Indian J. Applied & Pure Bio. Vol. 37(3), 945-956 (2022). **ISSN: 0970-2091**
A web of Science Journal

Toxicity assessment of chlorpyrifos and induced changes in the Ach and associated AchE activity and behavioral response in *Cyprinus carpio* (L.)

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Abstract

Chlorpyrifos (O, O diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate) second largest selling organophosphate insecticide, heavily used in the world to control agriculture and household pest. Chlorpyrifos (CPF) LC50 – 96 h was calculated as 0.318 mg/l using probit analysis based on LC50 value two sublethal concentrations of 1/5th of LC50 (0.0636 mg/l) and 1/10th of LC50 (0.0318 mg/l) were determined. The influence of CPF on the acetylcholinesterase (AChE) activity and behavioral response at both the sublethal concentrations for 7, 14, and 21 days of exposure and were allowed to recover in the pesticide-free medium for one week only after the 21st day of exposure behavioral responses and morphological deformities were studied in the experimental periods. Inhibition of AchE results in excess accumulation of acetylcholine (ACh) in cholinergic synapses leading to hyperstimulation and cessation of neuronal transmission attributed to paralysis. The carp were found under stress, there is significant mortality was observed in both the sublethal concentrations. Caudal bending and impaired morphological deformities were observed in the experimental periods. This may be a consequence due to the inhibition of brain and muscular AChE activity by chlorpyrifos-oxon via biotransformation of bioaccumulated chlorpyrifos in the tissues.

Chlorpyrifos (O, O diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate) is one of the earliest evolved biggest selling organophosphate insecticides, widely advocated to exterminate agricultural and household pests throughout the world[19]. In 1965 CPF was introduced to India[12], and categorized as a harmful pesticide[15]. It is one of the major insecticides

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found in fishery items. According to the report in 2012 about 314,000 tons and in 2015 about 200,000 tonnes of CPF were used, the rate at which pesticides were applied surged significantly as a result, it is anticipated that this insecticide will endure being in high demand universally, leading to widespread distribution. Hence allowable limit of CPF is surpassing in the environment. Earlier reports claimed that there were several hundred parts per billion of chlorpyrifos in the water associated with fish death instances, its half-life is 26.5 days[40] in water, because of its low perseverance and prevalence of CPF in the aquatic environment making it the most potent hazardous agent to exert negative impacts on nontarget organisms, especially fish[2]. For these reasons, it is an excellent applicant for toxic studies on the health of aquatic organisms[26].

Chlorpyrifos may disrupt the active serine hydroxyl group of acetylcholinesterase (AChE), thereby altering the enzyme structure[5]. Thus neutralizing and preventing the hydrolysis of the enzyme. CPF is a potent neurotoxin inhibitor of acetylcholinesterase enzyme activity in the brain[41]. AChE inhibition is irreversible it terminates the neurotransmitter Acetylcholine (Ach) by hydrolysis resulting in the release of choline and acetate[42]. This leads to loss of nerve impulse transmission at the synaptic cleft of cholinergic neurons thus ending the neurotransmission and causing neurotoxicity[47] due to CPF intoxication. thus AchE is a sensitive biomarker to assess pesticide toxicity to fish.

CPF is a potent neurotoxicant due to inhibition of AchE activity it may influence the behavioral pattern, Behavior offers a special viewpoint that connects an organism's physiology and ecology to its surroundings[20]. Behavior is a series of quantitative acts through the peripheral and central nervous systems[17] as well as the cumulative effect of physiological, pharmacological, and genetic processes vital for survival, such as eating, reproducing, and predator protection. Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment.

Pesticides used at sublethal levels in aquatic environments alter the morphological and functional characteristics of aquatic species more frequently than mortality[37]. One of the most sensitive indicators of environmental stress is a behavioral modification, and many of these changes have an impact on survival. Behavioral changes in fish, notably in non-migratory animals can also provide important indices for ecosystem assessment. Any change in the behavior of fish indicates the deterioration of water quality, as fish are the biological indicator and hence index of environmental suitability and the cost of survival.

There hasn't been much research done on chlorpyrifos effects on brain and muscle AChE activity-associated behavioral alterations and none has been done on *Cyprinus carpio*, commercially available and an ideal model indicator for toxicological investigations. hence an attempt has been made to assess the acute toxicity of chlorpyrifos and the sublethal effects of CPF on the AchE activity of the brain and muscle along with the behavior response in *Cyprinus carpio*.

The freshwater fish *Cyprinus carpio* (L.) (Family: Cyprinidae, Order : Cypriniformes) is edible and commercially valuable. Live fish of size 5-6 cm and weight 3.5-4.5g weight were procured from State Fisheries Department, Bhadra Reservoir Project, Karnataka State, India. To avoid dermal infections, fish specimens were bathed twice in 0.05 percent potassium permanganate (KMnO4) for 2 minutes. The specimens were then acclimatized for three weeks in semi-static systems under laboratory conditions.

During the acclimatization, period fish were supplemented with commercial fish pellets and rice bran twice a day. To decrease the ammonia content in the water, feces and other waste materials were drained off daily. The physicochemical characteristic of test water such as Temperature 25±1ºC, pH 7.2±0.2 at 25ºC, Dissolved Oxygen 6.7±0.8 mg/L, Carbon-dioxide 6.2±0.3 mg/L, Total Hardness 23.2±3.4 mg as CaCO3/L, Phosphate 0.37±0.002 µg/L, Salinity 0.01 ppm, Specific Gravity 1.001 and The conductivity of the water is less than 10 µS/cm. were examined following the standard method[3].

Pesticide (chemical test) :

For the present study, technical-grade of chlorpyrifos (50%EC) with the trade name 'Premain strong' (manufactured by ADAMA India Pvt. Ltd. Hyderabad) was purchased from the local market. It was found that chlorpyrifos (50%EC) grade chemical is heavily used in the agriculture fields.

Determination of acute toxic and sub-lethal concentration :

An acute toxicity bioassay was performed in the laboratory semi-static setup to estimate the LC50 – 96 h value of CPF. CPF was dissolved in acetone to make the stock solution. In the experimental medium, the maximum amount of acetone was less than 0.1ml/l. The test solution was changed every day to keep the chemical content consistent. A total of ten acclimatised fish specimens were randomly chosen and exposed to each of the seven concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7mg/L) of chlorpyrifos (50% EC) for 96 hours. These concentrations were chosen after range-finding acute toxicity tests, and the experiment was run in triplicate to determine the test chemical's 96-hour LC50 value for the species. To ensure the uniformity of the experimental approach, a negative control (without pesticides) was included. No feeding was done during the acute toxicity test. During the experiment, dead fish were removed and mortality rates were calculated after 24, 48, 72, and 96 hours. A basic program from the Probit analysis was used to calculate the LC50 and 95 percent confidence limits of chlorpyrifos for *Cyprinus carpio*[11].

In vivo sub-lethal exposure experiment :

For *Cyprinus carpio*, the 96-hour LC50 value of chlorpyrifos was determined to be 0.318 mg/l (L.) based on the LC50 – 96 h value, the two test concentrations of chlorpyrifos, sub-lethal 1 (1/5th of LC50, 0.0636 mg/l) and sub-lethal 2 (1/10th of LC50, 0.0318 mg/l) were selected following the methods[43]. and another group was kept in control. To keep the pesticide content constant, the fish specimens were exposed to these two test concentrations in a semi-static system with test water changed every other day. The exposure could last up to 21 days.

For the estimation of ACHE activity and Ach content Sublethal concentration 1 (1/ 5th LC50, 0.0636 mg/l)and Sublethal concentration 2 (1/10th LC50,0.0318 mg/l) were selected, as the nominal concentration for the analysis of AchE and Ach content in the brain and muscle tissue at the end of the exposure periods 7th,14th, and 21st-day of *Cyprinus carpio*.

Estimation of Acetylcholine (ACh) content:

ACh content of the brain and muscle was calculated using Hestrin's technique, as reported by[4]. After separating and weighing the brain and muscle tissue, it was teased and transferred to tubes that had already been placed in a boiling water bath for 10 min to inactivate the enzyme acetylcholinesterase and release bound ACh. After cooling the tubes, the contents were homogenized in 2.0 ml of distilled water. A total of 2.0 mL of alkaline hydroxylamine hydrochloride and 1.0 mL of dilute HCl with H_2O at a ratio of 1:1 were added. The contents were centrifuged, and the supernatant was treated with 1.0 ml of ferric chloride. The optical density of the sample was measured in a spectrophotometer at 540 nm in comparison to a blank.

Estimation of acetylcholinesterase (AChE):

The procedure given by Metcalfe[23] was used to calculate acetylcholinesterase activity in the brain and muscle. In cold 0.25 M sucrose solution, 3% of the homogenate of brain and muscle tissue was produced and homogenated. The enzyme test was performed using supernatant. A total of 3.0 ml of reaction mixture included 12 µm of acetylcholine chloride, 100 µm of sodium phosphate buffer (pH 7.4), and 1.0 ml of homogenate. After 30 minutes of incubation at 37°C, the reaction was halted by adding 2.0 ml of alkaline hydroxylamine hydrochloride solution, followed by 1.0 ml of HCl (1:1 HCl: H_2O). The mixture was properly blended and filtered. 1.0 ml of 0.37 M ferric chloride solution was added to the clear filtrate, and the color was measured at 540 nm in a spectrophotometer with a blank. Protein content in each homogenate was estimated[22] to determine the specific activity of the enzyme.

Statistical analysis :

The data obtained from the experimental investigation were statistically analyzed using Percent mortality data of fish after 96 hours of exposure analyzed using NCSS v. 22.0.3 software; For the estimation of Ache and Protein were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple range tests, and statistical significance was assessed at 5% ($P < 0.05$) levels using Graph Pad prism version (8.0) software.

Evaluation of Chlorpyrifos Acute toxicity Test :

The calculated acute toxicity values for Chlorpyrifos (50%EC) pesticide exposed for 96hr value were found 0.318mg/l and 1/5th and 1/10th sublethal doses were selected for the analysis. 96hr LC_{50} values with 95% confidence limits for chlorpyrifos upper bond (0.404) and the lower bond (0.250) were given in (Table-1). Percent mortality of fish *Cyprinus carpio* in different concentrations of chlorpyrifos

Table-1. LC_{50} values with 95% confidence limits for chlorpyrifos

Concentration	Exposed Fishes	% Mortality	Lower bound – Upper bound
0.318mg/L	10	50	0.250 - 0.404

Table-2. Percent mortality of fish *Cyprinus carpio* in different concentrations of chlorpyrifos at 96h exposure period.

Percentile	Probit	Log (Concentration)	Std. Error Log (Conc.)	Concentration	Std. Error Concentration
1	2.6737	-1.0305	0.1344	0.0932	0.0289
5	3.3551	-0.8743	0.1033	0.1336	0.0318
10	3.7184	-0.7911	0.0874	0.1618	0.0326
20	4.1584	-0.6903	0.0694	0.2041	0.0326
25	4.3255	-0.6520	0.0631	0.2229	0.0324
30	4.4756	-0.6176	0.0579	0.2412	0.0322
40	4.7467	-0.5554	0.0498	0.2783	0.0319
50	5.0000	-0.4974	0.0446	0.3181	0.0327
60	5.2533	-0.4393	0.0424	0.3637	0.0355
70	5.5244	-0.3772	0.0440	0.4196	0.0425
75	5.6745	-0.3428	0.0465	0.4542	0.0486
80	5.8416	-0.3045	0.0504	0.4960	0.0576
90	6.2816	-0.2037	0.0646	0.6257	0.0931
95	6.6449	-0.1204	0.0789	0.7579	0.1377
99	7.3263	0.0358	0.1085	1.0859	0.2713

at 96h exposure period was given in (Table-2), Probit mortality of fish *Cyprinus carpio* in different concentrations along with log concentration of chlorpyrifos at 96h exposure period was given in the (Table-3 and Fig. 1).

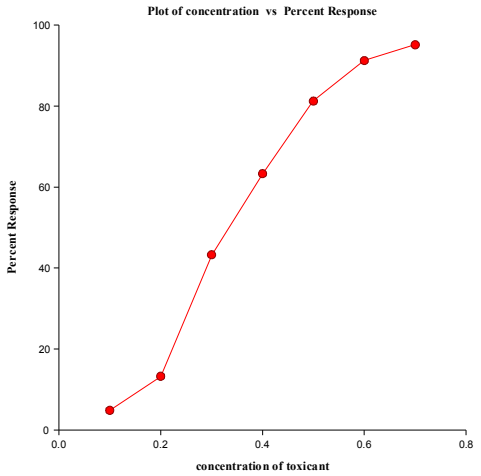

Fig.1. Percent response and concentration of chlorpyrifos.

Table-3. Probit mortality of fish *Cyprinus carpio* in different concentrations of chlorpyrifos at 96h exposure period.

Concentration mg/L	Actual Percent	Probit Percent	No. of Fish	Mortality Result	Expected Result	Difference	Chi-Square
0.1	4.90	1.41	10	0.49	0.14	0.35	0.87
0.2	13.30	18.95	10	1.33	1.90	-0.57	0.21
0.3	43.30	45.57	10	4.33	4.56	-0.23	0.02
0.4	63.30	66.78	10	6.33	6.68	-0.35	0.05
0.5	81.20	80.42	10	8.12	8.04	0.08	0.00
0.6	91.20	88.54	10	9.12	8.85	0.27	0.07
0.7	95.10	93.25	10	9.51	9.32	0.19	0.05

Total Chi-Square (χ^2) : 1.28
D.F. : 5
Prob Level : 0.94

An increase in the number of mortalities was observed as the concentration of chlorpyrifos toxicity increased (Table-3).

Estimation of Acetylcholinesterase :

The analysis of Acetylcholinesterase

alterations in fish has emerged as an important method for monitoring environmental exposure to pollutants both in laboratory and field research. The calculated values for *Cyprinus carpio* at a sub-lethal concentration of 1 and 2 of Chlorpyrifos after the 7th, 14th, and 21st days of exposure and percent change over control along with standard deviations are given. In the present study reduced AchE activity with increased Ach content was observed in the brain followed by muscle tissues of *Cyprinus carpio* for all the exposure time.

Brain AChE Activity :

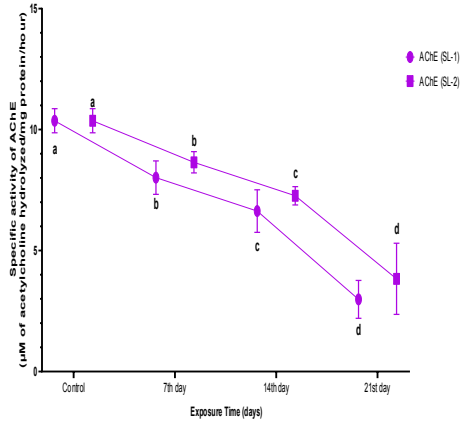

Fig. 2. Changes in the AChE content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of acetylcholine hydrolyzed/ mg protein/hour. Different alphabets within each tissue between exposure time denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant decrease in the AChE level of brain tissue of *C. carpio* with the different days of exposure in both the exposure concentrations (Fig. 2).

Acetylcholine(Ach) level in Brain

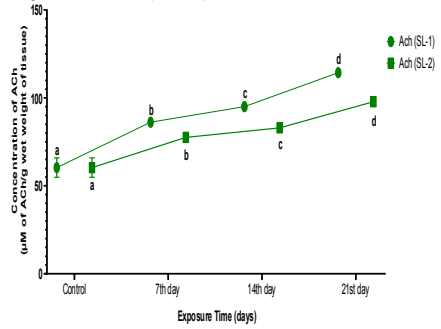

Fig. 3. Changes in the ACh content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of ACh/g wet weight of tissue. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant increase in the ACh level of brain tissue of *C. carpio* with the different days of exposure in both the exposure concentrations (Fig. 3).

Muscle AChE Activity

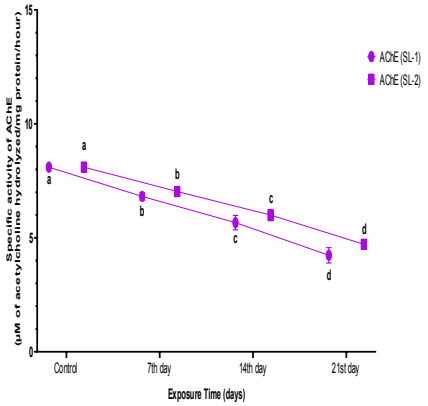

Fig. 4. Changes in the AChE content in muscle tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of acetylcholine hydrolyzed/ mg protein/hour. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant decrease in the AChE level of muscle tissue of *C.carpio* with the different days of exposure in both the exposure concentrations (Fig. 4).

Acetylcholine(Ach) level in Muscle

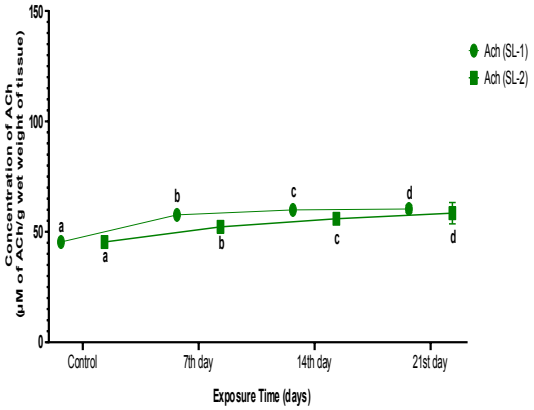

Fig. 5. Changes in the ACh content in muscle tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of ACh/g wet weight of tissue. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant increase in the ACh level of muscle tissue of *C.carpio* with the different days of exposure in both the exposure concentrations (Fig. 5).

Organophosphorus insecticides typically degrade more quickly and have a lower persistence level in the environment. However, with the extensive exploitation and use of chlorpyrifos in agriculture, its frequent detection has increased its residues in aquatic biota, soil, sediment, and water. Fish are frequently utilized as sentinel organisms for examining the toxicity level of aquatic pollutants.

Data obtained from Acute toxicity provides water quality guidelines for regulatory purposes[44]. the present study reveals that chlorpyrifos (50% EC) is toxic to fish. In addition, pesticide intoxication is dose and time-dependent. The current study's 96-hour LC_{50} value of 0.318 mg/L for *Cyprinus carpio* exposed to chlorpyrifos is slightly higher than the 96-hour LC_{50} values of 0.219mg/L of chlorpyrifos estimated in *Puntius chola* [50] and 0.280mg/L for *Ictalurus punctatus*[16]; 0.176mg/L for *Poecila reticulate*[39] and 0.136mg/L for *Cyprinodon variegatus*[7]; and for *Oreochromis mossambicus* 96 h LC_{50} value is 0.0022mg/L [28]; 0.0041mg/L for *Fundulus similis*[38]; our obtained 96 h LC_{50} value (0.318mg/L) is lower than 96 h LC_{50} value 0.35mg/L of *Gibelion catla*, 0.47mg/L for *Labeo rohita* and 0.65mg/ L for *Cirrhinus mrigala* [45] and 1.023mg/L for *Oreochromis niloticus*[10], 0.92mg/L for *Clarias gariepinus*[27]; 16.5mg/L for *Clarias batrachus*[32]; 1.57mg/L for Nile tilapia[13].

Many factors, including species, different conditions of pesticides, such as its manifestation, stereochemistry[48], and also water parameters can affect the concentrations at which a compound is lethal. Our results are in good consonance with the previous reports

validating the high toxicity of chlorpyrifos to various fish species[13,32,45].

In the present study, the level of AchE activity in gill, muscle, tissues of fish, *C. carpio* exposed to chlorpyrifos decreased suggesting the inhibitory effect of pesticide on the AchE system (Fig. 2 and 4). inhibition of AchE is higher in brain than that in muscle in Inconsonance with the decrease in the AchE activity, there is a corresponding increase in the Ach content of the tissues (Fig. 3 and 5) suggesting a decrease in the cholinergic transmission and consequent accumulation of Ach in the tissues. indicating a high accumulation of Ach in the synaptic cleft of nerves as a result of multiple neurotoxic effects and shuttered cholinergic transmission[24]. A comparable corroborative rise in Ach content as a result of a drop in tissue AchE levels was documented in different fish species[18,35,36].

Acetylcholinesterase is an enzyme that regulates the amount of neurotransmitter material present at neuronal junctions[25], as well as the ionic content[49]. The reduced ionic composition in *C. carpio* brain tissue may influence the inhibition of AchE and the rise of Ach concentration.Subsequent protein estimation was carried out to measure the specific activity of enzyme. The possible consequences of variations in total protein concentration within the brain tissue suggest induced pesticidal stress may interfere with the glial peptide bonding or total neuronal response to the cholinergic agonist resulting in alterations in the overall biochemical activity due to neural breakdown in the synaptic cleft.

In the present study, chlorpyrifos regulates nerve impulses by shunting the influx of sodium channels as a result multiple nerve impulses occur in place of a single nerve impulse, and these impulse release Ach neurotransmitters to activate more neurons, causing an accumulation of abundant ACh within the nerve impulse thus results in impaired neurophysiological activity attributed to multiple neurotoxic effects and reduced cholinergic transmission[24]. Similar results were obtained in tissues and other fish species[6,30].

The greater inhibition of AchE activity with a simultaneous rise in Ach content in the tissues implies stronger inhibition of central nervous system integratory activity and accumulated Ach in the brain tissue[31]. Damage to the central nervous system may have resulted in the uncontrolled release of hormones, and an animal's toll may be conceivable due to the degradation of numerous biochemical and physiological activities[8].

CPF is a potent neurotoxin[41] and an AchE inhibitor CPF was a more intensive inhibitor of AChE activity in muscle and brain. Pesticides bind the active site and prevent Ach breakdown[1,21], resulting in synaptic transmission blockage in cholinergic neuronal cells. Acetylcholine, a neurotransmitter molecule, is deactivated by AchE. Neurotransmitters are required to transmit nerve impulses flowing from one nerve cell to the next across the synaptic gap. AchE deactivates Ach nearly soon after the impulse is conveyed by breaking it down. If AchE is blocked, Ach, accumulates and nerve impulses cannot be halted, which leads to uncontrolled muscle contraction resulting in paralysis, which is evident in the present study of behavioral response of *cyprinus carpio* and also causes behavioral abnormalities, and widespread disruption in

neuronal physiology, ultimately leading to the organism's death.

Behavioural response :

In the present study effect of CPF, a potent neurotoxicant alters significantly the behavior of *Cyprinus carpio*. In the current investigation, the control fish were active for feeding and alert to even the smallest disruption with their well-synchronized motions. The behavior did not vary between the control groups, therefore, these results were taken as standards for the entire experimentation.

Chlorpyrifos-exposed carp displayed disruptive behavior and located at the test chamber's bottom, gulping air and swimming at the water surface were observed on the day of exposure to sublethal concentrations of chlorpyrifos. Gulping of air may help to avoid contact with the toxic medium and to ease respiratory stress. Even after a week's recovery intervals, the surfacing phenomena and simple predation persisted in both test concentrations. This represents the toxicant's disastrous effects. Easy predation is the most serious damage caused by a pollutant on sensitive species like fish, which ultimately decide the survival of a species in a given ecosystem. In the present study fish showed enlarged fins, fading of body color and fish body became lean towards the abdomen under stress were observed with time and concentration in experimental periods. Unfortunately, some of the fish failed to fight off chlorpyrifos stress during both the sublethal exposures and the recovery periods, sinking to the bottom with their tiniest opercular movements. Later phases in both test concentrations were accompanied by modest belly edema that lasted even throughout recovery intervals. Lean fish have lower appetites and a metabolism that is focused on responding to toxic stimuli. Fish food consumption was severely reduced and hampered. This remained even after periods of recovery and was especially obvious during the first fifth of sublethal exposure times. Reducing these animals' food intake in unfavorable environmental conditions might be beneficial to lessen the energetic expenditures of digesting for them. Fish commonly experience depression in appetite in reaction to stress, and extended durations of intermittent feeding can have a noticeable effect on growth and reproduction[33].

One of the most typical occurrences is Increased mucus synthesis in fish leads to a generalized defense against toxins, perhaps reducing toxin exposure. Mucus also serves as a barrier between the body and the toxic medium to decrease the irritating effect of the toxic medium or to scavenge it through epidermal mucus.[34] made similar observations following RPR-V (a novel phosphorothioate insecticide, 2-butenoic acid-3- [diethoxy phosphinothionyl] ethyl ester) exposure to euryhaline fish, Oreochromis mossambicus. A substantial growth reduction caused by toxicant stress has important implications for survival in natural situations.[9] indicated that the abnormalities in fish behavior observed in exposure to OP insecticides (chlorfenvinphos, chlorpyrifos, and diazinon) could be related to the failure of energy production or the release of stored metabolic energy, which may cause severe stress, leading to the death of the fish. Caudal bending, which significantly slowed down the typical swimming pattern, was seen in both toxicant concentrations over time and persisted even during recovery periods. There

was significant caudal bending and morphological deformities were noticed at both sublethal concentration of the 96-hour LC50 but maximum CPF effect was observed in the maximum toxicant concentration. Caudal bending may be due to paralysis a condition that may be caused by neural signals being blocked as a result of muscular AChE activity being suppressed. A brief period of rapid voluntary muscle jerking precedes the onset of paralysis[14,51]. Thus, chlorpyrifos leads to reduced instinctive behavioral responses and affected morphological features. Behavioral anomalies were evidenced right from the day of exposure to sublethal concentrations of chlorpyrifos and are due to inhibition of brain AChE [6]. Overall impairments in fish behavioral responses and morphological malformations may be caused by chlorpyrifos-inhibition oxon of brain musculature AChE activity through the biotransformation of sequestered chlorpyrifos in the storage organs. Due to the actions of their active oxygen analog chlorpyrifos-oxon (CPF-oxon), chlorpyrifos (CPF) inhibits AChE[46]. Cytochrome P450 may have undergone a desulfuration reaction to convert the sequestered chlorpyrifos into chlorpyrifos-oxon, which is the active oxygen analog of chlorpyrifos (CYP)[29] the presence of xenobiotic compounds enables the organisms to activate biotransformation enzyme systems enable to survive in sublethal exposure.

The abnormalities in fish behavior observed in this study could be related to the inhibitory action of chlorpyrifos on AchE and subsequent accumulation of Ach at the nerve endings.

Statements and Declaration :

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval :

Not applicable. As the fish used for the study used has a high economic food value in India.

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