Assessment Of Acute Toxicity Of Chlorpyrifos And Its Sub-Lethal Effects On Protein Patterns Of *Cyprinus Carpio*(L.) By Using SDS-PAGE

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Abstract— The present study was focused on investigating the acute toxicity of chlorpyrifos and its impact on electrophoretic protein patterns in the tissues of *Cyprinus carpio*, an acute toxicity bioassay was conducted in the semi-static system and the fingerlings of *C. carpio* were exposed to various chlorpyrifos(50%EC)concentrations ranging from 0.1mg/L to 0.7mg/L for 96 h, and acute toxicity was determined as 0.318mg/L. Based on the LC_{50} value, the *Cyprinus carpio* were exposed to two sub-lethal concentrations viz., SL-1 ($1/5^{th}$ of LC_{50}, 0.0636mg/l) and SL-2 ($1/10^{th}$ of LC_{50},0.0318 mg/l) of chlorpyrifos. Proteins have a unique role in cell metabolism and proteomic analysis provides valuable information when variations that occur within the proteome of organisms are compared as a consequence of biological perturbations or external stimuli. The study's goal was to investigate the influence of chlorpyrifos on electrophoretic protein patterns by performing SDS-PAGE on muscle, liver, gill, and brain tissues after exposing fish to two sublethal concentrations in a semi-static system for 3 weeks. The intensity of protein subunits in exposed tissues decreased, and some subunits disappeared, CPF influences both high-intensity protein bands and low intensity as well in both the sub-lethal concentrations. The study has suggested that the changes in the protein banding are more prominent in the SL-1 concentration of chlorpyrifos.The variations in protein subunits band patterns are due to protein deprivation of various proteins, proteolysis, or maybe insufficient amino acid incorporation into the polypeptide chain due to induced stress imposed by the toxicant.
Keywords: Chlorpyrifos, *Cyprinus carpio*, Protein pattern, SDS-PAGE

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1. Introduction

In India, Pesticides have become a vital constituent in agriculture progress since the tropical environment is incredibly advantageous to pest breeding. Indiscriminate use of this pesticide to enhance agricultural productivity and yield may pose a serious risk for non-target creatures, particularly aquatic life, and the environment. Pesticide pollution of the aquatic biota is a serious issue, and fish are more at risk of these pollutants. Every year, roughly 3 million incidences of pesticide poisoning occur, resulting in 220,000 global deaths (WHO 1992). Many of these substances are carcinogenic (Garaj-vrhovac and Zeljezic 2000; Kumar et al. 2009; Nwani et al. 2010), and have been associated with cancer development (Leiss and Savitz 1995), or may induce developmental abnormalities (Arbuckel and Server 1998). In India, Chlorpyrifos (O, O-diethyl O- 3, 5, 6-trichlor-2-pyridyl phosphorthioate) is widely used organophosphate which is used for controlling various pests belonging to the orders such as Diptera, Coleoptera, and Homoptera in soil or on vegetation (Tomlin, 2000). Likewise, it is also used in the control of domestic pests. However, due to its widespread use, it may cause adverse effects on the non-target organism, fish (Anita et al., 2016).

The extreme toxicity of chlorpyrifos to fish may affect their growth and reproduction (Levin *et al* 2004; Sledge *et al* 2011)) as well as their gills and liver (Xing *et al* 2012). Chlorpyrifos exerts several other effects *i.e.* affecting hepatic dysfunction, hematological changes, genotoxicity, and neurobehavioral and neurochemical changes. The activities of glutathione (GSH), catalase (CAT), and glutathione S-transferase (GST) have been

demonstrated to be significantly reduced after exposure to CPF (Poet *et al* 2003; Mehta *et al* 2008; Alagoa *et al* 2009; Slotkin *et al* 2005; Goel *et al* 2005).

The current study has been undertaken to examine the acute toxicity of chlorpyrifos (CPF) and its impact on electrophoretic protein patterns within the tissues of *C.carpio*. Proteins are the principal effector molecules in all living systems, and any adaptive responses to environmental, physiological, or pathological factors will be reflected in changes in protein activity or content (Bradley et al. 2002). As a result, global methodologies like proteomics provide effective tactics for toxicological investigations and are recognized as a potent tool for investigating cellular responses to environmental contamination such as pesticides (Dowling and Sheehan 2006). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), One of the most popular techniques in many scientific fields, such as molecular biology, biochemistry, forensic sciences, etc. can separate proteins on a gel, Depending on the length of their polypeptide chains. Thus SDS-PAGE, an effective technique is widely employed in various disciplines to classify proteins based on electrophoretic mobility. According to Muhammad (2018), SDS-PAGE analysis is an important biomarker for toxicological studies in fish.

The underlying principle is that a proteome varies from cell to cell and changes constantly due to biochemical interactions with the genome and environment. As a result, environmental factors influence the expression of a distinct collection of proteins in the exposed organism, tissue, or cell type (Nesatyy and Suter 2007).

To the best of our knowledge, no research has been undertaken as a result of the literature review to investigate the protein patterns of *C.carpio* tissues subjected to chlorpyrifos.

2. Materials and Methods

2.1. Experimental fish specimens and chemical

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, Shimoga District, Karnataka State, India. To minimize cutaneous 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 a semi-static system under laboratory conditions.

During the acclimatization period, fish were supplemented with commercial fish pellets and rice bran twice a day. To reduce the ammonia content in the water, feces and other waste constituents were drained off daily. The physicochemical quality of test water such has, 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±0.002µg/L, Salinity 0.01 ppm, Specific Gravity 1.001 and the conductivity of the water is less than 10µS/cm were examined following the standard method (APHA 2005).

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.

2.2 Acute Toxicity Test

To determine the 96-hour LC_{50} value of chlorpyrifos an acute toxicity bioassay was performed in a semi-static system in the laboratory, the stock solution was prepared by dissolving CPF in acetone and the working standards were prepared from this stock standard solutions. To keep the chemical content consistent the test solution was changed every day. A total of ten acclimatized fish specimens were randomly selected 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 selected after range-finding acute toxicity tests, and the experiment was run in triplicate to determine the 96-hour LC_{50} value for the species. To confirm the consistency of the experimental approach, a negative control (without pesticides) was included. 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 LC_{50} and 95 percent confidence limits of chlorpyrifos for *Cyprinus carpio,* Finney (1971).

2.3. *In vivo* sub-lethal exposure experiment

For *Cyprinus carpio*, the 96-hour LC_{50} value of chlorpyrifos was determined as 0.318 mg/L, based on the LC_{50} – 96 h value of the two test concentrations of chlorpyrifos viz; sub-lethal 1 (1/5th of LC_{50}, 0.0636 mg/l) and sub-lethal 2 (1/10th of LC_{50}, 0.0318 mg/l) were selected and another group was kept as controls. 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. Fish were sacrificed at the end of each exposure period, and organs such as the muscle, liver, gills, and brain were separated, and immediately processed for SDS-PAGE analysis.

2.4. Protein electrophoresis

The conventional method of Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to examine the change in protein fractions in the gill, brain, liver, muscle of control, and pesticide-exposed tissues Laemmli (1970) method (Fig.1).

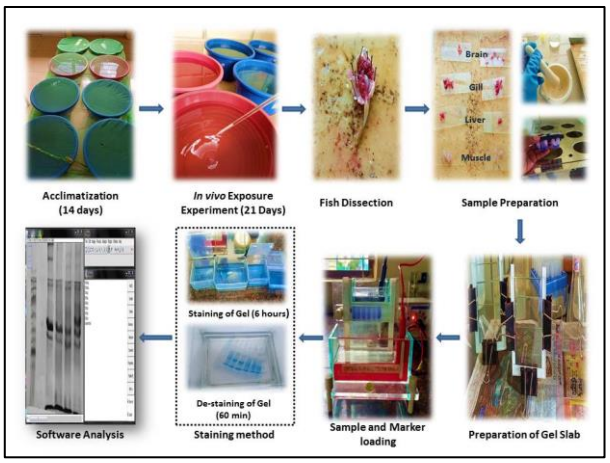

Fig.1. Experimental design of SDS-PAGE analysis.

2.4.1. Sample preparation

Muscle, liver, gill, and brain homogenates were prepared in 10% TCA and centrifuged at 8000 rpm for 10 minutes in the cooling centrifuge. The pellet was centrifuged for 10 minutes at 8000 rpm after being washed twice with ice-cold acetone. The pellet was dissolved in sample buffer (0.5M Tris-HCL, pH 6.8-2ml, 40% glycerol-1.6 ml) and heated in a water bath at 95ºC for 10 minutes.

2.4.2. Preparation of Gel Slab

The sandwich was made by clamping two clean glass plates together and pouring 1 mm Teflon gel solution of 12.5% (1.5 M Tris-HCL, pH8.8-2 ml, 30% Acrymalide-3.2 ml, 10% SDS-0.5 ml double distilled water-1.8 ml, TEMED-0.015 ml, Ammonium persulphate-0.5 ml)was prepared and emptied in between the clamped glass plates. The gel solution was overlaid with distilled water to eliminate any air bubbles. The plates were left unperturbed for 30 minutes to allow the gel to polymerize. Overlaid water was removed and rinsed with stacking gel buffer after gel polymerization. The 5% stacking gel solution was prepared (0.5 M Tris HCL, pH6.8-2 ml, 30% acrylamide-0.8 ml, 10% SDS-0.5 ml, double-distilled water1.2ml, TEMED-0.015ml, 1.5% APS 0.5ml) and poured over the polymerized resolving gel. The comb was precisely fitted , the gel arbor was left unperturbed for 15 min after loading the polymerization comb into the wells, and the gel was run at 60V.

2.4.3 Staining method

The Coomassie Brilliant Blue Stain (CBBS) was prepared, and the gel was incubated for 6 hours overnight in a staining solution containing 40% methanol, 10% acetic acid, and 0.025% Coomassie Brilliant Blue R-250, filtered through Whatman #1 paper and shaken on a rotary shaker. The destaining solution is the same as the staining solution but without the Coomassie R-250 dye powder. The gel was incubated in the destaining solution for 30-60 minutes before being rinsed many times in double-distilled water and stored in water.

2.4.4. Determination of molecular weight of the protein subunits separated on SDS-Runner.

The relative mobility of the individual protein subunits was estimated using the following formula to determine the molecular weight of the individual protein subunits.

$$\text{Relative mobility (Rm)} = \frac{\text{Distance traveled by the individual subunit}}{\text{Distance traveled by the marker dye}}$$

A standard curve was created by plotting the migration distances ('X'-axis) of known protein standards against their molecular weights ('Y'-axis) in Microsoft Excel. Based on the migration distance, the standard curve is used to compute the molecular weight of an unknown protein.

2.5 Statistical Analysis

Percent mortality data of fish after 96 hours of exposure were analyzed using NCSS v. 22.0.3 software; confidential limits were calculated using probit analysis. Quantification of SDS-PAGE bands was done using Image J (version 1.46) software.

3. Results

3.1. Acute toxicity test

The calculated acute toxicity values for Chlorpyrifos pesticide exposed for 96hr value were found 0.318mg/l and the lower and upper bound 95% lethal confidence limits indicated a range of values (0.250mg/L – 0.404mg/L) respectively were given in (Table.1). Mortality of fish *Cyprinus Carpio* in different concentrations along with log concentration of chlorpyrifos at 96hexposure period was given in the (Table. 2), Percentile mortality of fish *Cyprinus Carpio* in different concentrations of chlorpyrifos at 96h exposure period was given in (Table.3 and Fig.2). An increase in the number of mortalities was observed as the concentration of chlorpyrifos toxicity increased.

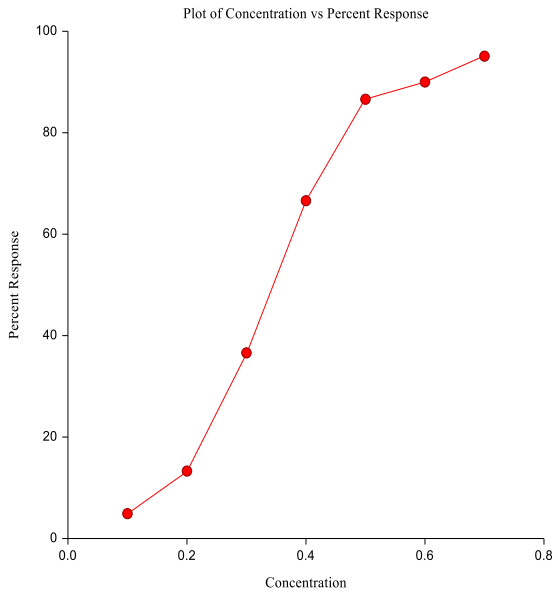

Fig.2. Percent response and concentration of Chlorpyrifos

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

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

The LC_{50} of chlorpyrifos to *C. Carpio* for 96 h of exposure was 0.318mg/L, and the lower and upper bound 95% lethal confidence limits indicated a wide range of values (0.250mg/L – 0.404mg/L) respectively.

Table 2: Mortality of fish *Cyprinus Carpio* in different concentrations of chlorpyrifos at 96 exposure period

Concentration (mg/L)	Actual Percent	Probit Percent	N	R	E(R)	Difference	Chi-Square
0.1	4.90	1.14	10	0.49	0.11	0.38	1.26
0.2	13.30	18.00	10	1.33	1.80	-0.47	0.15
0.3	36.60	45.28	10	3.66	4.53	-0.87	0.30
0.4	66.60	67.26	10	6.66	6.73	-0.07	0.00
0.5	86.60	81.21	10	8.66	8.12	0.54	0.19
0.6	90.00	89.33	10	9.00	8.93	0.07	0.00
0.7	95.10	93.91	10	9.51	9.39	0.12	0.02

Total Chi-Square (χ^2): 1.94

D. F.: 5 Prob Level: 0.86

Dataset Variables X = concentration, N = no of fish, R = percent mortality, E(R) =error percent mortality

Table 3: Concentration of Chlorpyrifos and Percentile Responses of *Cyprinus carpio*

Percentile	Probit	Log (Concentration)	Std. Error Log (Conc.)	Concentration	Std. Error Conc.
1	2.6737	-1.0107	0.1298	0.0976	0.0292
5	3.3551	-0.8601	0.1000	0.1380	0.0318
10	3.7184	-0.7798	0.0848	0.1660	0.0324
20	4.1584	-0.6826	0.0675	0.2077	0.0323
25	4.3255	-0.6457	0.0615	0.2261	0.0320
30	4.4756	-0.6126	0.0564	0.2440	0.0317
40	4.7467	-0.5527	0.0487	0.2801	0.0314
50	5.0000	-0.4967	0.0436	0.3186	0.0320
60	5.2533	-0.4407	0.0414	0.3625	0.0345
70	5.5244	-0.3808	0.0427	0.4161	0.0409
75	5.6745	-0.3477	0.0450	0.4491	0.0466
80	5.8416	-0.3108	0.0487	0.4889	0.0548
90	6.2816	-0.2136	0.0621	0.6115	0.0875
95	6.6449	-0.1333	0.0757	0.7357	0.1283
99	7.3263	0.0173	0.1040	1.0405	0.2493

The pesticide's toxicity was time and concentration-dependent, accounting for differences in LC_1-LC_{99} values obtained at different concentrations and times of exposure.

3.2. Protein Pattern Analysis

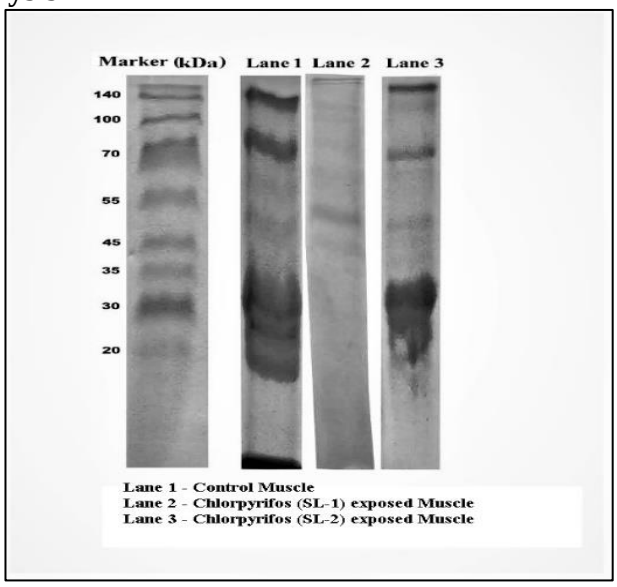

Fig 3: SDS-PAGE analysis of chlorpyrifos-induced protein profile changes in *C.carpio* of control muscle, and at sublethal concentrations of chlorpyrifos for 21 days.

Marker	Lane 1 - Control Muscle	Lane 2 – Chlorpyrifos (SL – 1) exposed Muscle	Lane 3 – Chlorpyrifos (SL – 2) exposed Muscle
0.03	-	-	-
0.10	-	-	-
-	0.16	0.16	0.16
0.22	-	-	-
-	0.29	0.29	-
0.31	-	-	-
-	-	0.36	-
-	0.39	0.39	0.39
0.42	-	-	-
0.50	-	-	-
-	0.59	-	0.59
0.60	-	-	-
-	0.64	-	0.64
0.70	-	-	-
-	0.75	-	-

Table 4: Changes in Rm values of protein subunits of *C.carpio* of control muscle, and at sublethal concentrations of chlorpyrifos for 21 days.

The electrophoretogram (Fig.3) represents the muscle protein subunits of Chlorpyrifos (SL-1) and Chlorpyrifos (SL-2) which showed a decrease in the intensity of banding patterns subunits when compared to the control. The Rm values (Table 4) of control muscle protein subunits 0.16, 0.29, 0.39, 0.59, 0.64, and 0.75 corresponded to ~87, ~62, ~47, ~27, ~24, and ~18kDa molecular weights respectively.

In chlorpyrifos-exposed tissue samples, the Chlorpyrifos (SL-1) muscle protein subunits had a lower intensity in the banding pattern than the Chlorpyrifos (SL-2) tissue sample. In the Chlorpyrifos (SL-1) exposed sample, the Rm values of protein subunits 0.59, 0.64, and 0.75 with a molecular weight closer to ~27, 24, and 18kDa respectively completely disappeared.

A protein subunit with an Rm value of 0.75 with a molecular weight closer to ~18kDa was absent in both concentrations of exposed tissue samples. A protein subunit with an Rm value of 0.36 was observed in (SL-1) which was not observed in (SL-2) and control tissue samples.

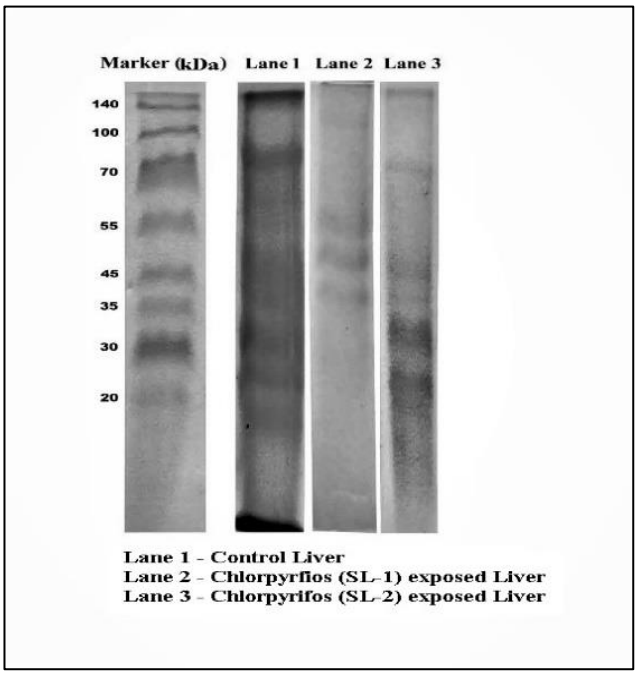

Fig.4: SDS-PAGE analysis of chlorpyrifos-induced protein profile changes in *C.carpio* of control liver and at sublethal concentrations of chlorpyrifos for 21 days.

Marker	Lane 1 - Control Liver	Lane 2 – Chlorpyrifos (SL – 1) exposed Liver	Lane 3 – Chlorpyrifos (SL – 2) exposed Liver
0.03	-	-	-
0.10	-	-	-
-	0.16	0.16	0.16
0.22	-	-	-
-	0.26	-	0.26
0.31	0.31	0.31	-
0.42	0.42	-	-
-	0.48	0.48	0.48
0.50	-	-	-
0.60	-	-	-
-	0.66	-	0.66
0.70	-	-	-
-	0.75	-	-

Table 5: Changes in Rm values of protein subunits of *C.carpio* of control liver, and at sublethal concentrations of chlorpyrifos for 21 days.

The electrophoretogram (Fig. 4) represents the liver protein subunits of Chlorpyrifos (SL-1) and Chlorpyrifos (SL-2) exposed samples, which showed a decrease in the intensity of liver protein subunits when compared to the control. The Rm values (Table.5) of control liver protein subunits 0.16, 0.26, 0.31, 0.42, 0.48, 0.66, and 0.75 corresponded to ~89, ~68, ~58, ~43, ~37, ~23, and ~18kDa molecular weights respectively. In chlorpyrifos-exposed tissue samples, the Chlorpyrifos (SL-1) liver protein subunits had a greater reduction in the intensity of the banding pattern than the Chlorpyrifos (SL-2) tissue sample. In the Chlorpyrifos (SL-1) exposed sample, the Rm values of protein subunits are 0.26, 0.42, 0.66, and 0.75 with a molecular weight closer to ~68,43 and 23,18kDa, respectively completely disappeared. A protein subunit with an Rm value of 0.75 with a molecular weight closer to 18kDa was absent in both concentrations of exposed tissue samples.

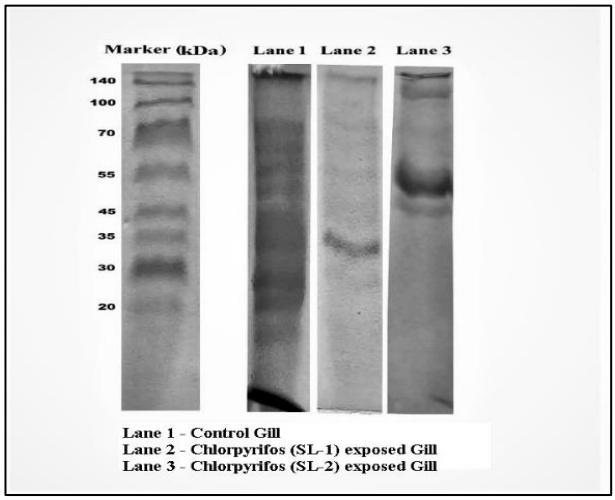

Fig. 5: SDS-PAGE analysis of chlorpyrifos-induced protein profile changes in *C.carpio* of control gill and at sublethal concentrations of chlorpyrifos for 21 days.

Marker	Lane 1 - Control Gill	Lane 2 – Chlorpyrifos (SL – 1) exposed Gill	Lane 3 – Chlorpyrifos (SL – 2) exposed Gill
0.03	-	-	-
0.10	-	-	-
-	0.17	-	0.17
0.22	-	-	-
-	0.23	0.23	-
-	0.30	-	0.30
0.31	-	-	-
-	0.36	0.36	0.36
0.42	-	-	-
0.50	-	-	-
-	0.53	0.53	0.53
0.60	-	-	-
-	0.64	-	-
0.70	-	-	-
-	0.71	-	-
-	0.80	-	-

Table 6: Changes in Rm values of protein subunits of *C.carpio* of control gill, and at sublethal concentrations of chlorpyrifos for 21 days.

The electrophoretogram (Fig.5) represents the Gill protein subunits of Chlorpyrifos (SL-1) and Chlorpyrifos (SL-2) exposed samples, which showed a greater decrease in the intensity of gill protein subunits when compared to the control. The Rm values (Table 6) of control gill protein subunits 0.17, 0.23, 0.30, 0.36, 0.53, 0.64, 0.71, and 0.80 corresponded to ~86, ~73, ~61, ~51, ~33, ~24, ~20, and ~15kDa molecular weights respectively. In chlorpyrifos-exposed tissue samples, the Chlorpyrifos (SL-1) gill protein

subunits had a lower intensity in the banding pattern than the Chlorpyrifos (SL-2) tissue sample. In the Chlorpyrifos (SL-1) exposed sample, the Rm value of protein subunit 0.17, and 0.64,0.71,0.80 with a molecular weight closer to ~86,24,20,15kDa was absent, and the protein band with Rm value 0.23 at ~73 shows light intensity in (SL-1). In the Chlorpyrifos (SL-2) exposed sample, the Rm values of protein subunits 0.23,0.64, 0.71, and 0.80 with a molecular weight closer to ~73, ~24, ~20, and ~15kDa, respectively completely disappeared.

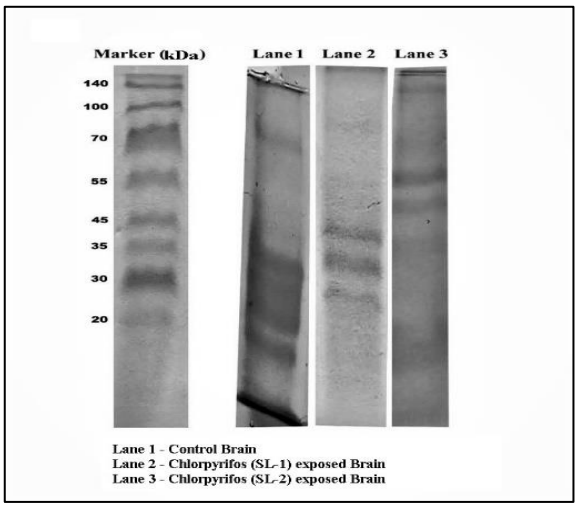

Fig 6: SDS-PAGE analysis of chlorpyrifos-induced protein profile changes in *C.carpio* of control brain, and at sublethal concentrations of chlorpyrifos for 21 days.

Marker	Lane 1 - Control Brain	Lane 2 – Chlorpyrifos (SL – 1) exposed Brain	Lane 3 – Chlorpyrifos (SL – 2) exposed Brain
0.03	-	-	-
0.10	-	-	-
-	0.19	0.19	0.19
0.22	-	-	-
0.31	-	-	-
0.42	-	0.42	-
0.50	-	-	-
-	0.56	0.56	0.56
0.60	-	-	-
-	0.63	0.63	0.63
0.70	-	-	-
-	0.72	-	0.72
-	0.81	-	-

Table 7: Changes in Rm values of protein subunits of *C.carpio* of control brain, and at sublethal concentrations of chlorpyrifos for 21 days.

The electrophoretogram (Fig.6) represents the brain protein subunits of Chlorpyrifos (SL-1) and Chlorpyrifos (SL-2) exposed samples, which showed a decrease in the intensity of brain protein subunits when compared to the control. The Rm values (Table 7) of brain control protein subunits 0.19, 0.56, 0.63, 0.72, and 0.81 corresponded to ~81, ~30, ~24, ~19, and ~15kDa molecular weights respectively. In chlorpyrifos-exposed tissue samples, the Chlorpyrifos (SL-1) brain protein subunits had a slightly lower intensity in the banding pattern than the Chlorpyrifos (SL-2) tissue sample. In the Chlorpyrifos (SL-1) exposed sample, the Rm values of protein subunits 0.72,0.81 with a molecular weight closer to ~19,15kDa completely disappeared whereas a protein subunit with Rm value 0.42 was observed only at (SL-1) of brain tissue. The protein band with an Rm value of 0.19 at ~81kDa shows a low-intensity banding pattern in the (SL-1) exposed sample.

4. Discussion

Acute toxicity test

Pollutant management in the aquatic ecosystem can be done using bioassays. The purpose of using bioassays is to monitor the levels of toxicity effects in the targeted biotope and to identify the low concentrations of toxicants that cause adverse effects (Kelso et al., 1990). These studies are critical in raising awareness about the potentially negative impacts of pesticides on the environment (Adedeji et al., 2008).

Data obtained from Acute toxicity provides water quality guidelines for regulatory purposes (*Sundaram et al.*,1994) the present study reveals that chlorpyrifos 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* (Verma and Saxena 2013) and 0.280mg/L for *Ictalurus punctatus* (Johnson and Finley 1980) ; 0.160mg/l *Cyprinus carpio* (Halappa and David 2009),0.176mg/L for *Poecila reticulate* (Sharbidre 2011) and 0.136mg/L for *Cyprinodon variegates* (Clark *et al.,* 1985) and for *Oreochromis mossambicus* 96 h LC_{50} value is 0.0022mg/L (Padmanabha 2015); 0.0041mg/L for *Fundulus similis* (Schimmel 1983) ; 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 *Cirrhinusmrigala* (Tilak *et al.,* 2004) and 1.023mg/Lfor *Oreochromis niloticus* (Díaz and Girón,2014) 0.92mg/L for *Clarias gariepinus* (Okechukwu *et al.,* 2013); 3.54mg/l and 2.15mg/l for *Cyprinus carpio* and *Liza abu* (Aitte 2018) 16.5mg/L for *Clariasbatrachus* (Reddy *et al*.,2012); 1.57mg/L for Nile tilapia Gul (2005).

Many factors, including species, different conditions of pesticides, such as its manifestation, stereochemistry (Ullah 2015), 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.(Tilak *et al.,* 2004; Díaz and Girón, 2014; Okechukwu *et al.,* 2013 ;Reddy *et al*.,2012; Gul, 2005).

In the current study, the 96hr LC50 value of chlorpyrifos to the fish *Cyprinus carpio* agrees with the findings of previous studies. According to the LC50 values mentioned above, it is clear that organophosphates are more toxic to fish. The selected *Cyprinus carpio* test fish is very sensitive to the toxic action of chlorpyrifos. The aforementioned numerous earlier reports and reported results to provide support for the current chlorpyrifos 50% EC toxicity results, resulting in the rating of the chosen test compound as Toxicity Category II.

Protein Pattern Analysis

Pesticides may either activate or inhibit other genes, depending on which genes are affected, resulting in the production of certain mRNAs that may then be translated into specific proteins known as stress-induced proteins. Under sublethal exposure, fish undergoes greater stress during the metabolic detoxification of the given toxin. Protein depletion is the physiological response of the treated fish to a changed metabolic system.

The present study reveals that chlorpyrifos influenced all molecular weight protein components in all tissues the variations in protein subunits indicate *Cyprinus carpio* is under stress. Subsequent fading or decrease in intensity of protein subunits expressions is found to be more in liver and gill tissues it might be due to the liver being the center of detoxifications whereas the gill is the respiratory site, influenced by any change in environmental water quality (Lyndon and Houlihan 1998). Toxicant found to influence the structural proteins and glycolytic enzymes of *Cyprinus carpio* treated with sublethal convergence(SL-1) of chlorpyrifos recommends the presence of high proteolytic action, impairment of signal transducing transcription factors, structural and metabolic proteins, mucus glycoproteins and oxidative stress which attributes to produce stress-induced proteins resulting in degradation of proteins causing reduced protein synthesis and also substantial changes in the expression of proteins results in greater decrement of intensity in protein bands or complete disappearance or fading of protein bands are found to be more in (SL-1) having a high concentration of chlorpyrifos than the (SL-2) of chlorpyrifos. In the present study, new protein subunits appeared in muscle and brain

tissue, this is evident that CPF significantly inhibits gene expression or it may activate some set of genes that synthesize mRNAs, to overcome toxicant stress, survive in stressful situations, mRNAs are translated into stress-induced proteins and also may be the alterations in the cytoplasmic proteins due to insecticidal inhibitory action on protein anabolism.

In the present study, high and low molecular weight proteins are denatured as a result of CPF toxicity, decrease in intensity of protein bands with higher molecular weights was accompanied by an increase in intensities of protein bands with some lower molecular weight proteins observed, this variation in the intensity of protein banding patterns and protein biosynthesis due to toxicant stress resulting to alters the functioning of cellular and structural proteins hence typical metabolic function is impaired. CPF affects all the protein subunits of high and low molecular weight proteins this could be due to peptides with high molecular weight proteins greater than 50kDa usually having multiple domains which fold independently (Erickson, 2009) whereas in low molecular weight 10 to 30kDa proteins, peptide folding results in a single domain. Each domain leads to a compact three-dimensional structure and is a functional, structural component and are homologous sequence unit of an evolutionary system that may act independently on a structural level. domain arrangements their structural modifications result in proteins with varied functionality (Wang et al., 2011) Thus CPF can degrade protein subsequently at both sub-lethal concentrations, it attacks low molecular weight proteins and high molecular weight proteins, that is multiple domain proteins and single protein domains as well. Throughout the exposure time, the protein subunits displayed a consistent decreasing trend in the intensity of all the fractions, indicating a chlorpyrifos inhibitory effect on *Cyprinus carpio,* therefore it is categorized as a hazardous insecticide in the world (ITRC, 1989).

The current findings are in agreement with the observations on the same pesticide CPF, Slaninova (2009)stated that as the concentration of pesticide chlorpyrifos in goldfish Carassius auratus (var.,) auratus was increased resulting in the alterations in protein banding patterns of liver tissue. Similar observations for other toxicants on different fishes, including a decrease in the intensity of protein banding pattern in the tissues and the fading/disappearance of some protein subunits. (El-Sherif et al., 2009; Suneetha et al., 2010; Bheem Rao et .al., 2018; Florence Borgia et al., 2019). Some observations show both the appearance and disappearance of new protein subunits (Firat and Kargin, 2010; Arivu et al., 2015; Sobha et al., 2017). All these reviews uphold our current examination, depletion in total protein and decreased expression of protein patterns in tissues exposed to chlorpyrifos implies a degradation of proteins due to the toxic stress of pesticides, and also it could be due to hormonal imbalance, impaired tissue repair which affects the protein levels in tissues, or maybe hepatocytic necrosis of cells which subsequently dysfunction the protein biosynthesis.

Conclusions

The comparison of the 96 h LC_{50} values between fish species shows that *Cyprinus carpio* is more sensitive to chlorpyrifos and the current study found that administering sublethal concentrations to fish is harmful and causes changes in their protein patterns. The considerable alteration of protein subunits shows that chlorpyrifos may interact with peptide sequences in *C.carpio* directly or indirectly thereby altering the structural and functional confirmations of cellular proteins. Changes in these characteristics may provide an early warning signal for determining pesticide toxicity and its impact on aquatic species. It would be very advantageous in assessing the associated environmental risk of these pesticides and thus establish subsequent management strategies for safeguarding aquatic organisms and their associated fauna.

References:

1. Anita B, Yadav AS, Cheema N (2016) Genotoxic effects of chlorpyrifos in freshwater fish Cirrhinus mrigala using micronucleus assay. Advances in Biology 1-6. https://doi.org /10.1155/2016/9276963
2. Adedeji, O.B., Adedeji, O.A., Adeyemo, O.K. and S>A>Agbede.(2008).Acute

toxicity of diazinon to the African catfish(*Clarias gariepinus*) African Journal of Biotechnology. 7(5): 651-654.

3. Aitte, S. A. (2018) . Study the Effect of the Duration Exposure for Lethal and Sub-Lethal Concentrations of Organophosphorus Chlorpyrifos Pesticide for Freshwater Fish Common Carp (Cyprinus carpio) (Linn) and Liza abu (Haeckel) Fishes. J. Chem. Pharm. Res., 10(6): 48-57.
4. Alagoa KJ, Ekweozor, IKE, (2009) Sub-lethal effect of the dispersant Goldcrew on selected blood parameters of the African catfish *Clarias gariepinus*. Toxicology and Environmental Chemistry 9: 339-343. https://doi.org/10.1080/02772240802138036
5. Arbuckel TE, Server LE (1998) Pesticide exposure and fetal death: a review of the epidemiological literature. Crit Rev Toxicol 28(3):229-270 https://doi.org /10.1080/104084 49891344218
6. Arivu, I, Muniyan M, Muthulingam M, Parthiban P, Ambedkar G, Kamalkanth S, Anbarasan R, (2015) Effect of 2, 4-dichloro phenoxy acetic acid on protein changes of freshwater fingerlings *Labeo rohita* (Hamilton) under SDS-PAGE gel separation. Int J Toxicol Appl Pharmacol 5:7-13.
7. APHA, AWWA and WPCP, 2005. Standard methods for the examination of water and wastewater. 21 ed. American Public Health Association, Washington, DC.
8. Bheem Rao T, Thirupathi K, Venkaiah Yanamala, Effect of Methyl Parathion (An Organophosphate) on Electrophoretic Patterns of Proteins in Gill and Muscle of Freshwater cat Fish Heteropneustes fossilis (Bloch). World J Pharm Res., 2018: 7(9):899-908.
9. Bradley BP, Shrader EA, Kimmel DG, Meille JC (2002) Protein expression signatures: An application of proteomics. Mar. Environ. Res 54(3), 373-377. https://doi.org/10.1016/s0141-1136(02)00115-0
10. Clark, J.R., James, M., Patrick, J., Douglas, P.M., James, C.M.(1985) Relative sensitivity of six estuarine fishes to carbophenothion, chlorpyrifos and fenvalerate. Ecotoxicol.Env. Saf,10:382-390. https://doi.org/10.1016/0147-6513(85)90083-1.
11. Díaz, R.K.J.G., Girón, P.M.I.(2014). Effect of chlorpyrifos on the immune response of Nile tilapia (Oreochromis niloticus). Rev. Bio Cien,3(1):59-64.
12. Dowling VA, Sheehan D, (2006) Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6(20):5597-560 https://doi.org/10.1002/pmic.200600274
13. Erickson HP, Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. Bill. Proceed. Online, 2009:11:32-51.
14. Firat Ö, Kargin F, (2010) Response of *Cyprinus carpio* to copper exposure: alterations in reduced glutathione, catalase and proteins electrophoretic patterns. Fish Physiol Biochem 36(4): 1021–1028 https://doi.org/10.1007 /s10695-010-9380-0
15. Finney, D.J., 1971. Probit Analysis. Cambridge University Press, Cambridge. 333.
16. Florence Borgia VJ, Thatheyus AJ, Murugesan AG, (2019) Impact of electroplating industry effluent on the electrophoretic protein pattern of serum in the freshwater fish *Cyprinus carpio*. Indian J Biochem Biophys 56(6):460-465
17. Garaj-Vrhovac V, Zeljezic D (2000) Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay. Pesticide genotoxicity revealed by comet assay. Mutat Res 469(2):277-285 https://doi.org /10.1016/s1383-5718(00)00092-9
18. Gül A.(2005) Investigation of acute toxicity of chlorpyrifos-methyl on Nile tilapia (Oreochromis niloticusL.) larvae. Chemo,59(2):163-166.
19. Goel A, Danni V, Dhawan DK (2005) Protective effects of zinc on lipid peroxidation Antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. *Chem. Biol. Interact* 156: 131-140. https://doi.org/10.1016 /j.cbi.2005.08.004

20. Industrial Toxicology Research Centre (ITRC), Toxicity Data Handbook: Pesticide-B TDS, 1989:192, 4, pp. 458–463.
21. Johnson, W.W and Finley, M.T.(1980). Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. Resources Publications 137, Fish and Wildlife Service, U.S.D.I., Washington, D.C:98.
22. Kelso, J.R.M., Shaw, M.A., Minns, C.K. and Mills, K.H.(1990). An evaluation of the effects of atmospheric acidic deposition on fish and the fishery resource of Canada. Can. J. Fish. Aquat. Sci., 47(3), 644-655.
23. Kumar R, Nagpure NS, Kushwaha B, Srivastava SK, Lakra WS (2009) Investigation of the genotoxicity of malathion to fresh water teleost fish *Channa punctatus* (Bloch) using the micronucleus test and connect assay. Arch Environ Contain Toxicol 58(1):123-130 https://doi.org/10.1007/s00244-009-9354-3
24. Laemmli UK, (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685 https://doi.org/10.1038/227680a0.
25. Levin ED, Swain HA, Donerly S, Linney E (2004) Developmental chlorpyrifos effects on hatchling zebrafish swimming behavior. *Neurotoxicol Teratol* 26:719-723. https://doi.org/10.1016/j.ntt.2004.06.010
26. Leiss JK, Savitz DA (1995) Horne pesticide use and childhood cancer: a case control study. Am J Public Health 85:249-253 https://dx.doi.org/10.2105%2Fajph.85.2.249
27. Lyndon AR, Houlihan DF, (1998) Gill Protein Turnover: Costs of Adaptation. Comp Biochem Physiol A Mol Integr Physiol 119A(1):27-34 https://doi.org/10.1016/s1095-6433(97)00409-1
28. Mehta A, Verma RS, Srivastava, N (2008) Oxidative DNA damage induced by chlorpyrifos in rat tissues. *Environ. Mol. Mutagen* 49: 426-433.https://doi.org/10.1002/em.20397
29. Muhammad, OI, Mahmoud UM, Fazio F, Sayed AEH. SDS-PAGE technique as biomarker for fish toxicological studies. Toxicology Report, 2018:5:905- 909.
30. Nesatyy VJ, Suter M.J-F, (2007) Proteomics for the analysis of environmental stress responses in organisms. Environ Sci Technol 41(20):6891-6900 https://doi.org/10.1021/es070561r
31. Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, Srivastava SK, (2010) Mutagenic and Genotoxic effects of Carbosulfan in fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem Toxicol 48(1):202-208 https://doi.org/10.1016/j.fct.2009.09.041.
32. Okechukwu, O.E,, Usman, .I.B, Jehu, A.(2013). Investigation of acute toxicity of chlorpyrifos-ethyl on Clariasgariepinus (Burchell, 1822) using some behavioral indices. Int. J. Basic Appl. Sci,2(2):176-183. https://doi.org/10.14419 /IJBAS. V2I2.778.
33. Padmanabha, A., Reddy, H.R.V., Khavi M Prabhudeva, K.N., Rajanna, K.B., Chethan, N. (2015). Acute effects of chlorpyrifos on oxygen consumption and food consumption of freshwater fish, Oreochromis mossambicus (Peters). Int. J. Rec. Sci. Res.,6(4):3380-3384.
34. Poet TS, Wu H, Kousba AA, Timchalk C (2003) *In vitro* rat hepatic and intestinal metabolism of the organophosphate pesticides chlorpyrifos and diazinon. *Toxicol. Sci* 72: 193-200. https://doi.org/10.1093/toxsci/kfg035
35. Ramesh Halappa, and Muniswamy David. (2009) . Behavioural Responses of the Freshwater Fish, Cyprinus carpio (Linnaeus) Following Sublethal Exposure to Chlorpyrifos. Turk. J. Fish. Aquat. Sci. 9: 233-238.
36. Reddy, N.M., Ghousia, B., Rajender K, Rao, J.V. (2012). Toxic impact of two organophosphate insecticides on biochemical parameters of a food fish and assessment of recovery response. Toxicol. Ind. Heal.,1-10. https://doi.org/10.1177/0748233711412423.
37. Schimmel, S.C., Garnas, R.L., Patrick, J.M., Moore, J.C.(1983). Acute toxicity, bioconcentration, and persistence of AC

222,705, benthiocarb, chlorpyrifos, fenvalerate, methyl parathion, and permethrin in the estuarine environment. J. Agri. Food Chem., 31(1):104-113. https://doi.org/10.1021/jf00115a027

38. Sledge D, Yen J, Morton T, Dishaw L, Petro A, Donerly S, Linney E, Levin ED (2011) Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. *Neurotoxicol Teratol* 33: 742-751. https://doi.org/10.1016/j.ntt.2011.06.005
39. Slaninova AM, Smutan H, Modra Z, Svobodova ZA. A review: oxidative stress in fish induced by pesticide. Neuro. Endocrinol. Lett,2009:30(1):2-12.
40. Slotkin TA, Olivier CA, Seidler FJ (2005) Critical periods for the role of oxidative stress in the developmental neurotoxicity of chlorpyrifos and terbutaline, alone or in combination. *Dev.Brain Res* 157: 172-180. https://doi.org/10.1016/j.devbrainres.2005.04.001
41. Sundaram, K.M.S., Sundaram, A., Hammock, B.D. (1994). Persistence of Bacillus thuringiensis deposits in a hardwood forest, after aerial application of a commercial formulation at two dosage rates. J. Environ. Sci. Health Part B,29(5):999-1052.
42. Sharbidre, A.A., Metkari, V., Patode P. (2011) Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, Poecilia reticulate. Pesticide Biochemistry and Physiology,101: 132-141. http://dx.doi.org/10.1016/j.pestbp.2011.09.002.
43. Sherif-EI MS, Ahmed MT, EI-Danasoury MA, Nagwa HK, El-Nwishy (2009) Evaluation of Diazinon toxicity on Nile Tilapia fish. J Fish Aquat Sci 4(4):169-177 http://dx.doi.org/10.3923/jfas.2009.169.177.
44. Sobha K, Yamini Sarada N, Anita Susan T (2017) An evaluation of the alterations in protein content, total free amino acids and protein profiles of some major tissues of the edible carp, *Labeo rohita* (Hamilton) exposed to nitrogenous compounds. Int J Fish Aquat Stud 5(5):417-424.
45. Suneetha, K., Kumar, K.G., and Veeraiah, K (2010).Changes in protein subunits induced by endosulfan and fenvaleratein fresh water fish Labeo rohita through SDSPAGE. Journal of Environmental Biology, 31(5):759-763.
46. Tilak, K.S., Veeraiah, K., Rao, D.K. (2004). Toxicity and bioaccumulation of chlorpyrifos in Indian carp Catlacatla (Hamilton), Labeo rohita (Hamilton), and Cirrhinusmrigala (Hamilton). Bull Environ Contam Toxicol,73(5):933-941.
47. Tomlin CDS, ed (2000) The pesticide manual. 12th ed. London: The British Crop Protection Council.
48. Ullah, S. (2015) Protective role of vitamin C against Cypermethrin induced toxicity in Labeo rohita(Ham.): Biochemical aspects. Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.
49. Verma, V.K., Saxena, A. (2013) Investigations on the acute toxicity and behavioral alterations induced by the organophosphate pesticide, chlorpyrifos on Puntius chola (Hamilton-Buchanan). Ind. J. Fish,60(3):141-145.
50. Wang M, Wang Y, Wang J, Lin L, Hong H, Wang D (2011) Proteome profiles in medaka (*Oryzias melastigma*) liver and brain experimentally exposed to acute inorganic Mercury. Aquat Toxicol 103(3-4):129-139 https://doi.org/10.1016/j.aquatox.2011.02.020
51. WHO Commission on Health and Environment & World Health Organization. (1992) Our planet, our health: report of the WHO Commission on Health and Environment. World Health Organization https://apps.who.int/iris/handle/10665/37933 (accessed on 11 April 2022).
52. Xing H, Li S, Wang S, Gao X, Xu X, Wang X (2012) Oxidative stress response and histopathological changes due to atrazine and chlorpyrifos exposure in common carp. *Pest. Biochem. Physiol* 103: 74-80. https://doi.org/10.1016/j.pestbp.2012.03.007

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Conflict Of Interest

The authors declare that they have no conflicts of interest.