Fishery Technology 62 (2025) : 52 - 62

Assessment of the Toxicological Effects of Cypermethrin Nanoformulation on *Hypselobarbus pulchellus* using Selected Biomarkers

Jesna Parakkandi[1], Basanta Kumar Das[2*], Dhruba Jyoti Sarkar[2], Kishore Kumar Krishnani[3], Narinder Kumar Chadha[4] and Kannur Hemaprasanth[5]

[1] *Regional Centre of ICAR- Central Inland Fisheries Research Institute, Bangalore, India-560089*
[2] *ICAR - Central Inland Fisheries Research Institute, Barrackpore, Kolkata, India- 700120*
[3] *ICAR - Indian Institute of Agricultural Biotechnology, Ranchi, Jharkhand, India-834003*
[4] *ICAR - Central Institute of Fisheries Education, Versova, Mumbai, India - 400061*
[5] *Regional Research Centre, ICAR- Central Institute of Freshwater Aquaculture, Bangalore, India-560089*

Abstract

Pesticides are widely used in aquaculture farms against ectoparasites. However, due to the development of resistance in ectoparasites against the traditional pesticide formulations, pesticides are being applied at a higher dose or in combinations. Nano formulation of pesticides with enhanced properties is a viable option to reduce the volume of traditional pesticides applied in aquaculture. Hence, the present study developed a nano formulation of the pesticide cypermethrin and evaluated the toxic effects in the fish *Hypselobarbus pulchellus*. The acute toxicity study recorded the mortalities at 24h intervals for 96h. The 96h LC_{50} value was estimated to be 0.003 mg L^{-1}. Chronic toxicity studies were also conducted exposing *H. pulchellus* to sublethal concentrations for 45 days. The brain acetylcholine esterase activity was significantly reduced ($p<0.05$) in the cypermethrin nano formulation exposed fish. The nano formulation exposure also caused a significant increase in the liver and kidney superoxide dismutase activity. Elevated catalase and glutathione-s-transferase levels were observed in the liver and gills, with no conspicuous change in the kidneys. The serum enzymes aspartate aminotransferase and alanine aminotransferase were elevated significantly by cypermethrin exposure. The serum triglycerides, cholesterol, and protein content were reduced significantly. Similarly, the red blood cell, haematocrit, haemoglobin, and platelet count also showed significant reduction on cypermethrin exposure. These findings on the toxic impacts may help to understand the effect of nano-formulated antiparasitic drugs in fin fish.

Keywords: Superoxide dismutase, glutathione-s-transferase, catalase, haematology

Introduction

Aquaculture has emerged as a critical sector for global food security, particularly in countries like India, where it significantly contributes to the economy and sustenance of rural livelihoods. However, the intensification of aquaculture practices has also led to the increased use of pesticides to manage ectoparasites and other pests. One such pesticide, cypermethrin, a synthetic pyrethroid, is widely employed in aquaculture due to its high efficacy against a broad spectrum of pests. Cypermethrin affects the normal operation of the nervous system, causing paralysis and death of the targeted pests (Akter et al., 2024).

The indiscriminate use of pesticides at high doses and the application of combination pesticides to combat resistant ectoparasites often leads to ecological imbalance (Talebi, Hosseininaveh, & Ghadamyari, 2011). In this scenario, nanotechnological interventions could be a viable option to develop pesticide formulations with enhanced properties compared to the traditional formulations. Nanoformulations of pesticides offer enhanced stability, controlled re-

Received 17 July 2024; Revised 7 August 2024; Accepted 9 August 2024

Handling Editor: Dr. B. Madhusudana Rao

*Email: basantakumard@gmail.com

lease, and improved efficacy (Elabasy, Shoaib, Waqas, Jiang, & Shi, 2019; El-Gendy, El-Banna, El-Sabagh, El-Kareem, & Ibrahim, 2020). However, the toxicological effects of these nanoformulations on aquatic biota remain insufficiently understood.

Toxicity assessments are conducted via acute and chronic exposure studies. Acute toxicity evaluates the lethal concentration (LC_{50}) of a substance required to cause mortality in 50% of a test population over a short period, providing crucial insights into the toxicity of the substance (Bhatt et al., 2024). In contrast, chronic toxicity studies examine the sub-lethal effects of prolonged exposure to toxicants, utilizing biomarkers such as enzymatic activities to assess physiological impacts. Biochemical biomarkers such as enzyme activities (acetylcholinesterase, catalase, superoxide dismutase) and glutathione levels provide insights into the organism's metabolic and oxidative stress responses due to the sub-lethal effects of contaminants, facilitating a comprehensive understanding of their toxicological impact (Livingstone, 2001). Studies have confirmed that the potential harmful effects of various pesticides on fish include antioxidative stress and the disruptions in antioxidant defense mechanisms (Atli & Canli, 2007; Mishra & Srivastava, 2015). Specifically, nanoformulations of pesticides, despite their enhanced properties, may pose unique risks due to their increased bioavailability and reactivity (Elabasy et al., 2019).

Hypselobarbus pulchellus, a native freshwater fish of India, has been successfully bred, and its larval rearing has been standardized (Sridhar, Raghunath, Hemaprasanth, Raghavendra, & Ekanath, 2014). As a candidate species for aquaculture, assessment of the impacts of using cypermethrin on its health and welfare can help in shaping such effects on other species and culture systems. In light of this, the current study investigates the toxicological effects of cypermethrin on *H. pulchellus* by employing biochemical, molecular, and physiological markers. The research aims to elucidate the potential risks posed by cypermethrin nanoformulation as a parasiticide in aquaculture systems.

Materials and Methods

For the preparation of cypermethrin nanoformulation, cypermethrin 90% TC was sourced from Tagros Chemicals India Pvt. Ltd. Polyethylene glycol (PEG) (molecular weight = 200 g mol^{-1}) was obtained from Merck, India. All other chemicals used in the study were sourced from Merck and Himedia, India.

The nanoformulation of cypermethrin was prepared following the method described by Sarkar, Bera, Baitha, and Das (2022) with modifications. In the current study, the cypermethrin was encapsulated using polyethylene glycol. In brief, cypermethrin (90%) was diluted with dichloromethane in a 1:1 ratio. PEG was then added and stirred for a minimum of 24h to ensure complete evaporation of dichloromethane traces. The resulting slurry (10% active ingredient) was added to deionized water to create a nanoemulsion at a concentration of 1000 mg L^{-1} of cypermethrin. After preparation, the characteristics of the nanoemulsion, such as the micellar size and zeta potential were measured using a Dynamic Light Scattering instrument, Zetasizer Ver. 7.12, Malvern Instruments Ltd. For testing short-term toxicity and chronic exposure studies, a 1000 mg L^{-1} stock solution was prepared and was serially diluted to achieve the required concentrations for the experiments.

H. pulchellus fingerlings were procured from ICAR-Central Institute of Freshwater Aquaculture, Regional Centre at Bangalore. After transportation, the fish underwent a two-week acclimatization period in 165 L glass tanks provided with continuous aeration. Fish were fed commercial pellet feed at 3% of body weight per day. Fingerlings used for the study were sourced from the acclimatization tank.

To ascertain the range of concentrations causing mortality, a range-finding test was conducted following Reish and Oshida (1987) protocols. The lethal concentrations were determined according to OECD (1992) recommendations. *H. pulchellus* fingerlings were exposed to successive doses of 0.001, 0.01, 0.1, 1.0, 5.0, and 10 mg L^{-1} on a log scale. Water temperature was maintained between 22.7°C and 23.2°C with dissolved oxygen levels at 6.5-7.6 mg L^{-1}. Ten fish were stocked per tank in triplicates, and mortality was monitored at 24h, 48h, 72h, and 96h intervals.

Following range-finding, the tanks were set with nanoformulation dosage of 0.001, 0.003, 0.005, 0.007, and 0.009 mg L^{-1}, along with a control in triplicate, to determine the median lethal concentration (LC_{50}). Based on the static bioassay approach (Reish & Oshida, 1987), ten fingerlings were placed in each tank, with no medium changes during exposure.

Tanks housing control fish were separated from test tanks to prevent cross-contamination, and all equipment used was meticulously cleaned. Mortality was assessed at 24, 48, 72, and 96h intervals, with careful observation for clinical signs and abnormalities. Dead fish were promptly removed during each observation to avoid contamination of the water. The safe application limit was determined as per Hart, Weston, and Demann (1948) guidelines.

The chronic exposure tests were conducted for a period of 45 days. Two sub-lethal doses were evaluated: $1/10^{th}$ (0.0003 mgL^{-1}) and $1/50^{th}$ (0.00006 mgL^{-1}) of the anticipated 96h LC_{50} (CP1 and CP2, respectively). To maintain control fish (control = CP), toxin-free water was utilized. The treatment and control fish were housed in 165 L glass tanks with 20 fish per tank. To ensure a consistent cypermethrin concentration, 50% of the medium was replaced in three days interval, with continuous aeration of the tanks. Fish were fed with commercial pellet feed at 3% body weight per day. Basic water quality indicators, including temperature, pH, specific conductivity, total dissolved solids, and dissolved oxygen, were monitored at 15 days interval using a multiparameter water quality probe (YSI ProDSS®). Additionally, total alkalinity and hardness were assessed in accordance with APHA (2012) standards.

Fish were regularly monitored for feed consumption and behavioural abnormalities, if any. Samples were collected in the beginning and followed by every 15 days over a period of 45 days. During sampling, the fish were carefully removed from the tanks, anaesthetized using MS-222 at a dose of 1 mg L^{-1}, and their tissues were collected meticulously for further analysis.

Fish were anaesthetized prior to blood collection to minimize handling stress. Blood was drawn from the vein at the caudal peduncle using a 1.5 mL hypodermal syringe containing 1% EDTA as an anticoagulant. Blood samples collected from 3–4 fish per tank were pooled for further analysis. A Sysmex XP-100 differential cell counter was used to estimate red blood cell (RBC) count, haemoglobin (HGB) content, haematocrit (HCT) value, white blood cell (WBC) count, platelets (PLT), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC). Serum biochemical characteristics were analyzed using a Transasia-Erba® EM-200 serum biochemical analyzer, assessing the metabolites, such as serum total protein, cholesterol (CHO), glucose, triglycerides, and the enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

The liver, brain, kidney, and gill samples were dissected out from the fish for enzyme analysis. Tissues were homogenized using a 0.25 M sucrose solution, centrifuged, and the supernatant was obtained and stored at -20°C for further analysis. Total protein content was determined (Lowry, Rosebrough, Farr, & Randall, 1951), and the activities of antioxidant enzymes such as superoxide dismutase - SOD, catalase - CAT, glutathione-s-transferase - GST and the enzyme acetylcholine esterase (AChE) were measured using standard methods following Misra and Fridovich (1972); Takahara et al. (1960); Mannervik et al. (1985); Dietz, Rubenstein, and Lubrano (1973) for assessing SOD, CAT, GST, and AChE, respectively. The change in absorbance was recorded using an ELISA-cum-Spectro reader (BioTekEpochTM2 microplate reader) and the enzyme activity was expressed as U mg^{-1} protein.

SPSS 16 software was used for finding the lethal concentration values. Mean cumulative mortalities were calculated for each experimental unit, and LC_{50} values were determined using probit analysis. The rest of the statistical analysis was performed by R-4.1.0 software. The significant differences ($p<0.05$) in means of the variables between the treatment and control groups at every time period and between the exposure periods were estimated by one-way analysis of variance (ANOVA). If the ANOVA was significant, the Tukey HSD (Tukey Honest Significant Differences) test was applied for multiple comparisons (pairwise) between the means of groups. The homogeneity of variances in the different treatment groups was tested by Levene test at 0.05 significance level, and the normality assumption was tested by Shapiro-Wilk's test. T test was followed to understand the difference in variables such as haematological and serum parameters between the time periods.

Results and Discussion

In cypermethrin nanoemulsion, the hydrodynamic diameter of the micelle was estimated to be 501.3±52.3 nm, with a polydispersity index of 0.142. The zeta potential was estimated to be -10.8±1.38

mV, indicating improved emulsion stability (Sarkar et al., 2022).

Selected fingerlings, averaging 7.7±1.62 g in weight and 9.6±1.2 cm in length, were chosen for the toxicity studies. From the mortality data of *H. pulchellus* exposed to cypermethrin nanoformulation, the 24h and 96h LC_{50} values were estimated to be 0.004 mg L^{-1} and 0.003 mg L^{-1} respectively. The safe application limit of cypermethrin nanoemulsion in *H. pulchellus* was estimated to be 0.00051 mg L^{-1}. Ural and Calta (2005) recorded 96h LC_{50} as 2-4 μg L^{-1} in *Cyprinus carpio* exposed to cypermethrin, which is in accordance with the present findings. During the acute toxicity studies, the fish showed erratic swimming activities and high opercular movement. A few minutes of abnormal swimming activities were followed by the termination of body movement. The elevated opercular movement was counted at up to 82-128 times $minute^{-1}$, which then reduced to 28-33 times $minute^{-1}$. By 48h, 100% mortality occurred in all three higher concentrations (0.005, 0.007, and 0.009 mg L^{-1}). The marked alterations in behavioural pattern of *H. pulchellus* were in agreement with the observations in *Channa punctatus* exposed to cypermethrin (Kumar, Singh, Singh, & Singh, 2007).

Table 1. Haematological parameters of *H. pulchellus* exposed to cypermethrin nanoemulsion at two sub-lethal concentrations: CP1 = $1/10^{th}$ of 96h LC_{50}, CP2 = $1/50^{th}$ of 96h LC_{50}, and CP = control, for 45 days

Haematological Parameters	Treatments	Initial	45th Day
WBC ($x10^3$ no. μL^{-1})	CP1	$168.76^{aA} \pm 8.9$	$164.3^{aA} \pm 9.13$
	CP2	$164.9^{aA} \pm 11.5$	$168.45^{aA} \pm 7.5$
	CP	$171.54^{aA} \pm 7.6$	$173.8^{aA} \pm 12.1$
RBC ($x10^6$ no. μL^{-1})	CP1	$3.12^{aA} \pm 0.3$	$1.89^{bB} \pm 0.16$
	CP2	$3.2^{aA} \pm 0.31$	$1.83^{bB} \pm 0.17$
	CP	$3.09^{aA} \pm 0.3$	$3.11^{aA} \pm 0.29$
HGB (g dL^{-1})	CP1	$5.1^{aA} \pm 0.34$	$4.05^{bB} \pm 0.32$
	CP2	$4.9^{aA} \pm 0.46$	$4.12^{bB} \pm 0.29$
	CP	$4.88^{aA} \pm 0.45$	$5.09^{aA} \pm 0.49$
HCT (%)	CP1	$25.05^{aA} \pm 2.12$	$14.33^{bB} \pm 1.5$
	CP2	$27.23^{aA} \pm 2.3$	$17.72^{bB} \pm 1.11$
	CP	$28.19^{aA} \pm 2.5$	$29.3^{aA} \pm 2.5$
MCV (fL)	CP1	$115.3^{aA} \pm 8.1$	$94.7^{bB} \pm 9.5$
	CP2	$102.5^{aA} \pm 7.9$	$95.3^{bB} \pm 4.53$
	CP	$118.9^{aA} \pm 10.2$	$106.3^{aA} \pm 9.1$
MCH (pg)	CP1	$23.9^{aA} \pm 2.2$	$21.44^{aA} \pm 2.1$
	CP2	$26.8^{aA} \pm 1.6$	$22.73^{aA} \pm 1.8$
	CP	$24.5^{aA} \pm 2.3$	$22.8^{aA} \pm 1.2$
MCHC (g dL^{-1})	CP1	$23.9^{aA} \pm 2.31$	$20.5^{aA} \pm 1.9$
	CP2	$23.5^{aA} \pm 1.98$	$21.4^{aA} \pm 2.1$
	CP	$21.89^{aA} \pm 1.8$	$23.18^{aA} \pm 2.1$
PLT ($x10^3$ no. μL^{-1})	CP1	$72.32^{aA} \pm 4.8$	$45.11^{bB} \pm 7.2$
	CP2	$74.91^{aA} \pm 6.2$	$43.8^{bB} \pm 4.1$
	CP	$71.48^{aA} \pm 5.8$	$73.14^{aA} \pm 4.2$

Mean (mean ± standard deviation) values with different alphabets a, b, and c denote the significant difference between CP1, CP2 and CP during the same exposure period, and the alphabets A, B, and C indicate a significant variation in the means of the same treatment at different exposure periods (95% confidence level). HGB = haemoglobin; HCT = haematocrit; WBC = white blood cells; RBC = red blood cells; PLT = platelets; MCH = mean corpuscular haemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular haemoglobin concentration.

Haematological parameters of *H. pulchellus* exposed to cypermethrin are shown in Table 1. The results show that there was no significant difference ($p>0.05$, one-way ANOVA) in the WBC count of nanoemulsion exposed fish compared to the control and to the initial values (as evidenced by the results of the t-test), but the RBC, HGB, HCT, MCV, and platelets showed significant reduction ($p<0.05$) in the exposed fish compared to the control (one-way ANOVA) on the 45th day and compared to the initial values (t test). However, MCH and MCHC values did not vary significantly ($p>0.05$) when compared to the control fish and the initial values.

Chronic toxicity is assessed through different biomarkers in fish. Haematological parameters are such indicators that are often used to assess fish health and welfare since they have been shown to be very sensitive (Kim & Kang, 2016) to a variety of factors such as nutrition, water quality, stress, and infections. Singh and Srivastava (2010) have well documented the use of haematological parameters as bioindicators of insecticide exposure. In the present study, the presence of the cypermethrin did not induce a considerable variation in the WBC count. The immune system of fish can be resilient to certain toxicants, showing no significant changes in WBC counts. A study by Yildirim and Benli (2013) found that exposure to sub-lethal concentrations of pesticides did not cause significant changes in WBC counts in fish, suggesting that the immune system might not be highly sensitive to these compounds at lower doses. However, the RBC, HGB, HCT, MCV, and platelets showed significant reduction, indicating an altered erythropoiesis and oxygen transport capacity due to the presence of the toxicant. Reduced HCT levels might be attributed to the haemolysis or impaired erythropoiesis. Martinez,

Table 2. Serum biochemical parameters of *H. pulchellus* exposed to nanoemulsion at two sub-lethal concentrations: CP1=1/10th of 96h LC_{50}, CP2=1/50th of 96h LC_{50}, and CP = control, for 45 days

Serum biochemical Parameters	Treatments	Initial	45th Day
Total protein (g dL^{-1})	CP1	$2.19^{aA} \pm 0.2$	$1.74^{bB} \pm 0.06$
	CP2	$2.57^{aA} \pm 0.13$	$1.9^{bB} \pm 0.13$
	CP	$2.43^{aA} \pm 0.18$	$2.51^{aA} \pm 0.22$
Triglyceride (mg dL^{-1})	CP1	$121.5^{aA} \pm 11.2$	$82.4^{bB} \pm 7.4$
	CP2	$119.65^{aA} \pm 9.6$	$85.3^{bB} \pm 8.1$
	CP	$124.7^{aA} \pm 10.4$	$123.8^{aA} \pm 10.2$
Glucose (mg dL^{-1})	CP1	$47.5^{aA} \pm 4.2$	$69.3^{bB} \pm 5.7$
	CP2	$43.6^{aA} \pm 3.1$	$70.9^{bB} \pm 6.2$
	CP	$46.3^{aA} \pm 3.1$	$48.19^{aA} \pm 4.1$
CHO (mg dL^{-1})	CP1	$124.2^{aA} \pm 12.1$	$92.1^{bB} \pm 5.1$
	CP2	$129.62^{aA} \pm 10.4$	$96.6^{bB} \pm 9.2$
	CP	$128.9^{aA} \pm 10.1$	$131.4^{aA} \pm 11.2$
ALT (U L^{-1})	CP1	$4.29^{aA} \pm 0.32$	$10.1^{bB} \pm 0.43$
	CP2	$5.45^{aA} \pm 0.51$	$9.26^{bB} \pm 0.41$
	CP	$4.53^{aA} \pm 0.17$	$5.09^{aA} \pm 0.51$
ALP (U L^{-1})	CP1	$26.5^{aA} \pm 2.5$	$30.4^{aA} \pm 2.55$
	CP2	$31.6^{aA} \pm 1.81$	$28.5^{aA} \pm 1.9$
	CP	$29.4^{aA} \pm 1.57$	$27.9^{aA} \pm 2.18$
AST (U L^{-1})	CP1	$245.6^{aA} \pm 12.9$	$356.64^{bB} \pm 14.4$
	CP2	$259.8^{aA} \pm 25.3$	$349.9^{bB} \pm 11.2$
	CP	$233.49^{aA} \pm 21.5$	$248.12^{aA} \pm 9.5$

Mean (mean ± standard deviation) values with different alphabets a, b, and c indicate the significant difference between CP1, CP2 and CP within the same exposure time, and the alphabets A, B, and C indicate a significant variation in the variable means of the same treatment during different exposure periods at 95% confidence level. CHO = cholesterol; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase.

Souza, and Valenti (2004) have demonstrated significant reductions in HCT values in fish exposed to various toxicants, indicating possible anaemic conditions or reduced red blood cell production. A reduction in platelet count can be indicative of a physiological response to stress. In accordance with the present observation, Kanu, Okoboshi, and Otitoloju (2023) recorded a significant decrease in platelets of *Oreochromis niloticus* and *Clarias gariepinus* exposed to various pesticides such as atrazine, chlorpyrifos, mancozeb, lambda-cyhalothrin, and their combination. Referencing these studies, it becomes evident that the results on haematological parameters are consistent with findings from other research on sub-lethal toxicant exposure.

The MCV measures the average volume of RBCs, while MCH indicates the average mass of haemoglobin per RBC. MCHC represents the average concentration of haemoglobin in a given volume of RBCs. Previous studies have shown that the absolute values of these haematological parameters in fish exposed to various pesticides do not follow a specific alteration pattern (Witeska, Kondera, & Bojarski, 2023; Barathinivas et al., 2022). Pesticidal intoxication can cause alterations in the size of RBCs as well as the shape, thus affecting these absolute values. RBCs with normal morphology exhibit standard MCV values, whereas enlarged or reduced RBCs display higher or lower MCV values, respectively. Such pesticide-induced changes in haematological parameters may result from disruptions in erythropoiesis, haematopoiesis, or iron metabolism (Sinha, Gour, Singh, & Nigam, 2022). In the present study, no significant alterations were observed in MCH and MCHC values. However, at the end of the exposure period, MCV values were significantly lower compared to the control.

Serum biochemical parameters of *H. pulchellus* are represented in Table 2. The results show that the total protein, triglycerides, and CHO were significantly reduced ($p<0.05$) in the cypermethrin-exposed *H. pulchellus* compared to the initial values (as evidenced by the results of the t-test) and compared to the control on the 45th day. The glucose level was significantly ($p<0.05$) higher than the control as well as the initial values. It was also observed that the ALT and AST enzyme activities increased significantly on cypermethrin exposure, while there was no significant ($p>0.05$) variation in ALP enzyme activity.

The reduction in total protein content can be attributed to the disruption of protein synthesis and metabolism due to toxic stress. For example, Al-Ghanim et al. (2016) observed that exposure to certain pesticides led to a significant decrease in total protein content in fish, indicating impaired protein metabolism as a stress response. Likewise, the reduction in triglycerides might be linked to alterations in lipid metabolism under stress conditions. Studies such as those by El-Sheikh and Mohamed (2017) have demonstrated that exposure to pesticides and other toxicants leads to decreased triglyceride levels in fish due to impaired lipid metabolism and increased energy demand to cope with the stress. A significant reduction in cholesterol content has been associated with the interference of lipid metabolism pathways. In consistent with the present findings, fish exposed to various contaminants exhibited decreased cholesterol levels, which were attributed to the effects on liver function and lipid processing (Abdel-Warith, Younis, & Al-Asgah, 2013). Pesticides can interfere with normal metabolic pathways, causing an imbalance in carbohydrate metabolism, leading to an increase in glucose production or a decrease in glucose utilization, both of which result in higher serum glucose levels. The present findings show that there is a significant increase in the serum glucose level in *H. pulchellus*, which is in agreement with the report by Akhtar, Khan, Tabassum, Ahmad, and Badshah (2021).

Elevated levels of serum enzymes ALT and AST indicate liver damage or stress, as this enzyme is released into the bloodstream when liver cells are damaged. Unchanged ALP levels in the presence of liver damage typically suggest that there is no cholestasis or biliary obstruction. In accordance to the present findings, Ural and Calta (2005) reported elevated serum enzyme levels in *L. rohita* exposed to carbofuran and cypermethrin.

The brain AChE activity in *H. pulchellus* exposed to nanoformulated cypermethrin exhibited a significant ($p<0.05$) reduction as represented in Fig. 1. There were around 36.9 and 41.4% reductions in CP1 and CP2 brains, respectively, as compared to the control values at the end of the experiment. There was a significant reduction ($p<0.05$) in the activity in CP1 and CP2 compared to the initial values as well as during the 15th, 30th, and 45th days.

Acetylcholinesterase is an important enzyme for neurotransmission, and its activity can be inhibited

by various pesticides and toxicants, leading to neurotoxic effects. Studies such as those by Fulton and Key (2001) have shown that exposure to certain insecticides and pesticides significantly reduces AChE activity in fish, indicating neurotoxicity and disruption of normal neural function. In the present study, there was a significant reduction in the AChE activity as a result of cypermethrin. Research by Monteiro, Almeida, Rantin, and Kalinin (2006) demonstrated that fish exposed to pesticides, including those with neurotoxic properties, exhibited a marked decrease in AChE activity. This reduction in enzyme activity is a common biomarker for pesticide exposure and neurotoxicity in aquatic organisms.

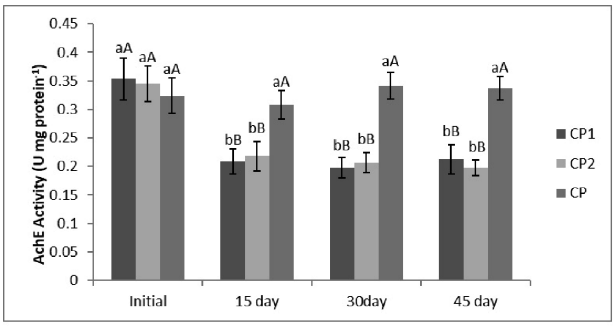

Fig. 1.Brain AChE enzyme activity in *H. pulchellus* exposed to cypermethrin nanoemulsion at two sublethal concentrations (CP1=1/10th of 96h LC_{50}, CP2=1/50th of 96h LC_{50}, and CP = control) for 45 days

Different alphabets a, b, and c indicate the significant difference between CP1, CP2 and CP within the same exposure time, and the letters A, B, and C denote a significant variation in the variable means of the same treatment at different exposure periods at 95% confidence level. The error bars denote standard deviation.

SOD is an enzyme that is responsible for the exclusion of the reactive oxygen species (ROS), the superoxide radicals, in cells. In the absence of SOD activity, these free radical forms hydroxyl radicals (Li et al., 2016) that are the most reactive ROS. As no antioxidant is capable of neutralizing the activity of hydroxyl radicals, the only viable approach is either to prevent their formation or to repair the damage they have already caused (Schieber & Chandel, 2014). The enzymes, CAT and glutathione peroxidase (GP), remove hydrogen peroxide (Devi et al., 2019), which is a non-radical species that can pass through cell membranes and has a longer half-life than the majority of the ROS radicals. In the presence of glutathione, GP can neutralize hydrogen peroxide (Monteiro, Rantin, Kalinin, & Alves, 2006). The enzyme GST acts by catalyzing the conjugation of reduced glutathione and increases the solubility of these molecules in water. Thus, these enzymes are of great significance in the cellular defense against oxidative stress.

Significant ($p<0.05$) induction of liver and gill CAT activity was recorded (Fig. 2a and Fig. 2b) from the

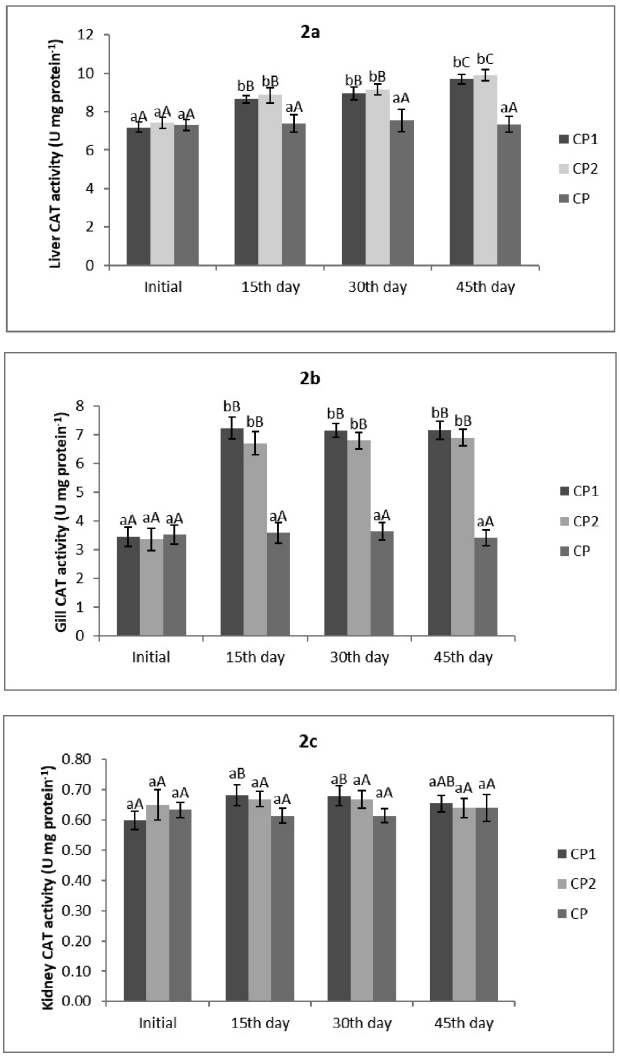

Fig. 2. CAT enzyme activity in liver (2a), gill (2b), and kidney (2c) of *H. pulchellus* exposed to sub-lethal doses of cypermethrin nanoemulsion (CP1=1/10th of 96h LC_{50}, CP2=1/50th of 96h LC_{50}, and CP= control) for 45 days

Different alphabets a, b, and c indicate the significant difference between CP1, CP2 and CP within the same exposure time, and the alphabets A, B, and C denote the significant variations in the variable means of the same treatment at different exposure periods at 95% confidence level. The error bars indicate standard deviation.

15th day compared to the initial values and compared to the control during each exposure period. The liver CAT activity was increased by 31.9 and 34.6%, and the gill CAT activity was increased by 109.9 and 102.1% in CP1 and CP2, respectively, compared to the control on the 45th day. There were no significant differences ($p>0.05$) in the kidneys CAT activity (Fig. 2c).

Significantly high SOD activity ($p<0.05$) was observed in the liver (Fig. 3a) and kidneys (Fig. 3c). When compared to the control, on the 45th day, the liver SOD activity was elevated by 48.9 and 38.2% in CP1 and CP2, respectively. In kidneys, 89.4 and 86.1% higher enzyme activity were estimated in CP1 and CP2, respectively. Compared to the initial values, liver SOD and kidney SOD values were significantly higher during the 45th day ($p<0.05$). Whereas there were no significant differences ($p>0.05$) in the gills as depicted in Fig. 3b.

In *H. pulchellus, the* GST activity increased significantly ($p<0.05$) in the liver (673.9 and 534.8% increase than control values in CP1 and CP2, respectively at the end of the experiment) (Fig. 4a) and gills (127.2 and 115.3% increase in CP1 and CP2, respectively) (Fig. 4b). The liver GST values were higher on the 15th, 30th, and 45th days than the initial values, while the gill GST values showed an exposure-period-dependent variation. There were no significant ($p > 0.05$) alterations in the kidney GST levels of the treatments and control fish as explained in Fig. 4c.

In the present study, the nanoformulation exposure caused a significant increase in the liver and kidney SOD activity. Whereas elevated CAT and GST levels were observed in the liver and gills with no conspicuous change in the kidneys. These observations are in line with the findings of other authors who tested the toxic effects of various pesticides on fish (Prusty et al., 2011; Abdelkhalek, Ghazy, & Abdel-Daim, 2015; Al-Ghanim et al., 2020). High SOD, CAT, and GST activities explain the effects of cypermethrin on stress enzymes that may help cope with the abnormal production of ROS in these tissues. Future research could be oriented to an in-depth analysis of the underlying mechanisms in the variations of the studied parameters in fish under different concentrations of cypermethrin for a comprehensive understanding of the toxic effect of cypermethrin nanoemulsion.

The environmental conditions during the trial were

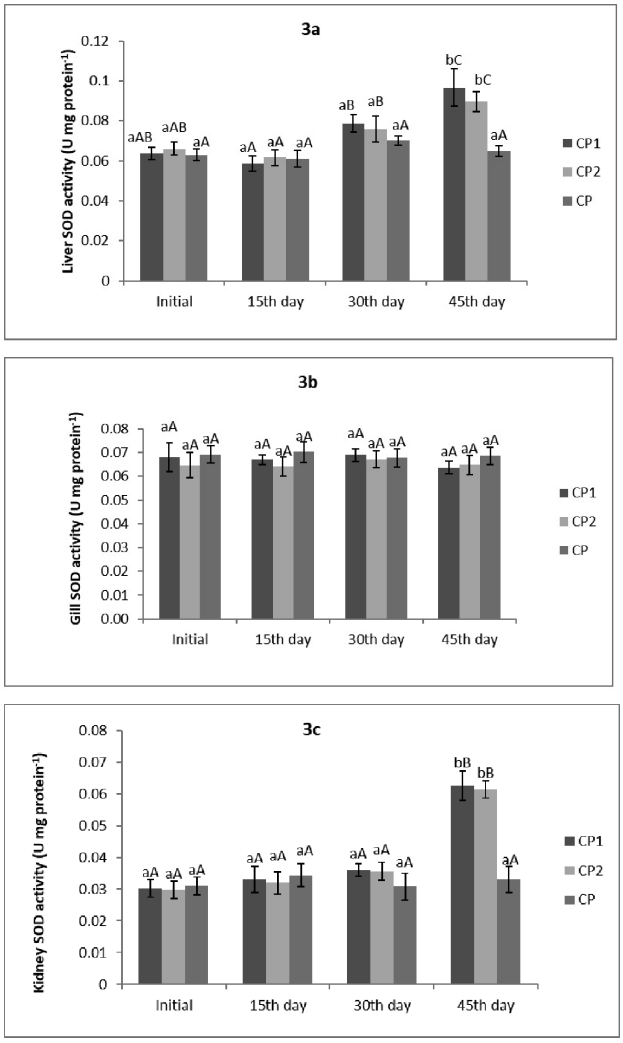

Fig. 3. The SOD enzyme activity in liver (3a), gill (3b), and kidney (3c) of *H. pulchellus* exposed to sub-lethal doses of cypermethrin nanoemulsion (CP1=1/10th of 96h LC_{50}, CP2=1/50th of 96h LC_{50}, and CP= control) for 45 days

Different alphabets a, b, and c indicate the significant difference between CP1, CP2 and CP within the same exposure time, and the alphabets A, B, and C indicate significant variation in the variable means of the same treatment at different exposure periods at 95% confidence level. The error bars denote the standard deviation.

as follows: temperature ranged from 22.6-23.3°C, dissolved oxygen was between 6.9-7.8 mg L^{-1}, and pH levels ranged from 7.3-8.1. The total alkalinity was 135-149 mg L^{-1} and the total hardness was 140-152 mg L^{-1}. It was made sure that all the parameters were in the desirable range for carp aquaculture.

The toxic effects of cypermethrin nanoformulation in *H. pulchellus* were assessed through both acute

and chronic exposure studies for a comprehensive understanding of the toxicity in fish. Chronic exposure studies indicated significant alterations in several biomarkers, particularly in tissue-specific enzyme activity. These findings are crucial for understanding the basic toxic effects on the fish, and similar effects are anticipated in other related species. This preliminary study provides foundational insights into the effects of cypermethrin nanoformulation, paving the way for future research aimed at assessing its efficacy in eradicating ectoparasites.

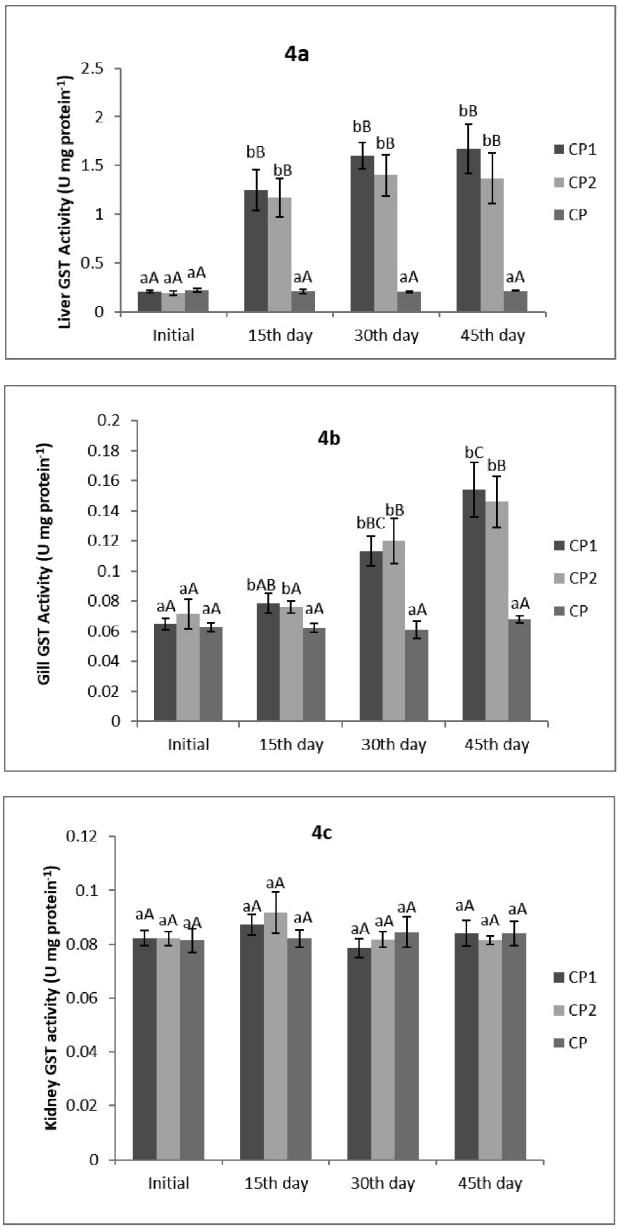

Fig. 4. GST enzyme activity in liver (4a), gill (4b), and kidney (4c) of *H. pulchellus* exposed to sub-lethal concentrations of cypermethrin nanoemulsion (CP1=1/10th of 96h LC_{50}, CP2=1/50th of 96h LC_{50}, and CP= control) for 45 days

Different alphabets a, b, and c indicate the significant difference between CP1, CP2 and CP within the same exposure time, and the alphabets A, B, and C indicate significant variation in the variable means of the same treatment at different exposure periods at 95% confidence level. The error bars denote the standard deviation.

Acknowledgements

The authors would like to thank the staff at the ICAR-Central Inland Fisheries Research Institute in Barrackpore and the Regional Centre in Bangalore for their assistance in carrying out the study.

Ethical guidelines

The experiment adhered to OECD (1992) guidelines and the National Research Council's Guide for the Care and Use of Laboratory Animals, with approval from the Institutional Animal Ethics Committee of the ICAR-Central Inland Fisheries Research Institute, Kolkata, India (Reference Number IAEC/2021/05, dated 10.12.2021).

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