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Biochemical studies on the effect of diclofenac on common carp (*Cyprinuscarpio*)

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ABSTRACT

The current study was carried out to investigate the biochemical investigations in common carp (*Cyprinus carpio*) when treated with diclofenac. The fishes are divided into four groups for this investigation. According to sub-lethal toxicity tests, fish exposed to one-tenth of the LC50 value of diclofenac. Diclofenac's 96-h LC50 in *C. carpio* was 11.71 mg/L. As a result, fish were exposed to 0.25, 0.50, and 1 mg/L concentrations of diclofenac and diclofenac separately for this experiment. In natural water, control fish were kept without any treatment. The biochemical estimation methods were used to investigate the influence of diclofenac on the biochemical contents of fish *C. carpio's* liver, gills, and brain, namely soluble protein, carbohydrates, and lipids. The treatment of diclofenac resulted in a considerable decrease in protein content in the liver, gills, and brain tissues. When compared to the other tissues, such as the liver and gills of the diclofenac-exposed fish, the decline in protein level was greater in brain tissue. When the time of exposure increased, the protein content decreased as the concentration of diclofenac rose. The percentage drop in glucose levels in diclofenac-exposed fish liver and brain tissues increased with exposure period. The fish, *C. carpio*, showed stress and reduced their food intake after being exposed to sublethal amounts of diclofenac. Based on these findings, it is inferred that the lipid content in all tissues *of C. carpio* in group IV animals decreased on the 21st day of diclofenac exposure.

Key words: Diclofenac, *C. carpio*, proteins, carbohydrates, lipids, biochemical.

INTRODUCTION

Global pharmaceutical consumption is increasing, resulting in greater levels of environmental contamination as well as increased risks of detrimental effects on animals. Although parent chemicals and their metabolites have been found in sewage treatment plant effluents as well as surface, drinking, and ground water, little is known about their potential effects on non-target species such as fish. Nonsteroidal anti-inflammatory medicines (NSAIDs) are among the most prevalent pharmaceuticals found in the aquatic environment. NSAIDs have been found in waste water, including surface waters, at quantities in the g/L range (Buser et al., 1998; Corcoran et al., 2010). These low environmental quantities are thought to represent only a little danger in terms of acute toxicity, but the situation after long-term exposure could be different.

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Although the effect levels for NSAIDs are often greater than the quantities normally seen in surface water, the risk to fish should be assessed. Pharmaceuticals are physiologically active substances that can act at low concentrations, and their continued discharge into the aquatic environment can cause long-term chronic exposure.

Diclofenac (sodium 2-[2-(2,6-dichloroanilino) phenyl] acetate) is widely used in human and veterinary medication, and as a result, it has been found in water in many countries. It is utilized all over the world, and its yearly production volume is estimated to be in the hundreds of tons (Buser et al., 1998). The amounts observed in wastewater are in the g/L range, with lower levels found in surface water (Heberer, 2002). The median concentration measured in wastewater is 0.8 g/L, with a maximum value of 2 g/L reported in both wastewater and surface water (Schwaiger et al., 2004). Diclofenac elimination rates at sewage treatment plants have been shown to vary between treatment plants (26–100 percent) (Fent et al., 2006). Photodegradation is critical in the removal of diclofenac from surface water (Buser et al., 1998).

Carbohydrates, proteins, and lipids are important energy precursors for fish under stress situations (Idler and Clemens, 1959). When using clinical diagnostic of fish physiology to evaluate the impacts of environmental stressors and hazardous chemicals, biochemical measures were frequently used (Osman et al., 2001). In general, metabolic profiles in stressed fish and other aquatic species serve as essential bioindicators in aquatic environment monitoring. Fish blood is frequently employed in toxicological research and environmental monitoring as a promising indicator of whole-body physiological and pathological changes (Velisek et al., 2010). Because blood plasma is the product of intermediate metabolism, it represents an animal's physiological state (Artacho et al., 2007), therefore blood plasma characteristics are crucial in diagnosing the structural and functional status of fish (Adhikari et al., 2004).

Keeping the foregoing in mind, the investigation was aimed to investigate the harmful effect of diclofenac on common carp (*Cyprinuscarpio*) under experimental conditions, with a focus on biochemical abnormalities.

MATERIALS AND METHODS

Fish preparation and adaptation

A 21-day experiment was conducted in India from July to September 2019 with common carp (C. carpio) as the test organism. Fish were caught and brought to the laboratory from fish hatchery ponds in Nanded, Maharashtra, India. Fish with an average body weight of 305 g and an average body length of 16.11.02 cm were stocked in aquaria (with a volume of 140 liters of water) two weeks before the experiment and aeration was provided with an air pump for 24 hours for adaption. Following the adaption phase, fish of similar mean weight were separated and a survival test with three replications was performed: Each replication employed 20 fish at a density of 3.5g L-1. At all periods, aeration was provided, and a photo period of 12:12 (L:D) was used. Fish were fed at a rate of 1% of their body weight, and 50% of their water was switched daily.

Test Compound

Diclofenac was chosen as a toxicant for this study partly because of the possibility of metabolic consequences. Due to their low water solubility, analytical grade Diclofenac sodium salt was acquired from Fischer Scientific India Pvt. Ltd, India, and 0.2 ml/l was used to make the stock solution at varying concentrations of 5, 10, 15, 20, 25, and 30 mg/L.

Acute Toxicity Study

Median lethal concentration

At room temperature, test systems consisting of 1208040-cm glass tanks filled with water reconstituted from the following salts: NaHCO3 (174 mg/L), MgSO4 (120 mg/L), KCl (8 mg/L), and CaSO4.2H2O (120 mg/L) were maintained with constant aeration and a natural light/dark photoperiod. Static systems were used, and no food was given to the specimens during their exposure.

The median lethal concentration (LC50) of diclofenac was determined to establish the target value to be employed in evaluating biochemical examination of tissue. To that end, six experimental systems containing varying concentrations of diclofenac (5, 10, 15, 20, 25, and 30 mg/L) in reconstituted water were set up, along with a seventh diclofenac-free control system, and ten carp were randomly selected from the stock (using the random number method) and placed in each system. The LC50 was calculated using 10 fish from each group.

The exposure period lasted 96 hours, after which the number of dead specimens in each system was counted. The assay was carried out in quintuplicate. Probit analysis was used to calculate the 96-hour LC50 of diclofenac as well as its 95 percent confidence limits (P0.05).

According to sub-lethal toxicity tests, fish exposed to one-tenth of the LC50 value of diclofenac. Diclofenac's 96-h LC50 in C. carpio was 11.71 mg/L. As a result, during this experiment, fish were exposed to diclofenac doses of 0.25, 0.50, and 1 mg/L. In natural water, control fish were kept without any treatment.

Experimental groups and dosage

Fish (n=40) were gathered and randomly placed into 4 glass aquariums after being fasted for 24 hours. Each tank had 10 fish and 25L of test fluid, and three tanks were utilized in each treatment group. The control fishes are in Group I. Group II fish were given 0.25 mg/L diclofenac for 21 days. Group III fish were given 0.50 mg/L diclofenac for 21 days. Group IV fish were treated to diclofenac at a concentration of 1 mg/L for 21 days. Except for group I, the remaining three groups of fish were exposed to their respective sub-lethal concentrations of diclofenac for 24, 48, 72, 96 hours, 10 days, and 20 days. Group I was kept as the control group. The meal was the same for all groups, and the other conditions were the same. Fish were slaughtered at the end of each exposure period, and parts such as the gills, liver, and brain were dissected and extracted. The tissues (10 mg) were homogenized in 80% methanol, centrifuged for 15 minutes at 3500 rpm, and the clear supernatant was utilized to analyze several biochemical parameters. The proteins, carbs, and lipids in the supernatant were then measured.

Biochemical Study

The following estimation methods have been applied to study the effect of diclofenac on biochemical contents i.e. soluble protein, carbohydrates and lipids in the liver, gills and brain of fish *C. carpio.*

Estimation of Protein

The total protein concentration was calculated using the Lowry et al. (1951) method, which is based on the following concept. Proteins in the sample form a compound with copper ions in alkaline media. The amino acids in copper protein complex with aromatic groups, tyrosin and tryptophane, combine with FolinCliocalteu phenol reagent to produce blue color due to phosphomolybdate reduction. The intensity of the produced color is proportional to the amount of protein present in the sample. The number is given in milligrams per gram of tissue.

Estimation of Carbohydrate

The quantitative estimation of carbohydrate in tissues was performed using Hedg's and Hofreiter's approach (1962). Using dilute hydrochloric acid, carbohydrates are first hydrolyzed into simple sugars. Glucose is dehydrated to hydroxymethyl furfural in a hot acidic media. This chemical, when combined with anthrone, produces a green product with a maximum absorbance at 630 nm.

Estimation of Lipid

The lipid was estimated using Richmond's (1973) approach, which is based on the following concept. Cholesterol esterase is a protein that hydrolyzes cholesterol esters to release free cholesterol and fatty acids. Cholesterol oxidase transforms cholesterol to cholest-4-en-3-one and hydrogen peroxide in the second step. Hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidise to create red quinoeimine dye with a maximum absorbance at 510 nm (500-530). The amount of total cholesterol in the material is related to the intensity of the red color.

Statistical Analysis

Sublethal toxicity assay findings were statistically analyzed using one-way analysis of variance in the acute toxicity assay (96-h LC50 of diclofenac) (ANOVA). Data from at least three different experiments were collected, statistically evaluated, and provided as mean SEM. The mean values of the control and treatment samples were then compared using one way ANOVA in Microsoft Excel's Statistical Package, with p 0.05 considered statistically significant.

RESULTS AND DISCUSSION

Determination of LC50

24, 48, 72 and 96 h median lethal concentrations (LC50) of Diclofenac for *C. carpio* are shown in Figure.1. The probit numerical values along with their 95% confidence intervals are also presented in Tables 2-9. The 96-h LC50 of Diclofenac was 11.71 mg/L in C. carpio.

Table.1. Median lethal concentrations (24-96 h LC50) of diclofenac for *C. carpio*

NSAIDs (mg/L)	Mean LC50 values			
	24 h	48 h	72 h	96 h
Diclofenac	20.48	16.87	15	11.71

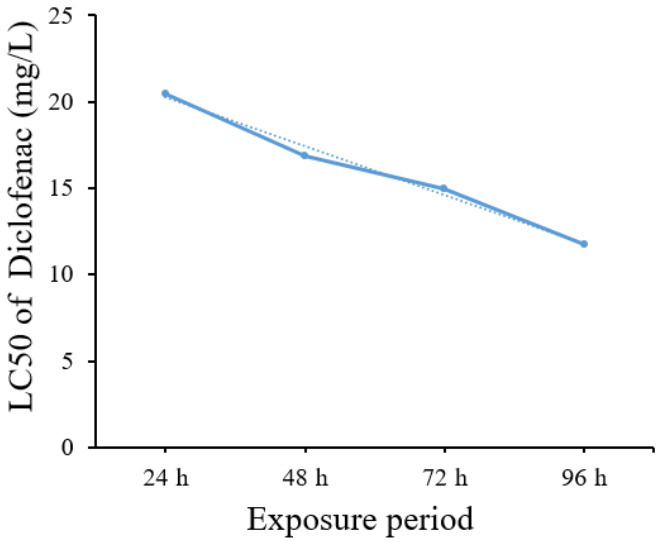

Figure.1 Median lethal concentration (24-96 h LC_{50}) of Diclofenac for *C. carpio*

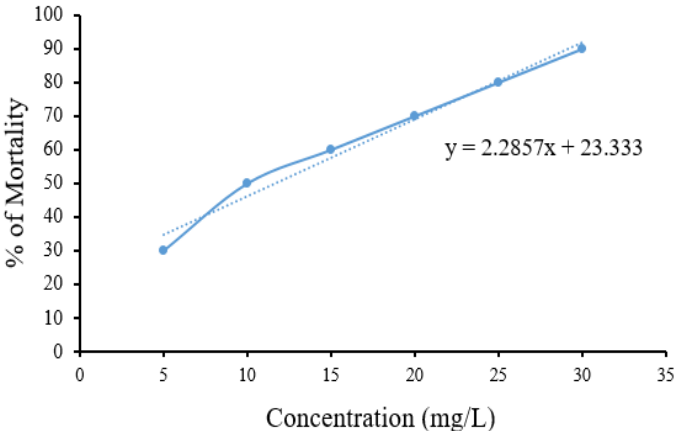

Figure-2. Linear graph of Percentage of mortality at different concentration of diclofenac

According to 96 h LC_{50}=11.71 mg/L of diclofenac in *C. carpio*, Fish were exposed to nominal concentration of 0.25, 0.50, and 1 mg/L of diclofenac to study the biochemical alterations.

Effect of diclofenac on Protein content:

The treatment of diclofenac resulted in a considerable decrease in protein content in the liver, gills, and brain tissues. When fish were treated to 0.25 mg/L diclofenac, the protein concentration in the liver reduced after 7 days from 14.9 to 12.1, 14 days from 14.8 to 10.1, and 21 days from 14.9 to 7.8 mg/g tissue. Protein content in the liver was shown to be lower in animals subjected to 1 mg/L diclofenac for 7 days (-31.3 percent), 14 days (-45.3 percent), and 21 days (-57.1 percent) compared to controls. (Table-2)

The percentage drop in protein in the gills of diclofenac-exposed fish was -11.4, -18.8, and -54.6 for 7, 14, and 21 days of exposure, respectively, whereas at 0.5 mg/L, the percentage decrease was -6.5, -12.1, and -48.6 for 7, 14, and 21 days of exposure, respectively.

The percentage decrease of protein in the brain of diclofenac-exposed fish at 0.25 mg/L was -12.9, -31.8, and -54.7 for 7, 14, and 21 days of exposure, respectively, whereas at 0.5 mg/L, the percentage decrease was -30.5, -54.5, and -60.2 for 7, 14, and 21 days of exposure, and at 1 mg/L, the percentage

Table-2. Effect of diclofenac in the Liver, Gills and Brain tissue of fish *Cyprinuscarpio*with reference to protein contents (mg/g) (Values are mean ± SE and % changes)

Tissue	Day of Exposure	Experimental Groups			
		Group I (Control)	Group II (0.25 mg/L)	Group III (0.5 mg/L)	Group IV (1 mg/L)
Liver	7	14.9+0.67	12.1+0.77 (-18.7)	11.5+0.38 (-22.8)	10.4+0.2 (-31.3)
	14	14.8+0.14	10.1+0.61 (-31.7)	8.5+0.44 (-42.5)	8.1+0.33 (-45.3)
	21	14.9+0.73	7.8+0.71 (-47.6)	7.7+0.96 (-48.3)	6.4+0.23 (-57.1)
Gills	7	18.3+0.74	17.5+0.73 (-5.4)	17.3+0.38 (-6.5)	16.4+0.55 (-11.4)
	14	18.1+0.95	15.1+0.41 (-16.5)	15.9+0.49 (-12.1)	14.7+0.63 (-18.8)
	21	18.5+0.14	13.2+0.43 (-28.6)	9.5+0.68 (-48.6)	8.4+0.56 (-54.6)
Brain	7	16.4+0.66	14.3+0.55 (-12.9)	11.4+0.65 (-30.5)	9.3+0.22 (-54.5)
	14	16.8+0.43	11.6+0.76 (-31.8)	9.3+0.34 (-54.7)	7.2+0.76 (-58.2)
	21	16.3+0.87	7.4+0.66 (-54.7)	6.5+0.88 (-60.2)	5.8+0.97 (-64.5)

Table-3. Effect of diclofenac in the Liver, Gills and Brain tissue of fish *Cyprinuscarpio*with reference to Carbohydrate contents (mg/g) (Values are mean ± SE and % changes)

Tissue	Day of Exposure	Experimental Groups			
		Group I (Control)	Group II (0.25 mg/L)	Group III (0.5 mg/L)	Group IV (1 mg/L)
Liver	7	13.5+0.87	11.7+0.17 (-13.3)	9.8+0.35 (-27.4)	8.7+0.43 (-64.4)
	14	14.0+0.13	10.4+0.81 (-25.7)	8.3+0.34 (-40.7)	6.3+0.23 (-55.0)
	21	13.6+0.63	8.9+0.81 (-34.5)	6.9+0.98 (-50.7)	3.4+0.28 (-75.0)
Gills	7	9.3+0.84	8.2+0.83 (-11.8)	7.3+0.28 (-21.5)	6.9+0.35 (-25.8)
	14	8.8+0.98	7.6+0.31 (-13.6)	5.8+0.39 (-34.0)	5.8+0.53 (-34.0)
	21	8.3+0.64	6.9+0.13 (-16.8)	4.7+0.78 (-43.3)	3.7+0.96 (-55.4)
Brain	7	11.5+0.73	10.4+0.56 (-9.5)	8.4+0.85 (-26.9)	6.5+0.25 (-56.5)
	14	12.8+0.53	11.2+0.79 (-12.5)	8.1+0.74 (-36.7)	5.5+0.26 (-57.0)
	21	11.9+0.52	9.6+0.36 (-19.3)	6.0+0.58 (-49.5)	4.9+0.98 (-58.8)

decrease was -54.5, -58.2, and -64.5 for 7, 14, and 21 days of

When compared to the other tissues, such as the liver and gills of the diclofenac-exposed fish, the decline in protein level was greater in brain tissue. Almost the same pattern was found with diclofenac-exposed fish of varying concentrations. As the concentration of diclofenac and the time of exposure increased, so did the protein content.

diclofenac exposed animals for 7 days (-64.4%), 14 days (-55.0%) and 21 days (-75.0%) in comparison to control. The decline in carbohydrate level in liver tissue is higher when compared to other tissues.

Due to the continuous exposure to the sublethal concentrations of diclofenac, the fish, *C. carpio*, has developed a stress and reduction in food intake resulted in the depletion of liver carbohydrate from the beginning

Table-4. Effect of diclofenac in the Liver, Gills and Brain tissue of fish *Cyprinuscarpio*with reference to lipid contents (mg/g) (Values are mean ± SE and % changes)

Tissue	Day of Exposure	Experimental Groups			
		Group I (Control)	Group II (0.25 mg/L)	Group III (0.5 mg/L)	Group IV (1 mg/L)
Liver	7	11.2+0.67	9.1+0.57 (-18.7)	8.6+0.55 (-23.2)	6.8+0.25 (-39.2)
	14	12.6+0.73	11.4+0.91 (-9.5)	10.5+0.35 (-16.6)	7.2+0.21 (-42.8)
	21	11.4+0.52	9.8+0.51 (-14.0)	8.0+0.95 (-29.8)	5.3+0.88 (-53.5)
Gills	7	8.5+0.82	7.2+0.85 (-15.2)	6.3+0.78 (-25.8)	4.7+0.55 (-44.7)
	14	9.4+0.53	8.7+0.24 (-7.4)	7.4+0.39 (-21.3)	4.8+0.63 (-48.9)
	21	9.1+0.49	8.8+0.83 (-3.2)	7.1+0.78 (-21.9)	3.6+0.86 (-60.4)
Brain	7	18.1+0.21	16.6+0.53 (11.1)	14.5+0.55 (-19.8)	11.0+0.95 (-39.2)
	14	15.4+0.78	13.2+0.55 (-14.2)	12.1+0.94 (-21.4)	9.4+0.66 (-38.9)
	21	14.7+0.51	12.5+0.57 (-14.9)	11.5+0.45 (-21.7)	8.3+0.75 (-43.5)

Effect of diclofenac on Carbohydrates in liver, gills and brain tissue:

Depletion of carbohydrate content of the liver, gills and brain (Table-3) of *C. carpio* exposed to the diclofenac for 7, 14 and 21 days in 0.25, 0.5 and 1 mg/L sublethal concentrations were estimated. Among these, the maximum depletion of carbohydrate was observed in liver during 21 days exposure. Generally, depletion in carbohydrate content is directly proportional to the exposure period of the toxicant. The obtained biochemical estimation values of the liver, gills and brain were subjected to statistical analysis and showed significant values at $P<0.05$.

In liver, the carbohydrate content is decreased after 7 days from 13.5 to 11.7, after 14 days decreased from 14.0 to 10.4 and after 21 days decreased from 13.6 to 8.9 mg/g in liver tissue when fish was exposed to 0.25 mg/L diclofenac. Noticeable decline of carbohydrate content in liver was observed in 1 mg/L concentration till the termination of the experiment. The percentage reduction in the carbohydrate level in both the liver and brain tissues of diclofenac exposed fish was high with the increase of exposure duration. The per cent reduction of carbohydrate level in the gills of diclofenac exposed fish was -11.8, -13.6, and -16.8 at 0.25 mg/L, whereas -21.5, -34.48 and -43.3 at 0.5 mg/L and -25.8, -34 and -55.4 at 1mg/L after 7, 14 and 21 days of exposure respectively. The percentage of reduction of carbohydrate level in the gills was high (-55.4) during the 21 day of exposure at 1 mg/L when compared to control.

Effect of diclofenac on Lipids:

The amount of lipid in the tissues determined after subjecting the fish to different Diclofenac drug exposure times is shown in Table-4. The treatment of diclofenac resulted in a considerable drop in lipid content in the liver, gills, and brain tissues.

At 1 mg/L diclofenac exposure for 7, 14, and 21 days, the lipid in the liver tissue decreased from 11.2+0.67 to

6.8+0.25 (-39.2%), 12.6+0.73 to 7.2+0.21 (-42.8%), and 11.4+0.52 to 5.3+0.88 mg/g (-53.5%), respectively.

There are no significant changes in lipid levels in fish exposed to diclofenac for 14 days. The lipid level in the gill tissue reduced to 15.2 percent (0.25 mg/L), 25.8 percent (0.5 mg/L), and 44.7 percent (1 mg/L) after 7 days of diclofenac exposure, and 3.2 percent (0.25 mg/L), 21.9 percent (0.5 mg/L), and 60.4 percent (1 mg/L) after 21 days.

When compared to control fish lipid levels, the percentage reduction in lipid levels in the brain after 7, 14, and 21 days of exposure was 11.1, 19.8, and 39.2 in concentrations of 0.25 mg/L diclofenac, 14.2, 21.4, and 38.9 in concentrations of 0.5 mg/L diclofenac, and 14.9, 21.7, and 43.5 in concentrations of 1 mg/L diclofenac.

The fish, C. carpio, showed stress and reduced their food intake after being exposed to sublethal amounts of diclofenac. This has resulted in the depletion of reserve lipid levels after 21 days of exposure, with no notable changes in lipid levels during the previous period of exposure (upto 14 days). The decrease in total protein content may be attributable to the breakdown of protein into free amino acids caused by diclofenac at lower exposure levels (Shakoori et al., 1994).

A decrease in protein content was detected after a 21-day rise in exposure at 0.25, 0.5, and 1 mg/L sublethal concentrations. These results show that diclofenac induces proteolysis in fish even under sublethal toxic stress, resulting in elevated levels of protein content; however, the degree of proteolysis appears to be time-dependent, as the decrease in protein levels progressed significantly at the 7th and 14th days of exposure, but regressed and attained almost normally at the 21st day.

As a result, elevated activity of protease, a lysosomal enzyme, in fish organs may be attributable to lysosomal damage caused by these medicines. Increased protease activity induced proteolysis, with the intensity increasing with the increase in exposure period between the 14th and 21st day. It is possible that the increase in free amino acid pool due to increased proteolysis would act as an osmotic and ionic effector to restore electrostatic equilibrium between the external medium and blood (Shashikant, 1986).

Carbohydrates are vital components of living cells and energy sources for animals. The current findings revealed a considerable decrease in carbohydrate content in all tissues tested. Imidacloprid exposure resulted in a considerable drop in glucose content, according to ShaziaQuadir et al. (2014). Tissue specific carbohydrate depletion, as seen in the current investigation, may be owing to its fast use to meet energy demands under the influence of the medication.
In the current investigation, an initial drop in total carbohydrate levels was observed in the liver, gills, and brain tissues. The disruption in glucose metabolism was regarded as one of the most notable biological lesions caused by pharmacological activity. The drop in carbohydrate content in the gills, liver, and brain could be attributed to glucose use to meet the increased energy requirement imposed by the acute anaerobic stress of mercury intoxication (Anon et al., 1975).

Another probable cause of tissue depletion is a reduction in glycogen production. Under hypoxic conditions, fish obtain energy via the anaerobic breakdown of glucose, which becomes accessible to the cells due to enhanced glycogenolysis. The observed carbohydrate depletion in the current study reflects the increased requirement for these molecules to provide energy for the cellular metabolic activity under hazardous manifestations.
Lipid is a typical bodily ingredient that is vital in the formation of cell membranes, the synthesis of bile acid, and the manufacture of steroid hormones. According to Remia et al. (2008), the drop in lipid may be attributed to the fish, Tilapia mossambica, using fatty deposits instead of glucose for energy purposes when exposed to Monocrotophos. According to Mohsen Abdel – Tawwab et al. (2013), there is a considerable drop in lipid content.

CONCLUSION

Furthermore, the chemical assessment of any persistent toxicant concentration in water and sediment may not provide information on the degree of contamination, particularly at sublethal levels. As a result, biological monitoring employing a series of assays in a "key species" has become unavoidable since it allows a sensitive technique to forecast the potential risk of persistent pollutants, which is useful in establishing "acceptable levels" of such bioaccumulative substances with hazardous potential. The first stage in evaluating the water quality needs and fish health is to conduct acute toxicity and biochemical investigations. These experiments clearly demonstrate the toxicant concentrations (LC50) that induce fish mortality even after a brief exposure and also aid in determining the metabolic changes that occur in fish as a result of toxicant exposure. As a result, research demonstrating the sensitivity of harmful effects of contaminants in aquatic creatures, particularly fish, is required. As a result of the current investigation, it is possible to conclude that fish are highly sensitive to diclofenac and that diclofenac is dose dependent.

Conflicts of Interest

Authors declare that there is no conflict of interests regarding the publication of this paper.

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