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Toxicological Effects of Chlorpyrifos and Lead on the Aquatic Snail *Helisoma duryi*

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Abstract

Aquatic reservoirs remain the ultimate sink of chemical pollutants emanating from anthropogenic activities such as agriculture, mining and industry. Freshwater biota undoubtedly is at risk from the adverse effects of these water pollutants and there is therefore, a need to monitor effects of these chemical pollutants in order to safeguard the health of aquatic biota. We investigated the oxidative stress effects of chlorpyrifos and lead on the freshwater snail *Helisoma duryi* to assess the potential of using this enzyme system as a biondicator of exposure to environmental pollutants. Groups of snails were exposed to 5 ppb lead acetate and 25 ppb chlorpyrifos for 7 days after which half of the snails were sacrificed and the other half were allowed to recover in clean water and sacrificed after another 7 days. Post mitochondrial fractions were used to measure the activities of the following antioxidant enzymes: superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and diphosphotriphosphodiaphorase. Both pollutants enhanced the activities of all the antioxidant enzymes suggesting a defensive mechanism by the snail to combat the oxidative stress due to the organophosphate chlopryrifos and metal pollutant lead. There was a significant recovery of the antioxidant defense system of the snails allowed to recover in clean water shown by the reduced alteration of the antioxidant enzyme activities of the snails allowed to recover for 7 days. This suggests the need to minimize exposure of aquatic biota to chemical pollutants and remediate the polluted water reservoirs in order to safeguard the health of aquatic life.

Keywords

Pollutants, Snails, Oxidative-Stress, Organophosphates, Metals

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1. Introduction

Anthropogenic activities undertaken by man in his quest to improve his livelihood have resulted in the production and release of chemicals in quantities that the environment cannot cope with. Increase in urbanization and industrial activities as well as uncontrolled exploitation of arable land has undoubtedly resulted in significant discharge of chemical pollutants and their byproducts which find their way into aquatic reservoirs which are the ultimate sink for environmental pollutants. Thousands of organic and inorganic materials such as pesticides, fertilizers, detergents and metals contaminate natural aquatic ecosystems daily and affect aquatic biota such as snails and fish. Many developing countries, including Zimbabwe, depend on agriculture to boost their economies; thus pesticides are likely to represent an important source of xenobiotics in contaminated rivers. Pesticides such as organophosphates, carbamates and pyrethroids are extensively used in agriculture and public health. In agriculture pesticides such as chlorpyrifos are used in the control of pests of cotton, corn and fruit trees such as apples and oranges [1]. Chlorpyrifos and other organophosphates are chemicals designed to target the nervous system of pest organisms in particular they inhibit the activities of cholinesterases, the enzymes required to ensure the smooth transmission of nervous impulses. Cholinesterases are thus termed useful biomarkers of exposure to environmental pollutants such as organophosphates and carbamates since these two classes of compounds share a common mechanism of toxicity [2]. Literature reports have however, shown that chlorpyrifos and other organophosphates are non-target organ/tissue specific. Wang *et al.* [3] reported the inhibition of the glutathione based antioxidant enzyme glutathione S-transferase (GST) of the goldfish (*Carassius auratus*) after exposure to chlorpyrifos and a carbamate isoprocarb supporting the fact that organophosphates and carbamates do not only target AChEs. Ferrari *et al.* [4] also observed alterations of antioxidant enzyme activities after exposure to organophosphates. The authors observed enhancement of superoxide dismutase (SOD) and GST in amphibian larvae exposed to the organophosphate, azinphos-methyl.

Mining and industrial activities also release byproducts such as metals and organic compounds which have stressful effects on the environment. In aquatic ecosystems these pollutants affect the wellbeing of aquatic biota. Netpae *et al.* [5] reported an increase in the activities of SOD, GST and glutathione reductase of the freshwater clam (*Corbicula fluminea*) after exposure to the heavy metal copper. Several studies have demonstrated that heavy metals can promote the formation of reactive oxygen species (ROS) in aquatic organisms [6]-[8]. Low degradability and bioaccumulation at different trophic levels through aquatic food webs make them major stress factors to aquatic organisms [2]. There is a need therefore to come up with early warning signals that can be used as preventive measures that protect the health of aquatic biota.

The objective of this study was to evaluate in laboratory conditions the oxidative stress effects of an organophosphorus insecticide, chlorpyrifos and the metal lead, as single entities on the aquatic snail species *Helisoma duryi*.

2. Materials and Methods

2.1. Chemicals

All enzymes, substrates, pesticides and chemicals were bought from either from Sigma Chemical Company or Aldrich Chemical Company, Germany. All other laboratory reagents, used in this study, were of analytical (ANALAR) grade.

2.2. Snail Breeding and Exposure

Snails were bred in outdoor cement aquaria containing tap water and fed on fresh garden lettuce according to the method of [9]. Juvenile snails (12 per group) were exposed to 25 ppb of chlopyrifos or 5 ppb of lead acetate for 7 days after which half of the snails were sacrificed and the other half placed in clean water for another 7 days. The exposures were performed in duplicates. After the exposure period the snails were sacrificed and post mitochondrial fractions prepared.

2.3. Preparation of Homogenates

Six whole snails from each experimental group were pooled and homogenized in (5X weight of soft tissue) ml of ice-cold homogenization buffer (0.1 M potassium phosphate pH 7.4). The homogenates were centrifuged at

10,000 × g for 10 minutes and the resultant supernatant (S-10) fraction stored at −80˚C until analyzed. Protein content in post mitochondrial fractions of the snails was determined using the method of [10] using bovine serum albumin as the calibration standard and absorbance at 750 nm.

All enzymes assays described below were performed on S-10 fractions of the whole snail homogenates.

2.4. Biochemical Assays

2.4.1. Superoxide Dismutase

Superoxide dismutase (E.C.1.15.1.1) activity in the S-10 fractions was measured following the method of [11]. Xanthine and xanthine oxidase were used to generate superoxide anion radicals, which react with 2-(4-indophenyl)-3(4-nitro-phenyl)-5-phenyl tetrazolium chloride (NBT) to form a red formazan dye. One enzyme unit of superoxide dismutase is defined as the amount, which inhibits the NBT reaction by 50%. Specific activity was defined as units/mg protein.

2.4.2. Catalase

Catalase (E.C. 1.11.1.6) activity was measured in S-10 fractions according to the method of [12] and expressed as nanomoles of hydrogen peroxide decomposed/min/mg protein.

2.4.3. Glutathione Peroxidase

Glutathione peroxidase (EC.1.11.1.9) activity was determined in S-10 fractions according to the method of [13] and was expressed as micromoles GSH oxidized/min/mg protein.

2.4.4. Glutathione S-Transferase

Glutathione S-transferase (EC.2.5.1.18) activity was measured in S-10 fractions according to the method of [14], and expressed as millimoles of 1-chloro-2-4-dintrobenzene-glutathione (CDNB-GSH) conjugate formed/min/mg protein.

2.4.5. NAD(P)H Quinone Oxidoreductase

NAD(P)H quinone oxidoreductase (EC.1.6.99.2) activity was measured in S-10 fractions following the method of [15] and was expressed as millimoles decomposed DCPIP/min/mg protein.

2.5. Statistical Analysis

Statistical differences between control and experimental groups were analyzed using the Dunnet test. Differences were considered significant at $^{*}p < 0.05$.

3. Results

3.1. Superoxide Dismutase Activity

Superoxide dismutase activity was significantly increased ($p < 0.05$) in both lead and chlorpyrifos exposed snails when compared to the controls, unexposed snails (**Figure 1**). Chlorpyrifos increased SOD activity by 93% while lead caused an increase of 165% in the enzyme activity. There was a significant difference in SOD activity between the exposed snails and the snails allowed to recover.

3.2. Catalase Activity

Catalase activity was significantly increased ($p < 0.05$) in all pollutant exposed snails when compared to the controls (**Figure 2**). Increases of 155% and 227% in enzyme activities were observed in snails exposed to chlorpyrifos and lead respectively. In the recovery period there were significant differences in enzyme activity between the exposed snails and the snails allowed to recover.

3.3. Glutathione Peroxidase

Glutathione peroxidase activity was significantly increased ($p < 0.05$) in lead and chloryrifos exposed snails

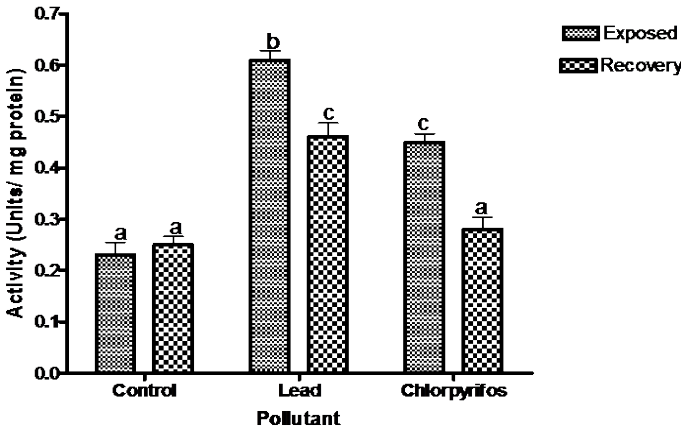

Figure 1. Effect chlorpyrifos and lead on superoxide dismutase activity of the freshwater snail *H. duryi.* = exposed and = recovery. Significance of results was ascertained at $p < 0.05$ and different letters express significant differences and bars with the same letters indicate that there is no significant difference.

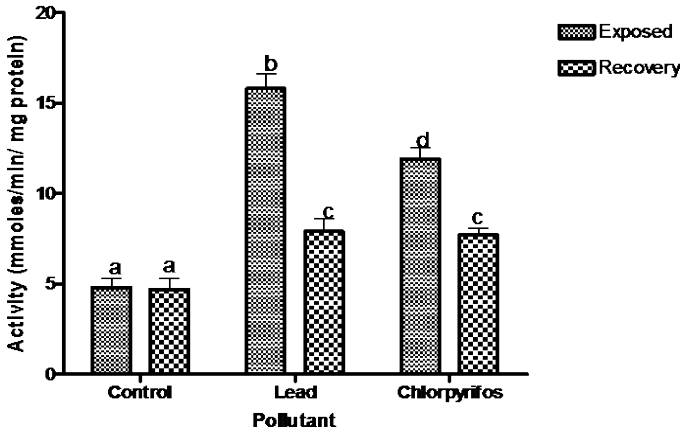

Figure 2. Effect chlorpyrifos and lead on catalase activity of the freshwater snail *H. duryi.* = exposed and = recovery. Significance of results was ascertained at $p < 0.05$ and different letters express significant differences and bars with the same letters indicate that there is no significant difference.

when compared to the controls (**Figure 3**). Activation of enzyme activities of 22% and 30% were observed in *H. duryi* snails exposed to chlorpyrifos and lead respectively. In the recovery period there was significant difference between the exposed snails and the snails allowed to recover.

3.4. Glutathione S-Transferase

Glutathione S-transferase (GST) activity was significantly increased ($p < 0.05$) in lead and chloryrifos exposed snails when compared to the controls (**Figure 4**). Chlorpyrifos activated GST activity by 66% while lead caused an increase in enzyme activity of 51%. In the recovery period there was significant difference between the exposed snails and the snails allowed to recover.

3.5. NAD(P)H Quinone Oxidoreductase

The activity of NAD(P)H quinone oxidoreductase was significantly increased ($p < 0.05$) in pollutant exposed snails when compared to the controls (**Figure 5**). Increases in NAD(P)H quinone oxidoreductase of 40% and 34% were observed in chlorpyrifos and lead treated snails respectively. In the recovery period there were no significant differences between the exposed snails and the snails allowed to recover.

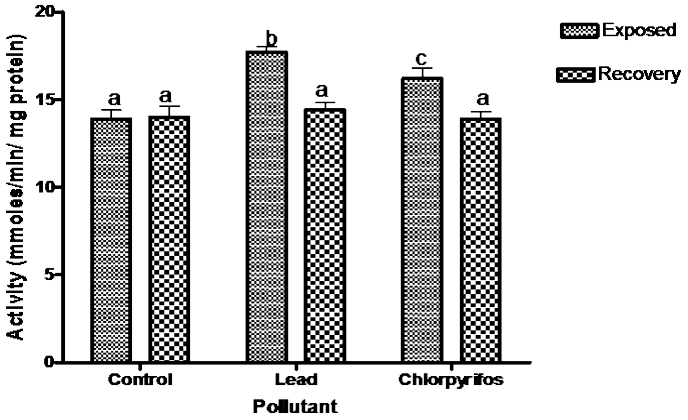

Figure 3. Effect chlorpyrifos and lead on glutathione peroxidase activity of the freshwater snail *H. duryi.* = exposed and = recovery. Significance of results was ascertained at $p < 0.05$ and different letters express significant differences and bars with the same letters indicate that there are no significant differences.

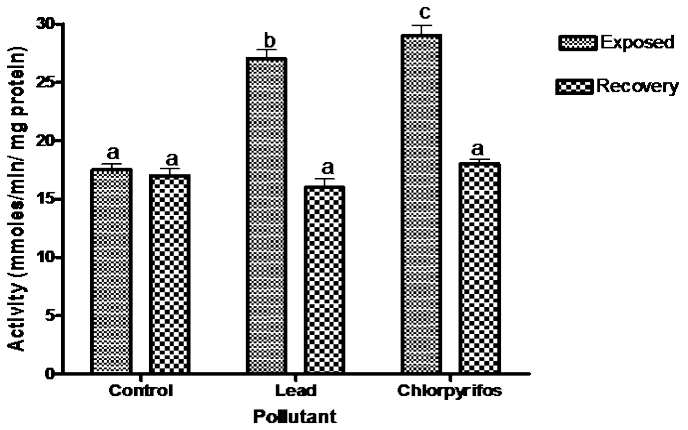

Figure 4. Effect chlorpyrifos and lead on glutathione S-transferase activity of the freshwater snail *H. duryi.* = exposed and = recovery. Significance of results was ascertained at $p < 0.05$ and different letters express significant differences and bars with the same letters indicate that there are no significant differences.

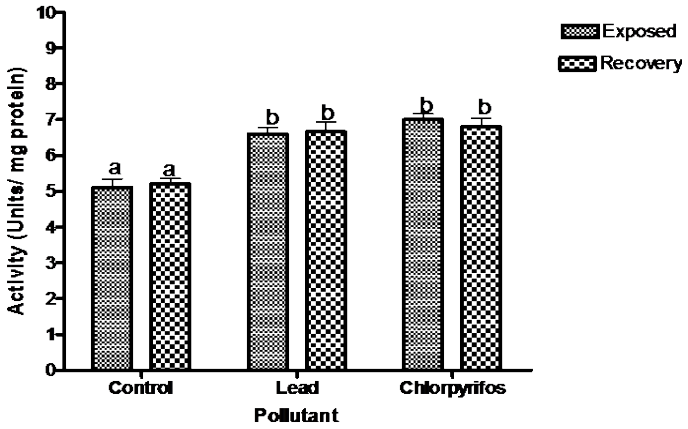

Figure 5. Effect chlorpyrifos and lead on NAD(P)H quinone oxidoreductase activity of the freshwater snail *H. duryi.* = exposed and = recovery. Significance of results was ascertained at $p < 0.05$ and different letters express significant differences and bars with the same letters indicate that there are no significant differences.

4. Discussion

Pollutants such as metals occur naturally in the environment. However, due to man's daily activities to improve his livelihood, their levels in ecosystems increase to levels that cause adverse effects to other organisms. Metals like lead are introduced in aquatic ecosystems as effluent from mining and industrial activities. Pesticides which are used by man to improve the quality and quantity of crops find their way in aquatic environments via aerial drifts during spraying, leaching and as runoffs after heavy rains. In fact literature shows that of the pesticides applied by farmers only 50% gets to the target organisms [16] and the rest end up on nontarget organisms where they usually cause deleterious effects on these organisms. Literature reports show that metals and pesticides induce oxidative stress in cells of living organisms and this result from an imbalance between the generation and elimination of reactive oxygen species [17].

Metals like lead probably cause production of reactive oxygen species (ROS) which if not eliminated effectively attack macromolecules such as lipids, proteins and carbohydrates in living organisms. The induction of oxidative stress in biological systems by lead is reported by several researchers however, mechanism of ROS induction is unknown [18]-[20]. The actual mechanism of elimination of ROS like superoxide anion radical is generally by the dismutation activity of SOD producing hydrogen peroxide. Our results showed that lead activated the activities of antioxidant enzymes in the range 30% to 227% (**Figures 1-5**) depending on the enzyme. The activation of the antioxidant enzymes observed in the present study suggests that lead has oxidative stress inducing effects which the snail's defense system counteracted by increasing the activities of the enzymes that protect living organisms from oxidative stress. Superoxide dismutases are a group of metalloenzymes that catalyse the dismutation of superoxide anion radicals ($O_2^{\cdot-}$) to form hydrogen peroxide (H_2O_2) and oxygen. Activation of SOD of 165% in lead exposed snails was observed in the present study. Altered SOD activity has been shown to be indicative of exposure to pollutants that induce oxidative stress [21]. The observed activation of SOD in pollutant exposed snails suggest that the detoxification of the pollutants by the snail's defense system probably produces superoxide anion radicals as byproducts.

Our results also showed activations of CAT and GPX of 227% and 30% respectively in snails exposed to lead (**Figure 2** and **Figure 3**). Induction of CAT and/or GPx is a protective response by the snail's system to combat the oxidative stress effects of hydrogen peroxide a product of the dismutation of superoxide anion radical produced during normal metabolism of pesticides [22]. Our results are consistent with the findings of [23] who also observed elevated CAT and GPX activities in snails exposed to metals such as lead, cadmium, iron and copper. Radwan *et al*. [18] also reported activation of CAT, GPX and GST in *Theba pisana* snails exposed to the heavy metals; lead, zinc, copper and cadmium. Induced catalase activity has been regarded as an adaptive response to reactive anion radicals generated during metabolism of various pollutants such as metal residues [5]. Catalase and GPX protect cells from H_2O_2 generated within them and it plays an important role in the acquisition of tolerance to oxidative stress [24]. The higher activations of CAT activity when compared to GPX activity observed imply that CAT is probably, the major enzyme detoxifying the reactive hydrogen peroxide a product of the dismutation of SOD. In fact the linked reactions involving SOD and catalase (CAT) provide the first line of defense against oxidative stress [25].

Activated GST activity shows the important role of this enzyme in protecting cells against oxidative stress [26]. Increased levels of GST activity of 51%, observed in this study, in snails exposed to the heavy metal lead reflect and suggest that the snail defense system managed to positively respond by attempting to protect itself against xenobiotic induced toxicity.

Activation of NAD(P)H quinone oxidoreductase, a detoxification enzyme, that reduces reactive quinones and quinone-imines to less reactive and less toxic hydroquinones in living organisms has been shown to be a defense strategy by living organisms to guard against the oxidative effects of reactive quinones which can be formed during detoxification reactions. In the present study we observed significant ($p < 0.05$) activation of NAD(P)H quinone oxidoreductase in all snails exposed to chlorpyrifos or the heavy metal, lead (**Figure 5**). The activation of NAD(P)H quinone oxidoreductase suggest the production of quinone like structures as intermediates or by-products in the detoxification mechanisms of studied environmental pollutants. Another probable reason for the activation of NAD(P)H quinone oxidoreductase activity in pollutant exposed snails is that the enzyme maybe involved in the detoxification of other ROS in the metabolism of xenobiotics. Activation of NAD(P)H quinone oxidoreductase as a defense mechanism has been reported in other polluted exposed aquatic organisms. Porte *et al.* [27] observed an increase in microsomal NADPH dependent quinone oxidoreductase in organic pollutant exposed *Mytilus galloprovincialis*.

The organophosphate chlorpyrifos is an insecticide, acaricide and miticide used to control foliage and soil-borne insect pests on a variety of food and feed crops. It controls pests such as cutworms, aphids, root borers, beetles, armyworms, spider mites and pink bollworms in crops that include cotton, soya beans, wheat, corn, citrus and tree nuts [28]. Organophosphorus pesticides like chlorpyrifos are normally associated with neurotoxicity and in particular this group of insecticides inhibits the activity of acetylcholinesterase thereby causing accumulation of acetylcholine molecules and affecting the normal functioning of nerves. Literature reports have however, showed that most pesticides, organophosphates included, affect organs/tissues other than their target organs/tissues [29]. Literature also shows that there is limited information on effects of pesticides on antioxidant enzymes in invertebrates such as snails. Salama *et al.* [30] reported an increase in levels of GSH and increased oxidative stress in the land snail *Helix aspersa* exposed to chlorpyrifos. Leomanni *et al.* [31] reported induction of the antioxidant enzymes SOD, CAT, GPX and glutathione reductase in the aquatic snail species *Cantareus apertus* exposed to the carbamate carbaryl.

In the present study chlorpyrifos activated the activities of SOD, CAT, GPX, GST and DTD in all pesticide treated snails. Activation ranges of 22% - 155% were observed depending on the antioxidant enzyme analyzed. Chlorpyrifos is a degradable pesticide, and a number of environmental forces may be active in its breakdown. In soil, water, plants and animals, the major pathway of degradation involves cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinol (TCP). This conversion to TCP is a detoxification step and the TCP can be further degraded via microbial activity and photolysis to carbon dioxide and organic matter. During normal metabolism of chlorpyrifos ROS may be produced and biological systems use antioxidant enzyme systems to protect themselves from effects of ROS. The results observed in the present study imply that probably in the metabolism of chlorpryifos in the freshwater snails *H. duryi*, ROS were indeed produced and probably that caused the defense mechanism of the snails to be activated (indicated by activation of CAT, GPX and GST) probably as an adaptive mechanism to overcome the oxidative stress induced effects of chlorpyrifos. Our results are supported by Khalil [32] who also reported induction of CAT, GPX and GST in the freshwater snail *Lanistes carinatus* exposed to the organophosphorus insecticide chlorpyrifos.

All exposed snails significantly recovered from the effects of the two pollutants chlorpyrifos and lead when allowed to recover in clean water for 7 days. The antioxidant enzymes of snails exposed to chlopryrifos recovered by 18% - 90% depending on the antioxidant enzyme. While, the snails exposed to lead recovered by 49% - 88% depending on the antioxidant enzymes in the recovery period. There are few studies that have shown how contaminated aquatic organisms can recover their biochemical status when the pollutants are removed. Ma *et al.* [33] reported activation of antioxidant enzymes SOD, CAT and GST of *Physa acuta* exposed to the pesticide abamectin and the enzyme activities returned to control levels during the recovery period. Recovery of altered biochemical endpoint parameters has also, been reported in other aquatic organisms exposed to water pollutants. A significant recovery in all enzymatic and non-enzymatic endpoints was noticed after 7 days of recuperation period in fish (*Oreochromis mossambicus*) exposed to the organophosphorus insecticide profenofos [34].

In the present study there was no significant recovery observed in the NAD(P)H quinone oxidoreductase activities for all pollutant exposed snails. The results suggest that probably NAD(P)H quinone oxidoreductase is an active enzyme in the scavenging of ROS during the detoxification of environmental pollutants. Our results are supported by Siegel *et al.* [35] who have shown that apart from catalyzing the reduction of quinones, NAD(P)H quinone oxidoreductases also have $O_2^{\cdot-}$ scavenging properties.

5. Conclusion

Our results have shown that the antioxidant enzyme system of *H. duryi* snail's is sensitive to the studied pollutants indicated by the consistent activations of the studied antioxidant enzymes and as such has a potential of being used as a non-specific biological indicator of exposure to environmental pollutants.

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