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IMPACTS OF MALATHION ON MORTALITY AND BIOCHEMICAL CHANGES OF FRESHWATER FISH *LABEO ROHITA*

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ABSTRACT
Malathion was used *in vivo* on fresh water fish *Labeo rohita* to study its toxicity. The acute toxicity tests were conducted during certain intervals in various concentrations (5, 10, 15, 20, 25 and 30 mg/L) of malathion. The physical and chemical analyses of water were carried out by following APHA methods. While treating with malathion, the percentage of fish mortality was assessed during 24, 48, 72 and 96 hours. The lethal and sub-lethal concentration of malathion were found to be LC_{100} (25 mg/L) and LC_0 (5 mg/L), respectively. The antioxidant enzyme activity (Catalase 43.1±2.3, 16.5±0.57, 23.9±0.17 µ moles of phenol liberated/min/100mg protein and Glutathion-S-transferase (GST) 270.5±0.16, 143.2±1.03, 215.5±0.72 µ moles of phenol liberated/min/100mg protein), in the liver, muscle and gill, respectively increased during the accumulation of malathion, whereas it decreased (Catalase 17±1.44, 7.9±0.23, 10.7±0.69 µ moles of phenol liberated/min/100mg protein and GST 219.5±1.12, 108.1±0.34, 160.2±0.46 µ moles of phenol liberated/min/100mg protein) in the liver, muscle and gill respectively during depuration period. The effects of malathion resulted in the gradual decrease of nucleic acids, protein, free amino acids (FAA) and glycogen. During recovery period, the levels of biochemical components progressively increased indicating a probable recovery from the disruption of internal organ. Hence, the pesticide intoxication has made defective consequences in the normal metabolic pathways which led increasing the rate of mortality in fish population.

Key words: *Labeo rohita,* Malathion, Protein, Nucleic acids and Antioxidant enzymes

INTRODUCTION
Deliberate or accidental contamination of ponds by widely utilized organophosphorous (OP) insecticides such as malathion is a potential problem for aquaculture in tropical countries. The pesticide, on reaching to aquatic systems, greatly influences the non target organisms such as fish and birds. The toxic effects of the chemicals may be physiological, biochemical and pathological in nature. The changes produced by these agents may be complex, damaging different organs, tissues or cells. Examinations of tissues from organisms after death may possibly reveal the causative agent.

Histological studies on fish have revealed that various toxicants have produced pathological changes in the tissues such as macrobiotic changes in the liver, tubular damage of kidneys, gill and lamellar abnormalities (Ramalingam, 2000). Due

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to growth of agriculture in and around fresh water bodies the pesticides are used abundantly during the cultivation season and found their way into water bodies.

The degree of toxicity produced by the poisonous substance is dose independent upon environmental conditions such as temperature, pH, oxygen content and presence of residue molecules (Capkin *et al*., 2006; Singh and Mishra, 2009; Gulfer *et al*., 2009). It is well known that protein, carbohydrates and lipid play a major role as energy precursors in fish under stress conditions. Enzymes play significant role in food utilization and metabolism. The proteolytic enzymes participate in the breakdown of protein molecules into amino acids and these amino acids are in turn oxidized to give energy for body function (Saravanan *et al*., 2000). Pollutants can produce metabolic changes at cellular levels by a way of influencing enzyme systems. Many authors have reported the changes in acid and alkaline proteases in fish exposed to sub lethal levels of pesticides.

The present study has been made to investigate the biochemical changes followed by mortality in the fresh water fish *Labeo rohita* induced by sub lethal dosages of the pesticide malathion.

MATERIALS AND METHODS

Fish (*Labeo rohita*) were collected and kept in aquarium. They were fed daily and acclimatized in laboratory for 30 days. The physical and chemical analyses of the water were carried out (APHA, 2005). Fish were divided into seven groups (each containing 10 fish) where six were experimental and one group as control. Acute toxicity study was carried out using the standard guidelines (EPA/ROC, 1998) to determine the lethal (LC_{100}), median (LC_{50}) and safe sub lethal (LC_0) levels of malathion in various concentrations (5, 10, 15, 20, 25 & 30 mg/L). The mortality of fish (%) was assessed during the interval of 24, 48, 72 and 96 hours.

1/3rd of median lethal concentration (5 mg/L) was taken to study the effect of malathion on the biochemical constituents and detoxifying ability of fish. The exposure was continued for 15 days at 28 ± 1°C with photoperiod of 12D:12L. The water was renewed freshly every day to produce constant effect of malathion on fish. At the end of 15 days exposure, the tissues such as liver, muscle and gill were collected by dissecting the animal and stored at - 20°C for biochemical parameters studies.

The remaining fish released into freshwater for 15 days to know the detoxifying ability of the fish. At the end of 30 days, tissues were collected again and one gram of muscle, liver and gill samples were suspended in 5mL of 0.1 M phosphate buffer of pH=7 and homogenized. These homogenates were stored for further studies at -20ºC.

Catalase activity assay was performed according to Beaumont *et al*., 1990 by following the H_2O_2 dismutation at 240 nm in a reaction mixture composed of 0.1 M phosphate buffer, pH=7, 50–100 mg protein and 18 mM H_2O_2. GST activity was measured at 37°C using 1 mM l-chloro-2,4 dinitrobenzene (CDNB) as substrate (Habig *et al*., 1974). The activity of acid phosphatase was assayed with the method of Tennis Wood *et al*., 1976. The tissue of liver, muscle and gill was homogenized in glass homogenizer separately, using 10 mL distilled water and centrifuged at 3000 rpm for 10 minutes. 0.5 mL of supernatant was taken in a clean test tube and 0.5 mL of the substrate solution (*p*-nitrophenyl phosphate) and 0.5mL of 0.1N citrate buffer were added. The test tube with the above solution was kept in water bath maintained at 37ºC for 30 minutes. After completion of 30 minutes, the reaction was arrested in the extracts by adding 3.8 ml of 0.1N sodium hydroxide. The color formed at the end was read at 415 nm in UV-visible spectrophotometer (Spectronic-20 Bausch and Lamb). Values were expressed in µ moles of phenol liberated/min/100mg protein.

The activity of alkaline phosphatase was assayed with the method of Tennis Wood *et al*., 1976. The tissue of liver, muscle and gill was homogenized in glass homogenizer separately, using 10mL of distilled water and centrifuged at 3000 rpm for 10 minutes. 0.5 mL of supernatant was taken in a clean test tube and 0.5mL of the substrate solution (*p*-nitrophenyl phosphate) and 0.5ml of glycine buffer were added. The test tube with above solution was kept in a water bath maintained at 37ºC for 30 minutes. After completion of 30 minutes the reaction was arrested in the extract by adding 10 mL of 0.2N sodium hydroxide. The color formed at the end was read at 415 nm in

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UVspectrophotometer (Spectronic-20, Bausch and Lamb).Values were expressed in µ moles of phenol liberated/ min/100 mg protein.

Known weights of the tissue were processed for the extraction of RNA and DNA. Subsequently, DNA and RNA concentrations of liver, muscle and gill were estimated (Schneider, 1957). Proteins levels were estimated by the method of Lowry *et al*., 1951 using bovine serum albumine as standard. Homogenates 2 ml (w/v) cold distilled water was prepared in 30% TCA; values are expressed as mg/100 mg wet wt of tissue.

Free amino acids (FAA) were estimated using the ninhydrin method (Moore and Stein, 1954). Homogenates (5% w/v) were prepared in 10% (w/v) TCA, centrifuged at 300 rpm and supernatant was used for amino acid estimation. FAA was expressed as mg/100 mg wet wt of the tissue. Glycogen was assayed by Anthrone method described by Carroll *et al.,* 1956. The values were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by LSD tests using the computer package SPSS 18.0 v and the significance of difference was set up at ($p< 0.05$).

RESULTS

The percentage of mortality of *Labeo rohita* exposed to malathion in 5, 10, 15, 20, 25 and 30 mg/L for 24h, 48h, 72h and 96h was assessed (Table 1). The median lethal concentration was observed as 15mg/L since it is caused 50% mortality in 96 h using the "Maximum likelihood method" (Finney, 1971). 1/3rd of median lethal concentration (5 mg/L) was taken to study the effect of malathion on the biochemical constituents and detoxifying ability of fish. The physico-chemical parameters were assessed in water and water containing different malathion concentration and tabulated (Table 2).

The activity of antioxidant enzymes in the liver, muscle and gill of *Labeo rohita* exposed to LC_0 concentration of 5 mg/L malathion during accumulation were observed (Catalase 43.1±2.3, 16.5±0.57, 23.9±0.17 µ moles of phenol liberated/ min/100mg protein and Glutathion-S-transferase 270.5±0.16, 143.2±1.03, 215.5±0.72 µ moles of phenol liberated/min/100mg protein) in the liver, muscle and gill, respectively increased during the accumulation of malathion whereas, it decreased (Catalase 17±1.44, 7.9±0.23, 10.7±0.69 µ moles of phenol liberated/min/100mg protein and GST 219.5±1.12, 108.1±0.34, 160.2±0.46 µ moles of phenol liberated/min/100mg protein) during depuration period (Table 3).

The concentration of acid phosphatase and alkaline phosphatase in tissue of *Labeo rohita* exposed to malathion were estimated (0.420±0.01, 2.249±0.00, 0.424±0.01 µm/mg protein/h and 0.850±0.02, 1.025±0.01, 1.825±0.01 µm/mg protein/h, respectively), Table 4. The sublethal effects of malathion on nucleic acid content in different tissues of *Labeo rohita* during accumulation and depuration periods were fluctuated (Table 5). Depletion on biochemical parameters like Protein, Glycogen and Free amino acid were evaluated during various periods of exposure (Table 6). Reduction on macro and micromolecules are directly proportional to the

Table 1: Mortality of Labeo rohita exposed to Malathion

S.No.	Concentrations (mg/L)	Exposure Periods (h)				96 h
		24	48	72	96	
1	5	N	N	N	N	15 mg/L
2	10	N	10 %	10 %	10 %	
3	15	10 %	20 %	30 %	50 %	
4	20	20 %	50 %	70 %	90 %	
5	25	60 %	100 %	N	N	
6	30	10 %	N	N	N	

Note: LC_0 - 5 mg/L, LC_{50} – 15 mg/L, LC_{100} – 25 mg/L and N – No mortality

Table 2: Physico-Chemical analysis of water

S.no.	Physico-Chemical Parameters	Malathion Concentration						
		Control	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L	30 mg/L
1	Temperature (^{0}C)	28	28	28	28	28	28	28
2	pH	6.2	5.8	5.8	5.8	5.8	5.8	5.8
3	Total solids (g/L)	81	72	63	52	68	86	78
4	Suspended solids (g/L)	80.5	69.3	64.5	57.3	62	75.3	64.1
5	Dissolved solids (g/L)	8.3	7	6	5	7.5	9	8
6	Dissolved oxygen (mg/L)	7	5.9	5.5	5.3	5	4	8
7	Free Carbondioxide (ppm)	7	7	4	4	3	2	2
8	Total alkalinity (mg/L)	110	107	90	81	79	70	61

Table 3: Antioxidant enzyme activity in the tissues of *Labeo rohita* during accumulation and depuration periods

Accumulation study (µ moles of phenol liberated/min/100mg protein)							
S.No.	Antioxidant enzymes	Liver		Muscle		Gill	
		Control	Day 15	Control	Day 15	Control	Day 15
1	Catalase	14± 1.15	43.1±2.3	7±0.11	16.5±0.57	6.9±0.17	23.9±0.17
2	Glutathion S-transferase	205±1.15	270.5±0.16	93.2±0.11	143.2±1.03	120±0.57	215.5±0.72
Depuration study (µ moles of phenol liberated/min/100mg protein)							
3	Catalase	39.03±1.13	17±1.44	10.5±0.75	7.9±0.23	22.7±0.86	10.7±0.69
4	Glutathion S-transferase	252.5±1.44	219.5±1.12	135.7±0.17	108.1±0.34	183±0.28	160.2±0.46

*The values are expressed as Mean ± SEM and the mean difference is significant at the 0.05 level

Table 4 : Concentration of Acid phosphatase and Alkaline phosphatase in tissue of *Labeo rohita* exposed to Malathion

S.No.	Enzyme	Sublethal concentration	Enzyme concentration (µm/mg protein/h)		
			Liver	Muscle	Gill
1	Acid phosphatase	Control	0.579±0.00	0.719±0.01	0.428±0.00
		35.0 ng/L	0.420±0.01	2.249±0.00	0.424±0.01
2	Alkaline phosphatase	Control	1.948±0.00	3.651±0.00	4.600±0.00
		35.0 ng/L	0.850±0.02	1.025±0.01	1.825±0.01

*The values are expressed as Mean ± SEM and the mean difference is significant at the 0.05 level

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Table 5: Sublethal effects of Malathion on Nucleic acid content in different tissue of Labeo rohita during accumulation and depuration periods

S.No.	Period of study	Liver	Muscle	Gill
	DNA (mg/g)			
1	Control (0 day)	1.7 ± 0.28	4.5 ± 0.23	1.6 ± 0.28
	Exposure (15 days)	1.9 ± 0.34	1.9 ± 0.11	1.2 ± 0.17
	Recovery (Control)	1.2 ± 0.23	3 ± 0.23	2.5 ± 0.23
	Recovery (15 days)	1 ± 0.23	2.5 ± 0.34	1.7 ± 0.34
	RNA (mg/g)			
2	Control (0 day)	5.3 ± 0.23	7.5 ± 0.34	5.4 ± 0.17
	Exposure (15 days)	4.4 ± 0.17	6 ± 0.11	4.9 ± 0.34
	Recovery (Control)	6.5 ± 0.23	7.9 ± 0.34	6.2 ± 0.23
	Recovery (15 days)	4.5 ± 0.40	5.9 ± 0.46	4 ± 0.17

*The values are expressed as Mean ± SEM and the mean difference is significant at the 0.05 level
FAA- Free amino acids

Table 6: Sublethal effects of Malathion on Protein, Glycogen and Free amino acids in tissue of Labeo rohita

S.No.	Organs	Biochemical Parameters	Control	Sublethal Concentration			
				24 h	48 h	72 h	96 h
1	Liver	Protein	225.5 ± 1.44	207.1 ± 1.21	188.3 ± 2.19	180.2 ± 0.46	142.5 ± 0.86
		Glycogen	11.4 ± 0.34	10.5 ± 0.17	9.7 ± 0.17	8.9 ± 0.23	6.4 ± 0.17
		FAA	28.2 ± 0.17	27.5 ± 0.17	35.4 ± 0.28	33.3 ± 0.23	28.3 ± 0.46
2	Muscle	Protein	189.4 ± 0.51	179.5 ± 0.40	140.1 ± 0.28	135.7 ± 0.11	129.6 ± 0.17
		Glycogen	9.9 ± 0.34	9.2 ± 0.34	7.2 ± 0.17	6.4 ± 0.28	5 ± 0.17
		FAA	17.5 ± 1.15	13.1 ± 0.23	39.2 ± 0.57	24.1 ± 0.11	20.5 ± 0.28
3	Gill	Protein	198.5 ± 0.34	162.5 ± 0.57	125.4 ± 0.63	95.7 ± 0.11	87.4 ± 0.17
		Glycogen	11.5 ± 0.23	8.3 ± 0.11	9.2 ± 0.05	6.9 ± 0.11	5.1 ± 0.17
		FAA	22.2 ± 0.17	24.2 ± 0.17	27.7 ± 0.51	29.1 ± 0.11	20.2 ± 0.23

Values are expressed as mg/100 mg wet wt of tissue.
*The values are expressed as Mean ± SEM and the mean difference is significant at the 0.05 level

concentration of malathion and exposure periods. The values were expressed as mean ± SEM and the significance of difference was set up at ($p< 0.05$).

DISCUSSION

During the acute toxicity tests, the fish were seen to exhibit several behavioural responses, such as fast jerking, frequently jumping, erratic swimming, spiraling, convulsions and tendency to escape from the aquaria. Following this state of hyper

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excitability, the fish became inactive and loss of orientation. There was loss of equilibrium and paralysis which ultimately resolved in death of the fish. These altered behavioral abnormalities were observed only at high concentration ranges (values higher than 96 h LC_{50}). Rao *et al*., 2005 reported that abnormal changes in behavior in mosquito fish *Gambusia affinis* in response to the sub-lethal exposure to chlorpyrifos. Acute toxicity of pesticides like Endosulfon, Malathion and Copper sulphate at different concentrations to fresh water prawns Macrobrachium rosenbergii were reported (Natarajan *et al.,* 1992). There are also reports of the effects of the herbicide 2, 4-D on the bottom fauna of fish ponds (Sarkar, 1991). These observations was in support with the study of Chattopadhyay *et al*., 2006 who reported that certain erratic behavioral patterns of fish was noticed during the exposure period to herbicides. The fish exhibited unrest and a peculiar tumbling motion before they died. Moreover, the herbicide butachlor persists in the aquatic system for a long period of time.

In the present study, the fish were treated with sublethal concentration of 5mg/L of malathion for a period of 15 days. One set of fish was sacrificed for enzyme analysis. Antioxidant enzymes such as catalase and glutathion S-transferase were analysed in different tissues of liver, muscle and gill of *Labeo rohita*. Similarly after 15 days exposure of malathion, the fish were released into fresh water for further 15 days (depuration study). At the day 30, the malathion depurated fish were sacrificed and antioxidant enzymes were analysed as it was in accumulation study. During the accumulation study, the catalase activity was more than double fold higher at day 15 in the liver of *Labeo rohita*, when compared to its control. Similarly glutathion S-transferase activity also increased at day 15 in the liver of fish, when compared to control (Table 3). fish when compared to control.

Increased responses of both enzyme activities were observed when released into fresh water for another 15 days. At day 30th tissues (Liver, muscle and gill) antioxidant enzymes were monitored. In the depuration study, It has been believed that enhanced antioxidant enzymes in malathion exposed fish would help in the removal of oxyradicals. During this depuration study the antioxidant enzymes were very low at day 16 when compared to control (Table 3). The level of GST in tissues of test fish was found to be higher than that in controls. The elevated level noted in prawns might reflect the formation of GST and malathion complexes as a mean of detoxification/ elimination (Arun and Subramanian, 1998). A similar report has been previously noted in *Macrobrachium malcolmsonii* exposed to heavy metals (Kabila, 1999).

The liver, muscle and gill tissues showed decreased level of acid phosphatase (ACP) and Alkaline Phosphatase (ALP) activities. In liver, the level of ACP slightly decreased, whereas in muscle it increased three fold and it was more or less similar in gill. In the case of ALP, the activity decreased two fold level in all tissues. Loss of ALP activity in the liver tissue of mercury intoxicated mice is a consequence of changes in the permeability of plasma membrane in addition to changes in the balance between synthesis and degradation of enzyme protein thus lowering the enzyme activity.

El-Dermerdash, 2001 stated that $HgCl_2$ intoxication significantly decreases the ACP and ALP activities in rats. In the liver, it is closely connected with lipid membrane in the canalicular zone, so that any interference with the bile flow, whether extra-hepatic or intra-hepatic leads to decrease in ACP and ALP activities. Shakoori *et al*., 1992 have suggested the decrease (or) inhibition of ACP and ALP activities are due to increased necrosis in the tissues like hepatocytes.

The effects of pesticide on nucleic acid contents in different tissues showed a remarkable observation. The tissues of liver, muscle and gill showed significant decrease in the nucleic acids content of RNA and DNA (1.9±0.34, 1.9±0.11, 1.2±0.17 mg/g and 1±0.23, 2.5±0.34, 1.7±0.34 mg/g), respectively during exposure period. However, during depuration period both RNA and DNA levels increased. The concentrations of DNA and RNA in tissues of test fish were found to be lower than those in controls (Table 5). Similar findings have been reported in the fish *Heteropneustes fossils* and *Brachydanio rerio* exposed to rogor and malathion (Borah and Yadav, 1995, a, b, c). This decrease in nucleic acid suggests the decrease in protein synthesis and further damage to the liver, which is the major metabolic organ of

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drug detoxification (Ramalingam *et al*., 2000).
Borah, 1996, stated that the decrease in DNA content of fish indicates the possible interference of Pollutant crude oil with nucleic acid synthesis. Usually RNA content indicates the intensity of protein synthesis in a tissue. High level of RNA reflects its involvement in cellular growth in control or normal fish. In view of the significant correlation of RNA and protein, a deficient synthesis of any type of RNA should have its reflection in corresponding failure of protein synthesis. Thus, the pollutant might decrease the synthesis of protein and DNA directed RNA formation. The gradual decrease in the protein content of treated fish suggests the disruption of carbohydrate metabolism, destruction of protein and protein synthesis machinery and inhibition of ATP synthesis. The protein, glycogen and free amino acids were decreased gradually compared to control, when the period of exposure increased. The depletion of protein may also be attributed to spontaneous utilization of amino acids in various catabolic reactions inside the organism in order to combat the stress condition (Borah, 1996). Increase of total free amino acids (TFAA) is an induction of stepped up proteolysis or fixation of ammonia into keto acids resulting in amino acid synthesis. Generally, these two processes contribute to the amino acid pool (Mohapatra and Noble, 1992). The glycogen and glucose contents in treated fish were found significantly reduced in both the tissues indicating the excess utilization of carbohydrate to withstand pollution induced toxicosis. The decrease in glycogen and glucose contents may result in impairment of carbohydrate metabolism due to toxic effect. It was observed that exposure of common carp *Cyprinus carpio* to sublethal concentrations of endosulfon showed decreasing in levels of haemoglobin and haemotocrit, significant elevation in blood glucose and little variation in the serum protein (Chandrasekar and Jayabalan, 1993). The carbohydrate reduction suggests the possibility of active glycogenolysis and glycolytic pathway to provide excess energy in stress condition (Reddy *et al*., 1993). In the present investigation, malathion causes reduction in total proteins in the various tissues of *L.rohita.* This is suggestive of degradation of proteins with the resultant increase of total free amino acids. This was correlated by the findings of Kabeer *et al*., 1984 in pesticide-treated mollusks. Nagabhushanam *et al*., 1983 have reported that the free amino acids serve as supplementary energy source under the condition of emergency during chronic stress.
The changes in biochemicals parameters such as carbohydrates, proteins and lipids are important to indicate the susceptibility of organ systems to pollutants by altering their function as indicated by Verma and Tonk, 1983. The present investigation shows biochemical changes due to sub lethal concentration of Malathion in total proteins, free amino acids (FAA) and glycogen in target organs and tissues significantly. The initial increase in FAA in tissues and later their sudden decline shows that these are utilized in the glycogenesis to compensate the energy demand under chemical stress. Thus the pesticides intoxification has disturbed the normal functioning of cells with the resultant alterations in the fundamental biochemical mechanisms in fish. This would in turn result in the mortality of fish on chronic exposure to the pesticide.
The Food and Drug Administration (FDA) and the EPA allow a maximum amount of 8 ppm of malathion to be present as a residue on specific things used as foods. There was a report carried out in herbaceous plant Centella asiatica to study the residual level of malathion (Latifah *et al.,* 2011). It suggested that the residue is lower than the Maximum Residue Limits (MRL), but regular consumption might be a threat to health.
Thus the pesticides intoxification has disturbed the normal functioning of cells with the resultant alterations in the fundamental biochemical mechanisms in fish. This would in turn result in the mortality of fish on chronic exposure to the pesticide. In addition, the pesticide might be deposited in fish accidentally or by means of contaminated water bodies with pesticide and it might lead to harmful consequences in human beings on continuous consumption of those fish.

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