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Toxicological Effects of Pendimethalin: Behavioral Changes and LC_{50} Analysis in *Cyprinus carpio*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The pre-emergent herbicide pendimethalin is a selective dinitroaniline compound that interferes with the production of microtubules, potentially contaminating groundwater. The current investigation sought to ascertain the acute toxicity levels and behavioral effects of pendimethalin (30% EC) on *Cyprinus carpio*. Finney's probit bioassay method estimated the lethal toxicity LC_{50} values for commercial-grade herbicide pendimethalin on *C. carpio*. This method entails fitting the data collected from experiments to a probit model, which converts the results obtained (such as mortality rates) into a normal distribution scale. It is a useful tool in toxicological research since it enables more precise estimations of the LC_{50}. The lethal toxicity (LC_{50}) values of pendimethalin to *C. carpio* exposed to 24, 48, 72 and 96-hr were determined to be 2.83µL/L, 2.59µL/L, 2.39µL/L and 2.20µL/L

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based on the mortality estimations. Pendimethalin-induced behavioral abnormalities in *C. carpio* were investigated using sublethal concentrations (i.e., 1/15th, 1/10th, and 1/5th of the 96-hour LC_{50} value) at intervals of 0, 7, 14, 21 and 28 days. Our research observed behavioral shifts such as reduced general activity, reduced foraging behavior, diminished schooling behavior, reduced reflex stimuli, increased opercula movements, irregular movements, mucus secretion, enhanced breathing rates and increased surface air gulping. Our studies showed that pendimethalin can produce both mortality and behavioral alterations in *Cyprinus carpio*, offering important insights into the pendimethalin fatal and sub-lethal effects. These results highlight how crucial it is to keep an eye on herbicide poisoning in aquatic habitats since even sub-lethal levels can have an effect on fish behavior and the general health of the ecosystem.

Keywords: C. carpio; pendimethalin; lethal toxicity (LC_{50}); behavioral alterations.

1. INTRODUCTION

The protection of the environment has gathered interest from a broad spectrum of individuals worldwide, and among researchers and academics who work in this field, it has grown to be a worldwide problem. In aquatic environments, pesticides are recognized as powerful toxins. A variety of manmade and natural processes provide pollutants to aquatic habitats. This is demonstrated by the numerous cases in which specific pollutants have had adverse effects on fish populations (Hamilton et al., 2016). Fish physiology, immunological response, and development are all negatively impacted by pesticide pollution (Ghafarifarsani et al., 2024). Pesticide impact on aquatic animals causes considerable loss in aquaculture productivity when their concentrations surpass the allowed levels. These effects may potentially have a detrimental effect on people's health in addition to lowering the overall wellness and diversity of aquatic ecosystems (Mustafa et al., 2024). There are many ways that herbicides can get into aquatic ecosystems including runoff, aerosol drift, volatilization, and sediment resuspension (Ribeiro et al., 2022).

A dinitroaniline class selective herbicide called pendimethalin (*N*-[1-ethylpropyl]-3,4-dimethyl-2,6-dinitrobenzenamine) is employed in both agricultural and non-agricultural areas to manage unwanted grasses and broadleaf weeds. Pendimethalin can be added to the soil before or after the stage of emergence. When pendimethalin seeps into waterways, it can lead to longer-term environmental problems, impacting aquatic ecosystems and perhaps getting into the human water supply. This makes its potential to pollute groundwater significant. Because of the potential for negative impacts on the environment and public health, it is imperative to evaluate and control the risks involved with its usage. Additionally, they are used in a variety of water bodies to prevent the emergence of many undesirable aquatic weeds. A portion of the aquatic vegetation provides fish with food and cover. Herbicide-induced eradication of these plants has a detrimental effect on both their food supplies and shelter (Ghafarifarsani et al., 2024).

Finding the lethal concentration 50, or LC_{50}, for fish subjected to herbicides is crucial for evaluating the hazards to aquatic environments and notifying regulatory bodies of the effects on species other than the intended targets. This information helps reduce environmental impact while guiding the appropriate use of herbicides in agriculture and promoting ecological investigations that examine its impact on fish and aquatic food chains. Finding the LC_{50} concentrations is therefore essential for encouraging sensible pesticide usage and protecting aquatic habitats.

A crucial tool for identifying the harmful impact that toxicants may have on organisms is behavior. Herbicides can alter the molecular structure of neurotransmitters and block the enzymes necessary for their manufacture or breakdown. Chronic usage of herbicides may negatively impact fish behavior, growth, and fertility. Fish tissues can absorb even minute levels of herbicides, leading to long-term damage (Naiel et al., 2020). Fish behaviors may range widely in complexity from really basic to rather complicated, and they are vulnerable to changes in their behavior brought on by dangerous toxicants and environmental contaminants, which could have lethal consequences. Herbicides can alter fish behavior in several ways. Fish that are less successful in discovering and gathering food due to herbicides are less effective feeders. This is one important factor that affects foraging (Khursigara et al., 2023). The specific behavioral assays employed in this study included: Swimming Activity, foraging behavior, movement patterns, social behavior, reactivity to stimuli, habitat depth selection, locomotive behavior, breathing rates and reactivity to stimuli.

Although earlier studies have examined Pendimethalin's acute toxicity to various aquatic species, little is known about its sub-lethal effects, especially regarding behavioral changes and the calculation of its LC_{50} in *C. carpio*. Therefore, the current study emphasizes finding the acute toxicity concentrations of pendimethalin in C. *carpio* and its impacts on various morphological and behavioral phenomena in carp.

2. MATERIALS AND METHODS

2.1 Collection of Test Animals

Living, well-formed individuals of *C. carpio*, weighing 60-70 grams, and generally measuring 14 to 17 centimeters, were retrieved from the Fish and Breeding Farm Deoli in Ghaggus (Himachal Pradesh). *C. carpio*, is frequently employed as a model organism in toxicological investigations for several reasons, most notably its ecological significance, simplicity of use in laboratory environments, and vulnerability to environmental contaminants. For two-three minutes, the fish were submerged in a 0.2% aqueous potassium permanganate (KMnO4) solution to disinfect them. The fish were subjected to laboratory settings for 15 days. Fish were acclimatized to laboratory conditions by providing appropriate environmental conditions like adjusting water temperature, dechlorinating aquarium water, providing natural photoperiods and minimizing stress through gentle handling. They were given commercial feed available on the market during this time, which included 15% crude ash, 4% fat, 4% protein, and 4% crude fiber. Throughout this time, the test water and the lab's normal physicochemical conditions were maintained.

2.2 Pendimethalin

Under the product name pendimol, the study chemical pendimethalin of purity 97% manufactured by Adarsh Agro Seeds, Gujrat, India was bought from the Shimla local market. The stock solution was made by dissolving 1mL of pendimethalin in 100mL of distilled water. Desirable concentrations were taken from this stock solution for further studies.

2.3 Bioassay test

The poisoning potential of Pendimethalin was assessed in freshwater fish, *C. carpio*, using a lethal toxicity LC_{50} test using Finney's probit bioassay method (Finney 1971). 100 fish had been used for the experimental setup. Fish were kept in 100- litre aquariums and were divided into 10 groups each group containing 10 fish. The first group served as control while the other 9 groups (90 fish) were exposed to different concentrations of pendimethalin for 96 hours. The water of the aquarium was changed every day and freshly prepared test solution was given to fish each day. The LC_{50} values for various times, including 24 hours, 48 hours, 72 hours, and 96 hours, were computed by assessing the mortality seen at various concentrations throughout that time. For analyzing the behavioral alterations *C. carpio* was administered with sublethal doses of pendimethalin i.e., $1/15^{th}$ (Minimum), $1/10^{th}$ (Moderate) and $1/5^{th}$ (Maximum) values of calculated 96-hrs LC_{50} value (i.e., 2.20 µL/L). Now, the fish were subjected to these doses for 7, 14, 21 and 28 days.

2.4 Statistical method

MS- Excel Data Analysis ToolPak was used for statistically analyzing the data obtained from present experiments.

3. RESULTS

3.1 Physio-chemical Characteristics of the Test Water

Table 1. The physicochemical characteristics of the water were calculated by APHA, (2005)

S. No.	Water parameter	Value
1	Temperature	25-28 ± 1.5°C
2	Dissolved oxygen	7.5 ± 0.7mg/L
3	Ph	7.3 ± 0.5
4	Total hardness	133 ± 2.5 mg/L
5	Total dissolved solids (TDS)	215-240 ppm
6	Chloride ion (Cl^-) concentration	300-330 mg/L
7	Alkalinity	350-380 ppm

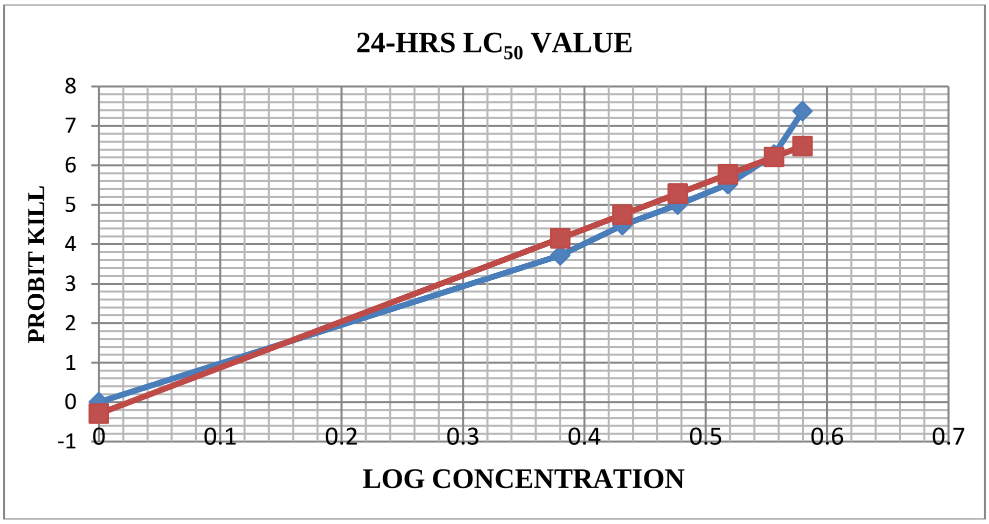
Table 2. Demonstrating probit values of mortality (PV's), pendimethalin (PND), mean mortality (Mt) and its percentage (%Mt)

				96-HOURS			72-HOURS			48-HOURS			24-HOURS		
S. NO.	**DOSE OF PND (µL/L)**	**Log Conc.**	**Fish**	**Mt**	**%Mt**	**PV's**	**Mt**	**%Mt**	**PV's**	**Mt**	**%Mt**	**PV's**	**Mt**	**%Mt**	**PV's**
1	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0
2	1.5	0.18	10	1	10	3.72	0	0	0	0	0	0	0	0	0
3	1.8	0.25	10	2	20	4.16	1	10	3.72	0	0	0	0	0	0
4	2.1	0.32	10	3	30	4.48	2	20	4.16	1	10	3.72	0	0	0
5	2.4	0.38	10	5	50	5	4	40	4.75	3	30	4.48	1	10	3.72
6	2.7	0.43	10	7	70	5.52	6	60	5.25	5	50	5	3	30	4.48
7	3	0.48	10	10	100	7.37	9	90	6.28	7	70	5.52	5	50	5
8	3.3	0.52	10				10	100	7.37	9	90	6.28	7	70	5.52
9	3.6	0.56	10							10	100	7.37	9	90	6.28
10	3.8	0.58	10										10	100	7.37

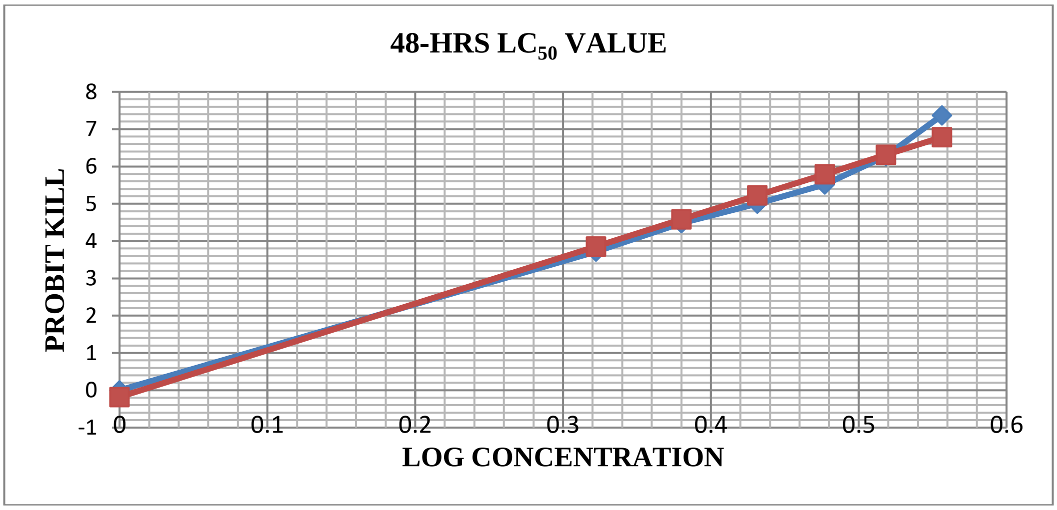
3.2 Lethal Concentration (LC_{50})

In the study of toxicology, the median tolerant range of any contaminant serves as a basic reference point (Ward and Parrish, 1982). Lethal toxicity values were determined according to Finney's probit bioassay for 24 to 96 hours. Different concentrations of toxicant were selected from the range test concentrations 0.001-0.1µL/L, 0.1- 1µL/L, and 1-10 µL/L to determine lethal toxicity values. No mortality was observed in the range 0.001-0.1µL/L and 0.1-1.0µL/L while mortality was observed in 1-10µL/L and based on this range 9 different concentrations (1.5µL/L, 1.8µL/L, 2.1µL/L, 2.4µL/L, 2.7µL/L, 3µL/L, 3.3µL/L, 3.6µL/L and 3.8µL/L) were selected for exposure. Data analysis was carried out by using the regression method. By using the probit method, the 24, 48, 72 and 96-hour LC_{50} values and 95% confidence limits for pendimethalin were determined to be 2.83µL/L, 2.59µL/L, 2.39µL/L and 2.20µL/L based on the mortality measurements (as shown in Table 1). The graphs between log concentration and probit mortality are shown (Graphs 1-4).

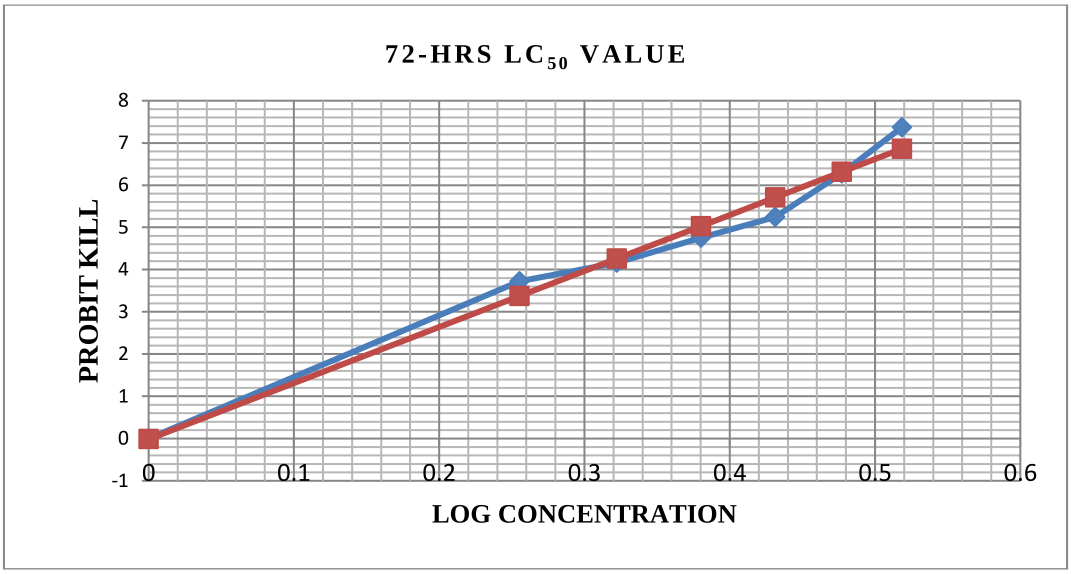
Graphs LC_{50} calculation:



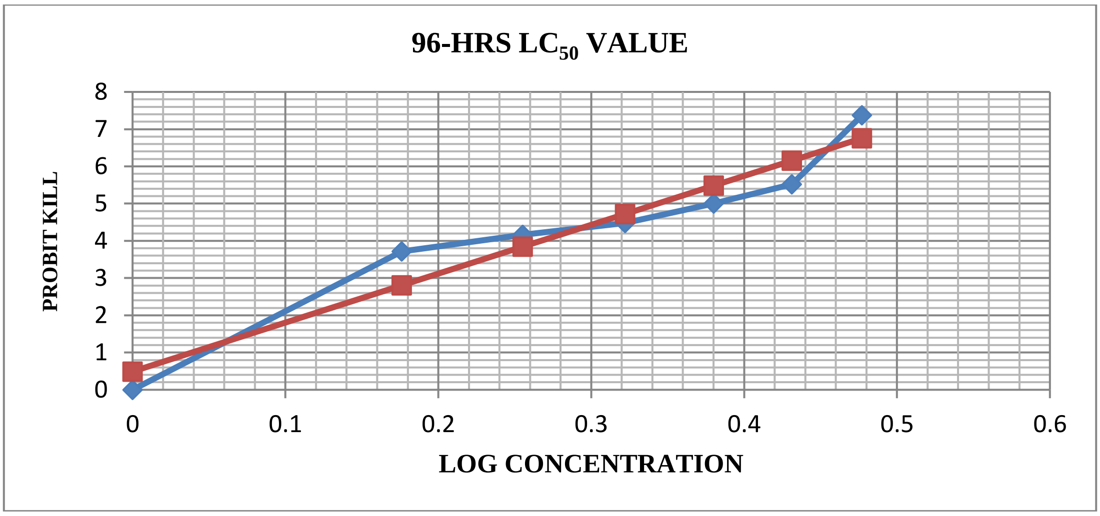
Graph 1. Showing relationship for log of concentration and probit mortality for 24-hrs LC_{50} value with 95% confidence limit. R-square value for this graph is 0.96. While the linear line equation is Y=11.69 + (-0.29)



Graph 2. Showing relationship for log of concentration and probit mortality for 48-hrs LC_{50} value with 95% confidence limit. R-sqare value for this graph is 0.98. While linear line equation is Y=12.52 + (-0.17)



Graph 3. Illustrating relationship between log of concentration and probit mortality for 72-hrs LC_{50} value with 95% confidence limit. R-sqare value for this graph is 0.98. While linear line equation is Y=13.26x + (-0.014)



Graph 4. Demonstrating relationship between log of concentration and probit mortality for 96-hrs LC_{50} value with 95% confidence limit. R-sqare value for this graph is 0.93. While linear line equation is Y=13.13x + 0.48

3.3 Behavioral Alterations at Sublethal Concentrations

Sublethal concentrations (i.e., 1/5th, 1/10th and 1/15th of 96-hrs LC_{50} value) were taken to study behavioral abnormalities caused by pendimethalin in *C. carpio* and various behavioral parameters such as general activity, feeding activity, movements dynamics, schooling behavior, aggression levels, breathing behavior and reflex towards stimuli were studied at 7, 14, 21 and 28-day intervals as compared to control.

General Activity: At minimum concentration, fish did not show much activity but at higher concentrations, fish showed a decrease in normal swimming patterns.

Foraging behavior: At high concentrations, at 21 and 28 days of pendimethalin exposure fish showed minimum foraging and sometimes avoided feeding as compared to normal feeding in the control group.

Table 3. Demonstrating various behavioral alterations caused by sublethal concentrations of pendimethalin exposed carp at 0 (Control), 7, 14, 21 and 28-day intervals

Behavioral Perspective	Period (In Days)	Baseline Concentration ($1/15^{th}$ LC_{50})	Mid-Level Concentration ($1/10^{th}$ LC_{50})	Elevated Concentration ($1/5^{th}$ LC_{50})
General Activity	Control	+	+	+
	7	+	+	+
	14	+	+	-
	21	+	-	--
	28	-	--	---
Foraging Behavior	Control	+	+	+
	7	+	+	-
	14	+	+	-
	21	+	-	--
	28	-	--	---
Movement Dynamics	Control	+	+	+
	7	+	+	+
	14	+	++	++
	21	+	++	++
	28	++	+++	+++
Social Behavior	Control	+	+	+
	7	+	+	-
	14	+	+	-
	21	-	--	---
	28	-	--	---
Reactivity to Stimuli	Control	+	+	+
	7	+	+	+
	14	+	+	+
	21	+	+	-
	28	+	-	--
Habitat Depth Select	Control	+	+	+
	7	+	+	+
	14	+	-	-
	21	+	-	--
	28	-	-	--
Respiration Rate/Breathing Frequency	Control	+	+	+
	7	+	+	++
	14	+	++	++
	21	+	++	+++
	28	++	+++	+++
Mucus secretion	Control	+	+	+
	7	+	+	+
	14	+	+	++
	21	+	++	++
	28	+++	+++	++++
Locomotive Behavior	Control	+	+	+
	7	+	+	+
	14	+	-	-
	21	+	-	--
	28	-	-	--

(+) Normal, (++) Slightly increased, (+++) Mildly increased, (++++) Severely increased, (-) Sightly decreased, (--) Mildly decreased and (---) Severely reduced.

Movement rates: With the increase in pendimethalin concentrations, carp showed increased movement rates as compared to normal movements of the control group. Fish showed erratic swimming behavior.

Schooling behavior: At elevated concentrations, fish social behavior was diminished, reduced social interactions and solitary behavior were seen.

Reactivity to stimuli: At high pendimethalin concentrations fish showed reduced response to external stimuli.

Habitat depth selection: At elevated pendimethalin doses fish seem to avoid depth, fish seem to prefer surface water for air gulping.

Respiration rate: With the increase in pendimethalin dose opercular movement speed was increased which showed increased breathing rates.

Locomotive behavior: Fish showed very little movement or no movement at elevated concentrations of pendimethalin.

Mucus secretion: With the increase in concentration of pesticide at 21 and 28 days fish showed higher secretion of mucus from the body.

4. DISCUSSION

This investigation aimed to assess the acute poisoning and behavioral reactions to herbicide pendimethalin exposure in *C. carpio* (Linnaeus). The findings unambiguously showed that fish exposed to pendimethalin had an increased death rate as the herbicide's dosage rose.

The standard measurement for lethal toxicity studies is the median lethal concentration (LC_{50}), which shows the quantity of an active ingredient that kills 50% of the sample organisms within 96 hours. Numerous investigations have shown the toxicity profiles of several pesticides, such as insecticides and herbicides, for a variety of fish species. Lethal toxicity values for *Clarias gariepinus* juveniles exposed to the glyphosate herbicide in an experiment by Sani and Idris, (2016) revealed 50% 96-hour death at 0.0072 mL/L. In similar line with our findings, *Channa punctatus* exposed to pendimethalin showed 96 hours 50% mortality values of 2.20 mg/L Kalita and Choudhury, (2018). Nearly similar results to our findings were seen when *Oreochromis niloticus* was exposed to stomp (pendimethalin), observed LC_{50} value was 3.55mg/L El-Sharkawy et al*.,* (2011). In another experiment in Nile tilapia exposed to pendimethalin, the 96-h lethal toxicity concentration was determined as 4.92 mg/L almost double as estimated by us El-Sayed et al*.* (2015). When treated with the herbicide pretilachlor, *Clarias batrachus* had LC_{50} values of 9.55, 8.57, 7.11, and 5.84 mg/L after 24, 48, 72, and 96 hours Soni and Verma, (2018). *C. punctatus* exposed to pendimethalin showed a 96-hrs LC_{50} value of 3.6 mg/L Ahmad and Ahmad (2016). Pendimethalin's 96-hour LC_{50} value is 199 ug/L in bluegill sunfish, 138 ug/L in rainbow trout, and 420 ug/L in channel catfish (Weed Science Society of America, 1994). Hence our findings suggested that even very low concentrations of pendimethalin can cause carp mortality.

Fish can be used as bio-indicators of water quality because of their ability to readily change in morphology, physiology, and behavior when placed in different environments. *C. carpio* exposed to sublethal amounts of pendimethalin for 28 days exhibited behavioral abnormalities including increased mucus secretion, erratic swimming, increased air gulping and opercular movement, decreased vigilance against predators, decreased schooling behavior, loss of balance, and increased surface breathing. These results were similar in line with the findings of Wang et al*.* (2022) who studied the effects of pendimethalin on *Hypophthalmichthys nobilis.* Our findings such as altered opercular activity and abnormal swimming behavior were similar to those observed by Yalsuyi et al*.* (2021), on *C. carpio* exposed to glyphosate. Similar results as observed by us such as abnormal gill movement, surface air gulping, abnormal swimming movements, loss of balance and increased mucus secretion were found by Baghfalaki et al*.*, (2012) in *Hypophthalmicthys molitrix*, *C. carpio* and *Rutilus rutilus caspicus* treated with tribenuron-methyl. In another experiment conducted by Neglur and David, (2021) on C. carpio showed altered opercula movement, fin movement and mucus secretion after treatment with Fenaxoprop-P-Ethyl similar to our findings.

5. CONCLUSION

In conclusion, the assessment of LC_{50} values and behavioral changes in C. carpio exposed to pendimethalin reveals the herbicide's substantial effects on aquatic life. The behavioral changes that have been noticed, such as adjustments to eating and swimming habits, erratic swimming, increased breathing rates, increased surface air gulping and reduced schooling behavior, point to a stress reaction to the exposure to chemicals. These alterations show that the chemical exposure caused a stress reaction. When assessing pendimethalin's toxicity, the determined LC_{50} value is a crucial metric. For regulatory actions and environmental risk evaluations, it offers vital data. These results highlight the requirement for more investigation into the ecological impacts of pendimethalin over the long term on fish populations and aquatic ecosystems, as well as the development of mitigation techniques for its use in polluted environments. In general, conservation efforts and the advancement of better aquatic ecosystems can be aided by a knowledge of *C. carpio* physiological and behavioral responses.

ETHICAL APPROVAL

The Institutional Animal Ethics Committee (IAEC) accepted this investigation, and IAEC criteria were rigorously followed throughout all operations. The study was carried out in compliance with ethical guidelines for responsible animal usage, with an emphasis on avoiding discomfort and guaranteeing the humane treatment of animals.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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