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STUDIES ON TOXICOLOGICAL ENDPOINTS OF FENOXAPROP-P-ETHYL ON BEHAVIORAL CHANGES IN FRESHWATER EXOTIC CARP *CYPRINUS CARPIO* (LINNAEUS)

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ABSTRACT
The present study was undertaken to determine the acute toxicity of the herbicide Fenoxaprop-P-ethyl (FPE) and the resulting behavioral alterations in the exotic freshwater carp *Cyprinus carpio*. The experimental fish were divided into groups of 10 (n=10) and exposed to different concentrations of the test pesticide for 24, 48, 72, and 96 hours. The 96-hour LC_{50} was found to be 300 μg/l. Sub-lethal concentration was fixed based on LC_{50}value is 37.5 μg/l (1/8th of LC_{50}) of the herbicide FPE for a period of 45 days. Regular observation was made throughout the exposure period to determine the long-term behavioral changes in the test fish. The fish displayed erratic swimming behavior that increased over the days of exposure. Behavioral anomalies such as whirling cork movement, altered opercular movement, altered fin movement and physiological changes such as dyspigmentation and altered mucus secretion were observed.

Keywords: *Cyprinus carpio*, Fenoxaprop-P-ethyl, Herbicide, Freshwater fish, Fenoxaprop acid

1. INTRODUCTION

Toxic chemicals have changed the quality of water that affects the well-being of fishes and other aquatic organisms. Increased use of chemical pesticides for agricultural purpose has resulted in the excess inflow of toxic chemicals into the environment, mainly into the aquatic ecosystems [1]. Herbicides are one among the more frequently used category of pesticides, because of their high herbicidal property, low mammalian toxicity, less persistence in the environment, and rapid biodegradability [2].To deal with the pests of agricultural crops, synthetic and chemical pesticides are being used on a large scale. This would perhaps enhance the quality and productivity of crops, and profit to the farmers. But at the same time, excess use of these chemicals has rendered the soil and water resources unfit for the non-target, non-pest species to thrive. Toxic chemicals that reach into the water bodies as a result of washing by rain greatly affect the aquatic invertebrates, fish, birds and mammals, and even plants. Degradation of pesticides also causes the release of its toxic derivatives/ by-products which in turn affect the growth and normal physiology of aquatic organisms. However, little information has been reported regarding the fate of Fenoxaprop-P-ethyl(FPE) post-application, especially in the complex environmental systems such as aquatic environments, and there is a need to consider the toxicity of degradation products.

Fishes are ideal sentinels to study the impact of exposure to various stressors and toxic chemicals on behavioral aspects of animals due to the following factors: 1) direct and constant contact with the aquatic environment where chemical exposure occurs over the entire body surface, 2) ecological relevance in many natural systems 3) ease of culture and maintenance, 4) ability to come into reproductive readiness [3], and 5) long history of use in behavioral toxicology studies. Fishes are able to uptake and retain different xenobiotics dissolved in water through active or passive processes. They can be used to detect and document the pollutants released into an aquatic environment. Sub-lethal concentrations of pesticides in aquatic environments may not kill the organism, but may cause devastating structural and functional changes in its morphology and physiology; and this is more common than mortality [4]. Behavioral alteration is one of the most sensitive indicators of environmental stress and may affect survival [5, 6]. Changes in fish behavior, particularly in non-migratory species, can also provide important indices for ecosystem assessment. Any change in the normal behavior of fish is a clear indication of the deterioration of water quality, and hence an index of environmental suitability and cost of survival. Behavioral studies provide a unique perspective in linking the physiology and ecology of an organism and its environment [7]. Behavior is both a sequence of quantifiable actions, operating through the central and peripheral nervous systems, and a cumulative manifestation of genetic, biochemical and physiologic processes essential to life such as feeding, reproduction, and avoiding predators [8]. Behavior allows an organism to adjust to the external and internal stimuli in order to best meet the challenges of surviving in a changing environment. Conversely, behavior is also the result of adaptation to environmental variables and is a selective response that constantly adapts through direct interactions with physical, chemical, physiological and social aspects of the environment. Evolutionary processes have conserved stable behavioral patterns in concert with the morphologic and physiologic adaptations in an animal. This stability provides the best opportunity for survival and reproductive success by enabling organisms to efficiently exploit the available resources and define suitable habitats [7]. Behavior is not a random process, but a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival of the individual in a given environment. Behavioral endpoints serve as valuable tools to discern and evaluate the effects of exposure to environmental stressors, both physical and chemical.

Fenoxaprop-P-ethyl or Ethyl (2R)-(+)-2-[4-(6-chloro-benzoxazolyl-2-oxy) phenoxy] propionate is one of the well-known herbicides used for the control of annual and perennial weeds in rice, wheat, onion, and many other important crop fields. It belongs to 2-(4-aryloxyphenoxy) propionic acids family and is used as post-emergence herbicide that exclusively impedes fatty acid synthesis in grasses via inhibition of the enzyme acetyl CoA carboxylase [9, 10]. Residue analysis experiments have indicated that the parent compound FPE continues to deteriorate into its residue Fenoxaprop-P for up to 20 days in wheat field soil [11]. Fenoxaprop acid has been shown to persist in the soil for 21 days with half-life of 7.3 days in Indian soil (pH 8.2). However, it was noted that soil pH played a major role in the degradation of the herbicide in the field [2]. Another study group demonstrated that the hydrolysis of FPE highly depended on pH and followed pseudo-first-order kinetics. Degradation rates were minimum at pH 6.0 while 6-fold higher at pH 4.0, and 270-fold higher at pH 10.0. Breakdown of FPE followed different mechanisms of hydrolysis at different pH levels. The acute toxicity of FPE to *Daphnia magna*, a sensitive crustacean, was found to be 14.3 µmol/L (48h EC_{50}) [12]. The evaluation of toxic impacts of FPE herbicide on porcine sperm parameters was carried out by exposing the sperm samples to a series of pesticide concentrations. The study concluded that FPE strongly affected the motility of pig sperm which was attributed to the damage of mitochondria in the sperm mid-piece [13]. The study gave new insights to the field of toxicology regarding the toxicity of FPE herbicide. The study was a clear demonstration of deleterious effects on non-target organisms. The study showed that the LC_{50} values of fresh and expired FPE were 12.0 mg/l and 10.56 mg/l respectively and indicated that the toxicity to aquatic organisms was very high. High concentrations of FPE caused cell death in the wing cells of *Drosophila*, however there was no sign of mutagenic activity [14]. Reports and data regarding the toxicity of FPE to aquatic organisms are meagre. Hence the present study was undertaken with the objective to determine the acute toxicity and behavioral impacts of FPE on the common freshwater carp *Cyprinus carpio*.

2. MATERIAL AND METHODS

2.1. Experimental animal and ethical clearance

Healthy and active fingerlings of *Cyprinus carpio* were procured from the Bhadra Fish Seed Farm, Shimoga, Karnataka. Fish were maintained in large cement tanks (6×3 feet) which were duly aerated. Water in the tanks was treated with 1% KCl solution prior to the introduction of the fish into the tank. Fish were fed with balanced nutritious food pellets (Nova, Aquatic P. Feed) and allowed to acclimatize for a period of 14 days at 24 °C temperature and 12-14 hours of photoperiod. Water in the tanks was renewed daily and the Physicochemical parameters of water were examined according to the guidelines of American Public Health Association [15]. All the experimental protocols performed in the present study abide by the guidelines of the Institutional Animal Ethics Committee (IAEC), Karnatak University and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines, New Delhi, India. All the animals used in the study were handled with extreme care.

2.2. Experimental pesticide

Fenoxaprop-P-Ethyl (6.9 % w/v EC) was procured from the local market of Dharwad, Karnataka, India, under the Bayer House, Central Avenue, Hiranandani Estate, Thane (West), India. The expiry date of the test substance was confirmed prior to the initiation of the exposure. Stock solution was prepared by mixing the calculated volume of the commercial solution with distilled water. Test concentrations for acute toxicity test (*i.e.* 100 μg/l to 500 μg/l) and sub-lethal behave-oral toxicity test (37.5 μg/l) were prepared by serial dilution of the stock solution using variable micropipette.

2.3. Acute toxicity test

Acute toxicity testing was carried out using semi-static renewal assay which involves daily renewal of water and test solution [16]. Range finding test was conducted to estimate the upper and lower concentrations of the test compound for the selected fish. This step was employed to minimize the unnecessary killing of the animals. Fingerlings weighing 10-12 gm and 7±2 cm in length were selected for the study. The fish were divided into 28 groups of 10 each and transferred to clean, pathogen-free glass aquaria. Feeding was withdrawn 24 hours prior to the exposure. All the test concentrations of FPE were maintained in triplicates. One group served as control. All the exposed fish were continuously monitored and mortalities were recorded over gradual intervals of time *i.e.* 24, 48, 72, and 96hours. Dead fish were removed from the experimental pool. Mean was calculated for the number of mortalities in each concentration group over each exposure duration and this value was used to calculate the LC_{50} of FPE by performing probit analysis [17].

2.4. Behavioral studies

Healthy *Cyprinus carpio* fingerlings (n=10) were introduced into a glass aquarium (volume 20ltr) and were exposed to the sub-lethal concentration of FPE. One eighth of LC_{50} (37.5 μg/l) was selected and fish were exposed for four different durations: 1, 15, 30, and 45 days. All exposures were maintained in triplicates. The fish were frequently observed for any morphological deformities and behavioral anomalies during the experimental period. Behavioral changes were recorded for further interpretation of the toxicant effects on *Cyprinus carpio*.

3. RESULTS AND DISCUSSION

3.1. Acute toxicity

The experimental fish were exposed to different concentrations of commercial grade Fenoxaprop-P-ethyl (FPE) 6.9% EC. Water in the exposure tanks was tested for the physico-chemical parameters to ensure the optimum and constant experimental conditions (Table 1).

Table 1: Physico-chemical parameters of water

SL. No.	Parameter	Obtained values
1.	Temperature	24±2°C
2.	pH	7.1±0.2 at 24°C
3.	Dissolved oxygen	9.6±0.8 mg/L
4.	Carbon dioxide	6.3±0.4 mg/L
5.	Total hardness	23.4±3.4 mg
6.	$CaCO_3$/L,	nil
7.	Phosphate	0.39±0.002 μg/L
8.	Salinity	nil
9.	Specific gravity	1.001
10.	Conductivity	less than 10 μS/cm

Toxicity of FPE to *Cyprinus carpio* exposed to different concentrations of the toxicant for 96 hours exhibited 100% mortality at 500 μg/L, no mortalities at 100 μg/L, and 50% mortality at 300 μg/L (Table 2).

Table 2: Mortalities of Cyprinus carpio fingerlings in different concentration of FPE at 96hrs exposure period

Sl. No.	Concentration of Pesticide μg/L	Log concentration of Pesticide	No. of fish exposed	No. of fish alive	No. of fish dead	Percent Kill (%)	Probit Kill
1.	100	2	10	10	0	Nil	0
2.	150	2.176	10	9	1	10	3.72
3.	200	2.301	10	8	2	20	4.16
4.	250	2.397	10	6	4	40	4.75
5.	300	2.477	10	5	5	50	5.00
6.	350	2.544	10	4	6	60	5.25
7.	400	2.602	10	3	7	70	5.52
8.	450	2.653	10	1	9	90	6.28
9.	500	2.698	10	0	10	100	0

Mortality rate gradually increased with increasing concentration of FPE- 150 μg/L to 500 μg/L. The LC_{50} values were calculated by probit analysis method [17]. Table 3 shows 95% confidence limit calculations for the obtained data. Fig.1 shows the percent mortality against FPE concentration and percent mortality converted to probit against log concentration respectively. Both the graphs are represented by straight lines and indicate the LC_{50} value of 300 μg/L. A sigmoid curve was obtained when percent mortality was plotted against the log concentration of FPE (Fig. 2).The data obtained from the experiment established the LC_{50}value of 300 μg/L and the upper and lower bound are shown in table 3.

Table 3: Acute toxicity (96 h LC_{50}) and 95% confidence limits of FPE to *Cyprinuscarpio*

Toxicant	96 h LC_{50} (μg/L)	95% confidence limits	
		Upper limit	Lower limit
Fenoxaprop-P-ethyl (FPE)	300	100	500

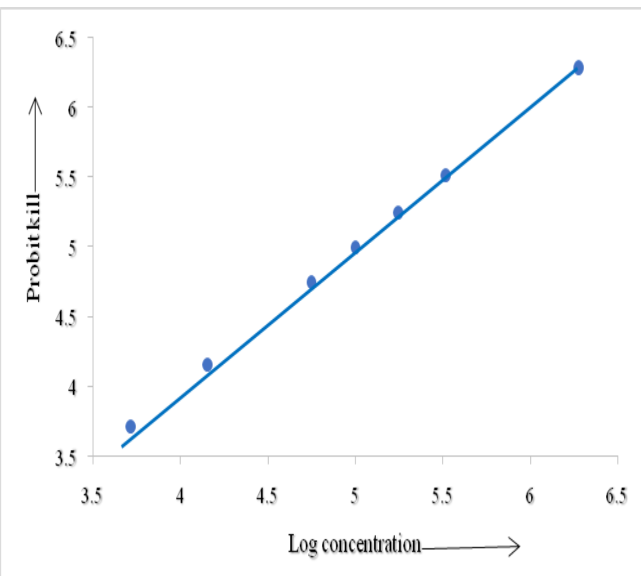

Fig. 1: Probit kill of *Cyprinus carpio* exposed to different concentrations of FPE

Behavioral anomalies were evidenced right from the day of exposure to lethal concentrations of FPE. Disrupted schooling behavior, gulping air, surfacing phenomenon (swimming at the water surface), this situation increased intensely throughout the test period, which is in accordance with the observations made by [18]. Surfacing phenomenon i.e. significant preference to upper layers of water in exposed fish may be a strategy to minimize the contact with the toxic medium [19]. Gulping of air may be due to the elevated demand for oxygen and may be seen as a means to ease the respiratory stress. Surfacing phenomenon and easy predation continued. This reflects the catastrophic impact posed by the toxicant.

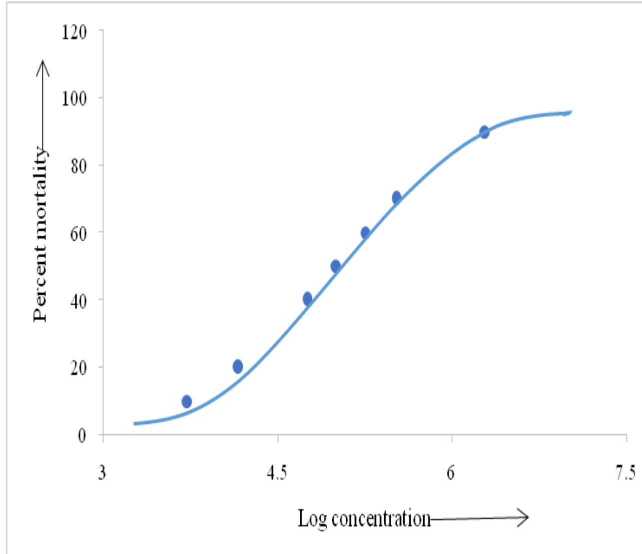

Fig. 2: Percent mortality vs log concentration of *Cyprinus carpio* exposed to different concentrations of FPE

Hyper-extension of fins, and dullness in body color were observed in fish under FPE stress. These phenomena increased with exposure time and FPE concentration. Intermittently, some of the fish sank to the bottom with extinguished opercular movements, failing to fight FPE stress in exposure periods. During the exposure period, some fish exhibited slight swelling of the abdominal region, which persisted even during the recovery. In general, fish poisoned with pesticides show signs of muscle paralysis (especially of the fins and respiratory organs), hyperactivity, and loss of balance [4]. Leaning of fish and reduction in body mass indicate reduced feeding and diversion of fish metabolism towards adaptability to the toxic media. Feeding preferences were affected and food consumption in fish was drastically reduced and impaired. This was more pronounced in lethal exposures. For these animals, it might be profitable to decrease the food intake under toxic environmental conditions to reduce the expenditure of energy on digestion. Intermittence of feeding for longer periods can have long-term impact on growth and reproduction [20]. Fish exposed to the lowest lethal concentration (150 μg/l) of FPE were alert and fed actively. Overall impairments in fish behavioral responses and morphological deformities even under recovery periods may be due to the continued inhibition of brain and muscle AChE activity by FPE via biotransformation of sequestered FPE in the storage organs, dearylation reaction utilizing the same enzymes and A-esterase [21]. Furthermore, physiological reactions such as activation of biotransformation enzyme systems in the presence of xenobiotic substances enable the organisms to survive in acute exposures.

3.2. Behavioral studies

Behavioral studies are the clinical observations that help to understand the overall response of an animal towards its environment.All the behavioral observations made in the study followed the guidelines of the Organization for Economic Co-operation and Development (OECD), directive no. 203 - "Fish acute toxicity test". In the present study, the control fish exhibited sensitive and quick responses to the disturbances in the environment. They were active for feeding and alert to slightest of the disturbance with their well-synchronized movements. Control fish exhibited normal schooling behavior, fright response, opercular beat, fin beat, and buccal movement. The behavior did not significantly vary between the control groups and hence these results were taken as standard for the entire experimentation. The experimental fish exposed to sub-lethal concentration of FPE on the other hand, showed various behavioral alterations and anomalies. Fish exposed to sub-lethal concentration of FPE displayed localization to the bottom of the aquarium, burst (spread out) swimming, reduced schooling behavior and fright response, increased opercular beat, fin beat and highly increased buccal movements on 15th day of exposure. Loss of coordinated movements and occupancy of twice the area as compared to that of control group were the early responses to FPE exposure. On exposure days 30 and 45, fish showed gradual increase of opercular beat and fin beat, heavy mucus secretion on the body surface and dyspigmentation of the body (Table 4).

Table 4: Behavioral changes in *Cyprinus carpio* exposed to different concentrations of FPE

Sl. No.	Observed behavior	Control	Exposure days		
			15 Days	30 Days	45 Days
1.	Lateral swimming	-	+	+ +	+
2.	Sinking phenomenon	-	+	-	+
3.	Schooling behavior	+++	+ +	+	-
4.	Fright response	+++	+ +	+	-
5.	Backward swim	-	+	+	+
6.	Dashing movement	-	+ +	+ + +	+
7.	Upward swim	-	+ +	+ +	-
8.	Whirling cork movement	-	+	+ + +	++
9.	Buccal movement	+	+ + +	+ + +	+
10.	Burst swimming	-	+ +	+ +	+
11.	Opercular beat	+	+ +	+ +	+ +
12.	Fin beat	+	+ +	+ + +	+ + +
13.	Mucus secretion	-	+	+ +	+ +
14.	Dyspigmentation	-	+	+ + +	+ +

Fishes exhibiting various behavior indicates (+) as low, (++) as medium &(+++) as high intensity of behavior upon exposure to FPE

Dyspigmentation of body surface was observed gradually from day 10 to day 30 in the exposed fish which coincided with the results obtained by Ramesh and David (2009), who worked on the effect of chlorpyrifos in *Cyprinus carpio*. These changes were more pronounced on 45th day of exposure. Behavioral alterations in FPE exposed fish were monitored daily and all the observations were video recorded.

Movement of the fish to the corners and the bottom of the test tanks can be viewed as a behavior to avoid the stressor molecules in the medium, in this case FPE. Further, behaviors such as erratic movements, irregular swimming, vertical hanging, and loss of body equilibrium may be attributed to the inhibition of acetylcholinesterase (AChE) in cholinergic synapses ensuing hyperstimulation. Inhibition of AChE activity is a typical characteristic of many widely used agricultural pesticides [23].

Fish exposed to sub-lethal concentration of FPE slowly became lethargic and secreted excess mucus all over the body surface. Intermittently, some of the test fish were restless, hyper-excited, and displayed erratic movements. These behavioral alterations persisted even during the recovery period. Increased mucus secretion is also one of the major observations in the experimental group compared to control group. The exposed fish secreted excess mucus that served as a barrier between the fish and its environment to minimize the chances of exposure to the toxicant. Similar observations were made by Mehmet Yilmaz *et al.* in the study of effect of 2, 4-D herbicide on the behavior of *Cyprinus carpio*. Excess secretion of mucus in fish forms a nonspecific response against toxicants, probably meant to form a barrier between the body surface and the toxic medium, thus reducing the contact between the toxicant and the body. Mucus may also minimize the irritation caused by the toxicant, and may even have a role in scavenging the toxicant. Rao made similar observations following RPR-V (a novel phosphorothioate insecticide) exposure in euryhaline fish, *Oreochromis mossambicus.*

Evaluation of toxicological impacts on aquatic life is one of the basic and important factors in order to introduce an eco-friendly herbicide. Fishes, being the key trophic level of feeding cycle in aquatic eco-systems, highly impact the health of other aquatic animals as well as terrestrial animals through food chain [24]. Technical grade FPE is reported to have acute toxicity (96 h LC_{50}) equivalent to 0.39 mg/L to *Oncorhynchus mykiss* and 0.19 mg/L for *Lepomismacrochirus* [25]. Present investigation assessed the behavioral changes in *Cyprinus carpio* exposed to sub-lethal concentrations of FPE. The fish exhibited erratic movements and hyper excitability when exposed to the toxicant for the first time. However, these changes normalized in subsequent exposure days and seem to be a part of adaptive system in the fish. During the study the control group maintained good co-ordination and normal behavioral patterns but the exposed groups of fish failed to cope up with the toxic stress and exhibited disruptions in schooling and fright response behavior. Burst swimming was found slowly evident from day 15 to day 45 of exposure which is a key indicator of damaged co-ordination between the muscles and the nervous system. Some of the fish displayed sinking phenomenon, dashing movements, backward swimming and vertical swimming which could be attributed to the loss of balance due to impaired nerve transmission or disturbances in the body physiology. Our findings were in accordance with the findings of M. David. *et al*., and Vijay Krishna Das.

During the study, the exposed animals exhibited increased buccal movement on of exposure. The opercular beat increased gradually till the day that might be attributed to the inability of the gills to supply enough oxygen in normal beat. Damage to the gills could be one of the reasons for the increased opercular beat as the fish physiology demands more oxygen at sudden stress conditions.

4. CONCLUSION

The results clearly demonstrate the profound toxicity of the herbicide on the fish behavior. The LC_{50} of the herbicide was found to be 300μg/L through acute toxicity studies and it is established that the herbicide FPE is highly toxic to the sensitive fish *Cyprinus carpio.* Further studies are required to assess the possible detrimental effect of FPE on growth, reproduction and other aspects of the fish *Cyprinus carpio* as well as that of other aquatic organisms of economical importance. This study offers the base for future research on freshwater carps and aquatic organisms in general.

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