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Toxicological Effects of Differently Polluted Dam Waters Spiked with Pesticides on Freshwater Snails *Lymnaea Natalensis*

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Abstract

Pesticides extensively used in agricultural fields to ensure high quality crop yields indirectly find their way to aquatic bodies where they affect aquatic biota. We investigated the effects of pesticides in different dam waters on esterase enzyme activity of the freshwater snail species *Lymnaea natalensis*. Groups of adult snails were exposed to 0.006 ppm chlorpyrifos and 0.003 ppm aldicarb in polluted water from Umguza dam and relatively pristine water from Hillside dam for 14 days. Carboxylesterase, acetylcholinesterase and arylesterase activities were measured. Both pesticides caused significant inhibition of esterase activity after the 14 day exposure period, with exposures to Umguza dam water showing higher inhibition as compared to exposures to Hillside dam water. Aldicarb and chlorpyrifos both showed a time-dependent inhibition of enzyme activity, the former causing a higher inhibitory effect as compared to the latter. Acetylcholinesterase was inhibited up to 80% following exposure to aldicarb while exposure to chlorpyrifos in Umguza water caused only 40% inhibition. Carboxylesterases were similarly inhibited with higher inhibition observed in snails exposed to Umguza dam water when compared to snails exposed to Hillside dam water, while arylesterases were inhibited in the range 80-90%, with an exception of chlorpyrifos spiked Hillside dam water which caused 45% inhibition. Contaminated Umguza dam water also appeared to enhance the effects of pesticides when compared to the relatively pristine Hillside dam water. Alteration of esterase activity can be used as an early warning signal indicating exposure to environmental pollutants. The results of this study therefore, highlight the adverse effects of pesticides on non-target aquatic organisms, evidenced by the inhibition of esterase activity.

Keywords: organophosphates, carbamates, esterases, snails, pollution

1. Introduction

Pesticides are applied widely to protect plants from diseases, weeds, fungal and insect damage. They usually come into contact with soil and aquatic systems, where they undergo a variety of transformations that provide a complex pattern of metabolites. Although the use of pesticides has resulted in increased crop production and other benefits, it has raised concerns about potential adverse effects on the environment and aquatic life (Aktar *et al*., 2009).

Carbamate and organophosphate pesticides such as aldicarb and chlorpyrifos respectively, are widely used for agricultural and non-agricultural purposes (Maltby and Hills, 2008; Wang *et al.*, 2010). These pesticides however are environmentally toxic and have a great potential for unintended adverse effects through contamination of rivers, lakes and dams (Lamers *et al.*, 2011). Runoffs and soil erosion after heavy rains carry the applied pesticides to rivers, ponds and dams. The pesticides may also enter aquatic systems as non-point source pollutants through spray drifts or leaching (Tiryaki and Temur, 2010).

The presence of agricultural pollutants in aquatic bodies disturbs the ecosystem health, which necessitates the detection of biological changes due to exposure to pollutants (Kaviraj *et al*, 2014). This is achieved through the use of measurable indicators known as biomarkers. Biomarkers are change in biological parameters related to exposure to environmental chemicals (Kaviraj *et al*, 2014). Organisms, termed bioindicators, are used to measure biomarker responses following exposure. The organisms include, fish and mollusks which have good filtration capacities, are sensitive to damage following exposure to chronic or sublethal concentrations and exhibit ease of caging. These bioindicators provide good model systems for the investigation of stress induced damage, cell response and repair mechanisms employed to ameliorate damage (Valavanidis *et al.*, 2006).

The biomarkers used in this study are the esterase enzymes; acetylcholinesterase, carboxylesterase and arylesterase. Esterases are found in all living cells and they catalyse the hydrolysis of ester bonds (Vioque-Fernández, 2007). The activities of the esterases have been found to be constantly inhibited by organophosphates `and carbamates (Vioque-Fernández, 2007; Sayali *et al*., 2013). Inhibition of acetylcholinesterases in aquatic organisms can be used as a warning signal of harmful effects of environmental pollutants such as pesticides on aquatic biota (Lionetto, 2013). Pesticides cause various toxicological effects on aquatic life. This was also highlighted by Anandhan *et al*., (2012) who reported that in addition to neurotoxicity, organophosphates also caused immunotoxic effects in aquatic organisms. Acetylcholinesterase inhibition in fish exposed to pesticides has been associated with impaired homing behavior and anti-predator alarm responses in Chinook salmon (Scholz *et al*., 2000) and decreased spontaneous swimming rates and feeding rates in coho salmon (Sandahl *et al*., 2005). This study therefore assessed the effects of pesticide spiked dam waters on esterases of the freshwater snail, *L. natalensis* and the potential of using this enzyme system as biomarkers of exposure to pesticides.

2. Materials and Methods

2.1 Chemicals

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

2.2 Water Sampling

Water samples were collected from two Bulawayo dams: Hillside and Umguza, in the month of February during the agricultural and rainy season. Hillside dam, situated in the Hillside recreational conservancy (Sebata, 2015), was selected because it is located upstream of effluent discharge zones of the city of Bulawayo and is therefore considered relatively pristine. Umguza dam on the other hand is located downstream of effluent discharges from industrial, domestic and agricultural activities of and surrounding the city of Bulawayo (Siwela *et al*., 2009; 2010; Dube *et al*., 2010). Two sampling sites were selected at each dam. The physico-chemical water quality properties were determined in situ using the Eutech Cyberscan Instruments water proof series meters (pH: 300 Series, conductivity: 410 Series), before the water was drawn into 25 litre containers and transported to the laboratory. The waterproof Eutech Cyberscan pH 310 meter was used to measure pH while the Eutech Cyberscan CON 410 meter was used to measure conductivity and total dissolved solids (TDS).

2.3 Snail Breeding and Exposure

The snail species used in this study were the *Lymnaea natalensis.* The snails were bred in cement tanks containing tap water and were fed on fresh garden lettuce twice a week. Prior to the exposures, the snails were collected from the cement tanks and brought to the laboratory where they were placed in tap water for 3 days to acclimatize. Groups of adult snails (21) were separately exposed to 0.006 ppm chlorpyrifos and 0.003 ppm aldicarb for 14 days. The exposures were done in I L volume of collected dam water, at room temperature and in duplicate. Seven snails were removed from the exposure tanks at time intervals (day 3, 7 and 14) for homogenization and preparation of the post mitochondrial fraction for biochemical analysis. The sublethal concentrations used for exposures were a third dilution of the LC50 obtained following several screening trials.

2.4 Sample Preparation of the Post Mitochondrial Fraction (PMF)

After the exposure period, snails were sacrificed. The shells were carefully removed using forceps and the soft tissue isolated on ice. The whole body tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Each pool of 7 snails was treated similarly. The snails were then homogenized in ice cold 0.1 M potassium phosphate buffer, pH 7. The volume used was 3x the weight of the snails. The homogenates were centrifuged at 10 000 x g for 15 minutes at 4ºC and the supernatant (PMF) collected and stored at -80ºC.

2.5 Protein Determination

Protein determination was carried out following a modified method of Lowry *et al.,* (1951) using bovine serum albumin (BSA) as a standard. Briefly, 5 ml of the alkaline solution (2.5 ml of 0.5 % $CuSO_4.5H_2O$ in 1% potassium sodium tartarate; 125 ml of 2 % NA_2CO_3 in 0.1N NaOH) was added to 0.5 ml of the test solution. The reaction mixture was then mixed thoroughly and allowed to stand at room temperature for 10 minutes. Following incubation, 0.5 ml of 1N folin-ciocalteau reagent was added, mixed rapidly and the reaction mixture further incubated for 30 minutes at room temperature. Absorbance was measured against an appropriate blank at 750 nm. The total protein concentration in the snail samples was obtained from the standard curve.

2.6 Assessment of Enzymatic Activity

2.6.1 Acetylcholinesterase

Determination of acetylcholinesterase activity was carried out following the method of Ellman *et al.* (1961) adapted for a microtitre plate reader as described by Kallander *et al*. (1997). Briefly, the following reagents were added to the microtitre plate:110 µl of 0.01 M Tri/HCl buffer pH 8.0, 20 µl of 3.2 mM 5.5 dithio-bis-(2-nitrobenzoic acid) (DTNB) and 50 µl of 1 mg/ml PMF. The mixture was incubated for 3 minutes before adding 20 µl of 10 mM acetylthiocholine iodide. The rate of production of a complex between thiocholine and DTNB was followed at 25ºC for 5 minutes at 412 nm using the SpectraMax 340 pc plate reader. Each sample was assayed in quadruplicate.

2.6.2 Carboxylesterase

Carboxylesterase activity was measured using the substrate 4- nitrophenyl acetate following the method of Mackness *et al.* (1983). The reaction mixture contained 200 µl of reagent A (0.027 M Tris/HCl buffer, pH 7.6 and 0.0096 M 4-nitrophenyl acetate in a ratio of 2:1 respectively). The absorbance was measured at 405 nm for 5 minutes at 25 °C using the SpectraMax 340 pc plate reader. Each sample was measured in quadruplicate.

2.6.3 Arylesterase

Arylesterase activity was measured using phenylacetate as the substrate, following the method described by Lorentz *et al.* (1979). Briefly, 20 µl of PMF was diluted with 2 ml of activator solution (20 mM calcium chloride, 155 mM sodium chloride). From this solution 25 µl was pipetted and placed into a test tube containing 3.4 ml of substrate (0.516 mM 4 aminoantipyrine, 4.02 mM phenyl acetate, 50 mM Tris acetate buffer). The mixture was incubated for 20 minutes at 25 °C before adding 100 µl of 213 mM potassium ferrocyanide. The absorbance was measured at 492 nm. Each sample was assayed in quadruplicate.

2.7 Statistical Analysis

The results obtained were analyzed using one-way Analysis of Variance (ANOVA) statistical with Dunnet's Multiple Comparison test found in the GraphPad Prism 5 statistical analysis software program, as well as Microsoft excel. The significance of the results was ascertained at *$p<0.05$ and **$p<0.01$.

3. Results

3.1 Water Quality

Analysis of the water quality parameters revealed the pH values were 9.07 and 7.44 for Umguza and Hillside dams respectively (Table 1). Conductivity and total dissolved solids recorded in Umguza were higher compared to that of Hillside dam water (Table 1), possibly due to surface runoffs from the agricultural areas which probably caused an increase in the amount of ions present.

Table 1. Water quality analysis of Umguza and Hillside dam water.

	pH	Conductivity (µS)	Total dissolved solids (TDS in ppm)
Umguza dam	9.07	685	344
Hillside dam	7.44	187	94

3.2 Acetylcholinesterase Activity

Chlorpyrifos or aldicarb spiked Hillside dam water caused a steady increase in acetylcholinesterase enzyme inhibition. Day 3 showed inhibition above 25%, which increased to 40-50% at day 7 (Fig 1). At day 14, degree of inhibition increased to 70-80% (Fig 1). This revealed that the longer the exposures, the greater the inhibition of esterase activity. Umguza dam water spiked with aldicarb, caused ~28% enzyme inhibition at day 3 which increased to a high of ~ 80% at day 7, with no significant change thereafter (Fig 1). Chlorpyrifos on the other hand caused enzyme inhibition ranging between 30-40% throughout the exposure period (Fig 1).

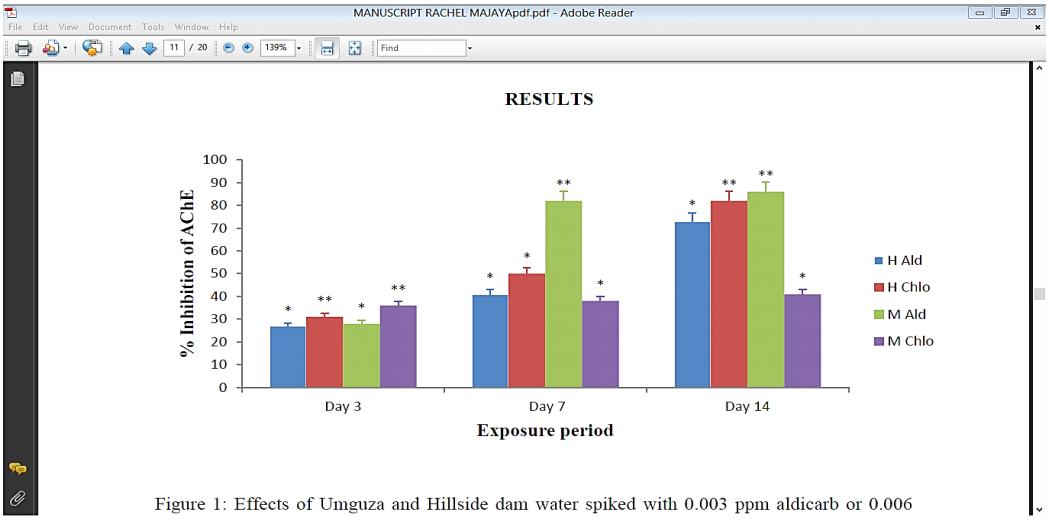

Figure 1. Effects of Umguza and Hillside dam water spiked with 0.003 ppm aldicarb or 0.006 ppm chlorpyrifos on acetylcholinesterase activity of the freshwater snail *L. natalensis.*

Acetylcholinesterase activity was measured using acetylcholine iodide as a substrate. H Ald = Hillside dam water with aldicarb; H Chlo = Hillside dam water with chlorpyrifos; M Ald = Umguza dam water with aldicarb; M Chlo = Umguza dam water with chlorpyrifos. Values represent the average of duplicate exposures and these are expressed as mean ± SD significantly different at *p<0.05 and **p<0.01.

3.3 Carboxylesterase Activity

Carboxylesterase activity following exposure to aldicarb spiked Hillside dam water was inhibited and the inhibition increased with increase in exposure duration in a nonlinear manner, with day 14 causing a highest inhibition of 80% (Fig 2). Similarly, exposure to chlorpyrifos caused a steady increase in inhibition, although the degree of inhibition was much lower than that of aldicarb (Fig 2). Umguza dam water spiked with aldicarb caused enzyme inhibition of above 60% at days 3 and 7 which later increased to ~90% at day 14 (Fig 2). Chlorpyrifos caused inhibition of up to ~70% at day 14 (Fig 2).

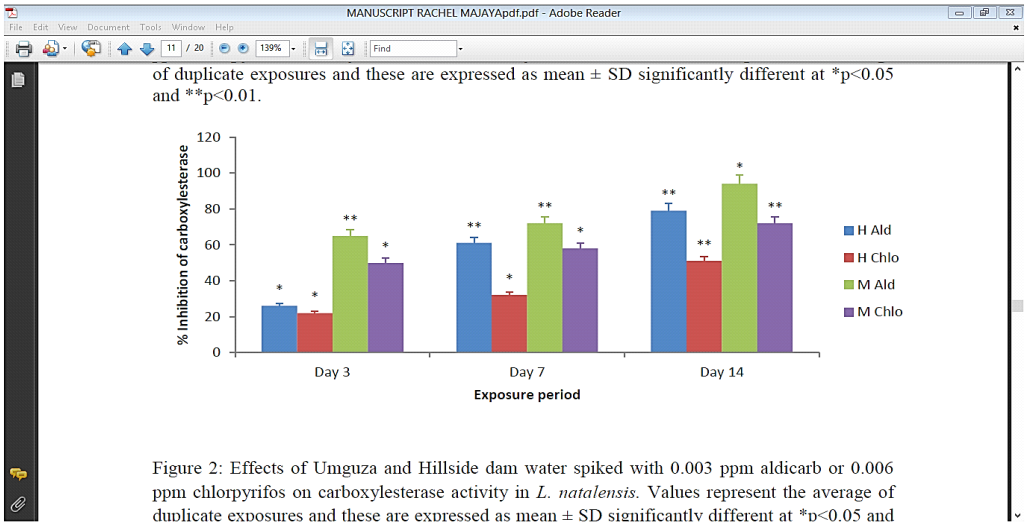

Figure 2. Effects of Umguza and Hillside dam water spiked with 0.003 ppm aldicarb or 0.006 ppm chlorpyrifos on carboxylesterase activity of the freshwater snail L. natalensis.

Carboxylesterase activity was measured using 4-nitrophenyl acetate as a substrate. H Ald = Hillside dam water with aldicarb; H Chlo = Hillside dam water with chlorpyrifos; M Ald = Umguza dam water with aldicarb; M Chlo = Umguza dam water with chlorpyrifos. Values represent the average of duplicate exposures and these are expressed as mean ± SD significantly different at *p<0.05 and **p<0.01.

3.4 Arylesterase Activity

Both dam waters spiked with pesticides inhibited arylesterase activity. Aldicarb caused a nonlinear increase in inhibition with the highest inhibition of ~ 80% at day 14 (Fig 3). Chlorpyrifos on the other hand caused a nonlinear dose dependent inhibition of arylesterase [above 45%] (Fig 3). Aldicarb spiked Umguza dam water caused enzyme inhibition of 80-90% throughout the exposure period while chlorpyrifos increased inhibition from ~60% at day 3 to ~80% at day 7 with insignificant change thereafter (Fig 3).

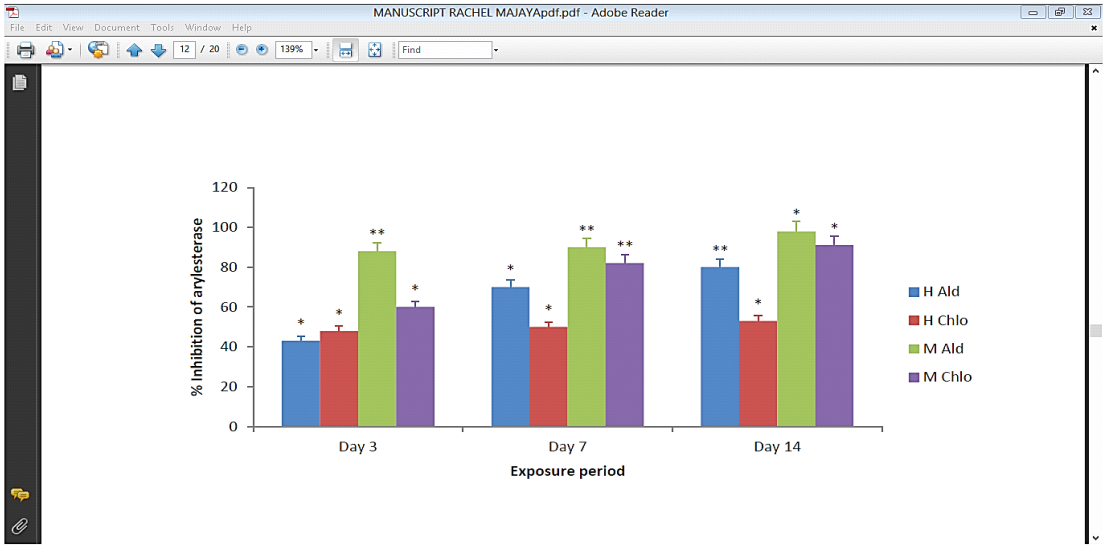

Figure 3. Effects of Umguza and Hillside dam water spiked with 0.003 ppm aldicarb or 0.006 ppm chlorpyrifos on arylesterase activity of the freshwater snail *L. natalensis.*

Arylesterase activity was measured using phenylacetate as a substrate. H Ald = Hillside dam water with aldicarb; H Chlo = Hillside dam water with chlorpyrifos; M Ald = Umguza dam water with aldicarb; M Chlo = Umguza dam water with chlorpyrifos. Values represent the average of duplicate exposures and these are expressed as mean ± SD significantly different at *$p<0.05$ and **$p<0.01$.

4. Discussion

Pesticides have an important use in the control of pests and weeds in agriculture and in the control of diseases in public health such as malaria. Unfortunately some of these pesticides are transported by air drifts when the pesticides are sprayed or washed off terrestrial surfaces as surface run-offs by rain into the aquatic systems. This, results in non-target species, which have physiologic or biochemical systems similar to those of target organisms, being affected (Tirello *et al*., 2013; Lekhani, 2015).

The water quality analysis of the water from Umguza and Hillside dams used in this study showed that the pH, conductivity and TDS were comparable with values for surface waters obtianed in literature (*Chapman et al.,* 1996). According to Chapman *et al.* (1996), surface waters conductivity normally ranges between 10-1000 µS and pH ranges between 6-8.5 with an allowance of +/- 1 for water designated as a habitat for fish and other aquatic life and water for irrigation (Bhatnagar and Devi, 2013). Environmental Protection Agency's maximum contamination level for TDS is 500 ppm (US, EPA) and water samples in this study from both dams had values below that value. Total dissolved solids levels in Umguza dam water were however, higher than that from Hillside which may be attributed to the fact that Umguza dam is a confluence of numerous streams which may be contaminated by various anthropogenic pollutants (Dube *et al*., 2010; Siwela *et al*., 2010). Overall pesticide exposures in Umguza dam water caused higher inhibition of esterase activity compared to exposures in Hillside dam water (with the exception of AChE activity following exposure to chlorpyrifos spiked Umguza dam water). It may therefore be inferred that higher TDS levels in water enhance the effects of pollutants, however, further studies would have to be performed with dams with an equivalent or even higher TDS values to make this conclusion.

Esterase sensitivity to both pesticides was observed. Exposures to the carbamate aldicarb showed greater enzyme inhibition, compared to the organophosphate chlopyrifos. This may be due to the fact that carbamates induce toxicity faster than organophosphates, as they do not need to be bioactivated to be potent inhibitors (Barata *et al.*, 2004). Despite this, carbamate pesticides are susceptible to hydrolysis by carboxylesterases and arylesterases (Peterson and Talcott, 2013). In the present study, however, the two enzymes showed less capability of hydrolyzing pesticides. This may have

been a result of the adaptability of the organism species to the chemical compounds. Some types of species are able to quickly hydrolyse / metabolise the compounds, while other species are resistant, failing to metabolise the compounds but rather become susceptible to inhibition by the toxicants (Wheelock *et al*., 2005). Acetylcholinesterases are known to be specifically inhibited by carbamate pesticides (Apilux *et al*., 2015). The present study showed higher inhibition caused by aldicarb with inhibition as high as 85% in Umguza dam water and 70% in Hillside dam water. Similar studies in literature have reported relatively high inhibitions of esterase activity due to carbamate exposure (Barata *et al*., 2004); Apilux *et al*., 2015). Kristoff *et al* (2006) reported inhibition of AChE activity of up to 65 % in the *Biomphalaria glabrata* snails, after exposure to a carbamate pesticide.

Some organophosphates, unlike carbamates, cannot directly inhibit acetylcholinesterases, but are metabolically activated by cytochrome P450 isoenzymes to form potent AChE inhibitors known as oxons (Dzul-Caamal *et al.*, 2012, Lionetto *et al.*, 2013). Exposures to chlorpyrifos in Hillside dam water showed increased inhibitions of up to 70% at day 14 while exposures in Umguza dam water only ranged between 30-40% throughout the exposure period. The observed difference in AChE inhibition may be due to the presence of chemical antagonists in the Umguza dam water, which may have reacted with the chlorpyrifos or its oxon form, subsequently producing a less toxic product. Antagonistic effects of pollutants were observed by Basopo *et al.* (2014) who showed inhibitions of esterase activity in snails exposed to pollutant mixtures being lower than in snails exposed to the individual chemicals.

Carboxylesterases are also targets of the oxon forms of organophosphates, however, their inhibitions do not result in the lethal cholinergic crisis observed in AChE inhibition (Ross *et al.*, 2010). Inhibition of carboxylesterases instead plays a protective role on the intrinsic target AChE as the carbooxylesterases scavenge the oxons generated within the organism, reducing the number of molecules available for inhibiting AChE (Barata *et al.*, 2004). In the present study, inhibition of carboxylesterases, following exposure to chlorpyrifos in both Hillside and Umguza dam water increased with prolonged exposure, suggesting enzyme increased affinity for the bioactivated chlorpyrifos. There is insufficient data, however, to conclude that CbE inhibition provided AChE protection. Further studies would have to be conducted as performed by Wheelock (2008) who, firstly, pretreated CbE with inhibitors and observed any potentiated toxicity of the organophosphate and secondly, considered any other protection contributed by other esterases such as butyrylcholinesterase.

Arylesterases are calcium dependent hydrolases that can detoxify active metabolites (oxons) of organophosphorus compounds (Aoki *et al*., 2014). The results in the present study showed 50% of arylesterase inhibition throughout the exposure period in Hillside dam water. Arylesterases are high density lipoprotein associated enzymes and so any disturbance in the lipid profile may decrease its activity (Gabrowny *et al.*, 2007). This may account for the low activity observed. The increased inhibition (90% at day 14) seen following exposures in Umguza dam water may be due to the presence of substances such as metal chlorides, that are known to inhibit A esterases (Arias-Almeida and Rico-Martínez, 2011; Prasad *et al*., 2009).

5. Conclusion

The present study showed that non-target organisms such as molluscs are potentially at risk to the toxic effects of pesticides. This is a cause of concern as the adverse effects of these agrochemicals may affect the aquatic ecosystem and biodiversity. Results from the i*n vitro* studies indicated that esterases from the exposed snails were consistently inhibited by exposure to pesticides and thus have a potential of being used as indicators of exposure to pesticide pollutants in aquatic ecosystems.

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