in order to get more representative results and to monitor changes in the water samples over time (seasonal changes).

7. CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated that the optimized assay using AChE from electric eel could effectively be used to detect pesticides at very low concentrations. In combination with chemometrics, this assay may be utilized to detect individual pesticides from mixtures. Whether this assay can be used as a screening tool to detect pesticide contamination in water, will, however, require further optimization. It is proposed that further investigation be done with regard to the effect of metals and salts on AChE activity enzyme that may be present in environmental samples and how these can be accounted for in the context of a neural network. We are currently also immobilizing AChE onto electrospun nanofibers with the aim of increasing the stability and the feasibility of using this enzyme for pesticide detection. Ms Tendai Mafuma, a Masters student is currently continuing with this work.

With regards to the potential endocrine disrupting effect of the pesticides tested, more tests will have to be carried out before all the pesticides can be ruled out as endocrine disruptors. This would entail standardization and implementation of bioassays as this will allow for suitable comparison of results across laboratories. Moreover, additional information also needs to be obtained as to whether the pesticides are receptor mediated, or have some other mechanism that could cause an effect on the endocrine system.

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WOLFF MS, TONIOLO PG, LEE EW, RIVERA M and DUBIN N (1993) Blood Levels of Organochlorine Residues and Risk of Breast Cancer. JNCI Journal of the National Cancer Institute **85 (8)** 648-652.

APPENDIX A: Raw data indicating Absorbance vs. Time for Eastern Cape Water Samples

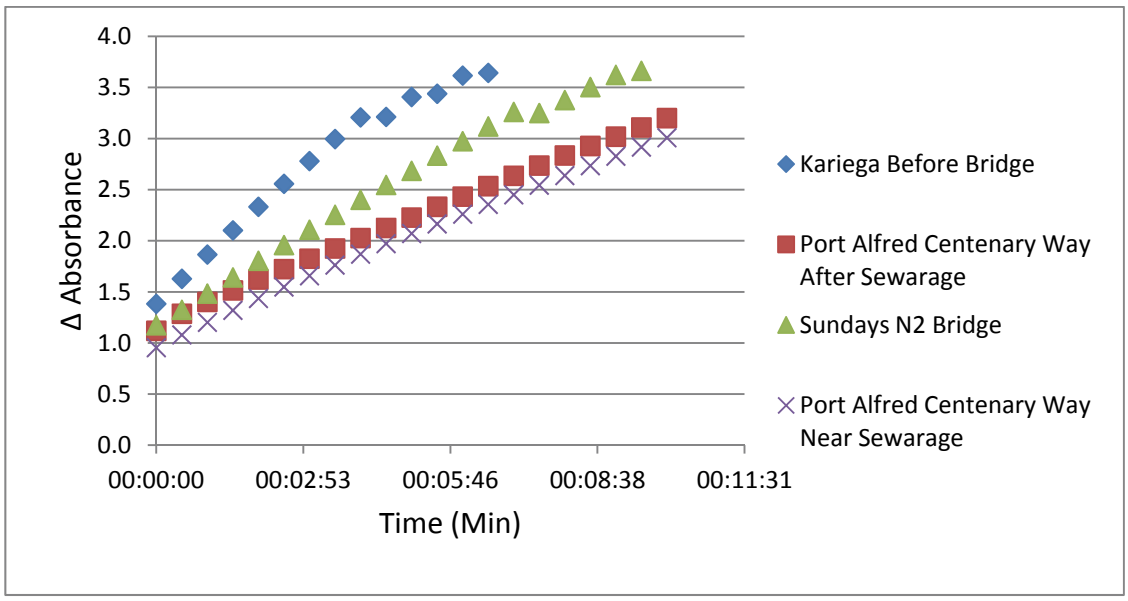

Figure A1: Raw data demonstrating the progressive color formation using the AChE assay for water samples from Centenary way in Port Alfred, Kariega and Sundays River. Data points represent the means (n=4).

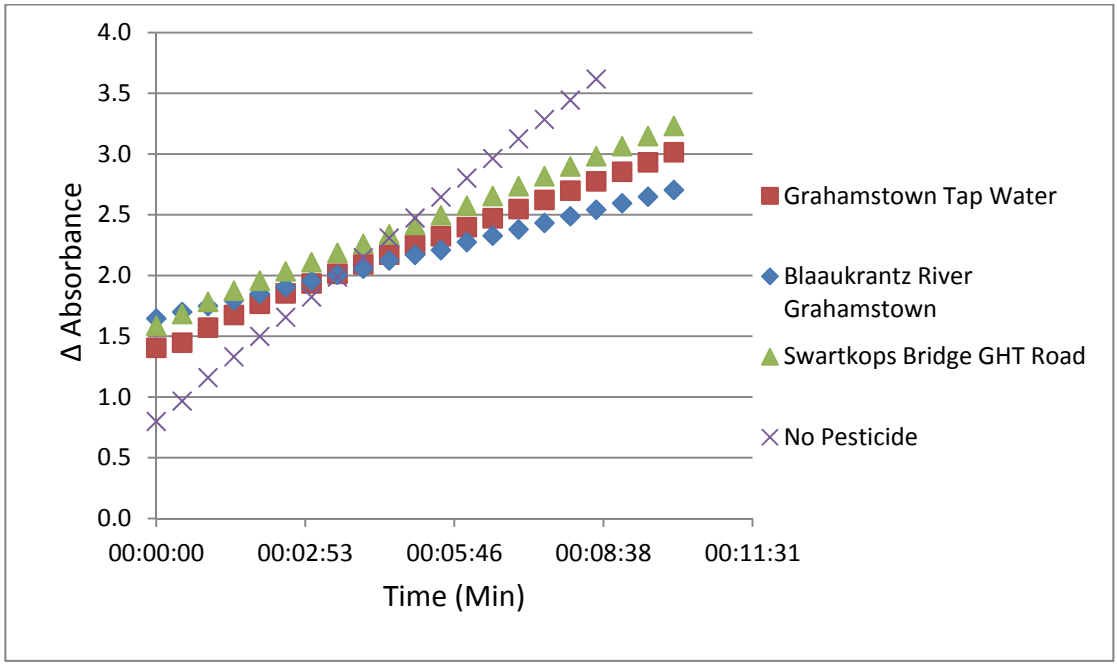

Figure A2: Raw data demonstrating the progressive color formation using the AChE assay for water samples from Swartkops River, Blaauwkrantz River in Grahamstown and Grahamstown Tap water. Data points represent the means (n=4).

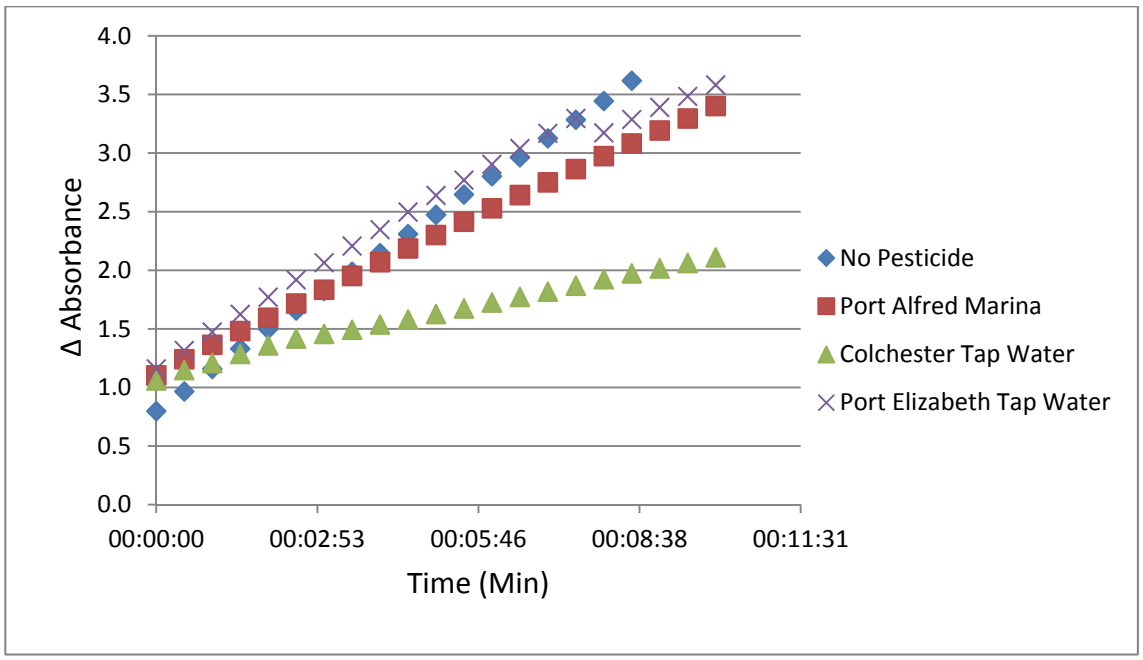

Figure A3: Raw data demonstrating the progressive color formation using the AChE assay for water samples from the Port Alfred Marina, Port Elizabeth and Colchester Tap water. Data points represent the means (n=4).

APPENDIX B: Copy of results received from South African Bureau of Standards for a second set of water samples.

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.

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

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