RAPID ENZYMATIC DETECTION OF ORGANOCHLORINE PESTICIDES IN WATER

Report to the
WATER RESEARCH COMMISSION

by

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WRC REPORT NO. KV 252/10
ISBN 978-1-4312-0040-5

OCTOBER 2010

The publication of this report emanates from a project entitled *Rapid enzymatic detection of organochlorine pesticides in water* (WRC Report No. K8/828).

EXECUTIVE SUMMARY

BACKGROUND

In recent years, increased public awareness and interest in environmental issues have highlighted the problem and the effects of the high levels of accumulated persistent pesticides and other toxins in the environment. The increased concern and attention around this issue has led to an increased need for effective methods of detection of these substances in potentially contaminated areas and systems (Iwata *et al.*, 1994). Current methods can offer highly sensitive and effective detection of toxicants; however, they also have their limitations. These methods include chromatography (gas chromatography, high performance liquid chromatography) (Muir and Sverko, 2006) and spectroscopy (UV spectroscopy, mass spectroscopy, fluorescence spectroscopy) (Pogačnik and Franko, 2001). The disadvantages of these methods are the requirement for expensive equipment and materials which makes it costly, as well as the need for highly skilled operators (Mazzei *et al.*, 2004; Swart and Pool, 2007). Further limitations include the lack of portability of this equipment to the contaminated site, and the fact that these methods can be time consuming, making it unsuitable for rapid, on-site detection (Mazzei *et al.*, 2004).

Enzymatic methods of detection offer a potentially simpler, more rapid and cost effective alternative for the detection of water contaminants. For the detection of pesticides, the inhibition of enzymes affected by specific pesticides can be monitored using enzyme assays, the degree of inhibition being proportional to and thus giving an indication of the concentration of pesticides in the water (Breuer, 1982). Such enzymes include cholinesterases (inhibited by organophosphorus and carbamate pesticides) and alkaline phosphatases (inhibited by heavy metal ions, as well as organophoshorus and organochlorine pesticides) (Garcia Sanchez *et al.*, 2003; Chouteau *et al.*, 2004; Gokcimen *et al.*, 2006). However, only a limited number of contaminants have been tested based on these enzymatic detection methods and there is therefore a need to determine the response of a wide range of contaminants on the activity of key enzymes.

It has been reported that significant levels of various pesticides have been detected in water and sediments in South Africa, including pyrethroids, organochlorines (DDT and DDE), organophosphates and carbamates (Slabbert *et al*., 1998; Sereda and Meinhardt, 2003; Burger and Nel, 2008; Slabbert *et al*., 2004); as well as polychlorinated dibenzo-ρ-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Vosloo and Bouwman, 2005). To ensure the ongoing identification and management of water pollution by pesticides, it is necessary to investigate the development of rapid and simple detection systems such as proposed in this study.

AIMS

The ultimate aim of this work was to develop a rapid enzymatic assay for the detection of organochlorine pesticides (OCPs). This was accomplished by addressing the following specific aims, which were:

a) To develop and establish a rapid enzyme assay for the detection of OCPs,
b) To test the reactivity of these OCPs, present either alone or in combination (i.e. in a mixture), in surface water,

c) To establish proof of concept for a subsequent, more detailed study (3-5 years) using environmental samples from around the Eastern Cape.

METHODOLOGY

A standard alkaline phosphatase (ALP) assay that made use of calf intestine ALP, with *p*-nitrophenyl (pNPP) where, in the reaction, the ALP cleaves a phosphate off the pNPP, yielding a yellow product, was used. The method was modified from that originally used by Gasser and Kirschner (Gasser and Kirschner, 1987). The effect of several OCPs (2, 4'-DDE (DDE), heptachlor (HEP), 2, 4'-DDT (DDT), toxaphene (TOX), dieldrin (DIE), aldrin (ALD), mirex (MIR), endosulfan (END), α-chlordan) on the ALP assay was investigated. Each OCP was detected and quantified separately using suitable inhibition kinetics. Once the effect of each OCP on the ALP assay had been established, the OCPs will be mixed in various combinations and the effect of these combinations on the ALP activity was evaluated. The effect of organophosphorus pesticides (OPPs), carbamate pesticides (CPs) and heavy metals on the ALP assay was also investigated, as reports in literature have hinted to the fact that these compounds may interfere with the assay.

RESULTS

This study showed that ALP was not an effective target enzyme for the design of a suitable bioprobe or biosensor product. ALP was not significantly inhibited by any of the OCPs investigated in this study, and in many cases a slight enhancement of the enzyme activity was observed. Slight enhancement of ALP was also observed in the presence of OPPs and CPs. No synergistic or even additive effects were noted when mixtures or combinations of the OCPs were assayed. The effect of various metal ions such as Cadmium, Mercury, Nickel and Zinc on ALP activity was also investigated. From the data obtained during the course of this study it was found that all these metal ions had a dramatic effect on the ALP enzyme, increasing the relative activity of the ALP by 200% even at very low concentrations of metals of 0.001 mg/l. This, perhaps, is the most significant result of our study. ALP activity was enhanced in the presence of several environmental samples (mainly rivers from around the Eastern Cape), indicating the potential presence of several metal ions in these samples.

CONCLUSIONS

This study, however, showed that ALP was not an effective target enzyme for the design of a suitable bioprobe or biosensor product. ALP was not significantly inhibited by any of the OCPs investigated in this study, and in many cases a slight enhancement of the enzyme was observed. No synergistic or even additive effects were noted when mixtures or combinations of the OCPs were assayed. ALP is probably more suitable as an enzyme target for the detection and monitoring of heavy metal compounds in water. In conclusion, we believe that the cholinesterases, such acetylcholinesterase and butyrylcholinesterase, present better target enzymes for the detection, especially for the newer generation pesticides (OPPs and CPs) classes.

RECOMMENDATIONS FOR FUTURE RESEARCH

Based on the results obtained during the course of this study, we would like to make the following recommendations:

a) ALP is probably more suitable as an enzyme target for the detection and monitoring of heavy metal compounds in water, and a follow-up study focussing on the use of ALP for heavy metal ion detection should be investigated.

b) The cholinesterases present better target enzymes for the detection of the newer generation pesticides (OPPs and CPs). These pesticides also have the added benefit of being more soluble and are more easily degraded in the environment. Currently, we are busy with a follow-on grant based on this approach (WRC 1902), extending the potential detection not only to the pesticides, but also to their degradation products in the water environment.

CAPACITY BUILDING

Biochemistry/Biotechnology degrees

2008: BSc Honours (Biochemistry). IL Cockburn. Title of thesis: "Rapid Enzymatic Detection of Endocrine Disrupting Compounds: Pesticides and Steroid Hormones"

Community involvement

Links with the local Grahamstown community was encouraged via the Rhodes University Experimental field station (EBRU) situated at the Grahamstown Municipal Sewage Works, Grahamstown. An academic and industrial resource base in the field of environmental and wastewater enzymology was established, with new knowledge contribution in this field.

KNOWLEDGE DISSEMINATION

Papers

Data from this study will be incorporated in a short communication and is currently being submitted to an internationally peer-reviewed scientific journal. The preliminary for this short communication title is: "Is alkaline phosphatase suitable for the enzymatic detection of pesticides and heavy metals in the environment?"

Technology transfer

Conference presentations were limited to those held locally in the region and Department, and were reports on general considerations already reported in literature, giving due concern to the protection of any potential intellectual property specifically inherent to this project.

Archiving of data generated during project

Data generated during the course of this project will be archived at Rhodes University, Grahamstown, South Africa in both hard and electronic format. All theses produced at the Honours level are archived in the Department of Biochemistry, Microbiology and Biotechnology at Rhodes University.

ACKNOWLEDGEMENTS

We would like to thank the Water Research Commission for the financial assistance provided during the course of this study. In particular, we would like to thank the Research Manager Ms APM Moolman, and the following people for their valuable input:

Ms Ansie Burger (Consultant, PO Box 427, Clanwilliam 8135)
Dr Sebastian Jooste (DWAF)
Ms L Slabbert (former CSIR and now private consultant)

The Project Leader (Dr BI Pletschke) would like the Project Team members for their contributions:

Dr Peter Adebiyi (Post-doctoral fellow),
Ms Ingrid Cockburn (BSc Honours student)

Ms J Susan van Dyk (Final preparation of the manuscript)

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AChCl	acetylcholine chloride
AChE	acetylcholinesterase
AChI	acetylthiocholine iodide
ALD	aldrin
ALP	alkaline phosphatase
BChCl	butyrylcholine chloride
BChE	butyrylcholinesterase
CAR	2,3-Dihydro-2, 2-dimethyl-7-benzofuranol N-methyl-carbamate
CP	carbamate pesticide
DDE	2, 4'-DDE
DDT	1,1,1-trichloro-2,2-di-(*p*-chlorophenyl) ethane
DIA	diazinon
DIE	dieldrin
EDC	endocrine disrupting compounds
ELISA	enzyme linked immunosorbent assays
END	endosulfan
FDA	fluorescein diacetate
FEN	fenitrothion
HEP	heptachlor
MAL	malathion
MIR	mirex
MUP	methyl-umbelliferyl-phosphate
OCP	organochlorine pesticide
OPP	organophosphate pesticide
PAM	paraoxon-methyl
PAR	parathion
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-ρ-dioxins
PCDF	polychlorinated dibenzofurans
p-NPP	p-nitrophenyl phosphate liquid substrate system
POP	persistent organic pollutants
PRO	propoxur
TOX	toxaphene
WHO	World Health Organisation

1. INTRODUCTION

In South Africa, as well as in the rest of the world, industrial and agricultural activities result in large amounts and a wide range of toxic compounds being released into and thus contaminating the environment, particularly the world's water resources (Chouteau *et al.*, 2004). Such toxic compounds include organophosphorus, organochlorine and carbamate pesticides (Chouteau *et al.*, 2004); steroid hormones excreted by humans and animals (Swart and Pool, 2007); polycyclic aromatic hydrocarbons (Kipp *et al.*, 1998), heavy metals (Rodriguez *et al.*, 2003) and phenols (Chouteau *et al.*, 2004) from industry; and polychlorinated biphenyls (Muir and Sverko, 2006). Although many of these groups of toxic compounds have adverse effects on the health of humans, animals and plants, the compounds of particular concern are those which display endocrine disrupting properties (Burger and Nel, 2008). Endocrine disrupting compounds (EDCs) are defined as compounds which interfere with the normal endocrine function in organisms, resulting in a large number of physiological problems, including cancer, infertility, birth defects and other reproductive and developmental problems (Burger and Nel, 2008). These effects are brought about by interference of these compounds with hormone-receptor complexes (Burger and Nel, 2008). EDCs found in environmental water include pesticides and steroid hormones (Burger and Nel, 2008).

1.1 Pesticides

Pesticides are generally used to defend plants against insects, fungi and weeds, to help to prevent the damage or depletion of mostly commercial crops by pests (Kaushik and Kaushik, 2007). Today, there are several major classes of pesticides: organochlorines, organophosphates, carbamates and pyrethroids (Yu-Xi *et al.*, 2007).

1.2 Organochlorine pesticides

Organochlorine pesticides generally have a ring structure with chlorine constituents (Yu-Xi *et al.*, 2007). A well known example of an organochlorine pesticide is 1,1,1-trichloro-2,2-di-(*p*-chlorophenyl) ethane, commonly known as DDT (Kaushik and Kaushik, 2007). In the 1930's, DDT's strong insecticidal properties were first discovered by Paul Muller in Switzerland (Kaushik and Kaushik, 2007). Very soon after that, DDT became a widely used pesticide due to the fact that it has broad spectrum effects, is relatively inexpensive, as well as being stable and selective as a pesticide (Kaushik and Kaushik, 2007). DDT has strong effects on a wide range of arthropods. It acts as a nerve poison to insects and mammals, causing hyperexcitability (due to over-stimulation of nerves), symptoms being tremors, convulsions, prostration and often death (Abdel-Aal, 1983). These effects of DDT are thought to be due to DDT binding to nerve membranes, and consequently interfering with neural transmission (Kaushik and Kaushik, 2007). Organochlorine pesticides have been linked to breast cancer, liver cancer, testicular tumours and lower sperm counts in humans,

and in particular, DDE, the most prevalent isomer of DDT, has been reported to have the ability to bind to the androgen receptor in male rats, resulting in endocrine disruption (Fatoki and Awofolu, 2002). Organochlorine pesticides are also known to have a high persistence, and accumulate in environmental systems (Suwalsky *et al.*, 1998, Garcia-Sánchez *et al.*, 2003).

1.3 Organophosphate pesticides (OPPs)

Another pesticide group, the organophosphate pesticides, includes compounds with one or more phosphorus atoms, as well as either a phosphoryl (P=O) or thiophosphoryl (P=S) bond (Gupta, 2006). Organophosphate compounds are also often used as flame retardants (Gupta 2006), and in World War II, they were used as nerve gas agents. As pesticides, they are toxic to insects and warm-blooded animals (Kaushik and Kaushik, 2007). Organophosphate pesticides show a significantly lower persistence in the environment than organochlorines, and thus have a lower chance of accumulating in environmental systems; however, they show a significantly higher toxicity than organochlorine pesticides (Garcia-Sánchez *et al.*, 2003). Examples of organophosphate pesticides used in agriculture today are triazophos and oxydemeton (Pogačnik and Franko, 2001). Triazophos has a relatively low toxicity (Pogačnik and Franko, 2001). Its toxicity, which is expressed as LD_{50} ("Lethal dose 50%": dose at which 50% of test subjects die) after oral dosage in rats is 57-59 mg/kg (Pogačnik and Franko, 2001).

1.4 Carbamate pesticides (CPs)

The first carbamate pesticide, a carbaryl, was produced on a large scale in 1956 (Kaushik and Kaushik, 2007). Apart from being used as pesticides, carbamate compounds are used as drugs against Alzheimer's disease (Gupta, 2006). Many carbamates are structurally similar to acetylcholine, a neurotransmitter, and thus some carbamates stimulate acetylcholine receptors in nervous systems (Gupta, 2006). Carbamates are also known to inhibit acetylcholinesterase (Gupta, 2006), resulting in an accumulation of acetylcholine in synapses, which results in neural problems (Podolska *et al.*, 2008). Carbamate pesticides frequently used in agriculture today include carbofuran and propoxur (Pogačnik and Franko, 2001). Carbofuran and propoxur have relatively high toxicities compared to that of triazophos given above (Pogačnik and Franko, 2001). Paraoxon, another carbamate pesticide, has an LD_{50} value of 5 mg/kg, for rats after oral dosage (Pogačnik and Franko, 2001).

1.5 Bans and restrictions on pesticides

The Stockholm Convention on Persistent Organic Pollutants (POPs) was initiated by the United Nations Environmental Programme (UNEP) in 1997 as a legally binding convention to eliminate or reduce the production or release of 12 POPs, namely aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, polychlorinated biphenyls

(PCBs), polychlorinated dibenzo-ρ-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF). The Convention was adopted in 2001 and requires parties to take measures to eliminate/reduce the release of these compounds into the environment (http://chm.pops.int/Convention/tabid/54/language/en-US/Default.aspx#convtext).
Ratification of the Convention is, however, voluntary for countries who wish to participate. Compounds such as DDT are still permitted for use by countries for disease vector control but its use is restricted in accordance to the World Health Organisation (WHO) guidelines and a register of countries using DDT is maintained.

Hamilton *et al.* (2003) gives a summary of the limits on pesticide residues set by the WHO and various countries, namely Australia, the United States, New Zealand, Japan, Canada, European Union and Taiwan. Different terminology is used in different countries but Table 1 below lists comparable limits for a few countries.

Table 1. Comparison of legal guidelines for pesticide residues in drinking water for various countries. Values are shown as µg/l. (Adapted from Hamilton *et al.*, 2003).

Pesticide	**WHO**	**New Zealand**	**Australia**	**Canada**
Aldrin/ Dieldrin	0.03	0.03	0.3	0.7
Carbofuran	7	8	10	90
Chlordane	0.2	0.2	1	
DDT	2			
Hexachlorobenzene	1	1		

1.6 Need for pesticide detection

In recent years, increased public awareness and interest in environmental issues have highlighted the problem and the effects of the high levels of accumulated persistent pesticides and other toxins in the environment (Iwata *et al.*, 1994). The increased concern and attention around this issue has led to an increased need for effective methods of the detection of these substances in potentially contaminated areas and systems (Iwata *et al.*, 1994). Significant levels of various pesticides have also been detected in South Africa (Sereda and Meinhardt, 2003). In Zululand, in KwaZulu-Natal, South Africa, pyrethroids, organochlorines (DDT and DDE), organophosphates and carbamates have been detected in water and sediments (Sereda and Meinhardt, 2003; Bornman *et al*., 2007, Burger and Nel, 2008, Bornman *et al*., 2010). Many of these residues are thought to result from agricultural spraying, as well from treatment of the sampling areas for malaria control or eradication (Sereda and Meinhardt, 2003).

1.7 Studies performed on environmental pesticide levels in the Eastern Cape, South Africa

In 2003, a study was conducted by Awofolu and Fatoki, to determine the levels of organochlorine pesticides in various water sources in the Eastern Cape, South Africa, using gas chromatography after liquid-liquid extraction procedures (Awofolu and Fatoki, 2003). Significant levels of organochlorine pesticides, including DDT and its analogue DDE, were found in the Buffalo, Keiskamma, Tyume and Swartkops rivers, as well as in the Sandile dam (Awofolu and Fatoki, 2003). Table 2 below indicates a few of the levels detected in the Buffalo River during the study (Awofolu and Fatoki, 2003).

Table 2: Levels of various organochlorine pesticides detected at a test site in the Buffalo river in January 2003; CV = Coefficient of variation (Table adapted from Awofolu and Fatoki, 2003).

Organochlorine pesticides:	**Detected pesticide concentration (ng/l ± CV)**
2,4'-DDT	260 ± 0.0006
2,4'-DDE	240 ± 0.0003
Heptachlor	171 ± 0.0008
B-BHC	450 ± 0.0002

According to a recent article on South Africa's drinking water standards in *The Water Wheel* in January 2008, the allowed maximum limit of pesticides in drinking water in South Africa is not available; however, the limits set by the European Union and by the Netherlands (known for very good water quality and stringent control on levels of contaminants) are 1.0 ng/ℓ for individual pesticides, and 0.5 ng/ℓ for total pesticides (Mamba *et al.*, 2008). Although the pesticide levels in Table 1 above are for river water, not drinking water, they still significantly exceed the acceptable levels set by the EU and the Netherlands (Mamba *et al.*, 2008), showing that the levels detected are significant, especially since, in general, water treatment doesn't remove pesticides (Awofolu and Fatoki, 2003).

Vosloo and Bouwman (2005) performed research on the presence of PCDDs and PCDFs in the aquatic sediments of various watercourses around South Africa. They found detectable levels of these compounds in sites across the country, although the levels did not exceed 50 ng/kg, which is the level at which the USA indicates as an action level.

1.8 Detection of endocrine disruptors in the environment

The harmful effects brought about by pesticide and estrogen contaminants in environmental water make it important to be able to detect the presence and the levels of these endocrine disruptors in water. Numerous methods of detection for both pesticides and estrogen compounds do exist, and are currently used. These methods include chromatography (gas chromatography, high performance liquid chromatography) (Muir and Sverko, 2006),

spectroscopy (UV spectroscopy, mass spectroscopy, fluorescence spectroscopy) (Pogačnik and Franko, 2001) and biosensors (Chen *et al.*, 2006). Pool (2008) has also suggested the use of ELISA based assays for the screening of estrogens and androgens in environmental samples.

1.9 Limitations of existing detection methods

The above mentioned methods can offer highly sensitive and effective detection of toxicants; however, they also have their limitations. The most prominent and most prohibitive factor of many of these methods is cost: the methods involve the use of expensive equipment and materials, making them unfeasible for use by many people and organisations (Mazzei *et al.*, 2004, Swart and Pool, 2007).

The second limitation is the fact that the detection methods often involve the use of high-tech and complicated equipment and procedures, and thus a high level of training and proficiency is required (Swart and Pool, 2007).

Time is another important factor: the harmful nature of endocrine disrupting compounds means that early and rapid detection is essential, making many of the existing methods unsuitable, as they can be extremely time-consuming (Swart and Pool, 2007).

Related to the factor of time is that of portability: techniques involving large and expensive equipment are not practical for rapid, on-site detection of toxicants in water, as well as detection of the compounds in rural or remote areas far away from suitable testing facilities (Mazzei *et al.*, 2004). Enzymatic methods of detection offer a potentially simpler, more rapid and cost effective alternative for the detection of water contaminants.

1.10 Enzymatic detection methods

For the detection of pesticides, the inhibition of enzymes affected by specific pesticides can be monitored using enzyme assays, the degree of inhibition being proportional to and thus giving an indication of the concentration of pesticides in the water (Breuer, 1982). Such enzymes include cholinesterases (inhibited by organophosphorus and carbamate pesticides) and alkaline phosphatases (inhibited by organochlorine and organophosphorus pesticides, as well as heavy metals) (Garcia Sanchez *et al.*, 2003, Chouteau *et al.*, 2004 and Gokcimen *et al.*, 2006).

Cholinesterase assays which can be used include acetylcholinesterase (AChE), with acetylcholine chloride (AChCl), acetylthiocholine iodide (AChI) and fluorescein diacetate (FDA) as substrates; as well as butyrylcholinesterase (BChE), with butyrylcholine chloride (BChCl) as a substrate), (Pogačnik and Franko, 2001; Chouteau *et al.*, 2004). These assays

can be monitored using colour reactions, conductometry, and fluorescence spectroscopy (Pogačnik and Franko, 2001; Chouteau *et al.*, 2004).

For alkaline phosphatase (ALP), monitoring enzyme activity involves the use of either *p*-nitrophenyl phosphate (*p*NPP) (Gasser and Kirschner, 1987) or methyl-umbelliferyl-phosphate (MUP) as a substrate (Chouteau *et al.*, 2004). When *p*NPP is used as the substrate, spectrophotometry is used to monitor the reaction, since the ALP cleaves a phosphate group off the *p*NPP, resulting in the formation of a yellow product (as shown in Fig. 1), i.e. it is a colorimetric assay (Chouteau *et al.*, 2004). When MUP is used as a substrate, the assay is monitored using fluorescence spectroscopy, since the reaction product formed is a fluorescent one (Chouteau *et al.*, 2004). Fluorescence spectroscopy and conductometry, although highly sensitive and suitable for quantification, are not necessarily practical in terms of cost, speed and portability. The use of colour reactions, however, is a useful alternative, allowing for on-site detection, potentially without the initial requirement for equipment such as spectrophotometers or conductometers.

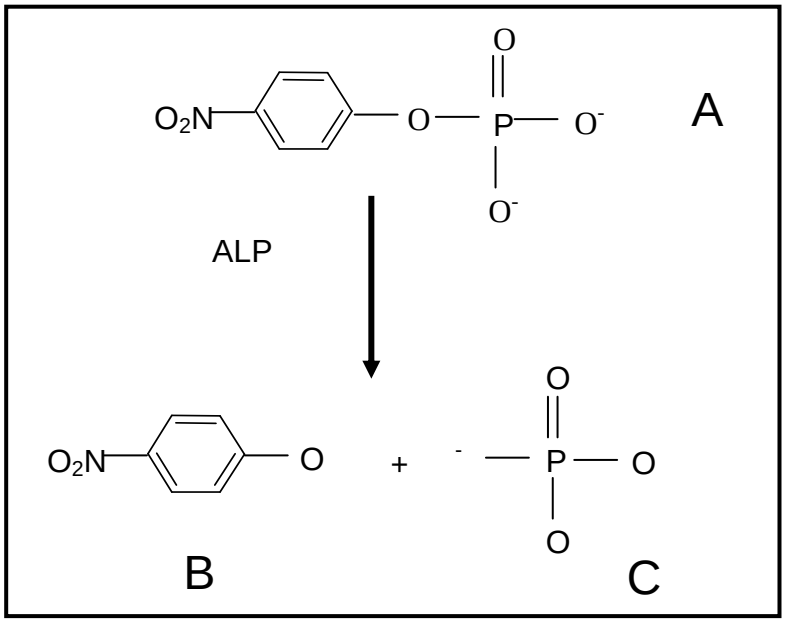

Figure 1: Representation of the reaction catalysed by ALP, where A = *p*-nitrophenyl phosphate; B = *p*-nitrophenyl and C = phosphate (Adapted from Chouteau *et al.*, 2004).

The rapidity, simplicity and relative low cost of the enzymatic methods described above makes them a highly feasible and potentially very useful alternative to existing methods used for the detection of endocrine disruptors, including pesticides and steroid hormones, in water.

2. PROBLEM STATEMENT

In South Africa, there are many compounds which contaminate the country's water sources. These contaminants, particularly OCPs, have numerous adverse effects on the environment; including plants, humans and other animals. This gives rise to the need for effective detection methods. Existing methods, though often very effective on terms of sensitivity and reliability, are in many ways impractical and have limitations, including cost, time, portability and complexity. There is therefore a need for rapid, inexpensive and simple methods for the rapid detection of OCPs in water.

3. HYPOTHESIS

Enzymatic methods can be implemented in the rapid for the rapid detection of organochlorine pesticides (and mixtures thereof) in water.

4. AIMS AND OBJECTIVES

The ultimate aim of this work was to develop a rapid enzymatic assay for the detection of organochlorine pesticides (OCPs). This was accomplished by addressing the following specific aims, which were:

a) To develop and establish a rapid enzyme assay for the detection of OCPs,

b) To test the reactivity of these OCPs, present either alone or in combination (i.e. in a mixture), in surface water,

c) To establish proof of concept for a subsequent, more detailed study (3-5 years) using environmental samples from the Eastern Cape, South Africa.

5. MATERIALS AND METHODS

5.1 Reagents and equipment

Alkaline phosphatase (ALP) from calf intestine (M.W. 140,000, specific activity, 1732.0 U/mg P) (Calbiochem), p-Nitrophenyl phosphate liquid substrate system (*p*-NPP) (Sigma). Organochlorine pesticides: 2, 4'-DDE (DDE), heptachlor (HEP), 2, 4'-DDT (DDT), toxaphene (TOX), dieldrin (DIE), aldrin (ALD), mirex (MIR), endosulfan (END), α-chlordan (Sigma), organophosphorus pesticides: malathion (MAL), parathion (PAR), fenitrothion (FEN), diazinon (DIA), 2, 4-D (24D), paraoxon-methyl (PAM); carbamates: 2,3-Dihydro-2, 2-dimethyl-7-benzofuranol N-methyl-carbamate (CAR), propoxur (PRO); heavy metals; cadmium chloride, nickel chloride, zinc sulphate and mercuric acetate; sodium arsenate and sodium chloride. All other chemicals and reagents were also of the highest analytical grade possible. All spectroscopic analyses were performed using Powerwave$_x$ plate readers, from Bio-Tek Instruments, Inc.

5.2. Methods

The standard ALP enzyme assay (originally described by Gasser and Kirschner, 1987) carried out at 25˚C consisted of 155 µl of 0.1 M sodium bicarbonate/carbonate buffer (pH 9.5), 10 µl of desired concentration of test sample, 10 µl of 0.000166 mg/ml ALP (prepared in buffer) and 25 µl substrate (p-nitrophenyl phosphate) resulting in a total volume of 200 µl in a 96-well microtiter plate. The reaction was initiated by the addition of substrate; the reaction ran typically for 10 min and was terminated by the addition of 50 µl of 3 M NaOH. The absorbance was measured at 405 nm by a microtiter plate reader. To account for the basal (chemical) breakdown of p-NPP into p-NP, the absorbance of the enzyme free blank was subtracted from that of the test samples.

Preparation of the test solutions

The test solutions were serially diluted in their respective solvents (hexane, methanol or acetonitrile) to provide a range of concentrations from 10 mg/l to 0.001 mg/l. The final incubation concentration in the assay solution ranged from 0.5 mg/l to 0.0005 mg/l.

Water samples

Water samples were collected in 500 ml-Schott bottles. Each bottle was washed with liquid soap, rinsed properly with distilled water and air-dried in the oven at 100˚C prior to sample addition. Sample preservation was accomplished by storing the bottles at 4˚C immediately after sampling prior to analysis.

Statistical analysis

The data were expressed as means of triplicate determinations. Statistical significance was assessed with one way analysis of variance (ANOVA) using Microsoft Excel Windows program. Treatment means were considered significantly different at $P \leq 0.05$.

6. RESULTS AND DISCUSSION

The selection of buffer was based on the study by Delory and King (1945), suitable for the investigation of ALP systems. In this method, activity of ALP was measured colorimetrically by the rate of production of *p*-nitrophenol (*p*-NP) from *p*-NPP.

Based on animal and other studies, DDT, dieldrin, endosulfan and lindane were listed as potential endocrine disrupting chemicals (Falconer *et al.*, 2006 and Bornman *et al*., 2010). In this report, the effects of nine endocrine disrupting pesticides (out of the twelve chemicals generally referred to as "The Dirty Dozen"), four heavy metals and two carbamate pesticides on ALP activity were investigated.

DDT, DDE, HEP and TOX were supplied in methanol with initial concentration of 100 ng/µl and 500 µg/ml respectively, while DIE, ALD and Mirex were supplied in acetonitrile with initial concentration of 100 ng/µl. α-Chlordan was supplied in hexane with initial concentration of 10 ng/µl, desired concentrations were made by diluting this stock further in hexane.

Figure 2 shows that there was no significant difference (P>0.05) in ALP enhancement between the control and α-chlordan at a concentration of 1 mg/l or below. A higher concentration of chlordane above 10 mg/l was not investigated because this high concentration is not typically found in the environment.

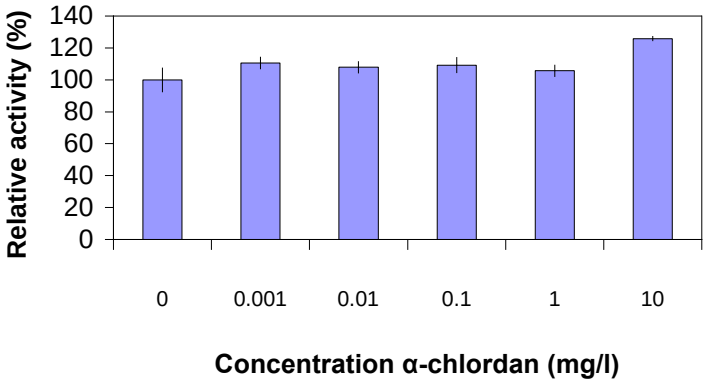

Figure 2: Enhancement of ALP by α-chlordan (α-chlordan was dissolved in hexane).
Data points represent means ± SD (n=3).

Figs. 3a and 3b show the result of ALP enhancement by eight OCPs. They were supplied in either methanol or acetonitrile solution. With the exception of heptachlor, there was a significant difference (P<0.05) between the methanol control (i.e. 0 mg/l pesticide concentration) and higher concentrations of the OCPs (Fig. 3a). Slightly higher enhancement of ALP was observed with dieldrin and endosulphan with increasing concentrations (Fig. 3b).

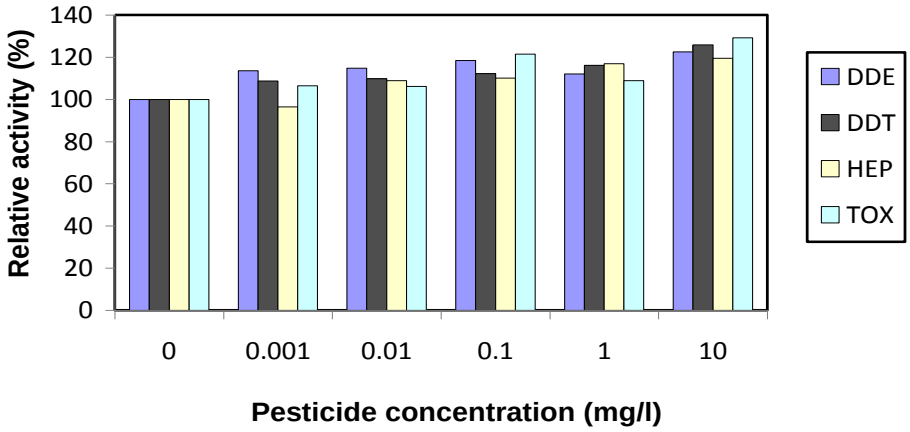

Figure 3a: Enhancement of ALP by organochlorine pesticides (pesticides were prepared in methanol). Data points represent means ± SD (n=3).

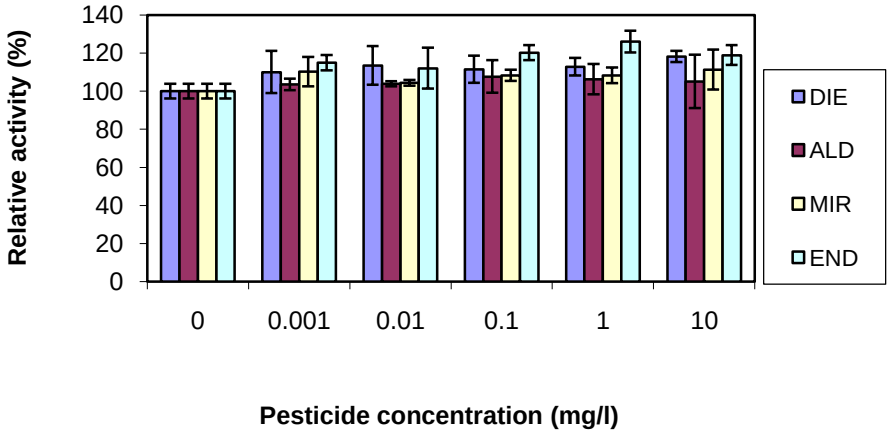

Figure 3b: Enhancement of ALP by organochlorine pesticides (pesticides were prepared in acetonitrile). Data points represent means ± SD (n=3).

Some studies have indicated that OPPs and CPs may have an effect on the ALP assay (Garcia Sanchez *et al.*, 2003). If this is true, the potential for interference resulting from these compounds should be considered. Enhancement of ALP by the organophosphorus pesticide paraoxon-methyl was very pronounced. Diazinon was the least activating (Fig. 4). The mean values obtained were significantly different ($P<0.05$) at all concentrations examined, except for diazinon at lower concentrations of 0.001 mg/l and 0.01 mg/l. Carbamate pesticides also activated ALP, and there was a significant difference ($P<0.05$) between the two carbamates examined and the control (Fig. 5).

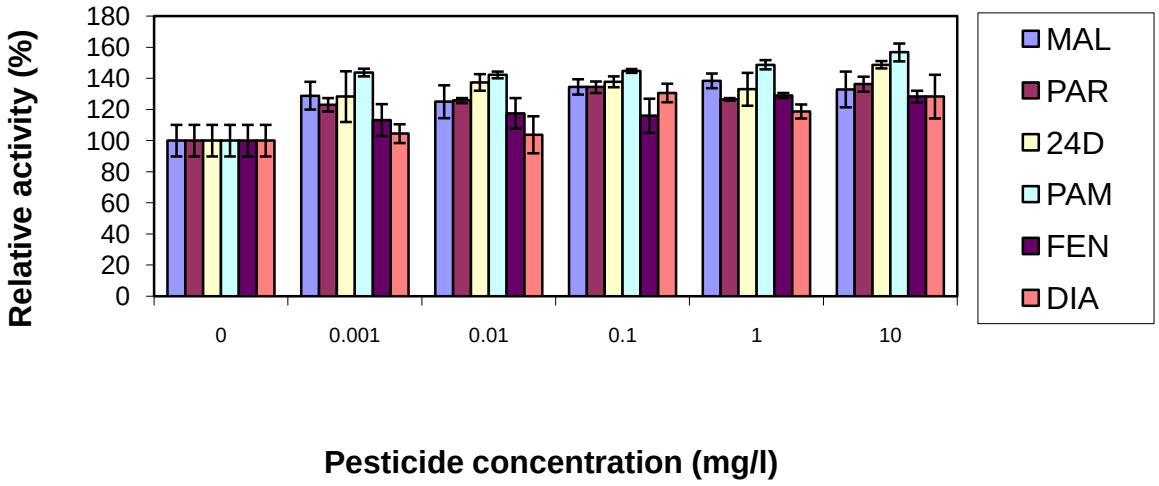

Figure 4: Enhancement of ALP by organophosphorus pesticides (pesticides were dissolved in methanol). Data points represent means ± SD (n=3).

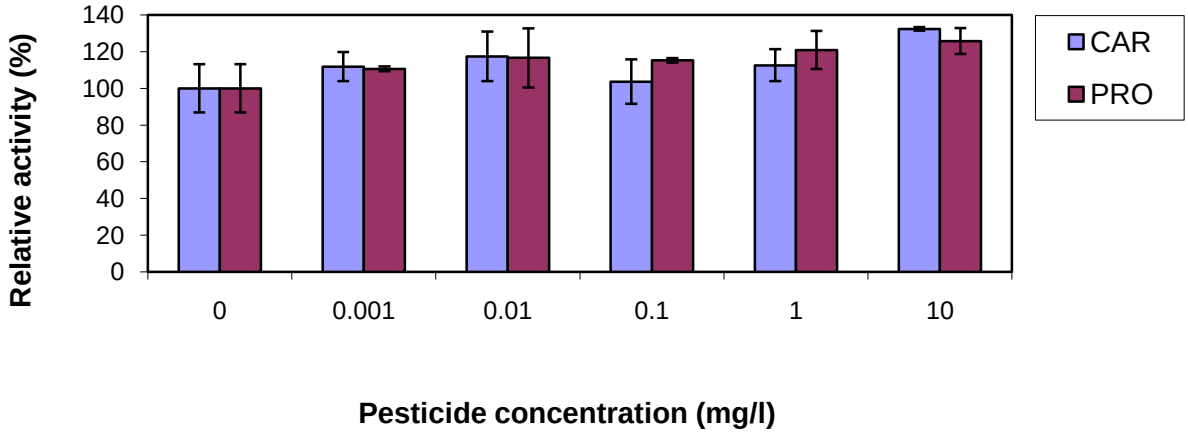

Figure 5: Enhancement of ALP by carbamates. Data points represent means ± SD (n=3).

Fig. 6 shows that ALP was strongly activated by heavy metals at all the concentrations examined. The mechanism of enhancement is unknown. The enhancement of ALP by heavy metals were significantly different ($P<0.05$) between the control and all the concentrations examined. Durrieu *et al.* (2003) stated that the concentration at which many heavy metals, phenols and some pesticides can be found in the field is 1 mg/l i.e. 1 ppm. In their investigation of the effects of three groups of chemicals on ALP of intact *Chlorella vulgaris*, pesticides had no marked inhibitory effect. However, contrary to our observation in this report, heavy metals showed marked inhibitory effect. Possibly, the biochemical and physiological characteristics of different organisms are quite different. In addition, enzyme sensitivity to a pollutant also depends on its nature as well as its origin (Chouteau *et al.*,

2004). In their study, Danzer and Schwedt (1996) reported that ALP is affected only by heavy metals like arsenic, bismuth and beryllium.

Sodium chloride enhancement was not significant (P>0.05) at 0.001 mg/l to 1 mg/l level, except at a higher concentration of 10 mg/l (Fig. 7). This is similar to the observation of Utida *et al.* (1967), that 500 mM NaCl enhanced the ALP activity in the homogenates of intestinal mucosa of rainbow trout, *Salmo gairdnerii*.

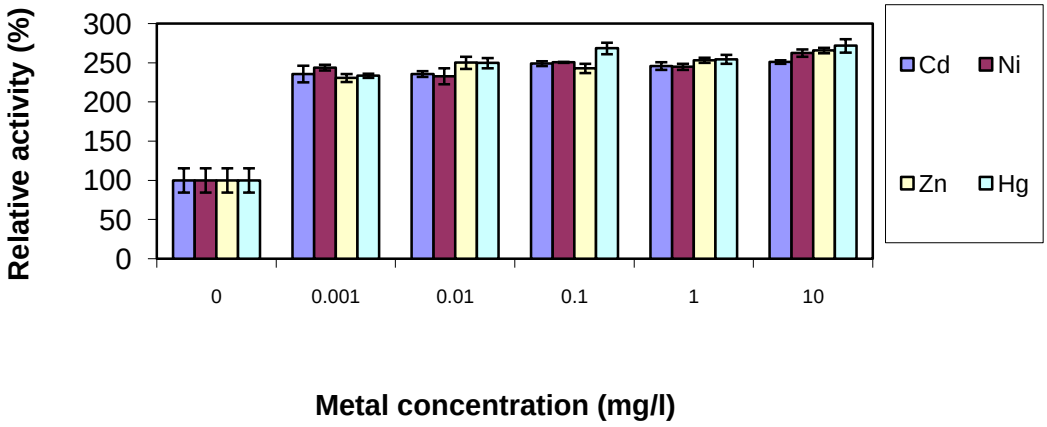

Figure 6: Enhancement of ALP by heavy metals [salts of the heavy metals were prepared in 0.1M Tris-HCl buffer (pH 8.4) containing 0.01M MgCl2]. Data points represent means ± SD (n=3).

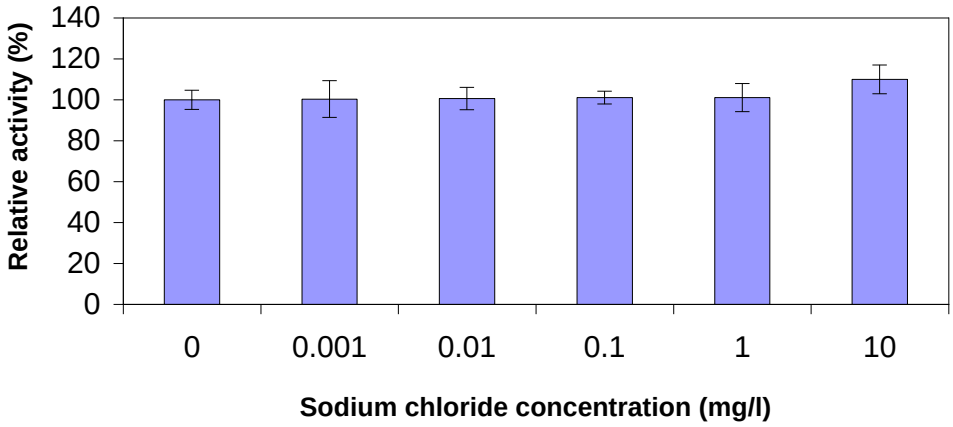

Figure 7: Enhancement of ALP by sodium chloride solution (control, distilled water). Data points represent means ± SD (n=3).

Different environmental water samples from the environment in the Eastern Cape were also collected and their effects on ALP activity were examined. Many pesticides are soluble in water out of necessity so that they can be applied with water and be absorbed by the target. It was observed that enhancement of ALP activity was pronounced in stagnant water samples from the Bloukrans River, Bellmont Valley, Grahamstown, and from Port Elizabeth (Fig. 8).

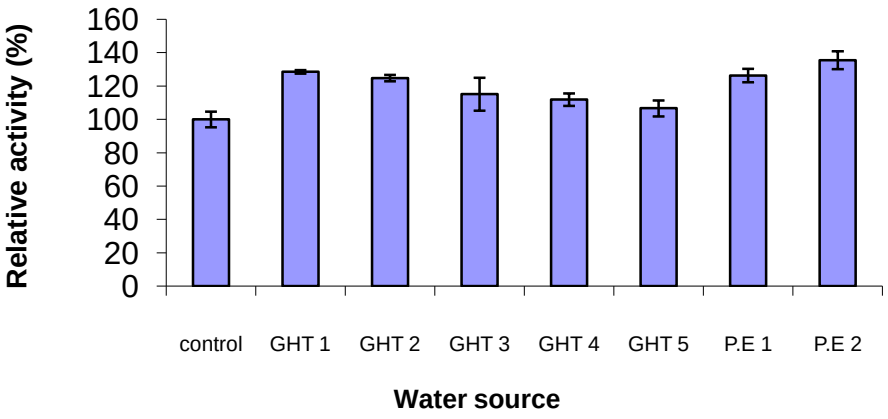

Figure 8: Enhancement of ALP by water from different sources. Control, distilled water; GHT 1, stagnant Bloukrans River water site 1; GHT 2, flowing Bloukrans River water site 1; GHT 3, stagnant Bloukrans River water site 2; GHT 4, flowing Bloukrans River water site 2; GHT 5, EBRU final effluent; P.E. 1, wastewater treatment plant from P.E.; and P.E. 2, river water from P.E.). Data points represent means ± SD (n=3).

In order to confirm that the ALP and its related assay were functioning properly, the total inhibition of ALP was monitored with 6000 mg/l (20 mM) sodium arsenate (Fig. 9). Reversible competitive inhibition of ALP by sodium arsenate was previously reported by Whisnant and Gilman (2002). Inhibition of ALP by amino acids was also reported by Gasser and Kirschner (1987). This inhibition is believed to be based on the ability of amino acids to form chelating complexes with divalent heavy metals.

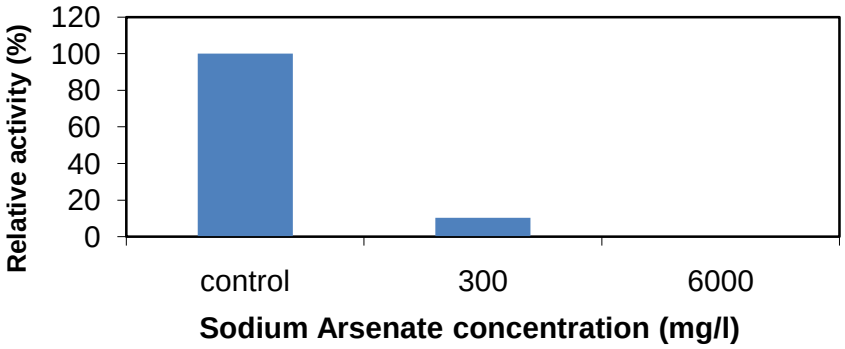

Figure 9: Inhibition of ALP by sodium arsenate (control, distilled water).

No additive or synergistic effects were observed on ALP enhancement at high concentrations of acetonitrile solubilised organochlorine pesticides (40 mg/l) (Fig. 10a) or even at very high concentration of pesticides 400 mg/l (Fig. 10b). Similar effects were observed for the methanol solubilised organochlorine pesticides (Figs. 11a and b). Data points represent means ± SD (n=3).

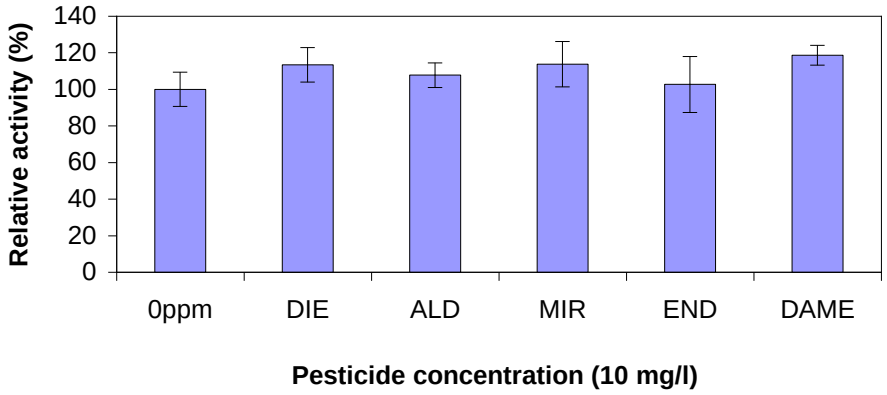

Figure 10a: Investigating the synergistic effects of pesticides at high concentrations (DAME indicates combined dieldrin, aldrin, mirex and endosulphan at a total of 40 mg/l).
Data points represent means ± SD (n=3).

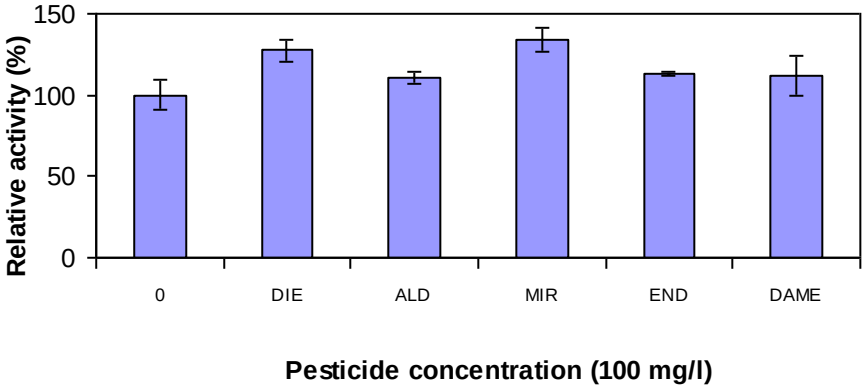

Figure 10b: Investigating the synergistic effect of pesticides at very high concentrations (DAME indicates combined dieldrin, aldrin, mirex and endosulphan at a total of 400 mg/l). Data points represent means ± SD (n=3).

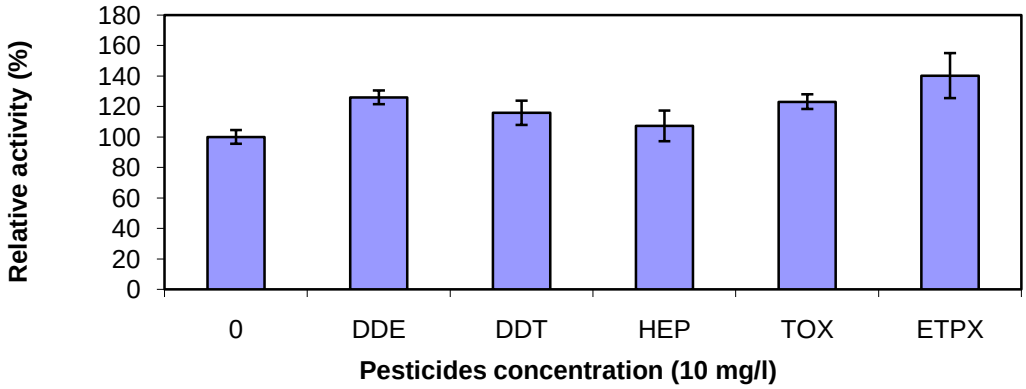

Figure 11a: Investigating the synergistic effects of pesticides at high concentration
(ETPX indicates combined DDE, DDT, Heptachlor and Toxaphene at a total of 40 mg/l). Data points represent means ± SD (n=3).

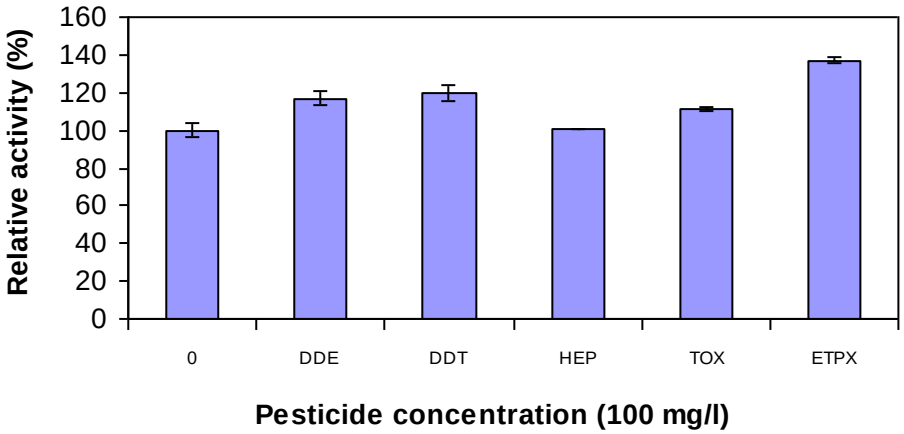

Figure 11b: Investigating the synergistic effects of pesticides at very high concentration (ETPX indicates combined DDE, DDT, Heptachlor and Toxaphene at a total of 400 mg/l). Data points represent means ± SD (n=3).

7. CONCLUSIONS

A standard ALP assay that made use of calf intestine ALP, with *p*-nitrophenyl where, in the reaction, the ALP cleaves a phosphate off the pNPP, yielding a yellow product, was used. The method was modified from that originally used by Gasser and Kirschner (Gasser and Kirschner, 1987). The effect of several OCPs (4'-DDE (DDE), heptachlor (HEP), 2, 4'-DDT (DDT), toxaphene (TOX), dieldrin (DIE), aldrin (ALD), mirex (MIR), endosulfan (END), α-chlordan) on the ALP assay was investigated. Each OCP was detected and quantified separately using suitable inhibition kinetics. Once the effect of each OCP on the ALP assay had been established, the OCPs were mixed in various combinations and the effect of these combinations on the ALP activity were evaluated.

This study, however, showed that ALP was not an effective target enzyme for the design of a suitable bioprobe or biosensor product for OCPs. This is in direct contrast to the findings of Garcia Sanchez *et al.* (2003). ALP was not significantly inhibited by any of the OCPs investigated in this study, and in many cases a moderate enhancement of the enzyme was observed. No synergistic or even additive effects were noted when mixtures or combinations of the OCPs were assayed.

The effects of OPPs, CPs and various heavy metal ions such as cadmium, mercury, nickel and zinc on ALP activity were also investigated. Some studies have alluded to the fact that some of these ions may have an effect on the ALP assay (Chouteau *et al.*, 2004; Mazzei *et al.*, 2004), and if this is true, then the potential for interference resulting from these compounds would have to be taken into account, reduced or removed altogether. From the data obtained during the course of this study it was found that all these metal ions had a dramatic effect on the ALP enzyme, increasing the relative activity of the ALP by 200% even at very low concentrations of heavy metals of 0.001 mg/l. ALP is probably more suitable as an enzyme target for the detection and monitoring of heavy metal compounds water, and it is suggested that a follow-up study on the use of ALP for metal ion detection is feasible. In contrast to the literature that states that metal ions inhibit ALP activity, we have found a strong stimulatory response on the enzyme.

ALP activity was enhanced in the presence of several environmental samples (mainly rivers from around the Eastern Cape), indicating the potential presence of several metal ions in these samples.

Bioelectrical or biosensor systems applying the principle of pesticides as inhibitors of ALP have been reported (Ayyagari *et al.*, 1995; Chouteau *et al.*, 2004; Mazzei *et al.*, 2004; Sanchez *et al.*, 2003); however, Durrieu *et al.*, 2003 have also reported the inhibition of ALPs by heavy metals and organophosphorus pesticides.

In conclusion, we believe that the cholinesterases, such acetylcholinesterase and butyrylcholinesterase, present better target enzymes for the detection, especially the newer generation of pesticides (See Appendix 1 for typical inhibition kinetic data for acetylcholinesterase). It has been established in literature that these enzymes are strongly inhibited by the organophosphorus and carbamate classes of pesticides. Currently, we are busy with a follow-on grant based on this approach (WRC K5/1902), extending the potential detection not only to these pesticides, but also to their degradation products present in the water environment.

8. RECOMMENDATIONS

Based on the results obtained during the course of this study, we would like to make the following recommendations:

a) ALP is probably more suitable as an enzyme target for the detection and monitoring of metal compounds water, and it is suggested that a follow-up study on the use of ALP for heavy metal ion detection is feasible. In contrast to the literature that states that these metal ions inhibit ALP activity, we have found a strong stimulatory response on the enzyme.

b) The cholinesterases present better target enzymes for the detection of the newer generation of pesticides such as OPPs and CPs. These pesticides also have the added benefit of being more soluble and more readily degradable in the environment. Currently, we are busy with a follow-on grant based on this approach (WRC grant K5/1902/3), extending the potential detection not only to the pesticides, but also to their degradation products in the water environment.

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APPENDIX 1

Acetylcholinesterase (AChE) assay

Another enzyme assay which was investigated was acetylcholinesterase, with acetylthiocholine iodide as the substrate. The assay was performed according to Ellman's method, which makes use of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB or Ellman's reagent). DTNB reacts with the product from the reaction, resulting in the formation of a yellow colour (Ellman *et al.,* 1960).

As with the alkaline phosphatase assay, the first objective in this assay was to optimise the conditions in order to obtain a suitable colour product as well as a linear range in the progress curve of the uninhibited reaction. The reaction mixture of the optimised, uninhibited reaction, with a total volume of 200 µl, contained the following:

- 0.001 mg/ml human erythrocyte acetylcholinesterase (freshly made up every day): 20.0 µl
- 0.1 M sodium phosphate buffer (pH 8.0): 160.0 µl
- 0.01 M DTNB (made up in 0.1 M phosphate buffer, pH 7.0, and stored for up to 2 weeks at 4 °C) 10.0 µl
- 0.075 M acetylthiocholine iodide substrate: 10.0 µl

The alkaline phosphatase assays were performed in microtitre plates at room temperature, and the reactions were initiated by the addition of the substrate. The assays were run for 20 minutes, and absorbance readings were taken every 2 minutes at 412 nm using a Powerwave$_x$ spectrophotometer. Each reaction was performed in triplicate.

Once the uninhibited reaction was optimised, inhibition studies were carried out on the assay. The effects of two carbamate pesticides, carbofuran and propoxur, as well as two organophosphate pesticides, triazophos and oxydemeton, on acetylcholinesterase activity were investigated, by introducing various concentrations of the pesticides to the reaction mixture. Stock solutions of the carbamate and organophosphate pesticides using methanol, and various quantities of these were added to the reaction mixtures (replacing buffer, so as to keep the total volume at 200 µl) depending on the desired concentration of pesticides in the reaction mixtures. An example of a set of assays run together is laid out in Table 3.

Table 3: Example of the various components and their volumes (µl) in a set of assays performed to investigate the effects of carbamate and organophosphate activity (PC = positive control, NC = negative controls, values 0.025 – 0.4 refer to final carbamate concentrations (mg/l) in reaction mixture).

	1	2	3	4	5	6	7	8	9
	PC	0.025	0.05	0.1	0.2	0.3	0.4	NC	BLANK
Buffer	160	157.5	155	150	140	130	120	180	180
Enzyme	20	20	20	20	20	20	20	20	-
DTNB	10	10	10	10	10	10	10	10	10
Carbofuran	-	2.5	5	10	20	30	40	-	-
Substrate	10	10	10	10	10	10	10	-	10
Total	200	200	200	200	200	200	200	200	200

The same assay setup was used for propoxur, triazophos and oxydemeton, each of the other three pesticides replacing carbofuran in the table above. In the inhibition studies, the pesticides were incubated together with the acetylcholinesterase, buffer and DTNB for 1 hour on ice prior to the addition of substrate.

In order to determine the inhibitory effects of the pesticides on the acetylcholinesterase activity, the progress curves of each of the reactions were plotted on Microsoft Excel. For each reaction, the equation of the linear portion of the progress curve was determined, and the slope of the line for each inhibited reaction was compared to that of the uninhibited reaction, where the slope of the uninhibited reaction was considered to be 100% acetylcholinesterase activity.

Results:

In the investigation of the effects of the carbamate carbofuran on acetylcholinesterase activity, carbofuran was found to have a significant inhibitory effect on the enzyme activity, as can be seen in Figure 12. At 400 ppb, the carbofuran completely inhibited the acetylcholinesterase activity.

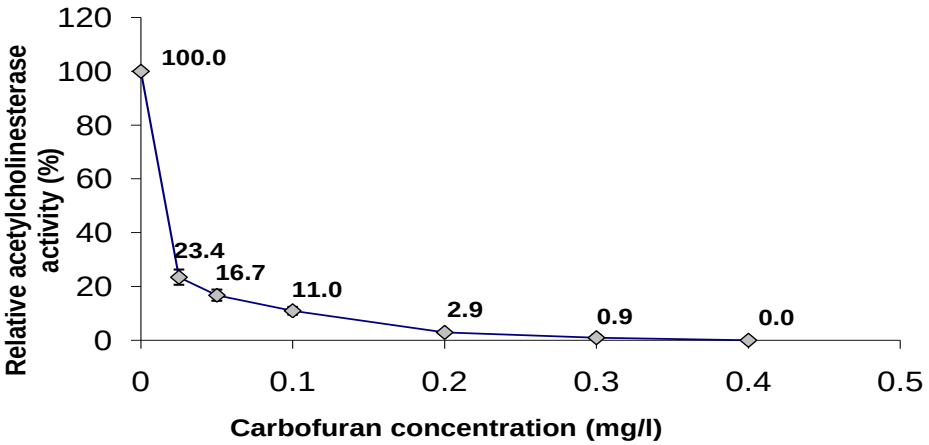

Figure 12: Plot of relative acetylcholinesterase activity vs. carbofuran concentration. Data points represent mean values ± SD (n = 3).

As shown in Figure 13, similar inhibitory effects on acetylcholinesterase activity were found for propoxur, the second carbamate pesticide investigated. In the case of propoxur, the enzyme activity was reduced to 13.1% at 400 ppb of propoxur, thus carbofuran showed a higher toxicity to acetylcholinesterase than propoxur. This is illustrated in Figure 14, which shows the estimated IC_{50s} of propoxur and carbofuran, IC_{50} being the concentration of pesticide which resulted in 50% inhibition of the enzyme activity.

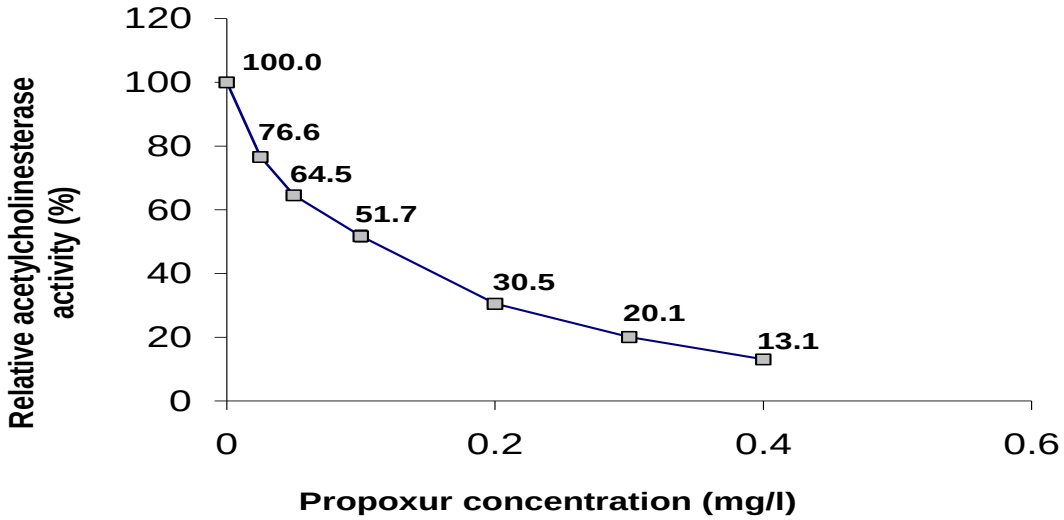

Figure 13: Plot of relative acetylcholinesterase activity vs. propoxur concentration. Data points represent mean values ± SD (n = 3).

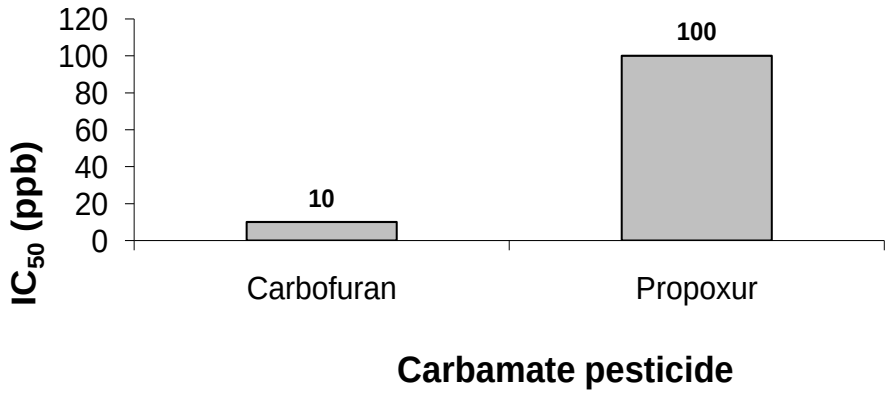

Figure 14: Plot of the estimated IC_{50s} of two carbamate pesticides inhibiting acetylcholinesterase activity.

In the investigation of the effects of organophosphate pesticides on acetylcholinesterase activity, triazophos and oxydemeton were found to inhibit the enzyme activity, as can be seen in Figures 15 and 16, for triazophos and oxydemeton, respectively. However, when compared to the carbamate pesticides discussed above, the two organophosphate pesticides were found to have a far lower toxicity to acetylcholinesterase. This becomes evident when looking at IC 50s, which for the carbamate pesticides were 10 and 100 ppb, whereas for the organophosphate pesticides, the IC 50s exceed 5000 ppb, since in the case of both triazophos and oxydemeton, at 5000 ppb of pesticide, the acetylcholinesterase activity was only inhibited to 67% and 71% respectively.

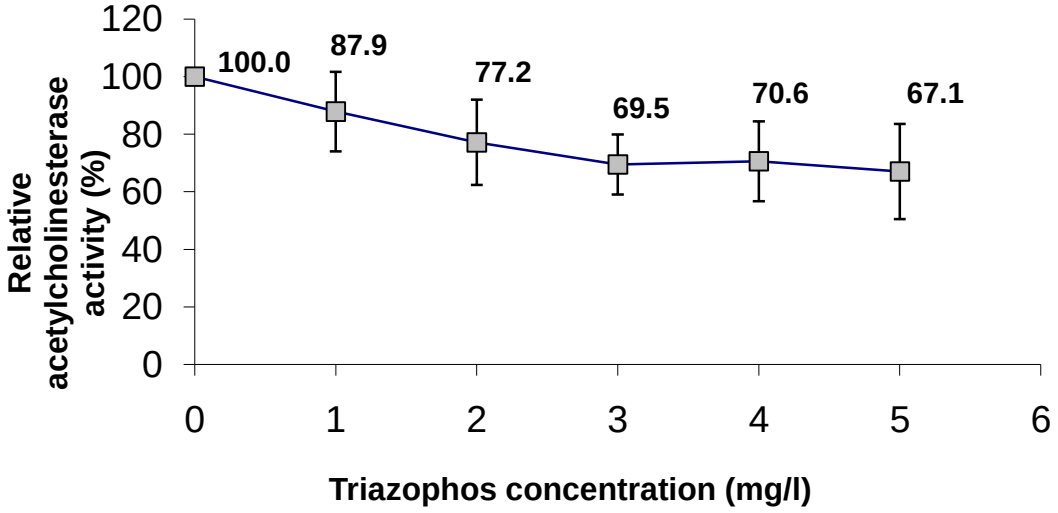

Figure 15: Plot of relative acetylcholinesterase activity vs. triazophos concentration. Data points represent mean values ± SD (n = 3).

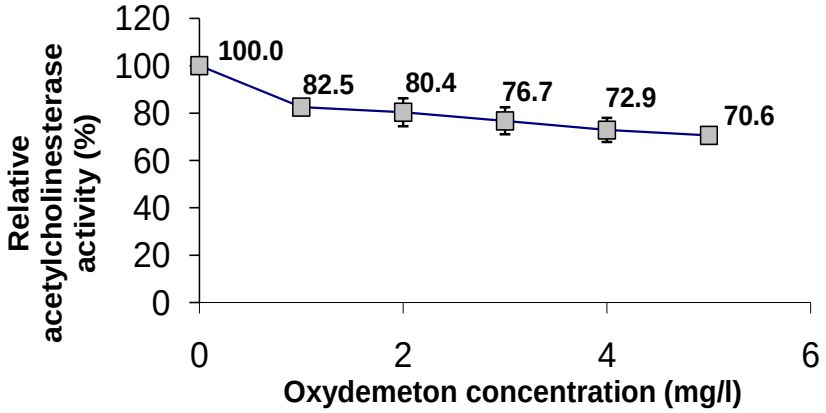

Figure 16: Plot of relative acetylcholinesterase activity *vs*. oxydemeton concentration. Data points represent mean values ± SD (n = 3).

From these results it can be deducted that the order of decreasing toxicities to acetylcholinesterase of the pesticides tested is:
Carbofuran > Propoxur > Triazophos > Oxydemeton, which agrees with findings in a study performed by Pogačnik and Franko (2001).

It can also be said from these results that the carbamates that were tested showed a far greater toxicity to acetylcholinesterase than the organophosphates tested.