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Estimation Of LC_{50} And Behavioral Modifications Of Cyprinus Carpio (L.) In Response To Isoproturon Herbicide

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Abstract

Isoproturon, a widely used Phenyl urea group herbicide for controlling weeds in cereal crops, frequently enters aquatic environments through agricultural runoff, posing risks to non-target species. This study examines the behavioral and morphological responses of Cyprinus carpio (L.), the common carp fingerlings following exposure to sub-lethal herbicide concentrations of the Isoproturon (75% WP). C. carpio was exposed to different concentrations of Isoproturon (0.10–0.200 mg/L) for 96 hours, with the median lethal concentration (LC_{50}) determined at 0.160 mg/L using a semi-static bioassay. Based on this value, two sub-lethal concentrations 0.0224 mg/L (1/7th of LC_{50}) and 0.0112 mg/L (1/14th of LC_{50}) were selected for subacute exposure studies lasting 1, 7, and 14 days. After the 14-day exposure period, the fish were transferred to a toxicant-free medium for a 7-day recovery phase. Exposed fish exhibited behavioral abnormalities, including erratic and darting swimming patterns, hyperactivity, loss of equilibrium, and a tendency to remain at the bottom. Morphological deformities, like caudal bending, discoloration of the body, and ocular disorders were observed throughout the exposure period. Although no significant mortality occurred at sub-lethal concentrations. Importantly, behavioral and morphological impairments persisted even after the recovery phase, indicating the long-lasting neurotoxic effects of Isoproturon. These results underscore the ecological threats posed by Isoproturon contamination in aquatic ecosystems, emphasizing the need for regulatory control and environmental monitoring to minimize its impact on aquatic life.

Keywords: *C. carpio, Isoproturon, LC_{50}, Stress, Behavioral Changes.*

INTRODUCTION

Herbicides disperse beyond their intended targets through atmospheric and hydrological processes such as wind and rainfall, contaminating aquatic environments, including lakes and rivers. The majority of applied herbicides contribute to environmental contamination, negatively impacting non-target organisms. Herbicide contamination has been linked to alterations in fish behavior, growth, and reproduction, leading to changes in population dynamics and biodiversity (van der Oost et al., 2003; Velmurugan et al., 2009). The detrimental effects of herbicides on aquatic ecosystems are well-documented, with increasing chemical pollution resulting in severe toxicological impacts on aquatic organisms (Livingstone, 2001; Matsumoto et al., 2006).

Among these herbicides, Isoproturon is a critical concern in aquatic environments. Isoproturon (IPU) (75% WP), a widely used phenyl urea-grouped herbicide in agriculture, is often detected in water bodies due to runoff and leaching. Like other herbicides, Isoproturon has been shown to impair aquatic organisms, including fish, by disrupting neurochemical pathways and inducing behavioral changes. Studies show that herbicides like Isoproturon can affect neurotransmitter systems, impairing fish locomotion, feeding, and predator avoidance (Naylor et al., 2004; Eissa et al., 2016). While much attention has been given to general herbicide contamination, herbicides such as Isoproturon are increasingly being recognized for their potential ecological risks, particularly regarding their effects on fish and other aquatic organisms.

(IPU), a selective herbicide is widely applied in agriculture to control broadleaf and grass weeds, particularly in wheat and barley crops. It inhibits photosynthesis by disrupting electron transport in photosystem II, thereby affecting plant growth. Since its introduction, (IPU) has been extensively used, but concerns about its environmental persistence and toxicity to non-target organisms, including aquatic life, have grown (Kumar et al., 2018). C.carpio, a significant freshwater species found in rivers, ponds, and reservoirs, is particularly vulnerable to contamination by (IPU) at sublethal concentrations. This exposure can lead to behavioral disruptions in fish populations, posing potential ecological risks. The present study examines the aquatic toxicity of (IPU), focusing on the behavioral responses of C.carpio exposed to sublethal concentrations of this herbicide. Understanding these behavioral changes is crucial for assessing the sublethal impacts of (IPU) on fish and its broader ecological implications.

Studies underscore a significant decline in fish populations, a vital ecological and economic resource, raising concerns about conservation efforts and the necessity of establishing safe thresholds for chemical pollutants in aquatic ecosystems. Among these pollutants, herbicides are particularly harmful to both environmental and human health. Herbicides, which are commonly used to control weeds in agricultural settings, have increasingly been found to contaminate water bodies, posing serious risks to aquatic organisms. These chemicals are highly persistent in the environment and can affect aquatic biodiversity, with some studies indicating that herbicide exposure leads to disruptions in fish behavior, growth, and reproduction, ultimately impacting population dynamics (Ayllon et al., 2015; Gauthier et al., 2016).

Fish are considered excellent indicators for behavioral assays when assessing the impact of various environmental stressors and chemical exposures. They offer several advantages: constant, direct contact with the aquatic environment, where chemical exposure affects the entire body surface, their ecological relevance in diverse natural systems (Little et al., 1993), ease of cultivation, ability to reach reproductive maturity (Henry and Atchison, 1986), and their long-standing use in behavioral toxicology studies. Recent advancements in behavioral monitoring technologies have further enhanced the ability to assess fish responses to environmental stressors, providing more precise insights into their physiological and neurological stress responses (Fu et al., 2022).

Behavior provides a valuable perspective on the link between an organism's physiology and its ecological role in the environment (Little and Brewer, 2001). It includes measurable actions driven by both the central and peripheral nervous systems (Keenleyside, 1979) and the collective result of genetic, biochemical, and physiological processes essential for survival, such as feeding, reproduction, and predator avoidance. Behavior allows organisms to adapt to internal and external stimuli, optimizing their chances of survival in a constantly changing environment. At the same time, it reflects the organism's evolutionary responses to environmental pressures. As such, behavior is a selective response that evolves continuously through interactions with the physical, chemical, social, and physiological components of the environment. Evolution has favored stable behavioral patterns that, together with morphological and physiological adaptations, improve survival and reproductive success by enabling organisms to efficiently exploit resources and find suitable habitats (Little and Brewer, 2001).

Fast research reports have highlighted the importance of using fish in behavioral toxicology due to their sensitivity to various pollutants and ability to exhibit notable behavioral changes even at sublethal exposure levels. Species Pimephales promelas (Fathead minnow) and Danio rerio (zebrafish) are commonly employed to study the neurotoxic effects of substances like heavy metals, herbicides, and pharmaceuticals (Zhang et al., 2010; Bundy et al., 2011). Behavioral changes in fish such as alterations in swimming patterns, aggression, feeding behavior, and predator avoidance are often used as early indicators of toxicity, sometimes appearing before visible morphological changes or mortality (Gauthier et al., 2015). Additionally, behavioral toxicology studies have increasingly integrated molecular techniques such as transcriptomic and proteomics to identify biomarkers of toxicity in aquatic species (Belfroid et al., 2015).

The ecological importance of behavioral assays is further demonstrated by their ability to predict long-term consequences at the population level. Behavioral alterations caused by herbicide exposure can result in reduced reproductive success, imbalances in predator-prey interactions, and modifications in species dynamics within aquatic ecosystems (Zhao et al., 2012). Consequently, behavioral assessments in fish have become essential tools for evaluating environmental risks and guiding regulatory measures regarding herbicide contamination in aquatic environments.

Behavior is not random but a structured sequence of actions that enhance survival and reproductive success. As such, behavioral responses serve as key indicators for assessing the effects of environmental stressors. Fish, due to their constant interaction with the aquatic medium, absorb and retain xenobiotics through both active and passive mechanisms. This makes them highly effective bioindicators for detecting pollution in aquatic habitats. Sublethal herbicide concentrations, which are commonly detected in water bodies, frequently induce structural and functional changes in aquatic organisms, often manifesting as behavioral modifications rather than direct mortality (Sancho et al., 2003). These behavioral shifts serve as sensitive markers of environmental stress and are closely linked to survival outcomes (Olla et al., 1983; Byrne and O'Halloran, 2001). In non-migratory fish species, behavioral changes offer critical insights into ecosystem health, as deviations from normal behavior indicate water quality deterioration. Fish thus serve as biological sentinels, with their behavioral patterns reflecting the ecological costs of environmental change.

Further the studies emphasize on the critical role of behavioral assays in aquatic toxicology, demonstrating their effectiveness in detecting early signs of environmental stress before morphological damage or mortality occurs. Exposure to sublethal levels of herbicide Isoproturon has been shown to disrupt swimming patterns and impair predator avoidance in freshwater fish (Nwani et al., 2013). These behavioral impairments can negatively affect fitness, increase susceptibility to predation, and reduce reproductive success, ultimately influencing population stability (Velisek et al., 2012). Consequently, behavioral monitoring is increasingly recognized as a valuable tool for assessing ecological risks associated with contaminants like Isoproturon (Sancho et al., 2009).

Recent advancements have further reinforced the importance of behavioral assays in toxicology. Zebrafish have become widely used in behavioral toxicology due to their sensitivity to neurotoxicants, allowing researchers to study pollutant-induced effects on sensory-motor functions, decision-making, and cognitive processes (Rajak et al., 2021). The incorporation of high-throughput screening (HTS) techniques has significantly improved the precision and efficiency of behavioral assessments, particularly through innovations in imaging, artificial intelligence, and machine learning (Kim et al., 2023). Additionally, recent neurotoxicity assessment strategies integrate behavioral assays with neuronal activity mapping and molecular analyses to provide a comprehensive understanding of contaminant effects on aquatic species (Lopez-Doriga et al., 2021). Moreover, bioanalytical system integration has further enhanced behavioral tests as effective tools for identifying early ecological disruptions caused by environmental pollutants (Fischer et al., 2020).

MATERIALS AND METHODS:

Sample Collection, Maintenance, and Acute Toxicity Assessment

Healthy and active Cyprinus carpio (L.) fingerlings were obtained from the Turvekere fisheries farm, Turvekere, Tumakuru. Upon arrival at the laboratory, the fish were carefully transferred into large aerated containers for transport. They were then acclimated for 30 days in 25L tubs under controlled conditions. Throughout the acclimatization period, the fish were fed commercial dry feed pellets to ensure proper nutrition and adaptation.

The fish with an average weight of 3 ± 0.33 g and a length of 4 ± 0.25 cm were further acclimated for 20 days in 25 L tubs at a maintained temperature of 24 ± 1°C. The tubs contained Dechlorinated tap water, which was analyzed for its physicochemical properties following APHA (2005) guidelines. The recorded water quality parameters were: temperature 24 ± 2°C, pH 6.9 ± 0.2 at 24°C, dissolved oxygen 9.4 ± 0.8 mg/L, carbon dioxide 6.3 ± 0.4 mg/L, total hardness 23.4 ± 3.4 mg as CaCO3/L, phosphate 0.39 ± 0.002 µg/L, salinity nil, specific gravity 1.001, and conductivity below 10 µS/cm. Water was replaced daily, and a 12-hour light/12-hour dark photoperiod was maintained throughout both the acclimatization and testing phases.

During both acclimatization and testing periods, fish were fed commercial fish pellets, with feeding suspended two days before the acute toxicity exposure. The test substance, Isoproturon (IPU), was procured from a local supplier in Tumakuru, Karnataka, India, under the trade name Srirama. Before use, the expiry date of the test substance was verified, and precise amounts of IPU were measured from the stock solution using a micropipette.

Range-Finding Test

In the range-finding test, groups of ten fish were exposed to varying concentrations of Isoproturon 75% WP (0.120 to 0.200 mg/L) in 20 L of test medium. Each test concentration, along with a control group, was tested in triplicate. The water was replaced every 24 hours, with fresh Isoproturon added to maintain the appropriate exposure levels. The final concentrations for the definitive toxicity tests were selected based on the highest concentration that resulted in 0% mortality and the lowest concentration that caused 100% mortality (Table 1).

Table 1. Mortality of fingerlings in different concentrations of (IPU) at 96 h exposure periods

Conc. of Isoproturon (mg/L)	Log conc.	No. of fish alive out of ten	% Corrected mortality	Probit kill
0.120	-0.900	10	0	--
0.130	-0.826	9	10	3.72
0.140	-0.843	8	20	4.16
0.150	-0.823	7	30	4.48
0.156	-0.804	6	40	4.75
0.160	-0.745	5	50	5.00
0.165	-0.762	4	60	5.25
0.170	-0.749	3	70	5.52
0.178	-0.747	2	80	5.84
0.188	-0.713	1	90	6.28
0.200	-0.688	0	100	--

Mortality was monitored at 24-hour intervals, with deceased fish promptly removed. The number of fatalities at each concentration was recorded for up to 96 hours. Mortality data, expressed as arc sine-transformed proportions (dead fish/total fish), were analyzed using Duncan's multiple range test (Duncan, 1955) following the estimation of residual variance via repeated measures ANOVA (Winner, 1971). Exposure time served as the repeated measure factor, while treatment (concentration and control) was the second factor. The 96-hour LC50 value was calculated using probit analysis (Finney, 1971), with a 95% confidence interval.

Exposure Durations and Contaminant Levels

For subacute studies, sublethal concentrations of (IPU) were selected based on one-seventh (0.0224 mg/L) and one-fourteenth (0.0112 mg/L) of the 96-hour LC_{50} (0.160 mg/L). Fish were exposed to these concentrations of (IPU) for 1, 7, and 14 days. Following the 14th day of exposure, a recovery phase of seven days was implemented in a toxicant-free medium. The control group, consisting of fish exposed solely to toxicant-free water, and the Isoproturon-exposed fish were continuously monitored for behavioral responses and morphological deformities throughout the study period.

Statistical Analysis

Experimental data were presented as mean values derived from three replicates. Differences among treatment groups were assessed using Duncan's multiple-range test (Duncan, 1955). Statistical analyses were performed using SPSS (Version 20), with significance set at $p < 0.05$.

Results and Discussion

The 96-hour LC_{50} value of Isoproturon (IPU) for C. carpio was determined to be 0.160 mg/L, with 95% confidence limits, indicating its high toxicity to fish. However, no significant mortality was observed at any of the sublethal concentrations throughout the experiment.

Previous studies have reported varying 96-hour LC_{50} values for IPU across different fish species. (Gluth and Hanke, 1985) reported an LC_{50} of 0.30 mg/L for Oncorhynchus mykiss (rainbow trout), while (Sanchez et al., 2006) found a lower value of 0.14 mg/L for Danio rerio (zebrafish). (Jabeen et al., 2015) determined an LC_{50} of 0.125 mg/L for Oreochromis niloticus (Nile tilapia) using a semi-static bioassay. These variations in IPU toxicity likely result from species-specific differences in metabolic rates, physiological adaptations, and environmental conditions, emphasizing the importance of species-specific risk assessments when evaluating pesticide toxicity in aquatic ecosystems.

Behavior of the Control and Exposed Fish

In this study, the control fish were highly active, demonstrating alertness to even minimal disturbances and exhibiting well-coordinated movements. There were no significant behavioral variations observed among the control groups, and these observations served as the baseline standard for the entire experiment.

In contrast, C. carpio exposed to Isoproturon displayed significant behavioral disturbances. Initial responses included altered schooling behavior, with the fish congregating near the bottom of the test chamber and swimming independently, spreading out to cover an area approximately twice the size occupied by the control group. Over time, exposed fish migrated to the corners of the chamber, showing avoidance behavior towards the Isoproturon-contaminated water. The fish also exhibited irregular, erratic, and darting swimming movements, followed by a loss of equilibrium and a tendency to hang vertically in the water column.

These findings align with previous research, such as that by (Hulya et al., 2006), who reported similar behavioral changes in Oreochromis niloticus following exposure to sublethal levels of diazinon, an organophosphate pesticide. Further supporting this, (Nwani et al., 2013) observed comparable behavioral impairments in Danio rerio exposed to sublethal concentrations of Isoproturon, noting symptoms like erratic swimming and reduced social interaction. Additionally, (Jabeen et al., 2015) documented altered feeding behavior and impaired predator avoidance in Oreochromis niloticus after Isoproturon exposure, highlighting the herbicide's neurotoxic potential in aquatic species.

Behavioral Effects of Isoproturon Exposure

(IPU), a Phenylurea herbicide, primarily disrupts plant photosynthesis by inhibiting photosystem II, thereby interfering with electron transport. However, in aquatic organisms, its toxicity is primarily linked to oxidative stress, neurotoxicity, and metabolic disturbances, which manifest as both behavioral and physiological impairments (Velisek et al., 2012; Nwani et al., 2013). One of the key mechanisms of IPU toxicity in fish is acetylcholinesterase (AChE) inhibition, which disrupts neurotransmission by preventing the breakdown of acetylcholine (ACh) at synaptic junctions (Guilherme et al., 2014). The resulting accumulation of ACh leads to neuromuscular overstimulation, causing irregular swimming behavior, loss of coordination, and other neurological dysfunctions. These effects highlight the potential ecological risks associated with IPU contamination in aquatic environments, emphasizing the need for continuous monitoring and regulatory measures to mitigate its impact on non-target organisms.

Fish exposed to (IPU) exhibited various symptoms, including lethargy, restlessness, and excessive mucus secretion. These non-specific defense responses likely serve as protective mechanisms to minimize toxicant absorption and shield the skin from irritation (Rao, 2006). Additionally, episodes of hyperactivity and erratic swimming were observed, followed by a gradual loss of equilibrium, suggesting neurotoxic effects. Notably, these behavioral disturbances persisted even after the exposure period, indicating potential long-term neurological impairment. Increased mucus secretion was particularly evident, possibly facilitating the elimination of toxicants and acting as a protective barrier against chemical exposure (Rao, 2006).

Exposed fish also exhibited disrupted shoaling behavior, frequent air gulping, and increased surface swimming. These behavioral changes, commonly observed under toxic stress, may serve as adaptive responses to reduce toxicant exposure and alleviate respiratory distress. Air gulping at the surface, often seen in fish exposed to contaminants, likely indicates an attempt to compensate for impaired oxygen uptake (Katja et al., 2005). These responses became more pronounced over time and were associated with increased vulnerability to predation, raising ecological concerns regarding the survival of affected fish populations.

A particularly notable observation was caudal bending, characterized by an abnormal curvature of the tail. This deformity persisted throughout the recovery phase and was most evident in fish exposed to higher concentrations ($1/7^{th}$ of the 96-hour LC_{50}). Caudal bending is likely a result of neuromuscular dysfunction, where AChE inhibition disrupts coordinated muscle contractions, leading to partial paralysis (Ware, 1989; Habig and DiGiulio, 1991). Similar deformities have been documented in fish exposed to neurotoxic pesticides and herbicides, further highlighting the significant neuromuscular toxicity of IPU (Velisek et al., 2012).

Other morphological and physiological abnormalities observed in (IPU)-exposed fish included fin hyperextension, Hyperpigmentation, Hemorrhage, Ophthalmic disorder, Agape, Thoracic perforation, Caudal perforation, and a lean abdomen. Over time, some fish sank to the bottom of the tank, displaying minimal opercular movements, which suggests severe metabolic stress. Mild abdominal swelling was also noted, and this persisted into the recovery phase. These symptoms align with previous findings on Phenylurea herbicide toxicity, which highlight its role in causing muscular weakness, hyperactivity, and impaired equilibrium (Sancho et al., 1998). A significant decline in feeding behavior was observed at higher exposure levels, likely due to metabolic shifts aimed at energy conservation under stress. Reduced feeding activity is a well-documented survival strategy in fish exposed to environmental contaminants (Rice, 1990).

Fig. 1: Hyperpigmentation

Fig. 2: Hemorrhage

Fig. 3: Agape and Ophthalmic disorder

Fig. 4: Thoracic perforation

Fig. 5: Caudal flexion

Herbicide-induced reductions in feeding and growth can have severe consequences for fish survival and reproductive success. (Dembele et al., 2000) Note that exposure to Phenylurea herbicides disrupts energy metabolism and physiological homeostasis, which can ultimately lead to stress-induced mortality. In the present study, fish exposed to the lowest sublethal concentration (1/14th of 96 h LC_{50}) exhibited normal feeding behavior and greater activity, suggesting a dose-dependent toxic response to (IPU) exposure.

Behavioral disruptions were apparent from the first day of exposure, likely due to the inhibition of brain AChE activity, which leads to cholinergic toxicity. (Chawanrat et al., 2007) Demonstrated that brain AChE inhibition occurs early in fish exposed to herbicides, supporting the observed behavioral impairments in C. carpio. The persistence of behavioral and morphological abnormalities beyond the exposure period suggests that Isoproturon toxicity is linked to long-term biochemical and physiological disruptions. Isoproturon undergoes metabolic biotransformation, generating toxic intermediates that may further exacerbate its effects (Guilherme et al., 2014). The conversion of (IPU) into more toxic metabolites is catalyzed by cytochrome P450 (CYP) enzymes, increasing its neurotoxic potential (Poet et al., 2003). Additionally, physiological detoxification mechanisms may enable fish to withstand subacute exposures, which could explain the lack of significant mortality in this study.

CONCLUSION

The present study demonstrated that (IPU), a commonly used herbicide, is highly toxic and has a significant detrimental impact on the behavioral responses of C. carpio at sublethal concentrations. Exposure to (IPU) impaired the fish's ability to adapt to their environment, as evidenced by: an increased time to learn to escape from or avoid external noxious stimuli, a decreased sensitivity to subtle changes in the environment, interference with the animals' ability to retain previously learned behaviors, and a general decline in coordination and motor skills.

Additionally, (IPU) exposure resulted in reduced feeding efficiency, indicating a disruption of normal metabolic processes. The impairments in behavioral responses observed even during recovery periods may be attributed to the inhibition of brain acetylcholinesterase (AChE) activity by (IPU), the active metabolite formed through the biotransformation of bioaccumulated (IPU) in tissues. Neurological impairments leading to muscle weakness, erratic swimming patterns, and reduced locomotive ability were also prominent throughout the exposure and recovery phases. These behavioral changes and morphological alterations could serve as reliable indicators in biomonitoring programs for assessing the Ecotoxicological risks of (IPU) to aquatic species.

Furthermore, the study highlights the potential of Isoproturon exposure to induce long-term neurological effects, even after cessation of direct exposure. These findings emphasize the importance of considering not only the acute toxicity of herbicides but also their potential for sublethal, chronic effects on non-target aquatic organisms. Given the widespread use of (IPU) in agriculture, further research is needed to investigate its long-term environmental impact and cumulative effects on freshwater ecosystems. This study underscores the need for enhanced regulatory measures to protect aquatic biodiversity from the harmful effects of herbicide contamination.

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