Inhibition of Esterases of the Freshwater Snails *Helisoma duryi* and *Lymnaea natalensis* by Mixtures of Pesticides

Norah Basopo[1], Yogeshkumar S Naik[2]

Department of [1]Applied Biology and Biochemistry and [2]Environmental Sciences and Health, National University of Science and Technology, P.O. Box AC 939, Ascot, Bulawayo, Zimbabwe

[1]norah.basopo@nust.ac.zw; [2]yogeshkumar.naik@nust.ac.zw

***Abstract*-Pesticides indirectly reach aquatic reservoirs via aerial drifts and as runoffs where they affect aquatic organisms. Aquatic reservoirs receive different pesticides from the different fields which usually surround them. However, studies investigating effects of pesticide mixtures on aquatic biota is limited. The effects of six pesticides and their mixtures on esterase activity of two freshwater snail species were investigated. Groups of snails were exposed to individual as well as mixtures of the pesticides for 96 hours before analysing for esterase activity. All pesticides inhibited esterase activity in exposed snails. Binary mixtures of pesticides caused additive or synergistic inhibitions of esterase activities when compared to effects of individual pesticides. The results indicated the importance of chemical interactions on the overall effects of pesticides on aquatic organisms.**

Keywords-pesticides, binary mixtures, snails, esterases, inhibition

I. INTRODUCTION

Pesticides reduce loss of crops due to effects of weeds and pests that attack agricultural crops. The types of pesticides applied as well as the frequency of applications are determined by the types of soils and crops planted. Some pesticides are applied as mixtures to ensure effective action against particular pests and weeds. Undoubtedly these pesticide mixtures end up in aquatic ecosystems where they affect non target aquatic biota. Aquatic ecosystems, therefore being the ultimate sink of effluent from agricultural, industrial and domestic activities receive different chemicals and components which are difficult to characterize. Pollution levels of aquatic ecosystems can be determined by assessing the health of aquatic organisms such as fish [1] and benthic macroinvertebrates communities [2], aquatic plants [3] and assessing the toxicity of different chemicals that exist in various components of aquaecosytems. Information on the potential adverse effects of pollutants can only be obtained by combining chemical biomonitoring with evaluation of biomarkers that represent early indications of biological effects [4; 5]. Biomarkers which are essentially alterations in selected biological parameters are used in monitoring effects of xenobiotics in both aquatic and terrestrial environments. Molluscs have been used extensively as bioindicators of pollution in aquatic systems because of their biodiversity and semi sedentary nature which ensures that point source pollution can be detected.

Molluscs have the ability to accumulate pollutants because of their filter feeding method and particulate substances filtered from the water are metabolized and selectively concentrated in their soft tissue or in their shells [6]. Protective biochemical responses in molluscs such as catalase (CAT), superoxide dismutase (SOD) and other parameters indicative of pollutant induced oxidative stress such as levels of thiobarbituric acid reactive substance and reduced glutathione are exploited as bioindicators of environmental quality [7]. In the present study the freshwater snails *Helisoma duryi* and *Lymnaea natalensis* were chosen as experimental organisms based on the fact that they satisfy the essential characteristics required for bioindicator species [8] which include the capacity to accumulate relatively large amounts of pollutants without being killed by them and are of high geographical distribution within the natural water bodies in Zimbabwe.

The purpose of this study was to investigate the effects of binary mixtures of chemicals representing different pesticide classes including insecticides organophosphates (OP), carbamates (CM), pyrethroids (PT) or chloronicotinyls (CNs) with atrazine a herbicide (HB) or thiabendazole a fungicide (FG) on the esterase activity of two freshwater snails in order to assess the potential use of esterases as biomarkers of exposure to pesticide mixtures.

II. MATERIALS AND METHODS

A. Chemicals

All the pesticides, substrates and standards were bought from Sigma Chemical Company, Germany. All other laboratory reagents were of analytical grade.

B. Snail Breeding and Exposure

Two species of snails, Helisoma duryi and Lymnaea natalensis were bred in cement tanks containing tap water and were fed on fresh garden lettuce according the method of Naik and Hasler, [9]. Groups of adult snails (20) were exposed to 25 ppb of carbofuran, 1 ppm of atrazine or thiabendazole and mixtures of carbofuran & atrazine and carbofuran & thiabendazole with the concentration ratio of the mixture constituents at 25 ppm: 1 ppm. The exposures were performed in quadruplicate while food, water and pesticide were refreshed every 24 hours. On the fourth day, post-mitochondrial fractions were prepared. The exposures were repeated 3 times and each time carbofuran was replaced by chlorpyrifos, imidacloprid or lambda cyhalothrin.

C. Post Mitochondrial Fraction Preparation.

Snails from each group were washed once with tap water to remove leaf particles and other dirt. The snails were deshelled, pooled and homogenized, with ice-cold homogenization buffer, 0.1 M potassium phosphate pH 7.4 using a glass teflon homogenizer. The volume of the buffer

used was equivalent to 3 times the weight of soft tissue of the snails. The whole organism homogenates were centrifuged at 10 000 *g* for 10 minutes using a Juan refrigerated high-speed centrifuge and the resultant supernatant fraction referred to as the post mitochondrial fraction (PMF), stored at -80^{o} C until required for enzymatic assays.

D. Protein Determination

The protein concentration was measured following the method of Lowry *et al*., [10] and bovine serum albumin was used as the standard. The PMFs were used as the enzyme source in the determination of esterase activity

E. Non-Cholinesterase Activity

Non-cholinesterase activity was measured using the substrate α-naphthyl acetate following the method of Mackness *et al.*, [11]. The reaction mixture contained 4 mL of reagent A (reagent A contained a mixture of 1 mL of 25 mg/ml α-naphthyl acetate plus 100 mL of 50 mg Fast Blue RR salt dissolved in 0.1 M Tris/HCl buffer pH 7.4) and 20 µL of 0.1 mg/mL PMF. The mixture was incubated for 10 minutes in the dark. The reaction was stopped by addition of 1 mL of 20% (v/v) acetic acid and absorbance was measured at 605 nm.

F. Cholinesterase Activity

Cholinesterase activity was measured using the substrate acetylthiocholine iodide according to the method described by Ellman *et a*l., [12] that was modified for a plate reader by Kallander *et al.*, [13]. The reaction mixture contained 50 µL of 0.1 mg/mL PMF, 110 µL of 0.01 M Tris/ HCl buffer pH 8.0 and 50 µl of 0.4 mM 5,5 dithio-bis-(2 nitro benzoic acid) DTNB. The mixture was incubated for 3 minutes before adding 30 µL of 0.5 mM acetylthiocholine iodide. The rate of production of a complex between thiocholine and DTNB was followed for 3 minutes at 412 nm using a SpectraMax 340pc plate reader from Molecular Devices and SoftMax Pro programme version 3.1.

III. RESULTS

All pesticides as individuals and as mixtures significantly reduced esterase activity in both snail species ($p<0.05$, $p<0.01$ or $p<0.001$).

The effects of binary mixtures of carbofuran with atrazine or thiabendazole are shown in Figure 1. Carbofuran caused higher inhibition of esterase activity when compared to the effects of either the herbicide or fungicide. Pesticide mixtures caused higher inhibitions of esterase activity when compared to effects of individual pesticides in both snail species.

Figure 2 shows the effects of binary mixtures of chlorpyrifos with atrazine or with thiabendazole. Considering the effects of individual pesticides only, chlorpyrifos caused highest inhibition of esterase activity up to 56% followed by thiabendazole which caused inhibition up to 32% and lastly atrazine which inhibited esterase activity by up to 17% depending on the snail species and substrate used in analyzing enzyme activity.

Figure 3 shows the effects of binary mixtures of the imidacloprid with atrazine or with thiabendazole. The insecticide imidacloprid caused higher inhibitions of esterase activity when compared to effects of the fungicide thiabendazole or herbicide atrazine. Pesticide mixtures caused inhibitions which more than doubled the inhibitions caused by individual pesticides in some exposed snail samples. Inhibition of cholinesterase caused by binary mixtures of pesticide reached as high as 77% and varied depending on the pesticides and snail species.

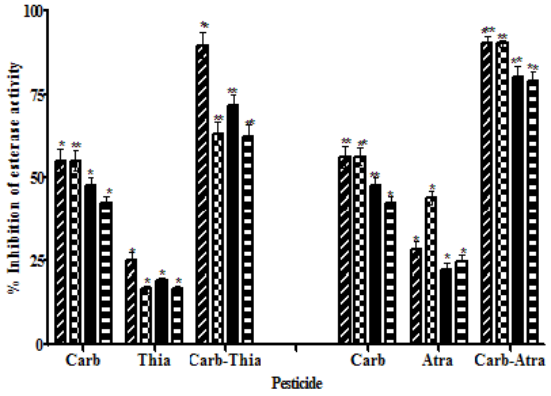

Figure 1 Effect of carbofuran, thiabendazole and atrazine as individual and binary mixtures on esterase activity in freshwater snails. Esterase activity was measured using acetylthiocholine iodide (ATChI): (*L.natalensis-ATChI), (H. duryi-ATChI) or α-naphthyl acetate (ANA): (L. natalensis-ANA), (H. duryi-ANA) Significantly different from control (*P<0.05; **P<0.01; *P<0.001*).

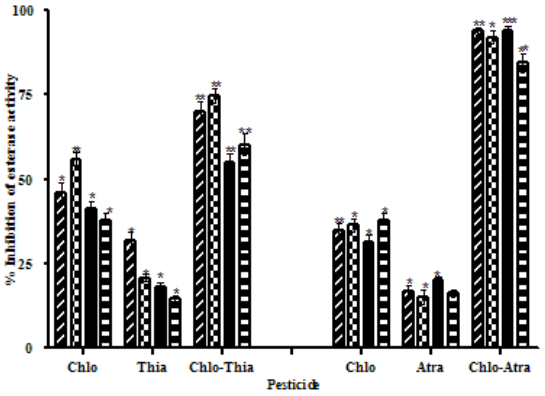

Figure 2 Effect of chlopyrifos, thiabendazole and atrazine as individual and binary mixtures on esterase activity in freshwater snails. Esterase activity was measured using acetylthiocholine iodide (ATChI): *(L.natalensis-ATChI), (H. duryi-ATChI) or α-naphthyl acetate (ANA): (L. natalensis-ANA), (H. duryi-ANA) Significantly different from control (*P<0.05; **P<0.01; *P<0.001).*

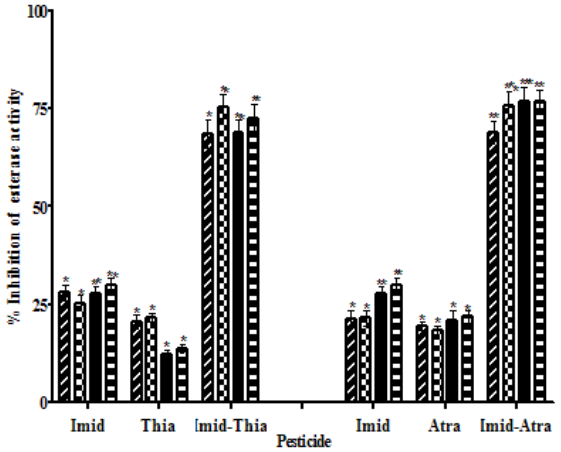

Figure 3 Effect of imidacloprid, thiabendazole and atrazine as individual and binary mixtures on esterase activity in freshwater snails. Esterase activity was measured using acetylthiocholine iodide (ATChI): (*L.natalensis-ATChI), (H. duryi-ATChI) or α-naphthyl acetate (ANA): (L. natalensis-ANA), (H. duryi-ANA) Significantly different from control (*P<0.05; **P<0.01; *P<0.001*).

The effects of binary mixtures of lambda-cyhalothrin with atrazine or thiabendazole are shown in Figure 4. Lambda-cyhalothrin caused inhibition of esterase activity of up to 30% while thiabendazole caused inhibition of up to 19% and atrazine inhibited esterase activity by up to 21%. The inhibition varied with snail species. Binary mixtures of pesticides caused inhibition of up to 80% depending on the pesticides and snail species.

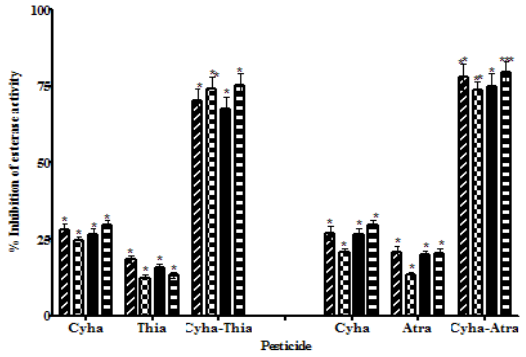

Figure 4 Effect of lambda cyhalothrin, thiabendazole and atrazine as individual and binary mixtures on esterase activity in freshwater snails. Esterase activity was measured using acetylthiocholine iodide (ATChI): *L. natalensis-ATChI), (H. duryi-ATChI) or α-naphthyl acetate (ANA): (L. natalensis-ANA), (H. duryi-ANA) Significantly different from control (*P<0.05; **P<0.01; *P<0.001*).

IV. DISCUSSION

The pesticides studied are commonly used as agricultural chemicals with intensive and overlapping applications in Zimbabwe [14]. All pesticides used in this study inhibited both choline and non-cholinesterase activity in both snails species. The highest inhibition was observed in snails exposed to pesticide mixtures. The observed combined effects of atrazine and chlorpyrifos on esterases in the aquatic snails are supported by the study done by Reference [15] who demonstrated that triazine herbicides individually are not toxic to the larvae of the midge *Chironomus tentans*, but when combined with OPs they enhanced OP toxicity considerably. Reference [15] also reported that atrazine in combination with chlorpyrifos significantly inhibited acetylcholinesterase activity in *Chironomous tentans* compared with chlorpyrifos on its own. The authors observed that the extend of this potentiation of inhibition of enzyme activity was concentration and type of triazine herbicide and OP dependent. Reference [16] also reported enhanced toxicity of chlorpyrifos when combined with cyanazine or atrazine in the *C. tentans* of similar magnitude. The authors noted that chlorpyrifos toxicity was enhanced by 1.8 and 2.2 fold in the presence of atrazine and cyanazine, respectively.

When studying effects of pesticide mixtures, it is important to appreciate the interactions that exist when chemicals are mixed. An understanding of such interactions will provide an insight on the overall effects of pesticides on aquatic biota. Reference [17] suggested that atrazine increased the enzymes involved in biotransformation of OPs, thus converting them into more toxic o-analog metabolites. Thionphosphates such as chloryprifos require oxidative activation by monooxgenases resulting in AChE inhibitors that are more potent than the parent compound. The hypothesis that atrazine induced the cytochrome P450 responsible for the conversion of some OPs to their reactive analogues is supported by Reference [18] who showed the induction of a 45-kDa protein (with properties similar to Cyt P450) in atrazine-treated midges. It is possible that the enhanced inhibition of AChE by chlorpyrifos in presence of atrazine observed in the present study may also be cytochrome P450 dependent.

The actual mode of action of the combined effects of imidacloprid or lambda cyhalothrin with atrazine is not known. The reason for the potentiated response to binary mixtures containing imidacloprid or lambda cyhalothrin with a herbicide could be that atrazine in some way increases the uptake of imidacloprid or lambda cyhalothrin by the snail enzyme. Further investigation on effects of mixtures of imidacloprid or lambda cyhalothrin with herbicides and fungicides on the health of the two snail species and other aquatic organisms are warranted in order to better understand the toxicity of these pesticides.

V. CONCLUSION

The present study shows that binary mixtures of pesticides from different pesticidal classes can potentiate toxicological effects of the individual pesticides on aquatic organisms, resulting in enhanced pesticide included alterations of biochemical parameters that are used to measure the wellbeing of aquatic organisms.

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