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Toxic Impact of Phenthoate on Protein and Glycogen Levels in Certain Tissues of Indian Major Carp *Labeo rohita* (Hamilton)

K. Somaiah [1], P.V.V. Satish[2], K. Sunita [3*], B. Nagaraju[4] and O. Oyediran Oyebola[5]

[1] *(Department of Environmental Sciences, Acharya NagarjunaUniversity, India)*
[2,3] *(Department of Zoology and Aquaculture, Acharya Nagarjuna University, India)*
[4] *(Department of Biochemistry, Acharya Nagarjuna University, India)*
[5] *(Department of Aquaculture and Fisheries Management, University of Ibadan, Nigeria)*

Abstract: *The organophosphorus pesticide Phenthoate is one of the most widely used insecticide in agriculture sector to control the varieties of pests and ectoparasites. The acute toxicity bioassay tests were conducted at various concentrations ranging from 0.4 and 4.0 mg/l with an interval of 0.4 mg/l on Indian major carp Labeo rohita for 24, 48, 72 and 96 h. The acute toxicity values were determined as 3.0, 2.6, 2.3 and 2.1 mg/l under laboratory conditions respectively. These LC_{50} values showed that the chemical is highly toxic to the fish. Then the fish were exposed to sub lethal concentrations ($1/10^{th}$ of 96 h LC_{50}) for 1, 4 and 8 days and studied the significant changes in total proteins and glycogen levels of the fresh wet tissues. The total protein content reduced in various tissues like Liver, muscle, kidney, brain, gill and gut by the percentage of 22.46%,17.91%,12.39%, 8.66%, 8.36% and 8.16 % and glycogen content depletion in the order of kidney, liver, muscle, gill, gut and brain tissues by 57.92%, 49.11%, 48.75%, 34.88%, 32.00% and 28.81% respectively when compared to control group. It was noticed that Phenthoate resulted in the gradual decrease of glycogen and protein levels in the vital organs of fish L. rohita, which led increase in the mortality rate.*

Keywords: *Behavioural alterations, Glycogen, Labeo rohita, Pesticide, Protein*

I. Introduction

The organophosphorus (OPs) pesticides widely used in agriculture to prevent the disease causing pests, parasites due to their lower persistence in the environment. However, the intensive use of these chemicals in agriculture and public operations has changed the ecological balance of many non target organisms like fishes [1]. Unsafe spraying and improper handling of the chemical pesticides may cause high risk of the health hazards. Aquatic ecosystems are the ultimate sinks for agricultural residues as well industrial pollutants and it has become a global environmental problem in recent days [2,3]. There are 234 kinds of pesticides used in India in which 24 are used widely and another 28 of them have been banned in India and other countries due to their intensive toxicity towards the non target organisms. The environmental condition like temperature, pH and dissolved oxygen play major role to increased pesticide toxicity in the presence of residual molecules.

The biological activity of Phenthoate which directly inhibit enzyme, acetyl cholinesterase (Ache) activity [4] in fish, increased protein turn over [5] and enhanced the glycolytic activity [6]. *Labeo rohita* is one of the most preferred edible species in Andhra Pradesh where the present study was carried out. However, its performance in terms of growth is slower compared to other species in the multispecies culture system. The most of synthetic organic pesticides of organophosphates, carbomates and organochlorides are extremely toxic to non-target species of freshwater fauna, which damage the population dynamics, complex food-web and food web energetics [7, 8]. Fishes are particularly very sensitive to the water contamination. Hence, pollutants such as insecticides, herbicides may significantly affect some physiological and biochemical processes when they enter into the organs of fishes [9-13]. More over the insecticides mainly impacts on liver of fish [14] and also decline glycogen content in liver and the intestine of *Ophiocephalus punctatus* exposed to sub lethal concentration of cypermethrin, these values of glycogen showed disturbance in carbohydrate metabolism due to toxic stress [15]. The mode of action of toxicants and causes for death of poisoned aquatic animal is better understood from biochemical investigations besides mortality studies. Several workers have investigated the impact of various heavy metals on biochemical constituents of various aquatic organisms [16]. Hence the present study was undertaken to evaluate the toxicity of Phenthoate (50% EC), on glycogen and protein levels of liver, kidney, gill, brain, muscle and gut in freshwater Indian major carp *Labeo rohita* (Hamilton) to sub-lethal and lethal concentrations for 1 day, 4 day and 8 day of exposure.

II. Materials and methods

2.1 Fish and Acclimatization:

Healthy juvenile fish *Labeo rohita* measuring with 7.5 ± 1.5 cm in length and 8.5 ± 0.5 gm in body weight were obtained from Local fish farm from Buddam village at Bapatla Mandal, Guntur distinct of Andhra Pradesh, India. The fishes were acclimatized to laboratory condition for two weeks in large plastic pools of 100 L capacity containing well aerated unchlorinated ground water, previously washed with 0.1% KMnO4 solution to free walls from microbial infection and physico-chemical characteristics of water were carried out by standard methods [17]. The estimated values of the water quality parameters are given in TABLE 1. During acclimatization fishes were fed with fish pellets and rice bran on every day after renewing the water.

2.2 Stock Solution Preparation and Acute toxicity study:

Phenthoate Stock solution was prepared by dissolving 1 gram of pesticide in 100 ml of acetone and the required quantity of Phenthoate was drawn from the stock solution to maintain the concentration of 1 mg/L in the container.

The fish were separated into several groups, each containing 10 individuals (n=10), pilot experiments were conducted to determine the safe sub lethal (LC_0), Lethal (LC_{100}) and Median (LC_{50}) concentrations for acute toxicity estimation range from 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6 and 4.0 mg/l for 24, 48, 72 and 96 h in the experiment (TABLE 2). During the whole process a control group (without toxicant) was maintained with acetone for comparison. The lethal concentration values obtained by the following method given by the committee of aquatic toxicity [18]. The experimental design included three replicates. The fish were not fed on the day before the beginning of the experiment. Each day number of dead fish were counted and removed immediately from the test container. The percentage mortality was calculated and the values were pooled up into probit scale. These values were determined and analyzed by using Finney's probit analysis method [19]. Then ten fish were exposed to sub lethal concentrations of $1/10^{th}$ of 96 h of LC_{50} value (i.e., 0.21mg/L) for 1, 4 and 8 days respectively. A suitable control was also maintained to nullify any other effects that likely to affect the fish. Then the fishes were dissected immediately. Fresh wet tissues of vital organs viz., brain, gill, liver, kidney, gut, muscle were isolated and preserved in 5% to 10% formalin. The control fish tissues (n=10) were also processed in the same method for biochemical estimation of the total proteins and glycogen by the standard recommended protocols [20, 21].

2.3 Total protein estimation:

The total Protein content of the pesticide exposed tissue samples were estimated according to modified standard method of Lowery et al. [20]. 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 was added. To this 0.5% ml of 50 % folin phenol reagent was added. In the time following 30 minutes, the optical density was measured at 540 nm against a blank. The standard graph was plotted by using Lowry method with bovine serum albumin. The values were expressed as mg/g wet weight of the tissue.

2.4 Glycogen estimation:

The glycogen was estimated by the standard method of Kemp et al. [21], 5% homogenate of gill, brain, muscle, gut 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 [22] and is expressed as mg of glycogen/g wet weight of the tissue.

2.5 Statistical analysis:

The data was subjected to one-way analysis of variance (ANOVA) using Microsoft Excel-2007 and the significance difference was set up at $p < 0.05$. These values were expressed as Mean ± SD for all parameters in the experiment.

III. Results:

The percentages of dead fishes for different Phenthoate doses of 0.6, 1.2, 1.8 2.4, 3.0, 3.6, 4.2, 4.8, 5.4 and 6.0 mg/L were determined for 24 h (TABLE 3 and Fig. 1) and 96 h LC_{50} (TABLE 4 and Fig. 2) was found in fingerling of *Labeo rohita.* With increasing concentration of the pesticide the mortality rate of the fingerlings

has gradually increased. Then the lethal (LC_{100}), median (LC_{50}) and safe sub lethal (LC_0) levels of the toxicant values were evaluated during 24-96 h exposure period of experimentation (TABLE 2).

3.1 Behavioral manifestation:

The fish showed normal behavior such as well-coordinated with active movements, static equilibrium, active swimming, normal gill movement, free gulping of air at the surface water, horizontal hanging in the water with natural body color and zero mortality rates were observed in the control group. During the exposure of pesticide for different time periods, at the lower concentration of toxicant i.e., 0.6, 0.8,1.0 and 1.2 mg/l, the fish showed normal behavioral responses and no mortality was found but at higher concentration i.e., 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 mg/l, a number of behavioral changes were observed i.e., fishes frequently coming to the surface of water, tried to jump out of the water, loss of equilibrium, erratic and darting swimming movements, rapid gill movement, vertical hanging, fading of their body colour, increased opercular movements, being lethargic and sluggish, excess mucus secretion all over the body and restlessness. Finally fish stays motionless and open their mouth prior to death in the bottom of the container throughout the test tenures. Lastly at 3.6, 4.2, 4.8, 5.4 and 6.0 mg/l concentration toxicant caused 100% mortality in the experimental group when compared to the control groups for 24, 48, 72 and 96 h respectively.

3.2 Biochemical analysis of total protein and glycogen:

The biochemical parameters viz., total glycogen and total proteins were estimated by standard procedures in six tissues i.e., brain, gill, kidney, liver, muscle and gut of all control fish groups, exposure groups to lethal and sub lethal concentration. $1/10^{th}$ of Median lethal concentration (0.21 mg/L) was taken as Sub lethal concentration and evaluated the rate of depletion in total proteins and total glycogen contents along with the percentage changes in wet tissues of the fish for 1, 4 and 8 days respectively (TABLE 5 & 6, Fig. 3 & 4). No mortality was observed at the exposed sub lethal concentrations of toxicant. From the results it is clear that there is an appreciable decline in different biochemical constituents of the fish under Phenthoate stress.

IV. Discussion

Fishes are the excellent models for monitoring environmental contamination in aquatic system [23, 24]. In the present study during acute toxicity test, the fishes exhibited several abnormal behavioral responses such as swimming much more frequently jumping, erratic and darting swimming movements, rapid gill movement, vertical hanging, fading of body colour, increase in opercula movements, lethargic and sluggish, excess mucus secretion all over the body and restlessness. The similar symptoms were observed in mosquito fish, *Gambusia affinis* in response to the sub-lethal exposure to chlorpyrifos [4]. These behavioral alterations might have caused from the nervous and respiratory manifestations due to Phenthoate exposure. Similar findings were reported in guppy fish, *Poecilia reticulata* [25, 26] and in rainbow trout, *Oncorhynchus mykiss* [27], when acutely exposed to different concentrations of the synthetic pyrethroids like deltamethrin and cypermethrin. These behavioral alterations can also be considered as symptoms of stress in the treated fish. It is proved that in the present study, the fishes after intoxication with Phenthoate were similar to those observed in other studies by peer researchers [28, 29].

The biochemical parameters either increase or decrease in the metabolic rate depending on the site of action. Most of the chemicals pesticides acts as metabolic depressor in the environment and generally causes pressure on biologically active molecules such as proteins, glycogen, carbohydrates and lipids [30]. Proteins are the building blocks of the animal's body, and it is most fundamental biochemical substance to maintain the blood glucose and energy source during the stress period. Proteins play a major role in the interaction process of the cellular medium in the organisms [31]. It is also considered as diagnostic tool and involved in various phases of physiological events. Proteins perform a vast array of functions within living organisms including catalyzing metabolic reaction. In the present study, the fish were exposed to sub lethal concentration (0.21 mg/l) of Phenthoate for a period of 1, 4 and 8 days. The treated group fish was sacrificed for biochemical alterations in different tissues like brain, gill, kidney, liver, muscle and gut of *Labeo rohita.*

Hence the total protein content of all tissues have been decreased gradually with the percent change 5.03%, 6.38%, 8.92%, 11.76%, 8.45%, and 7.62% in the fish *Labeo rohita* when subjected to toxicant for the period of 1 day. In this study the maximum depletion was exhibited in liver (149.57 to 132.03 mg/g) followed by kidney (116.6 to106.20), muscle (132.20 to 112.15), gut (108.4 to 100.15) and brain (113.78 to 108.06mg/g) and the percent variation was significant over control in all tissues. The proteins reduction might be due to the impaired or low protein synthesis under the toxic stress condition and enhancement of photolytic activity in the organisms. The similar results have been recorded by the previous workers [32-35]. More over the different catabolic reactions are also responsible for reduction of protein content inside the organism during various stressful conditions. It is also proved in the case of freshwater fish *Heteropneustes fossilis* when exposed to rogor (30% w/v dimethoate) [36].

After 4 days (96h) period of exposure the total protein content was progressively depleted with the percent change of 11.90%, 11.69%, 14.92%, 20.29%, 15.17%, and 14.8 % in this six tissues whereas on 8th day protein percent change have been deducted to 18.43%, 18.98%, 28.36%, 39.13%, 26.45%, and 21.02% in the tissues with increase the time period. In both 4 and 8days of exposure period the protein levels have fallen much in the liver and muscle followed by the kidney, gill, brain and gut. The decline in protein content in the different tissues might be due to degradation of proteins into free amino acids for various metabolic activities, which is supported by Kumar and Gopal [37]. There are three reasons to degrade protein content under stress condition i.e., i) increasing proteolysis, ii) lipoproteins formation in the repairing of cell damages and iii) energy requirement in cells [38]. It is reported that the *Clarias batrachus* fish exposed to the sub lethal concentrations of Malathion, a significant change was observed to increase total protein content in the kidney during the first week and later a gradual reduction was seen in the periods of exposure [39]. Baruah et al. [40] reported that decline trends in muscle protein content are due to meet the energy demand in catabolic process in the extremely stressful environment.

Glycogen is a sub divisional polysaccharide and major storage of Glucose that serve as a form of energy in animals. It plays an important role in the glucose cycle that can be quickly mobilized to meet a sudden need for glucose [41]. The glycogen content in various tissues of L. rohita was decreased with increased toxicant concentration in the present experiment. On the 1st day of sub lethal exposure of phenthoate, the maximum glycogen reduction was (23.48%) in muscle followed by brain (14.88%), kidney (12.58%), gill (8.39%) and minimum percentage (2.25%) was in gut of *L. rohita* compared to over control. Depletion of glycogen content in all the tissues might be due to the utilization of carbohydrates for energy production as a result of toxicant induced hypoxia. In the fish skeletal muscle glycogen is very important but it attains only 1-2% wet weight compared to liver [42]. Muscle and Liver tissues are a major glycogen parts in animals, which contains full of carbohydrates. The function of muscle glycogen is to act as a readily available source of hexose units for glycolysis within the muscle itself [43]. In the present study the glycogen content was found to be significantly reduced in all tissues indicating the excess utilization of carbohydrate to withstand pollution induced toxicosis. The similar report was observed in common carp *Cyprinus carpio* exposed to sub lethal concentrations of endosulfon showing a decrease in levels of blood glucose and little variation in the serum protein [44]. The carbohydrate reduction also showed the possibility of active glycogenolysis and glycolytic pathway to provide excess energy in stress condition [45].

On 4th day sub lethal exposure, the glycogen levels rapidly decreased in liver (38.08 to 13.83), kidney (15.36 to 5.718) and muscle (22.13 to 9.920) compared to normal values of the fish where as during 8 days exposure period the higher glycogen depletion found to be 80.47 in kidney and followed by muscle (73.86%), gill (68.56%), brain (61.76%) and gut (61.32%) lower levels was in liver (78.62%). The sudden decline of glycogen content in liver, kidney, gill, brain, muscle and gut tissues is an indication of typical stress response with pesticide concentrations in fish**.** Venkataramana also reported that the glycogen content was decreased (47.93%) in the muscles of fish to 0.5 ppm Malathion exposure during 24 to 96 h [46]. The similar results of muscle glycogen could have resulted decline due to anaerobic stress [47]. In the kidney, liver and muscle tissues glycogen content has been reduced progressively in the present study due to the entry of toxic substances into the body and failure of the normal functional mechanism of routine metabolism processes. The similar observation has been recorded in *Nemacheilus botia*, freshwater fish exposure to Meothrin [48]. Someone has recorded that the fall of glycogen content in freshwater prawn *Macrobranchium kistensis* when exposed to TDTL chemical [49]. Lomate and Mule [50] also recorded the effect of rogar on glycogen contents of freshwater snail, *Melenoides tubercultus*. Similar findings were assessed in liver glycogen levels in *Colisa fasciatus* and *Sarotherodon mossambicus* exposure to various toxicants [51, 52] and several studies proved that the depletion of glycogen content in liver, muscle, kidney and gill tissues was observed when exposed to various sub lethal concentration of pesticides in aquatic organisms [53]. Plasma glucose is used as sensitive indication of environmental stress in biochemical alterations of the fish, reduction of which might be due to the hypoxic condition created by pesticides during the period of experimentation. Similar findings have been recorded in different species when exposed to thiodin as well as arsenate [54, 55].

V. Conclusion

In the present study, we have observed the abnormal behavioral consequences in the treated fish. Then the sub lethal exposure of the Phenthoate, an organophosphorus pesticide proved to be moderately toxic to fish *L. rohita* and which effects on the total proteins and glycogen levels of vital organs like brain, gill, liver, kidney, gut and muscle etc. Thus the significant alterations were observed during the exposure for 1, 4 and 8 days respectively. The decline in both total proteins and glycogen levels could be the possible effects (impact) on the enzyme mediated bio-defense mechanism of fingerlings, which pose serious threats to the aquatic environment as well as human life through food chain.

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Table 1: Physico-chemical analysis of water for bench scale experimentation

Water Quality Parameter	**Calculated Value**
Temperature	28 ± 2^0 C
Turbidity	7.5 silica units
pH value at 28°C	7.12
Total Hardness as($CaCO_3$)	170 (mgl^{-1})
Total Suspended Solids (TSS)	4 (mgl^{-1})
Chemical Oxygen Demand (COD)	Nil
Biological Oxygen Demand (BOD)	8 -10ppm
Sulphates as (SO_4)	Trace amount
Phosphates	Trace amount
Dissolved Oxygen (DO)	5 - 6mgl^{-1}

Table 2: Estimation of LC_0, LC_{50}, and LC_{100} values of *Labeo rohita* exposed to Phenthoate for 24, 48, 72, and 96 days

S. No.	**Period of study**	**LC_0 values (mgl^{-1})**	**LC_{50} values (mgl^{-1})**	**LC_{100}values (mgl^{-1})**
1	24 h	0.6	3.0	6.0
2	48 h	0.52	2.6	5.2
4	72 h	0.46	2.3	4.6
4	96 h	0.42	2.1	4.2

Table 3: Effect of Phenthoate on survival of *Labeo rohita* for 24 hour exposure time

S. No.	Conc. of toxicant (mgl^{-1})	Log Conc.	No. of Exposed	No. of Dead	Percent of mortality	Probit Mortality
Control	-------		10	0	0	--
1	0.6	-0.2218	10	0	0	--
2	1.2	0.0791	10	0	0	--
3	1.8	0.2550	10	1	10	3.72
4	2.4	0.3802	10	3	30	4.48
5*	3.0	0.4771	10	5	50	5.00
6	3.6	0.5563	10	6	60	5.25
7	4.2	0.6232	10	7	70	5.52
8	4.8	0.6812	10	8	80	5.84
9	5.4	0.7323	10	9	90	6.28
10	6.0	0.7781	10	10	100	8.09

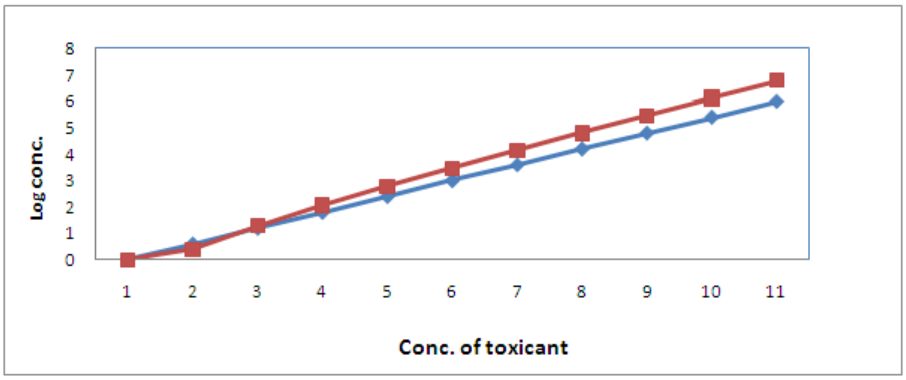

Fig 1: Concentration of Phenthoate Vs. Log concentration for 1 day (24 hours)

Table 4: Effect of Phenthoate on survival of *Labeo rohita* for 96 hours in the experimentation

S. No.	Conc. of toxicant (mg/l)	Log Conc.	No. of Exposed	No. of Dead	Percent of Mortality	Probit Mortality
Control	-------		10	0	0	---
1	0.42	-0.3767	10	0	0	---
2	0.84	-0.0757	10	1	10	3.72
3	1.26	0.1003	10	3	30	4.48
4	1.68	0.2041	10	4	40	4.75
5*	2.1	0.3222	10	5	50	5.00
6	2.52	0.4014	10	6	60	5.25
7	2.94	0.4683	10	7	70	5.52
8	3.36	0.5263	10	8	80	5.84
9	3.78	0.5774	10	9	90	6.28
10	4.2	0.6232	10	10	100	8.09

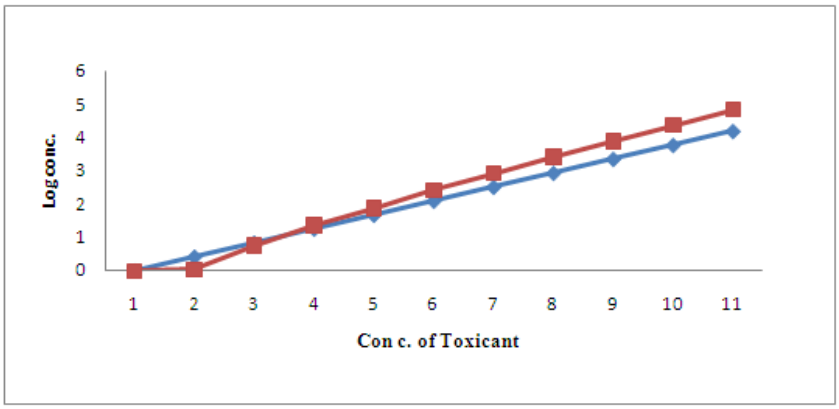

Fig 2: Concentration of Phenthoate Vs. Log concentration for 4 days (96 hours)

Table 5: Estimation of total proteins and total glycogen in different tissues of *L. rohita* on sub lethal exposure of Phenthoate for 1, 4 and 8 days

Biochemical Constituent	Tissues (mg/g)	Control (X ± SD)	Treated (X ± SD)		
			1 day	4 day	8 day
Total Proteins (mg/g)	Brain	113.78	108.06	100.24	92.81
	Gill	110.02	102.99	97.16	89.13
	Kidney	116.60	106.20	99.2	83.53
	Liver	149.57	132.03	119.22	91.05
	Muscle	132.20	112.15	112.15	97.23
	Gut	108.40	100.15	93.14	85.61
Glycogen (mg/g)	Brain	10.88	9.26	7.09	4.16
	Gill	12.99	11.91	7.928	3.898
	Kidney	15.36	13.43	5.718	3.00
	Liver	38.08	30.70	13.83	8.142
	Muscle	22.13	16.94	9.92	5.784
	Gut	10.11	9.88	7.27	3.91

Results are mean (X ± SD) of 5 observations indicates the standard deviation values and are significant at $P < 0.05$

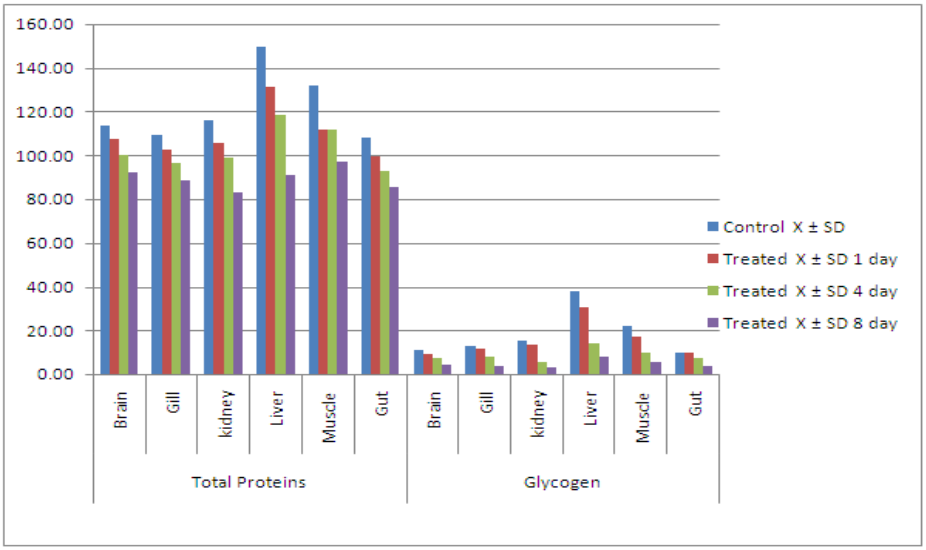

Figure 3: Quantity (mg/g) of total protein and glycogen levels in different tissues of *L . rohita* on sublethal exposure of Phenthoate for 1, 4 and 8 days

Table 6: Percent change of total proteins and glycogen in *Labeo rohita* exposed to Phenthoate for 1, 4 and 8 days

Biochemical Constituent	Tissues (Mg/g)	Percent change		
		1 day	4 day	8 day
Total Proteins	Brain	5.03	11.90	18.43
	Gill	6.38	11.69	18.98
	Kidney	8.92	14.92	28.36
	Liver	11.76	20.29	39.13
	Muscle	8.45	15.17	26.45
	Gut	7.62	14.08	21.02
Glycogen	Brain	14.88	34.83	61.76
	Gill	8.39	38.56	68.56
	Kidney	12.58	62.77	80.47
	Liver	19.38	63.67	78.62
	Muscle	23.48	55.17	73.86
	Gut	2.25	28.09	61.32

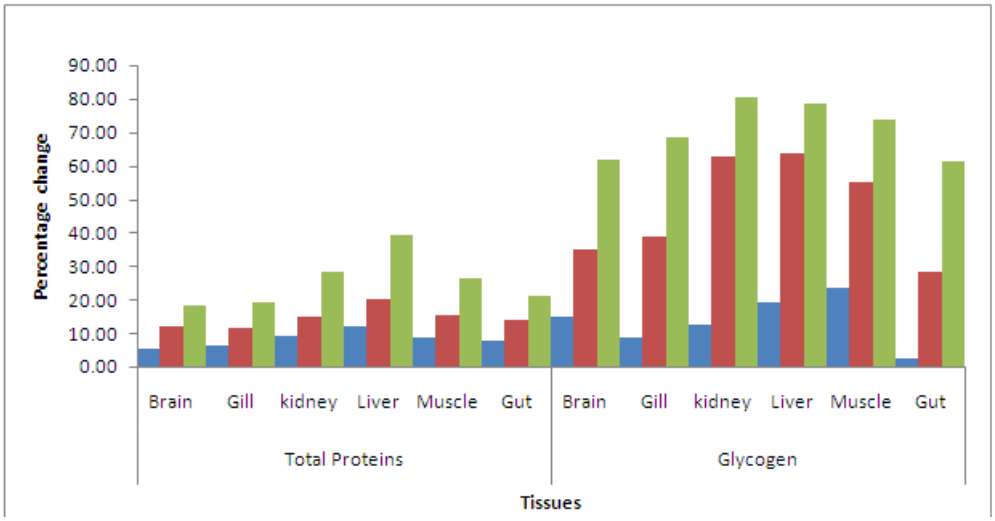

Figure 4: Sub lethal effects of Phenthoate on total protein and glycogen levels in wet tissue of *L. rohita* exposed to 1, 4 and 8 days