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Neurotoxic effects of sublethal Isoproturon exposure in the freshwater fish *Cyprinus carpio* (L.)

Devika Rani H K[1], Parimala B[2*]
[1] Research Scholar, DOSR in Zoology, University College of Science, Tumkur University, Tumakuru, Karnataka, India
[2] Associate Professor, Department of Zoology, University College of Science, Tumkur University, Tumakuru, Karnataka, India

Abstract
Isoproturon is a widely used agricultural herbicide that can easily reach nearby water bodies through runoff, where it may affect non-target aquatic organisms. This study focuses on understanding the neurotoxic effects of sublethal Isoproturon exposure in the freshwater fish *Cyprinus carpio* (L.). Fish were exposed to two sublethal concentrations (1/5th and 1/10th of the 96-hour LC_{50}) for 7, 14, and 21 days. Neurotoxicity was evaluated by analyzing changes in acetylcholine (ACh) levels and acetylcholinesterase (AChE) activity in the brain. The results showed a clear and significant reduction in AChE activity, along with a corresponding increase in ACh levels, and these effects became more pronounced with higher concentrations and longer exposure periods. Such changes indicate disruption of normal nerve signal transmission, suggesting that Isoproturon interferes with neural functioning. The accumulation of ACh may be associated with altered ionic balance and stress within brain tissue. These findings demonstrate that even low, non-lethal levels of Isoproturon can adversely affect the nervous system of fish. Neurochemical changes observed in *Cyprinus carpio* highlight its usefulness as a sensitive bioindicator for detecting herbicide-induced neurotoxicity in freshwater ecosystems. The study emphasizes the need for careful regulation and monitoring of Isoproturon to prevent long-term neurological damage to aquatic organisms.

Keywords: Isoproturon, *Cyprinus carpio*, neurotoxicity, acetylcholine, acetylcholinesterase

Introduction

Isoproturon is a selective phenyl urea herbicide extensively applied for weed management in cereal crops, particularly wheat and barley. Due to its widespread agricultural use, Isoproturon frequently contaminates nearby aquatic ecosystems through runoff, spray drift, and improper handling. Residues of this herbicide have been detected in surface and groundwater at concentrations that may pose risks to aquatic organisms, even though the compound undergoes partial degradation through photolysis and microbial activity (Leppert *et al.*, 1983; Sao *et al.*, 2008) [12, 22]. Despite moderate environmental persistence, Isoproturon remains biologically available and can exert sublethal effects on non-target aquatic fauna.

Fish are especially vulnerable to herbicide contamination because of their continuous exposure to the aquatic environment and comparatively slow metabolic detoxification. As a result, prolonged exposure to low concentrations of Isoproturon can lead to bioaccumulation and functional impairment of sensitive organs such as the brain (IPCS, 1986). Consequently, fish are widely used as model organisms and bioindicators for evaluating the neurotoxic effects of agrochemicals in freshwater systems (Burkepile *et al.*, 2000; van der Oost *et al.*, 2003) [4, 29].

Although Isoproturon is not designed to target animal nervous systems, increasing evidence indicates that it can interfere with neural function in fish. Neurotoxicity associated with Isoproturon exposure is mainly attributed to the inhibition of acetylcholinesterase (AChE), a key enzyme involved in the regulation of synaptic transmission. AChE rapidly hydrolyzes acetylcholine (ACh) at synaptic junctions, thereby preventing continuous nerve stimulation. Inhibition of this enzyme leads to excessive accumulation of ACh, resulting in prolonged neuronal excitation and impaired neuromuscular coordination (Quinn, 1987; Fulton and Key, 2001) [7, 18].

Several experimental studies have demonstrated significant suppression of AChE activity in fish exposed to sublethal concentrations of Isoproturon and related herbicides. (Reddy and Rani, 2023) [19] Reported reduced AChE activity and marked behavioral abnormalities in *Labeo rohita*, including erratic swimming and loss of equilibrium. Similar neurobehavioral disturbances have been observed in other fish species exposed to phenyl urea herbicides, suggesting a common mode of action involving cholinergic dysfunction (Fulton and Key, 2001; Sanchez-Hernandez, 2001) [7].

Environmental factors such as temperature, dissolved oxygen, and the presence of additional stressors can further influence the neurotoxic effects of herbicides. (Mukherjee and Bhat, 2024) [16] Demonstrated that exposure to herbicides under elevated temperature conditions intensified physiological stress and behavioral impairment in *Gambusia affinis*. Such findings indicate that climate-related stressors may exacerbate herbicide-induced neurotoxicity in aquatic organisms (Scholz *et al.*, 2013) [23].

Behavioral alterations are among the earliest visible indicators of neurotoxicity and are closely linked to biochemical disruptions in the nervous system. Reduced swimming performance, impaired feeding behavior, and delayed escape responses have been widely associated with AChE inhibition in fish exposed to agrochemicals (Scott and Sloman, 2004; Tierney *et al.*, 2010) [24, 27]. These sublethal neurotoxic effects can significantly reduce survival and reproductive success in natural populations.

Emerging research suggests that Isoproturon-induced neurotoxicity may also involve indirect mechanisms such as oxidative stress. Increased production of reactive oxygen species can damage neuronal membranes, disrupt ionic balance, and alter enzyme activity in brain tissue, thereby

contributing to AChE inhibition and neurotransmitter imbalance (Livingstone, 2001; Mukherjee and Bhat, 2024) [13, 16]. Such oxidative disturbances further compromise neural integrity and physiological stability.

Overall, the reviewed literature clearly indicates that sublethal exposure to Isoproturon can disrupt neural function in fish through cholinergic enzyme inhibition and associated behavioral changes. Alterations in acetylcholine levels and acetylcholinesterase activity are therefore considered sensitive and reliable biomarkers for early detection of herbicide-induced neurotoxicity. These findings emphasize the ecological risks associated with Isoproturon contamination and highlight the importance of continuous environmental monitoring and responsible herbicide management to protect freshwater ecosystems.

Materials and Methods

Experimental Fish and Chemical Details

Cyprinus carpio (L.), belonging to the family Cyprinidae and order Cypriniforms, was selected as the test species for the study. Specimens were procured from the Fishery Department of the Turvekere, Tumakuru, and Karnataka. The fish used in the experiment weighed between 3.5 to 4.5 grams and measured approximately 5 to 6 cm in length. To prevent dermal infections, all fish were rinsed twice in a 0.05% potassium permanganate ($KMnO_4$) solution for 2 minutes each time.

Following this, the fish were acclimated for two weeks under laboratory conditions in semi-static water systems. During the acclimatization period, the fish were provided with commercially available fish pellets twice daily. Uneaten feed and metabolic wastes were regularly removed by siphoning to maintain low ammonia levels in the water and ensure optimal water quality.

The test chemical, a commercial formulation of Isoproturon (75% WP), marketed under the trade name 'Srirama' and manufactured by FMC India Private Limited, was sourced locally for experimental use. Throughout the exposure period, the physicochemical properties of the water were monitored and maintained according to the standard methods outlined by APHA (2005).

In vivo experiment

The acute toxicity test was conducted under semi-static laboratory conditions following the OECD Test Guideline No. 203 for fish acute toxicity (OECD, 1992). The 96-hour median lethal concentration (LC_{50}) of Isoproturon for *Cyprinus carpio* was calculated using probit analysis as described by (Finney, 1971). The LC_{50} was determined to be 1.64 mg/L, with associated 95% confidence limits. Based on this value, two sublethal concentrations were selected for chronic exposure: SL-1 (0.68 mg/L, 1/5th of LC_{50}) and SL-2 (0.34 mg/L, 1/10th of LC_{50}).

These concentrations were employed to evaluate neurotoxic effects, which were assessed by measuring alterations in brain acetylcholine (ACh) levels and acetylcholinesterase (AChE) activity. Such neurochemical biomarkers are widely recognized as sensitive early-warning indicators of herbicide-induced neural stress in fish and have been extensively used in neurotoxicity studies involving agrochemical exposure (Fulton and Key, 2001; Reddy and Rani, 2023; Scott and Sloman, 2004) [7, 19, 24].

Fish were reared under a semi-static exposure system, with the test solutions replaced every alternate day to maintain stable and uniform exposure conditions. The experiment was conducted for a total period of 21 days, and samples were collected at regular intervals on days 7, 14, and 21 to monitor time-dependent biological changes. This experimental design has been widely adopted in recent studies evaluating the toxic effects of herbicides and pesticides in fish (Singh *et al.*, 2023; Reddy and Rani, 2023) [19, 26]. Such approaches have proven effective in demonstrating that alterations in acetylcholinesterase (AChE) activity and nuclear integrity serve as reliable biomarkers for assessing chronic toxicant exposure in aquatic organisms.

Neurotoxicity Assessment

Estimation of Acetylcholine (Ach) Levels

Brain acetylcholine (ACh) levels were estimated using a modified Hestrin colorimetric method as described by (Augustinson, 1957) [3]. After dissection, brain tissues were carefully removed from fish, accurately weighed, and gently homogenized for further analysis. The tissues were immediately transferred into preheated test tubes and placed in a boiling water bath for 10 minutes. This heat treatment was carried out to inactivate acetylcholinesterase (AChE), thereby preventing enzymatic breakdown of ACh and ensuring complete release of the neurotransmitter.

After cooling to room temperature, the tissues were homogenized in 2.0 mL of distilled water. To the homogenate, 2.0 mL of alkaline hydroxylamine hydrochloride solution and 1.0 mL of dilute hydrochloric acid (1:1 with distilled water) were added. The samples were mixed thoroughly and centrifuged, and the clear supernatant was collected. Subsequently, 1.0 mL of ferric chloride solution was added, leading to the formation of a purple-colored complex due to its reaction with hydroxamic acids. The absorbance of this complex was measured at 540 nm using a spectrophotometer, and ACh levels were calculated against a reagent blank.

This method is widely regarded as reliable for estimating ACh concentrations and has been extensively used in neurotoxicity studies. Similar biochemical approaches have been employed by (Reddy and Rani, 2023 and Mukherjee and Bhat, 2024) [16, 19] who reported that alterations in ACh levels serve as sensitive indicators of cholinergic dysfunction in fish exposed to herbicides.

Estimation of Acetylcholinesterase (AChE) Activity

Acetylcholinesterase (AChE) activity in brain tissue was determined following the method originally described by (Metcalf, 1951) [15]. Brain tissues were homogenized to prepare a 3% homogenate in ice-cold 0.25 M sucrose solution to minimize enzyme degradation. The homogenate was centrifuged, and the supernatant was used for enzymatic analysis.

The AChE assay was conducted using a 3.0 mL reaction mixture containing acetylcholine chloride (12 µM) as the substrate, sodium phosphate buffer (100 µM, pH 7.4), and 1.0 mL of the enzyme extract. The reaction mixture was incubated at 37°C for 30 minutes to allow enzymatic hydrolysis of the substrate. The reaction was then stopped by adding 2.0 mL of alkaline hydroxylamine hydrochloride solution, followed by 1.0 mL of dilute hydrochloric acid (1:1 with distilled water). After thorough mixing, the mixture was filtered to remove debris. To the clear filtrate, 1.0 mL of 0.37 M ferric chloride solution was added,

producing a colored complex. The absorbance was measured at 540 nm using a spectrophotometer, with a reagent blank used for calibration.

AChE activity was expressed as specific activity by normalizing the enzyme activity to protein content. Total protein concentration in the brain homogenate was estimated using the method of (Lowry *et al.*, 1951) [14]. This normalization allowed meaningful comparison across treatment groups and exposure durations. The methodology remains widely used in aquatic toxicology studies and has been successfully applied in recent investigations evaluating herbicide-induced neurotoxicity in freshwater fish (Reddy and Rani, 2023; Sharma *et al.*, 2022) [19, 25].

Results

No mortality was observed among the fish during the acclimatization period prior to Isoproturon exposure. Likewise, throughout the *in vivo* experimental period, all fish survived, and the feed supplied was readily consumed in all treatment groups. However, exposure to Isoproturon significantly influenced the growth performance of the fish.

At the end of the experiment, fish exposed to Isoproturon exhibited only a 3% increase in body weight, whereas the control group showed a 10% increase in body weight.

The physicochemical characteristics of the experimental water remained within acceptable limits throughout the study. The recorded parameters included a temperature of 24±1°C, pH of 7.4±0.3 at 25°C, dissolved oxygen of 6.9±0.54 mg/L, carbon dioxide concentration of 5.9±0.6 mg/L, total hardness of 22.4±2.4 mg as $CaCO_3$/L, total ammonia nitrogen of 0.02 mg/L, phosphate level of 0.35±0.004 µg/L, salinity of 0.01 ppm, specific gravity of 1.001, and conductivity below 10 µS/cm.

Neurotoxicity Experiments

ACh

Since P<0.05, there was a significant increase in the ACh level in the brain tissue of *C. carpio* with increasing days of exposure at both exposure concentrations. Increased concentrations of Isoproturon from SL-2 to SL-1 showed significant alterations in ACh levels (Fig. 1).

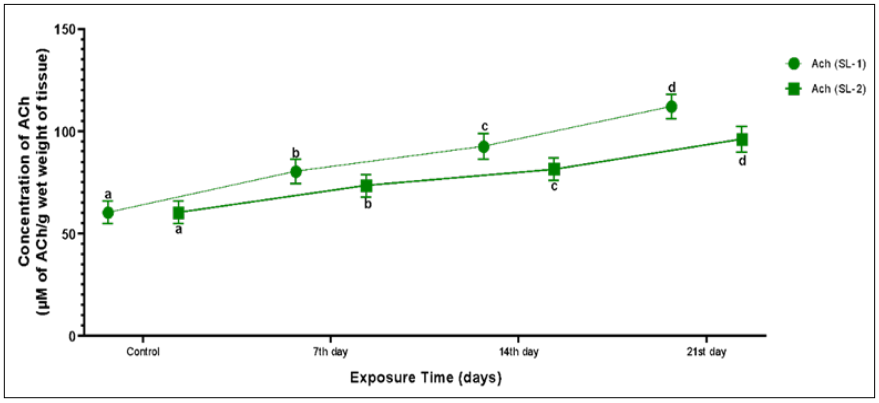

Fig 1: Alterations in the ACh content of brain tissue in *Cyprinus carpio* exposed to sublethal concentrations (SL-1 and SL-2) of Isoproturon for 7, 14, and 21 days were recorded. Each value represents the Mean ± SD of six individual fish (n=6) and is expressed as µM of ACh/g wet tissue weight. Different superscript alphabets within each concentration across exposure periods indicate statistically significant differences at P<0.05, whereas identical alphabets indicate non-significant differences.

AChE

Since P<0.05, a significant decline in the AChE activity was observed in the brain tissue of *C. carpio* with increasing duration of exposure at both exposure concentrations. Higher concentrations of Isoproturon from SL-2 to SL-1 produced significant alterations in AChE activity levels (Fig. 2).

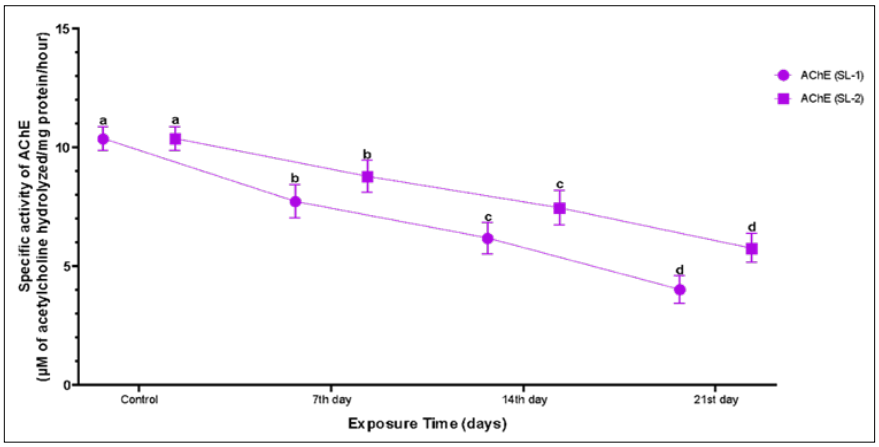

Fig 2: Variations in the AChE activity of brain tissue in *Cyprinus carpio* exposed to sublethal concentrations (SL-1 and SL-2) of isoproturon for 7, 14, and 21 days. Each value represents the Mean ± SD of six individuals (n=6). The results are expressed as µM of acetylcholine hydrolyzed/mg protein/hour. Different superscript alphabets within each concentration across exposure durations indicate statistically significant differences at the 5% level (P<0.05), whereas identical alphabets indicate non-significant differences.

Discussion

Toxicity assessment plays a vital role in evaluating the impact of herbicide on aquatic ecosystems, as toxic chemicals can alter the behavior, physiology, and survival of aquatic organisms. Such studies provide fundamental information regarding the adverse effects of pesticides on fish and other aquatic biota, thereby helping to understand their ecological consequences and environmental risks Ecotoxicology. Earlier investigations have emphasized that pesticide contamination may severely disturb aquatic biodiversity and ecosystem stability (Kaushal and Mishra, 2013; Adedeji *et al.,* 2008) [1, 11]. Recent studies have also reported that herbicides such as isoproturon induce oxidative stress, neurotoxicity, and metabolic disturbances in freshwater fishes and other aquatic organisms (Anitha *et al.,* 2025[2]; Hu *et al.,* 2026).

Neurotoxicity Experiments

Acetylcholinesterase (AChE) is an essential enzyme involved in regulating neurotransmission by hydrolyzing acetylcholine (ACh) at neuronal synapses after nerve impulse transmission. Proper functioning of AChE is necessary for maintaining normal nerve activity and ionic balance within nervous tissues (O'Brien, 1967; Van der Kloot, 1956) [17, 28]. Inhibition of AChE results in the accumulation of ACh at synaptic junctions, leading to continuous nerve stimulation, impaired cholinergic transmission, paralysis, behavioral abnormalities, and disruption of normal physiological functions.

In the present investigation, exposure to Isoproturon caused a marked reduction in AChE activity along with a significant increase in ACh content in the brain tissue of *Cyprinus carpio* (Fig. 1 & 2). The inhibition of AChE and accumulation of ACh suggest impairment of cholinergic neurotransmission and suppression of central nervous system activity. Similar observations were reported by Reddy *et al*., who stated that elevated ACh levels reflect stronger inhibition of integratory nervous functions. Disturbance in neural regulation may further affect hormonal balance and several biochemical and physiological processes, eventually reducing the overall health and survival of the organism (Corbett, 1974) [6].

The findings of the present study are consistent with earlier reports demonstrating pesticide-induced neurotoxicity in fishes. (Capkin *et al.,* 2014) [5] Observed significant inhibition of AChE activity in rainbow trout exposed to pesticides, while Adinarayan and Kishore documented increased ACh accumulation associated with decreased AChE activity in *C. carpio*. Recent investigations have also confirmed that exposure to herbicides, including Isoproturon, can alter neurotransmitter regulation and induce neurobehavioral abnormalities in freshwater fishes (Burch *et al.,* 2025; Hu *et al.,* 2026).

Conclusion

The present study demonstrated that exposure to Isoproturon caused significant neurotoxic effects in *Cyprinus carpio*, as evidenced by marked alterations in ACh and AChE levels in the brain tissue. A significant increase in ACh content accompanied by a reduction in AChE activity indicates disruption of cholinergic neurotransmission and impairment of normal nervous system functioning. The inhibition of AChE may be associated with changes in the ionic composition of brain tissue, leading to the accumulation of ACh and continuous nerve stimulation. These neurochemical disturbances suggest that Isoproturon adversely affects the central nervous system of fish in a concentration- and duration-dependent manner. Therefore, alterations in ACh and AChE activity can serve as sensitive biomarkers for evaluating herbicide-induced neurotoxicity in aquatic organisms. Continuous monitoring and cautious use of Isoproturon are essential to minimize its harmful effects on aquatic ecosystems and fish health.

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