Research Article

Studies on the Biochemical Responses in the Tissues of Freshwater Fish *Labeo rohita* Exposed to the Organophosphate, Phenthoate

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ABSTRACT

The sublethal and lethal toxic potential of an organophosphate pesticide, Phenthoate shows its effect on some biochemical parameters parameters (Glycogen, Proteins, Lipids, Carbohydrates and FAA) in an Indian major carp *Labeo rohita* was investigated under laboratory conditions. The Acute toxicity value (96 h LC50) of Phenthoate was found to be 2.1 mgl^{-1} calculated by Finney's probit method. Fish were exposed to sublethal concentration of $1/10^{th}$ 96 h LC50 (0.21 mgl^{-1}) for the period of 1 day and 10 days respectively and the biochemical alterations were assessed by using respective method. A significant ($p < 0.05$) decline in total glycogen, total proteins, total lipids, total carbohydrates and free amino acids was observed in Phenthoate exposed fish during lethal and sublethal concentrations. The reduction in biochemical component of different organs of fish was expressed in terms of percent change over the control. The maximum % change in total glycogen was observed in liver (72.48) during 1 Day lethal exposure and the maximum % change in total proteins was observed in intestine (70.37) during 10 Day sublethal exposure. The maximum % change in total lipids, total carbohydrates and free amino acids was observed in liver i.e., 71.49, 40.318 and 42.048 respectively during 10 Day sublethal exposure. Whereas the minimum % change in total glycogen, in total lipids, in total carbohydrates and free amino acids was observed in intestine i.e., 21.548, 20.22, 1.231 and 11.347 respectively during 1 Day sublethal exposure. The minimum % change in total proteins was observed in gill (25.85) during 1 Day sublethal exposure. Thus, the minimum % change in biochemical constituents was observed during 1 Day sublethal exposure.

Keywords: Toxicity, *Labeo rohita*, Total Glycogen, Total proteins, Total lipids, Total Carbohydrates, and Free amino acids.

INTRODUCTION

The chemical pesticides and fertilizers have been utilized in agriculture and public health operation to improve the food production by eliminating unwanted insects and protect the crops from the vector borne disease.[1,2] The rapid industrialization, power station, mining activities and urbanization also led to aquatic pollution.[3] These uncontrolled use of agrochemicals create a serious ecological problems and which causes many threats to non-target organisms like fish, prawn, frog and birds in the environment.[4] Among the all aquatic organisms, fish constitutes a very important group due to their rich nutritive values and which has a great economical source to humans.

The organophosphates are one of the most preferred insecticides to control agriculture pests as well as ectoparasites in aquaculture due to their low cumulative ability, higher insecticidal property and less persistence in the environment.[5,6]

Phenthoate is a non-systemic organophosphorus insecticide, moderately toxic to mammals with no residual activity, whose formulation has a board spectrum of effectiveness on crop pests especially against a wide range of chewing, piercing and sucking phytophagous insect pests on vegetables such as rice, cotton, pulses etc. Phenthoate has a strong pungent odour which acts as a repellent for adult moths due to which egg lying can be prevented.

The commonly sprayed organophosphorus pesticides are very strong neurotoxins resulting in neurotoxicity on acute or chronic exposure to the fish and other aquatic biota.[7,8] The large portion of the scattered pesticides may cause environmental pollution through the residues on the leaf surfaces of the crops to the surface water by rainwater pathway.[9,10] The evaluation of the ecotoxicological threats caused by pesticides based on the toxicity and impose the many non-target organisms with wide range of consequences.[11,12] The changes in biochemical contents in different tissues of the fish due to toxic effects of various organophosphorus pesticides have been reported by peer researchers.[13-15] The biochemical studies are good parameters and serve as bioindicator of water quality, which help to observe the adverse effect of toxicants on metabolic activities of the fish.[16,17] The effect of pesticides on muscle and kidney proteins have been studied.[18] The progressive decline in glycogen content in the tissues of gill, kidney, intestine, brain, muscle and liver was noticed when exposed to organophosphate.[19] In the fish *L. rohita* slight decline of free amino acids (FAA) was observed in the brain, muscle, kidney and gill exposed both to lethal and sub lethal concentrations of the toxicant.[20]

Hence, the present study was undertaken to estimate and to the know percent change of biochemical parameters like glycogen, proteins, lipids, carbohydrates and free amino acids in different tissues i.e., gill, intestine, muscle, kidney, liver and brain of the Indian major carp *Labeo*

rohita on exposure to lethal and sub lethal concentration of pesticide Phenthoate (50% EC) in comparison with the values of control group.

MATERIALS AND METHODS

Acclimatization of fish and LC_{50} value

Fingerlings of *L. rohita* (Hamilton) weighing 6.5 ± 0.5 gm and with mean body length of 8.5 ± 0.5 cm were obtained from the local pond at Buddam of Guntur district in Andhra Pradesh state, India. Fish were brought to the wet laboratory and acclimatized for the period of 10-15 days prior to conducting experimentation. The dechlorinated tap water was used throughout the course of the experiments and the physico-chemical parameters of water determined according to American Public Health Association (APHA).[21] The values are as follows: temperature, 28 ± 2°C; pH, 7.12; total hardness, 170 mgl^{-1} (as CaCO3); total suspended solid (TSS), 4 mgl^{-1}; turbidity, 7.5 Silica units and dissolved oxygen concentration, 5-6 mgl^{-1}. The LC_{50} value was estimated in the laboratory conditions as per the method of Finney's probit analysis (1971) starting with minimum range for acute toxicity trials. The acute toxicity for 96 h LC_{50} was found to be 2.1 mg/l and $1/10^{th}$ of 96 h LC_{50} was 0.21mgl^{-1}. The concentration of $1/10^{th}$ of 96 h LC_{50} was taken as sub lethal for experimentation.[22]

Experimental Groups

The total acclimatized fish of one hundred and fifty *L. rohita* fingerlings were selected for the present study and were divided into different experimental groups, each containing 15 fingerlings. The first group was exposed to lethal concentration of 2.1 mgl^{-1} of Phenthoate (O, O-diethyl, O-quinoxalin-2-ylphosphorothiate, in acetone) for one day, second and third group were exposed to sub lethal concentration of 0.21 mgl^{-1} of pesticide for one day and 10 days respectively. The fourth and fifth groups were maintained as control groups by adding the same volume of acetone using free tap water for one day and ten days respectively. The exposed and control groups were fed with palliated feed prepared in the laboratory (rice bran and peanut waste in an equal ratio) twice a day, and the water in the large circular tubs was changed every day (for 24 hours) to maintain a constant concentration of Phenthoate during the period of exposure. Continuous aeration was provided to each tank. No mortality was observed in all the groups during the entire experimental period except in one day lethal exposure. The total fish was taken from each group and were scarified on day 1 and day 10. The gill, liver, kidney, muscle, intestine and brain tissues were separated and frozen in ethanol until used (not more than 1 h). Then toxicant impact on biochemical parameters was estimated by following the standard methodologies.

Estimation of Total Glycogen

The glycogen was estimated by the standard method of Kemp[23], 5% homogenate of gill, brain, muscle, intestine and 2% homogenate of liver and kidney tissues were prepared in 80% methanol and centrifuged at 3000 rpm for 10 minutes. The tissue residue was suspended in 5 ml of trichloroacetic acid (TCA), boiled for 15 minutes at 100^0C, and then cooled in running water. The solution was made up to 5 ml with TCA to compensate the evaporation and then centrifuged. From this, 2 ml of supernatant was taken into the test tube and 6 ml of concentrated H_2SO_4 was added and the mixture was boiled for 10 minutes. The mixture was cooled and the optical density was measured at 520 nm. The standard graph was plotted with D-glucose by using the aforesaid method. The glucose was converted to glycogen by the multiplication factor of 0.98 and is expressed as mg of glycogen/g wet weight of the tissue.[24]

Estimation of Total Protein

The total Protein content of the pesticide exposed tissue samples were estimated according to modified standard method of Lowery et al.[25]. The Quantity of 5% homogenate of brain, muscle, gill and 2% of kidney, intestine tissue were isolated and precipitated with 5% trichloro acetic acid (TCA) and centrifuged at 3000 rpm for 15 minutes. The precipitate was dissolved in 1 ml of 1 N NaOH solution and 0.2 ml of extract taken into test tube and mixed with 5 ml of alkaline copper solution (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. Then samples were allowed to stand for 10 min, at the end of which 0.5 ml folin phenol reagent (diluted with double distilled water in 1:1 ratio before use) was added. After 30 minutes, the optical density was measured at 540 nm in a spectrophotometer (Elico Model SL207) against a blank. The standard graph was plotted using bovine serum albumin (BSA) as standard. The values were expressed as mg/g wet weight of the tissue.

Estimation of Total Lipids

Lipids were estimated according to the method of Barnes and Blackstock.[26] 50 mg of tissue was homogenized with 10 ml water in a warring blender in chloroform: methanol mixture (2:1). The homogenates were filtered through Whatmann No. 1 filter paper and the residue was re-homogenized as before and then filtered. The non-lipid matter from pooled filtrate was removed by shaking vigorously with 0.88% KCl (added as one fourth of the volume). 1 ml of filtrate was taken in a test tube and evaporated under nitrogen and 1 ml of concentrated H_2SO_4 was added and boiled for 10 min. For estimation of total lipid, 0.2 ml of solution was taken and 2 ml of vanillin reagent was added. The developed color was read in spectrophotometer at 520 nm against reagent blank. The standard graph was plotted by the above method with cholesterol powder. The values were expressed as mg/g wet weight of the tissue.

Estimation of Total Carbohydrates

Carbohydrates were estimated by Trevelyan and Harrison method.[27] The freshly prepared anthrone reagent (5 ml)

was pippeted into thick walled pyrex tubes (150 x 25 mm) and chilled in ice water. The solution under test (1 ml) was layered on the acid, cooled for a further 5 min, and then thoroughly mixed while still immersed in ice water. The tubes were loosely fitted with corks, heated as required in vigorously boiling, constant level water bath and then cooled in water for 5min. Then it was made up to 10 ml with water and optical density was determined in a spectrophotometer. The standard graph was plotted with D-glucose by using the above said method. The values were expressed as mg/g wet weight of the tissue.

Estimation of Free Amino Acids (FAA)

A free amino acid level was estimated by the Ninhydrin method as described by Moore and Stein.[28] To 1 ml of supernatant, 2.0 ml of ninhydrin reagent was added and the contents were boiled for exactly 5 minutes. They were cooled under tap water and the volume was made up to 10 ml with distilled water. The optical density of the color developed was measured using a spectrophotometer at a wavelength of 570 nm against a reagent blank. The standard graph was plotted with Amino Acid standard using the aforesaid method. The values were expressed as mg/g wet weight of the tissue.

A blank using distilled water and amino acid standards were also prepared and measured similarly.

Statistical Analysis

The results are expressed as mean (X) ± standard deviation. The n values were same for control and test groups.

The data was analyzed using 'Graph pad instat (Data set 1, SD) software student t-test was conducted for pair-wise comparisons to determine the significant difference at 95% level of confidence.

For all the tests, values of results with $p<0.05$ were considered to be of statistical significance. The graphs were drawn using MS Excel 2007. The percent change in the biochemical constituent of the exposed fish over the control was calculated as follows:

$$\%\ Change = \frac{Exposed\ value - Control\ value}{Control\ value} \times 100$$

Table 1: Changes in Biochemical constituents in different organs of fish exposed to the toxicant for 1 Day lethal concentration of Phenthoate

Biochemical Constituent	Organ	1 Day Control (X±SD)	1 Day Lethal (X±SD)	% Change
Total Glycogen (mg/g)	Gill	42.70 ±0.158	23.66±0.336	44.590
	Liver	75.52 ±0.295	20.78±0.485	72.48
	Kidney	39.52±0.192	25.46±0.321	35.57
	Muscle	55.36 ±0.270	20.14±0.351	63.61
	Intestine	27.38±0.192	20.38±0.192	25.566
	Brain	46.04±0.207	23.86±0.397	48.17
Total Proteins (mg/g)	Gill	135.58±0.311	85.38±0.192	37.02
	Liver	169.48±0.286	81.38±0.259	51.98
	Kidney	97.38±0.277	49.40±0.770	49.27
	Muscle	205.32±0.164	93.10±1.570	54.65
	Intestine	49.62±0.292	32.50±0.351	34.50
	Brain	154.24±0.167	74.48±0.327	51.71
Total Lipids (mg/g)	Gill	75.36±0.305	27.30±0.158	63.77
	Liver	97.42±0.311	28.46±0.230	70.78
	Kidney	86.42±0.192	25.38±0.192	70.63
	Muscle	74.54±0.270	23.44±0.288	68.55
	Intestine	30.36±0.207	19.54±0.270	35.63
	Brain	43.36±0.207	26.46±0.208	38.97
Carbohydrates (mg/g)	Gill	18.06±0.207	13.78±0.517	23.698
	Liver	19.06±0.497	12.62±0.259	33.788
	Kidney	17.4±0.158	13.66±0.321	21.494
	Muscle	18.58±0.259	13.04±0.439	29.817
	Intestine	14.62±0.239	13.60±0.158	6.976^{NS}
	Brain	16.4±0.158	14.38±0.311	12.317
FAA (mg/g)	Gill	12.14±0.207	10.12±0.259	16.639
	Liver	14.60±0.292	10.46±0.534	28.356
	Kidney	14.30±0.158	11.22±0.876	21.528
	Muscle	13.92±0.303	10.88±0.283	15.789
	Intestine	11.28±0.192	9.66±0.192	14.361
	Brain	13.65±0.192	11.57±0.390	15.201

The values are expressed as Mean±SD and the mean difference is significant at $p < 0.05$; NS- Decrease in the content is not significant.

Table 2: Changes in Biochemical constituents in different organs of fish exposed to the toxicant for 1 Day sublethal concentration of Phenthoate

Biochemical Constituent	Organ	1 Day Control (X±SD)	1 Day Sublethal (X±SD)	% change
Total Glycogen (mg/g)	Gill	42.70 ±0.158	25.52±0.286	42.102
	Liver	75.52 ±0.295	22.02±0.606	70.84
	Kidney	39.52±0.192	28.16±0.559	28.74
	Muscle	55.36 ±0.270	21.68±0.277	60.83
	Intestine	27.38±0.192	21.48±0.286	21.548
	Brain	46.04±0.207	25.84±0.573	43.87
Total Proteins (mg/g)	Gill	135.58±0.311	100.52±0.207	25.85
	Liver	169.48±0.286	95.52±0.342	43.63
	Kidney	97.38±0.277	51.48±0.159	47.13
	Muscle	205.32±0.164	145.48±0.295	29.14
	Intestine	49.62±0.292	35.56±0.311	28.33
	Brain	154.24±0.167	82.50±0.394	46.51
Total Lipids (mg/g)	Gill	75.36±0.305	30.36±0.207	59.71
	Liver	97.42±0.311	35.42±0.286	63.64
	Kidney	86.42±0.192	31.38±0.239	63.68
	Muscle	74.54±0.270	26.36±0.385	64.63
	Intestine	30.36±0.207	24.22±0.311	20.22
	Brain	43.36±0.207	29.60±0.158	31.73
Carbohydrates (mg/g)	Gill	18.06±0.207	16.04±0.358	11.184
	Liver	19.06±0.497	15.22±0.606	20.146
	Kidney	17.4±0.158	15.52±0.335	10.804
	Muscle	18.58±0.259	14.78±0.517	20.452
	Intestine	14.62±0.239	14.44±0.288	1.231^{NS}
	Brain	16.4±0.158	14.70±0.158	10.365
FAA (mg/g)	Gill	12.14±0.207	10.46±0.451	13.838
	Liver	14.60±0.292	11.50±0.337	21.232
	Kidney	14.30±0.158	10.38±0.493	27.412
	Muscle	13.92±0.303	11.20±0.114	13.312
	Intestine	11.28±0.192	10.00±0.292	11.347
	Brain	13.65±0.192	11.34±0.808	16.923

The values are expressed as Mean±SD and the mean difference is significant at $p < 0.05$; NS- Decrease in the content is not significant.

Table 3: Changes in Biochemical constituents in the different organs of fish exposed to the toxicant for 10 Day sublethal concentration of Phenthoate

Biochemical Constituents	Organ	10 Day Control (X±SD)	10 Day Sublethal (X±SD)	% Change
Total Glycogen (mg/g)	Gill	35.320±0.239	17.42±0.311	50.67
	Liver	50.520±0.327	18.46±0.230	63.46
	Kidney	35.440±0.297	12.72±0.224	64.10
	Muscle	47.340±0.344	19.24±0.114	59.35
	Intestine	22.420±0.259	10.30±0.158	54.05
	Brain	40.420±0.259	14.44±0.313	64.27
Total Proteins (mg/g)	Gill	128.580±0.259	54.30±0.158	57.76
	Liver	157.440±0.297	78.66±0.207	50.03
	Kidney	85.340±0.336	40.55±0.238	52.48
	Muscle	190.620±0.110	91.22±0.130	52.14
	Intestine	35.580±0.286	7.34±0.182	79.37
	Brain	140.580±0.311	76.82±0.217	45.35

Total Lipids (mg/g)	Gill	65.240±0.207	25.38±0.239	61.095
	Liver	92.600±0.255	26.40±0.274	71.49
	Kidney	81.500±0.224	24.35±0.256	70.12
	Muscle	62.400±0.274	25.50±0.158	59.13
	Intestine	24.380±0.192	17.60±0.316	27.80
	Brain	40.860±0.321	23.52±0.228	42.43
Carbohydrates (mg/g)	Gill	14.340±0.207	10.44±0.207	27.196
	Liver	17.56±0.669	10.48±0.295	40.318
	Kidney	13.400±0.274	10.24±0.378	23.582
	Muscle	15.460±0.241	10.58±0.661	31.565
	Intestine	11.440±0.297	8.22±0.148	28.146
	Brain	12.480±0.192	8.16±0.114	34.615
FAA (mg/g)	Gill	10.380±0.192	7.62±0.249	26.589
	Liver	13.080±0.239	7.58±0.540	42.048
	Kidney	12.620±0.192	7.72±0.303	38.827
	Muscle	12.300±0.158	7.60±0.332	38.211
	Intestine	9.600±0.158	7.54±0.230	21.458
	Brain	11.620±0.192	7.72±0.550	33.562

The values are expressed as Mean±SD and the mean difference is significant at $p < 0.05$.

RESULTS AND DISCUSSION

The estimated mean value of biochemical parameter of freshwater fish, *L. rohita* exposed to lethal (96 h of LC_{50}, 2.1 mgl^{-1}) and sublethal concentration ($1/10^{th}$ 96 h of LC_{50}, 0.21 mgl^{-1}) of organophosphate pesticide, Phenthoate for a period of one day and ten days; along with standard deviation (SD) and percent change over the control are represented in Table 1, 2 and 3. All the biochemical constituents in pesticide exposed fish decreased significantly at $p < 0.05$ when compared with the control fish without pesticide exposure. But the total carbohydrate content in intestine of 1 Day lethal exposed fish and 1 Day sublethal exposed fish did not show significant reduction ($p > 0.05$).

According to Figure 1, the maximum % change in total glycogen was observed in liver (72.48) during 1 Day lethal exposure and the maximum % change in total proteins was observed in intestine (70.37) during 10 Day sublethal exposure. The maximum % change in total lipids, total carbohydrates and free amino acids was observed in liver i.e., 71.49, 40.318 and 42.048 respectively during 10 Day sublethal exposure. Whereas the minimum % change in total glycogen, in total lipids, in total carbohydrates and free amino acids was observed in intestine i.e., 21.548, 20.22, 1.231 and 11.347 respectively during 1 Day sublethal exposure. The minimum % change in total proteins was observed in gill (25.85) during 1 Day sublethal exposure. Thus, the minimum % change in biochemical constituents was observed during 1 Day sublethal exposure and the maximum % change in the biochemical constituents was observed during 10 Day sublethal exposure. Thus there is a marked reduction in the biochemical contents in different organs of fish due to pesticide accumulation.

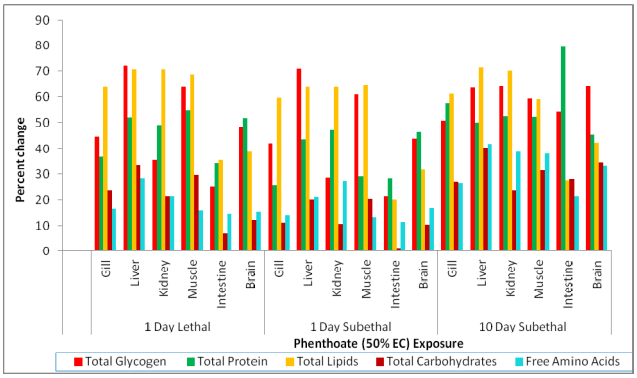

Figure 1: Percent change of biochemical constituents in different organs of fish during Phenthoate exposures.

In the present study, the fingerlings of *L. rohita* exposed to sub lethal and lethal concentrations of Phenthoate which generated various biochemical alterations in total glycogen, total proteins, total lipids, carbohydrates and free amino acids of gill, intestine, muscle, kidney, liver and brain tissues.

A major fall in glycogen content was observed in liver and muscle; moderate decline was noticed in brain and gill whereas slight change found in kidney and intestine during 1 Day lethal and sublethal exposures. After 10 Day chronic sub lethal exposure of Phenthoate, significant decline in the glycogen content in all the vital tissues at a pesticide concentration of 0.21 mgl^{-1} was noticed. The aquatic organisms need large amount of energy for active functioning of vital organs, which can be supplied from reserve food material of glycogen through glycogenolysis process.[19] A fall in glycogen level clearly indicates its rapid utilization to meet the enhanced energy levels in pesticide treated organs through glycolysis process or hexose monophosphate pathway.[29] These reductions might also be due to the prevalence of hypoxic or inhibition of the enzyme glycogen synthetase.

The similar results were noticed by peer researchers regarding biochemical parameters.[14,30,31] The glycogen levels in intestine also changed in both lethal and sub lethal effects of Phenthoate. Ganeshwade also observed these results in the fish *Puntius ticto* when applied to dimethoate pesticide.[19]

Proteins are very important organic substances. In the present study, the results show major fall in protein content in the tissues of liver, muscle and brain; moderate decline in gill, kidney and intestine during lethal and sublethal studies whereas more significant change was observed in all the tissues during 10 day exposure period. The decrease in protein content could also be attributed to spontaneous utilization of amino acids inside the organism by various catabolic reactions in order to combat the stress condition.[32]

The muscle protein reduction might be due to the extreme stress in metabolic process and impairment of protein synthesis process in the internal organs.[33]

Similar study was reported that the effect of the pesticidal mixture (Endosulfan, Malathion and Agrafun in 1:1:1) on total protein content in the intestine, stomach and ovary of the fish, *Clarias batrachus* during acute (96 h) sub chronic (7 and 14 days) and chronic (21 days) period of exposures and found to be reducing proteins.[34] The similar results were obtained in the same fish on exposure to Malathion confirms the present work.[35]

The lipid content of liver, kidney, gills, and muscle has decreased in the present report during lethal and sub lethal exposures of Phenthoate.

During the time of low availability of carbohydrates; lipids serve as a source of energy for supporting the physiological functions of the body. Hence, the decline in the lipid content was due to the utilization of lipids for meeting the energy demand under the pesticide stress. Similar results support the present work that the lipid levels were changed when fish had exposed to toxicant, Endosulfan.[36,37]

The Carbohydrates are the main source of energy in the cells and play a vital role in the cellular metabolism by acting as fuel and providing energy to the body cells. In the present study, maximum carbohydrate depletion was observed in liver and muscle during lethal and sublethal concentrations. The changes in carbohydrate metabolism that would meet the changing energy demands may be subjected to stress.[38,39] In vertebrates, generally from fishes to mammals; blood glucose level corresponds to the standard metabolic rate.[40] These alterations support that carbohydrate metabolism is effected by the toxicant.[41-43]

Amino acids are essential intermediate substances in the process of protein synthesis and its degradation products appear in the form of various nitrogenous compounds. The present study revealed that, free amino acids in the liver, kidney, gills, muscle showed a continuous reduction with both lethal and sub lethal concentrations.

Durga Prasad and Veeraiah reported the effect of cypermethrin on protein metabolism of the fish *L. rohita* and found that total protein levels reduced in all the tissues tested whereas the free amino acid levels were increased.[44]

The changes in biochemical constituents such as glycogen, carbohydrates, proteins, free amino acids and lipids are important to indicate the susceptibility of organ systems to toxicant by changing their function.[45]

CONCLUSION

The present study indicates that organophosphate pesticide, Phenthoate caused alterations in all the biochemical parameters of fish *L. rohita*, treated at different sub lethal and lethal exposure periods in fish tissues; which shown low levels of glycogen, protein and lipid contents when compared to untreated fish tissues, might be caused by intoxication of pesticidal stress in the intermediary metabolism of the fish.

It is concluded that Phenthoate poisoning causes considerable changes in glycogen, carbohydrate, protein, amino acid, and lipid contents in the freshwater fish *L. rohita*.

The declined biochemical values indicate the change in the rate of synthesis and degradation under the impact of accumulation of chemical pollutant. Moreover in the present investigation, Phenthoate is showing more toxicity on biochemical parameters of fish *L. rohita.* Hence, it is concluded that the utilization of these pesticides should be minimized and should create the awareness among fish farmers about the appropriate toxicity levels of pesticides and insecticides that cause harmful effects to the organisms.

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