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Oraganophosphorus Pesticides Induced Enzymological Responses in the Various Tissues of Freshwater Fish Koi Carp (*Cyprinus carpio*)

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ABSTRACT

The ornamental fish, Koi carp fingerlings were exposed to sub-lethal concentrations (1/5th of 96 h LC50) of Malathion (7.2 mg/l) and Glyphosate (13.2 mg/l) along with control group for a period of 30 days. Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Aspartate aminotranferase (AST), Alanine aminotransferase (ALT) and Acetylcholinesterase (AChE) were assayed in different tissues at the end of 15th and 30th day. The results infer that, AChE shows strong significant (P<0.05) inhibition in gills than that of muscle in both treatments at the end of the 30th day. Whereas ALP activity in brain, gills and muscle of fish shows fluctuation between the treatment groups, while on the other hand ALT, AST and LDH levels were significantly declined in all the tissues (P<0.05) when compared to control. Enzymological alterations observed in the present study indicated the fact that Malathion and Glyphosate are toxic to Koi carp fingerlings at the tested concentrations. Therefore, the findings of the present investigation could be considered as possible biomonitoring tool for the assessment of pesticide contaminations in aquatic ecosystem.

Keywords: Malathion, Glyphosate, Enzymology, Pesticide pollution

INTRODUCTION

During the past few decades, the usage of synthetic pesticides in intensive agricultural activities has been manifold increased worldwide. Among different groups of pesticides, organophosphorus pesticides are widely used to control agricultural pests in many countries including India [1,2]. The indiscriminate uses of these pesticides in agricultural activities reaches the aquatic ecosystems either via runoff, aerial spraying drift and leaching, where it poses detrimental effects to the non-target organisms and finally find the way to the food chain threatening the ecological balance and the biodiversity of the nature [3-5]. Organophosphorus pesticides (Ops) were derived from phosphoric acid and the active part of the Ops is the phosphate group sharing a double bond with either oxygen or a sulphur group. They are unstable and quickly breakdown in the environment but they were found to inhibit the enzyme acetylcholinesterase (AChE EC 3.1.1.7), which plays a significant role in neurotransmission at cholinergic synapses by rapidly hydrolyzing the neurotransmitter, acetylcholine to choline and acetate [6-8] and also involved in the development of neurons and network formation [9-11]. However, they can also cause bronchospasm, bradycardia, muscle weakness, hypertension, central nervous depression, blurred vision, abdominal cramps and tremors etc. in human beings. In aquatic organisms, Ops are known to produce genotoxic, immunotoxic effects and oxidative insult which incapable of increasing the levels of reactive oxygen species [12-18].

Malathion (0, 0-Dimethyl S-(1, 2-dicabethoxyethyl) phosphorodithioate) is a systemic organophosphorus insecticide, being used in public health, residential and agriculture against wide variety of insect pests [19,20] due to their quick degradation period and low tendency to bioaccumulate in organisms [21,22]. In addition, it has been used as disinfectant for the control of ectoparasites in finfish and shellfish during aquaculture practices. Glyphosate (N-(Phosphonomethyl) glycine) is a broad spectrum organophosphate herbicide commonly used to control noxious weeds both in agricultural and non-agricultural areas. In general glyphosate and its products have a short half-life, high water solubility, strong adsorption to soil and sediments and are rapidly metabolized [23-25]. However, due to over usage of these pesticides in agricultural and non-agricultural areas ultimately finds their way into aquatic ecosystems and poses potential toxicological risks to the aquatic organisms.

Biological indicators are species that can be used to monitor the health of an environment or biota. They are any biological species or group species whose function, population or status can reveal what degree of ecosystem or environmental integrity is present. Bio-monitoring programs usually involve the use of biomarkers, which represent behavioural, histological, physiological or biochemical variations determined in tissues, biological fluids or whole organisms [26-28]. Fish have proved to be of significance as bioindicators of the aquatic environment so-called ecological integrity [29,30]. It can provide quantitative information on the ecological integrity and its health. Therefore, fishes are successful bioindicators.

During stress conditions fish undergoes certain changes and adapt their metabolic functions [31] and the inhibition or reduction of the enzymes such as AST, ALT, ALP and LDH can be used to indicate tissue damage [32,33]. Enzymes like AST, ALT, ALP and LDH not only function as link enzymes between carbohydrate and protein metabolism, but also serve as an indicator of chemical stress. Alterations in metabolic enzyme activities are serving as a sensitive tool for the identification of environmental pollutants which in turn form the basis for an improved understanding of underlying toxic processes and an interpretation of the toxic related effects [34,35]. The available data on the AST, ALT, ALP, LDH and AChE activities in ornamental fish under pesticide stress are still scanty. Amongst ornamental fishes, *C. carpio* koi has the highest market value and hence they have been selected as the bioindicator model for pesticide pollution in the current study. So far not much study has been carried out in ornamental fishes. So the present investigation is carried out to evaluate the toxic effects of Malathion and Glyphosate on certain enzymological parameters in the brain, muscle, liver and gills of the freshwater fish Koi carp.

MATERIALS AND METHODS

Experimental animal

Fingerlings of *C. carpio* koi with uniform size (7-8 cm) were procured from Solai Aquarium fish farm Kolathur, Chennai, Tamil Nadu, India. The test organisms were brought to laboratory in the plastic bags with least disturbance and were washed with 0.1% $KMnO_4$ solution to get rid of dermal infection. Healthy fingerlings were selected and acclimated to the laboratory condition in large plastic tubs using chlorine free tap water for a period of 15 days. During the experimental period, they were fed with commercial feed twice a day satiation. The water in aquarium tank was renewed periodically to maintain water quality (Temperature=28 ± 0.5°C; pH=7.5; Dissolved Oxygen=6.7 mg/l; Salinity=0.5 ± 0.05 ppt). Feeding was stopped one day prior to the experiment.

Chemicals

Commercial grade pesticides of Malathion (Milathion 50% EC, manufactured by insecticides India Private Limited, New Delhi) and Glyphosate (Round up 41% SL, manufactured by Monsanto India Private Limited, New Delhi) were taken for evaluation of its toxicity.

Experimental design

Bioassay tests were carried out for the determination of 96 h LC_{50} value of the Malathion and Glyphosate to *C. carpio* koi. After completion of the range finding tests, five desired concentration of Malathion (20, 30, 40, 50 & 60 ppm) and Glyphosate (40, 60, 80, 100 & 120 ppm) were selected. 10 fishes were introduced in each concentration of Malathion and Glyphosate experimental tanks. The animals without toxicant served as control; three replicates per test concentration were used. During exposure no food was offered to the test organisms. The number of fish died in each concentration after 96 h exposure periods was recorded and discarded. The percentage of mortality was converted to Probit's by following the Finney method [36]. Chronic toxicity tests were carried out over a period of 30 days. Test animals were introduced into 50 L plastic tubs containing 35 L of water. Based on 96 h LC_{50} (Malathion: 36.2 mg/l; Glyphosate: 66 mg/l), the fishes were exposed to a sub-lethal concentrations of ($1/5^{th}$ of 96 hrs LC_{50}) Malathion (7.2 mg/l) and Glyphosate (13.2 mg/l). A control without any pesticide was also concurrently maintained. Triplicate of each group were carried out.

Group I with 20 fishes was kept as control, Group II with 20 fishes was exposed to $1/5^{th}$ of Malathion and Group III with 20 fishes was exposed to $1/5^{th}$ of Glyphosate. The concentration of the toxicant in the water medium of the fishes were renewed every 24 h. During the period of continuous exposure to Malathion and Glyphosate, the fish were fed with commercial feed every day. At the end of 15th and 30th day 10 fishes were collected from each tub. The tissues (gills, liver, muscle and brain) were vivisected and subjected to enzymatic studies.

Enzymological analysis

The activity of AChE (nmol/mg/min) was determined colorimetrically following the method of Ellman et al. [37]. The Lactate dehydrogenase (EC 1.1.1.27) activity was assayed according to the method of McQueen et al. [38] following the absorbance of sodium pyruvate at 340 nm and expressed as µmole formazone formed/mg of protein/ min. Alkaline phophatase (EC 3.1.3.1) activity was assayed by the method of King and Annstrong [39] using p-nitrophenyl phosphate as a substrate and was measured at 405 nm. Aspartate aminotranferase (AST) (EC 2.6.1.1) was assayed by the method of Mohun and Cook [40]. Alanine aminotransferase (EC 2.6.1.2) was assayed by the method of Mohun and Cook [40].

Statistical analysis

Significant differences between the control and experimental groups were calculated by using One way analysis of Variance (ANOVA) followed by Tukey's post-hoc test ($P<0.05$). Data expressed in Mean ± SD. All analysis was done using IBM SPSS Statistical Package (Ver. 20).

RESULTS

The results of the enzyme activity [Acetylcholinesterase (AChE), Alkaline phosphatase (ALP), Aspartate aminotranferase (AST), Lactate dehydrogenase (LDH) and Alanine aminotranferase (ALT)] levels in the brain, muscle, liver and gills of freshwater fish Koi carp fingerlings exposed to sublethal concentration of Malathion and Glyphosate for 15 and 30 days were shown in Figures 1-5.

AChE activity

Fish exposed to sublethal concentration of glyphosate showed a significant increase ($P<0.05$) of AChE activity in brain, liver and slight decrease in gills and muscle was observed during 30th day compared to control. Whereas the fishes treated with malathion showed significantly ($P<0.05$) decreased activity in brain and liver. At the same time, increased activity in gills and muscle was noticed during 30th day when compared to control (Figure 1).

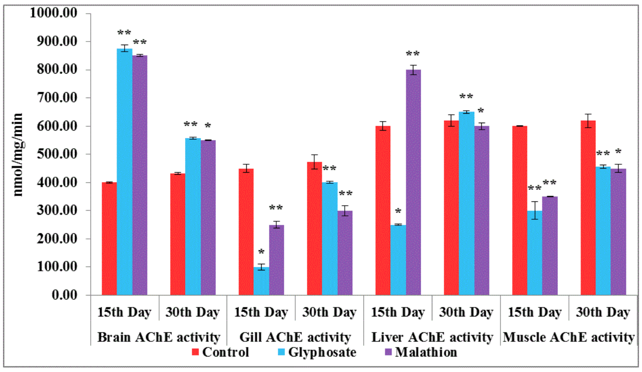

Figure 1: AChE activity in different tissues of koi carp treated with malathion and glyphosate; Significant different ($P<0.05$) from the control are indicated by asterisks

ALP activity

The ALP activity in different tissues of koi carp treated with malathion and glyphosate showed significant variations ($P<0.05$) (Figure 2) compared to control. Highest ALP activity was found in brain and gills in both the treatments at the end of 30th day. Whereas significant decrease ($P<0.05$) in ALP activity was observed in liver and muscle in both the pesticides.

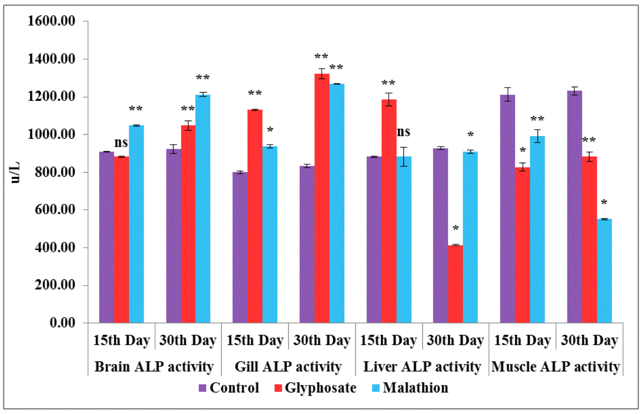

Figure 2: ALP activity in different tissues of koi carp treated with malathion and glyphosate; Significant different (P<0.05) from the control are indicated by asterisks

AST activity

AST activity in different tissues of koi carp treated with malathion and glyphosate showed significant variations (P<0.05) compared to control (Figure 3). In the malathion and glyphosate treated groups, tissues such as brain, gills, liver and muscle showed significantly declined activity (P<0.05) at the end of 30th day compared to control.

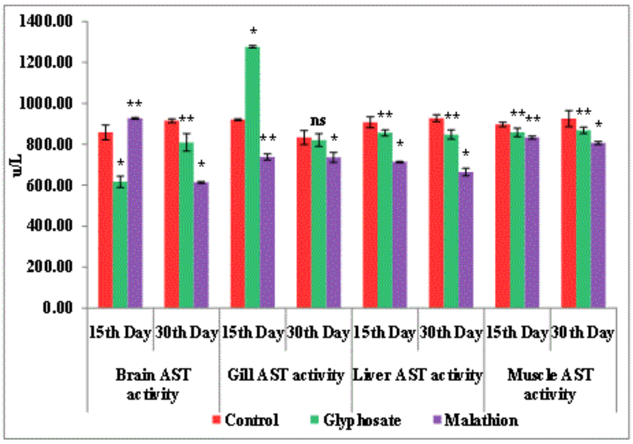

Figure 3: AST activity in different tissues of koi carp treated with malathion and glyphosate; Significant different (P<0.05) from the control are indicated by asterisks

LDH activity

The LDH activity in different tissues of koi carp exposed to malathion and glyphosate showed significant variations (P<0.05) compared to control. Brain of malathion treated group showed significantly increased activity (P<0.05) whereas the LDH activity in tissues such as gills, liver and muscle showed significantly decreased activity (P<0.05) when compared to control. At the same time, the LDH activity in gills of fish exposed to glyphosate showed significantly increased activity (P<0.05). However, the LDH activity in tissues such as brain, liver and muscle showed decreased activity (P<0.05) than that of control (Figure 4).

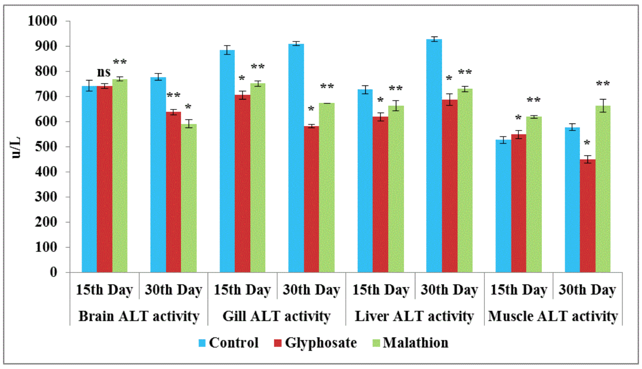

Figure 4: ALT activity in different tissues of koi carp treated with malathion and glyphosate; Significant different (P<0.05) from the control are indicated by asterisks

ALT activity

ALT activity was found to show significant variations (P<0.05) in all treated groups when compared to control (Figure 5). In the glyphosate treated group, tissues such as brain, gills, liver and muscle showed a statistically significant decline activity (P<0.05) during 30th day compared to control. Whereas the ALT activity of brain, gills and liver of the malathion treated group showed a significant decline activity (P<0.05) during 30th day compared to control. Muscle of malathion treated group showed a statistically significant increased activity (P<0.05) at both 15th and 30th days of exposure when compared to control.

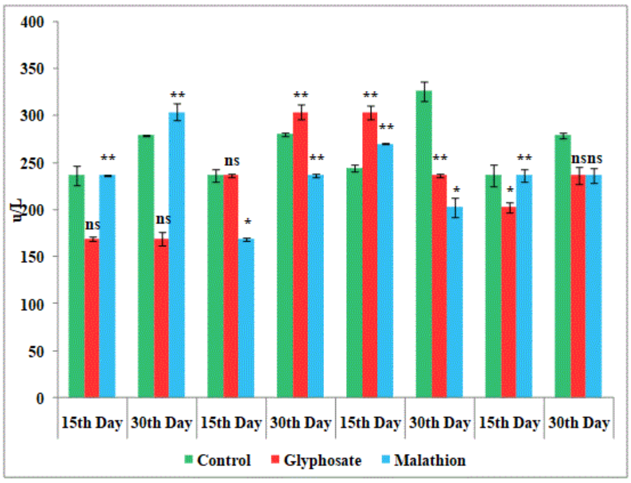

Figure 5: LDH activity in different tissues of koi carp treated with malathion and glyphosate; Significant different (P<0.05) from the control are indicated by asterisks

DISCUSSION

Evaluation of enzyme activities in aquatic organisms has been considered as a relevant stress indicator [41-44]. Therefore, activities of AChE, ALP, AST, LDH and ALT have been widely used in the diagnosis of finfish and shellfish diseases as well as detection of tissue damage caused by environmental pollution. An increase or decrease of these enzyme activities indicates stress based tissue impairment. Generally, the activities of AChE, ALP, AST, LDH and ALT may indicate degeneration changes of tissues as the toxicants effects on the cells are in the form of tissue damage in which cellular enzymes are released from the cells into the extracellular fluids or blood as a result it leads to impaired metabolism associated with disturbed homeostasis. For afore mentioned reasons, enzyme analysis stands pivotal role in toxicological point of view. The organophosphate pesticides alters the activity of several enzymes including antioxidant and oxidative enzymes in several aquatic organisms [28,45,46] .

Acetylcholine (AChE) is the most important neurotransmitter in most animals. It is resulted by a stimulated nerve cell into the synapse, or neuromotor junction with another nerve cell. Once Ach has been secreted into the synapse it binds to receptor sites on the next nerve cells, causing the latter to propagate the nerve impulse through the synapse, AChE secreted after the first impulse must be hydrolyzed by the AChE in the junction. Several studies on the mode of action and inhibition of AChE have been carried out. Indeed, inhibition of this enzyme is the focal target for most of the current synthetic pesticides. It has been established that the AChE enzyme unit consists of a negative subsite, which attracts the quaternary group of choline through both columbic and hydrophobic forces and an esterase subsite, where nucleophilic attack occurs on the acetyl carbon of the substrate [47]. The catalytic mechanism resembles that of other serine esterases (e.g. Alkaline phosphatases), where a serine hydroxyl group is rendered highly nucleophilic through a charge relay system involving the close apposition of an imidazole group and presumably a carboxyl group on the enzyme. Changes in brain, liver, gill and muscle AChE activity observed in koi carp exposed to Malathion and Glyphosate probably reflected in locomotory behavior. Similar changes were observed in fathead minnows (*Pimephales promelas*) exposed to Carbofuran [48].

Marked decrease in AChE activity in the tissues of European eel (*Anguilla anguilla*) was reported in response to Thiobencarb pesticide [49]. Pan and Dutta [50] studied the inhibition of brain AChE activity of juvenile largemouth bass, *Pterus salmoides* by sublethal concentrations of diazonian. A decrease in AChE activity in the brain and muscle of *Prochilodus lineatus* was observed by Modesto and Martinez [51] when exposed to glyphosate. Inhibition of AChE resulted in abnormal accumulation of acetylcholine, which causes eventual paralysis of the muscle. Death may occur as a result of asphyxia caused by the paralysis of respiratory muscle in due course [52].

Alkaline phosphatase is a brush boarder membrane enzyme, plays a significant role in hydrolysis of phosphate molecules and in membrane transport as well as act as a very good bio-indicator of stress in biological systems [34,35]. Alkaline phosphatases are microsomal enzyme involved in the process of mineralization of calcium carbonate in many invertebrates. The involvement of ALP in active transport [53,54], carbohydrate metabolism [55], protein synthesis [56,57] synthesis of some enzymes and secretory activities was reported [58,59].

The alteration of ALP activity in the present study might be due to tissue disorder in the cellular membrane of koi exposed to pesticides. The findings of the present investigation are in accordance with the report of Gill et al. [60] on *Puntius conchonius* exposed to aldicarb, phosphamidon and endosulfan. Similar to this, previous studies on carbon tetrachloride treated English sole, *Parophrys vetulus* [61] Carbofuran exposed *Channa punctatus* [62], Diethylphthalate treated freshwater fish, *Cirrhinus mrigala* [63], Copper exposed ornamental fish *Carrassius auratus* [64] showed significant increase in the ALP activity.

Malathion and Glyphosate exposure in koi carp lead to changes in AST activity in the brain, gills, liver and muscle it might be due to inactive or ineffective transamination and oxidative deamination processes in these organs. These findings can also indicate a breakdown of the promotion of glyconeogenesis from amino acids as well as disruption in aminotransferases activities in the organs all of which could be as a result of tissue damage. These findings are in agreement with Gill et al. [60] in the liver and muscle of *Cyprinus carpio* exposed to diazinion for 90 h. The decrease in the AST activity may be due to disturbances in the Kerb's cycle intermediate enzymes. The decrease in enzyme activity in the present investigation indicates that there might be tissue damage, because AST enzyme one of the most sensitive biomarkers employed in the diagnosis of liver damage since they are cytoplasmic in nature and are released into blood circulation after cellular insults [65].

Lactate dehydrogenase is the key enzyme mainly participated in anerobic pathway of carbohydrate metabolism. This enzyme is one of the most sensitive enzymes to environmental pollutants. An alteration in its function would indicate the occurrence of pathological conditions in the organisms. The increase and decrease of LDH activity is a diagnostic index widely used to recognize increases of anerobic metabolism resulting from depletion of energy under anerobic and environmental stress conditions [34,66].

The results of this study showed increasing trend in LDH levels in various tissues of koi in both pesticide treatments. At the same time, significant elevation of LDH levels in gills and liver was observed. This might be due to tissue damage and shift from aerobic respiration to anerobic respiration promoting the glycolytic rate and conversion of pyruvic acid to lactic acid under pesticides stress. The increase in plasma LDH activity in benomyl treated fish *Oreochromis niloticus* indicate that the toxicity may be produced through anaerobic mechanism [67] and suggested that LDH an important glycolytic enzyme is inducible by oxygen stress and therefore, the activity of regulatory enzymes gets altered to meet the required energy demands under toxic stress including the activity of LDH, which sustains the continued process of glycolysis under anaerobic conditions [41,68]. Thus, the observed elevated LDH activity can be interpreted as a shift in the respiratory metabolism from aerobic to anerobic in order to meet the enhanced energy demand under the toxic stress [69,70].

This present study reveals that the activity of alanine aminotransferase (ALT) declines in brain, gills, liver and muscle of koi exposed to glyphosate, at the same time when the fish exposed to malathion shows elevated ALT activity in muscle. The decrease in level of ALT indicates the tissue damages in the brain, liver and gills [71,72]. Toxic action of pesticide combine with an enzyme to form an enzyme inhibition complex which reacts with functional groups of the enzymes inhibits the normal enzyme activity of major metabolic site [72]. The results obtained in the present study indicate that organophosphorus pesticides though principally a specific inhibitor of AChE, altered carbohydrate, protein and lipid metabolism.

CONCLUSION

To the best of our knowledge, this is the first study focusing on the enzymological responses in the freshwater Koi carp exposed to sublethal concentration of Malathion and Glyphosate under laboratory conditions. This investigation clearly indicates that Malathion and Glyphosate have significantly altered the enzymological (AChE, ALP, AST, LDH and ALT) responses. The enzymological parameters in this study could be used as potential biomarkers in assessing toxic effects of organophosphorus pesticides and also other pollutants. The findings of the present investigation can ascertain a safer level of these pesticides in the aquatic biota.

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