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Studies on effect of fipronil on behavioural aspects and protein metabolism of freshwater fish *Oreochromis mossambicus*

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Abstract
Pesticide application plays a crucial role in modern day agriculture. Leaching and mixing of chemicals from agricultural practices have a direct impact on the aquatic systems posing great threat to the aquatic life forms. Indiscriminate usage of new class insecticide fipronil (FPN) has been found to affect freshwater fish. The present work was aimed to elucidate the toxicity of FPN on freshwater fish, *Oreochromis mossambicus*. The acute toxicity was evaluated and 96 hr LC_{50} was found to be 3.0 mg/L. Further, two sub-lethal concentrations (0.5 mg/L and 1.0 mg/L) were selected for the assessment of behavioural toxicity and protein metabolism investigations. The duration of exposure selected for each sublethal concentration was 7 and 14 days. Changes in behavioural responses were noticed in fish exposed to FPN and found to affect the fish in dose and duration dependent pattern. Protein levels were estimated in terms of soluble, structural and total proteins and were found to significantly ($p<0.05$) decline with the increase in the days of exposure. Based on the outcome of the present study, it is inferred that FPN is highly toxic as it affects the behavioural aspect and protein biochemistry within the exposed fish. It is therefore suggested that care must be taken when FPN is used and disposed in the proximity of aquatic habitats.

Keywords: Ecotoxicology, fipronil, fish, tilapia

1. Introduction
By virtue, pesticides have turned out to be an integral utility in agricultural practice [1]. The environmental impact of pesticides is often greater than what it is intended by the users. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water, sediments and even food [2]. Assessing the toxicological impact on aquatic organisms is therefore one of the preliminary requirement for maintaining a balanced ecosystem [3]. Environmental monitoring plays a major role which provides a frame-work for the controlled usage of chemical pesticides for agriculture, household and veterinary uses. Fishes are one of the economically important natural resources which are known to play a crucial role in ecological sustenance [4].
The scarcity in the fish population is a matter of concern that has attracted many studies in the view of their conservation, proper health and growth which is important under aquaculture practice. Additionally, fishes serve as a form of cheap protein and also constitute to the major minerals and nutrients which otherwise act as an important component in the diet [5]. Fishes are directly exposed to the environmental stressors, the impact of which can be assessed at various levels viz., behavioural [6], histopathological [7], and biochemical outcome [8]. This makes them ideal organisms to analyze the impact of toxicants that are released into the aquatic systems by the water flow from the fields and disposed off from the industries and storage tanks. Few of these toxicants are potentially biodegraded if they have a short half-life, whereas some persist for a longer duration without undergoing degradation. The level of persistence is also attributed to the by-products of pesticides that cause more harm than the parent compound.
Fipronil (5-amino-1-[2, 6- dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile] (FPN) is the first and a highly active, broad spectrum pesticide from the phenylpyrazole chemical family effective against a wide range of economically important pests [9]. FPN was first registered for use in the United States in 1996. This pesticide is designed to specifically inhibit insect gamma amino-butyric acid (GABA) receptors in the

neurons of the central nervous system [10]. It potentially causes adverse health effects as it is used both commercially and to control household pests [11]. FPN is reported to be highly toxic to non-specific target organisms which include both economically important honey bees and other aquatic invertebrates also vertebrates like fishes, amphibians, aves and mammals as it is often used in or near aquatic environments affecting sensitive vertebrate life stages [12]. The half-life of FPN in water is 14.5 days [13] which makes it suitable for the current investigation.

2. Materials and Methods

2.1 Collection and maintenance of fish

Healthy *Oreochromis mossambicus* were procured from the State Fisheries Department, Dharwad, India and were acclimatized to laboratory conditions for 15 d at 24 °C. Further they were held in de-chlorinated tap water in large cement tanks which was previously washed with potassium permanganate to free the walls from any microbial growth. Fish were fed regularly and 12-16 h of photoperiod daily during acclimation. Water was renewed daily, whose physico-chemical characteristics were analyzed following the methods mentioned in APHA [14].

2.2 Acute toxicity test

A static renewal assay test was employed as a method of exposure for each acute toxicity test. A range finding test was performed prior in order to find the upper and lower limits of acute toxicity value of FPN against *Oreochromis mossambicus*. This step was carried out in order to minimize the animal killing. In all (six) sets, experiments were conducted simultaneously. The experimental fish (10 each) were transferred to the aquaria consisting of different concentrations of FPN in eleven different groups. Among these, one set however, served as a control group, which were being placed in de-chlorinated tap water without any traces of FPN (n = 10). For each concentration, including the control, 3 replicates were maintained and the mean values of these were taken into consideration for determining the test results. Observations were made at every 24 h intervals during the 96 h of exposure period. After every 24 h, the number of dead animals was recorded and the same were removed from the aquaria. Mortality data from the replicate samples from each FPN concentration were pooled prior to calculating the LC_{50} value and the 95% confidence limits.

2.3 Behavioural studies

To understand the behavioural responses clearly, a fish was transferred to a polypropylene container with 3000 ml of water along with its respective concentration of the toxicant. This helped to achieve the better understanding of swimming pattern and the other behaviour anomalies they possessed. The behaviour of all the fish in a group (n=6) was considered and the data was pooled together and interpreted.

2.4 Biochemical studies

The soluble, structural and the total proteins in the liver were estimated using the Folin-phenol reagent method as described by Lowry *et al*. [15]. 1% homogenate (W/V) was prepared in an ice-cold 0.25 M sucrose solution. For soluble and structural proteins, 1.0 mL of the homogenate was taken and centrifuged at 3000 rpm for 10 min. The supernatant was separated and to both the supernatant and residue, 3.0 mL of 10% trichloroacetic acid (TCA) was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentation. For total proteins, 1 mL of homogenate was taken; to it 3 mL of 10% TCA was added and centrifuged at 3000 rpm. The supernatant was discarded and the residue was taken for experimentation. All three residues were dissolved in 5 mL of 0.1N sodium hydroxide and to 1 mL of each of these solutions, 4 mL of reagent -D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to stand for 10 min, at the end of which 0.4 mL of Folin-phenol reagent (diluted with double distilled water in 1:1 ratio before use) was added. Finally, the optical density of the colour developed was measured using a spectrophotometer (Secomam, Anthelie advanced 2) at a wavelength of 600 nm.

2.5 Statistical Analysis

The biochemical status are reported as the mean ± standard error of the mean (SEM) obtained from triplicates. The data were subjected to one-way analysis of variance and further subjected to Tukey's test for post hoc analysis by defining the significance level at $P<0.05$.

2.6 Ethical committee

All procedures implemented in the present study were in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC). The animals subjected to experimentation were handled as per the guidelines issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

3. Results

3.1 Acute toxicity

The physicochemical parameters of the water used in the experimentation was carried out and the results are presented in table 1. The toxicity of FPN to *O. mossambicus* exposed for 96 h showed no mortality at 2.0 mg/L. The mortality rate increased with increase in the concentration of FPN and 100% mortality was observed at 4.0mg/L (Table 2). The 96 h LC_{50} value was determined by Probit method [16]. This 95% confidence limits was also determined and is presented in table 3. The percent mortality after transforming to Probit was plotted against log concentration of FPN. In this a straight line was obtained and the LC_{50} value obtained from the graph plotted was 3.0 mg/L (Figure 1 and 2). When percent mortality was plotted against log concentration of FPN, a sigmoid curve was obtained (Figure 3). The upper and lower 95% confidence limits are presented in Table 2. The 96h LC_{50} of FPN on *O. mossambicus* was found to be 3.0 mg/L.

Table 1: Physico-chemical characters of water used for the present investigation

Parameters	Values obtained
Temperature	24±1 °C
pH	7.1±0.3
Dissolved oxygen	6.1±0.4 mg/L
Total hardness	37.3±3.1 mg as CaCO3/L
Salinity	Nil
Specific gravity	1.003
Calcium	21.31±0.3 mg/L
Phosphate	0.9±0.0 mg/L
Magnesium	0.85±0.3 mg/L

Table 2: Showing mortality of *Oreochromis mossambicus* exposed to different concentrations of commercial grade fipronil

Sl. No.	Concentration mg/L	Log concentration of fipronil	No of Fish exposed	No of Fish alive	No of Fish dead	Percent mortality	Probit mortality
1	2.0	0.0	10	10	--	0	0
2	2.2	0.342	10	9	1	10	3.72
3	2.4	0.380	10	8	2	20	4.16
4	2.6	0.414	10	7	3	30	4.48
5	2.8	0.447	10	6	4	40	4.75
6	3.0	0.477	10	5	5	50	5.00
7	3.2	0.505	10	4	6	60	5.25
8	3.4	0.531	10	3	7	70	5.52
9	3.6	0.556	10	2	8	80	5.84
10	3.8	0.579	10	1	9	90	6.28
11.	4.0	0.602	10	00	10	100	7.33

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

Toxicant	96h LC (mg/L)	95% Confidence limits	
		Upper limit	Lower limit
Fipronil	3.0	2.0	4.0

Table 4: Behavioural changes in freshwater fish of *Oreochromis mossambicus* on exposure to sub-lethal concentrations of Fipronil

Sl. no	Behaviour observed	Control	Exposures			
			E1	E2	E3	E4
1.	Schooling behaviour	++ +	++	+ +	-	-
2.	Fright response	+ + +	+ +	+ +	-	-
3.	Dashing movement	-	++	++	+	+
4.	Backward swim	-	+	+	+	+
5.	Sinking phenomenon	-	+	-	+	+ +
6.	Upward swim	-	+ +	+	-	-
7.	Lateral swimming	-	+	+ +	+	+
8.	Loss of equilibrium	-	+ + +	+	+	-
9.	Whirling cork moment	-	+	+ +	+++	+
10.	Burst swimming	-	++	+ +	+ +	+
11.	Buccal movement	+	+	+ + +	+ +	+ +
12.	Opercular beat	+	+ +	+ +	+ + +	+
13.	Fin beat	+	+ + +	+ + +	+ +	+ +

Fishes exhibiting various behavioural anomalies, indicate (+) as Low, (+ +) as Medium and (+ + +) as high intensity of behaviour on exposure to Fipronil.

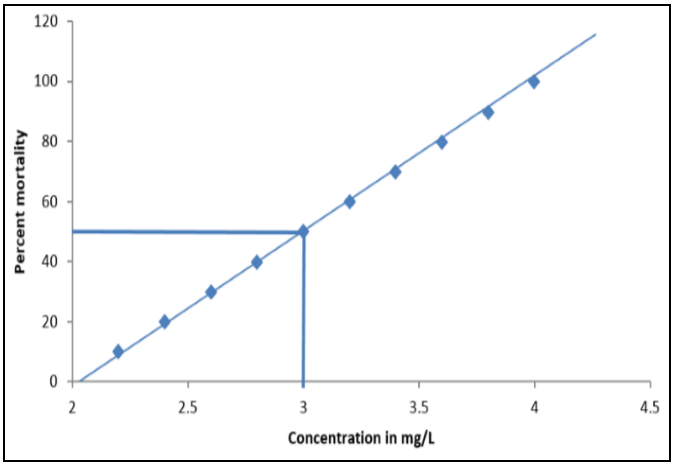

Fig 3: Showing mortality of *Oreochromis mossambicus* exposed to different concentrations of Fipronil

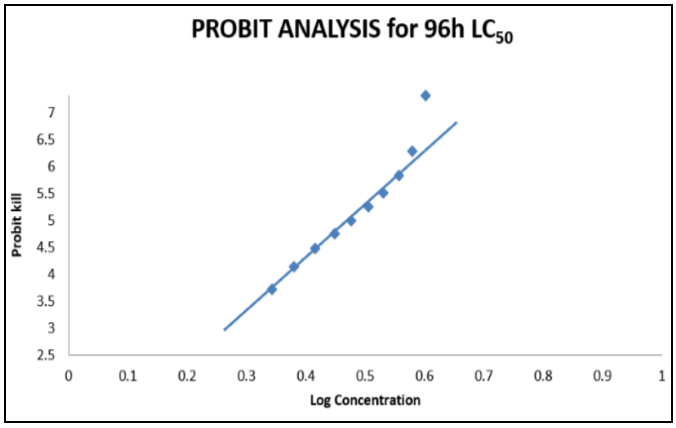

Fig 2: Mortality of *Oreochromis mossambicus* exposed to different concentrations of Fipronil

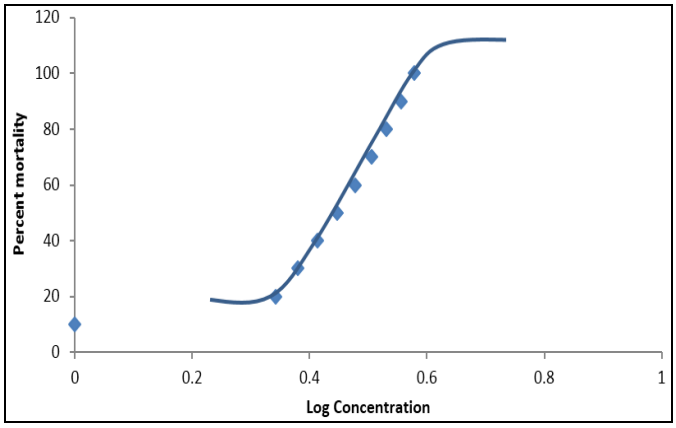

Fig 3: Showing mortality of *Oreochromis mossambicus* exposed to different concentrations of fipronil

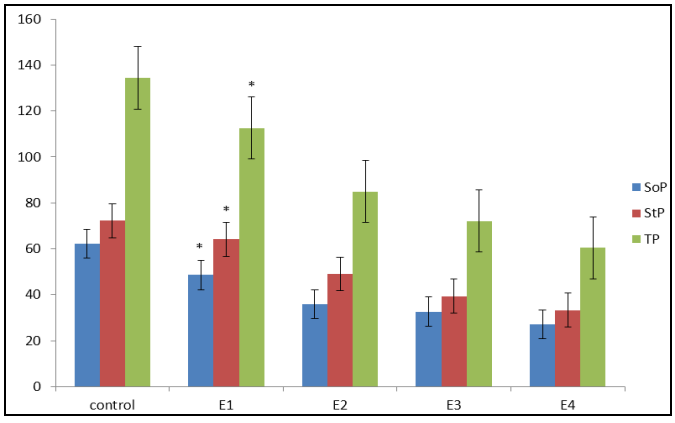

Fig 4: Protein levels in the liver of freshwater fish, *Oreochromis mossambicus* following exposure to sub-lethal concentration of fipronil

3.2 Behavioural studies

In the present study, the control fish were alert to slightest of the disturbance with their well-synchronized schooling movements. The opercular beat, fin beat, buccal movement, swimming patterns, pigmentation, the behaviour did not vary between the control groups; therefore, these results were taken as standards during the course of experimentation. The behavioural responses in the current study showed no changes in the control group. However, fish exposed to sub lethal concentrations of FPN exhibited impaired swimming patterns. The changes were driven by FPN and were noticed from 7th day of exposure. For instance, the possible movement of fishes were towards the direction in which the caudal fin was bent. The other findings include erratic and darting movements, hanging vertical in water and loss of equilibrium. All these findings were recorded from day 7 and progressed with the duration of exposure. However, no changes were witnessed during the 1st day exposure. Few fish at 7 and 14 days of exposure demonstrated whirling cork-screw like movements or darting movements (Table 4). Fishes exhibiting various behavioral anomalies, indicated (+) as low, (+ +) as medium and (+ + +) as high levels of impaired behavior due to intoxication of commercial grade FPN. The fishes in control batch exhibited normal behavior throughout the exposure period, with less or no variation in the swimming patterns.

3.3 Protein metabolism

The results from the present study indicate variations in biochemical levels. The protein levels were found to be depleted significantly (p<0.05) throughout the exposure periods. However no changes were observed in control group of fishes. The depletion in protein levels have been shown in Figure 4, in the liver of the fish exposed in batches. In contrast to the normal the E1 showed depleted levels of soluble protein (Sop), structural protein (Stp) and total protein (Tp). The soluble proteins were more depleted whereas the structural proteins were less depleted. The total protein levels were between those of the Sop and Stp. There was enhanced depletion in levels of protein under E2 than that of E1; wherein the Sop levels are more depleted than the Stp levels. The total protein levels are between that of Sop and Stp. There is a significant decrease in the levels of protein of E2 when compared to that of the normal. The Sop being - 21.96 (E1), -42.35 (E2), -47.52 (E3) and -56.33% (E4). The changes in structural proteins were also found to significantly vary with percent change of - 11.18, -31.98, -45.44 and -53.87% respectively for E1, E2, E3 and E4. Similar trend was noticed for total protein with percent change of -16.18, -36.78, -46.41 and -55.01% for E1, E2, E3 and E4 respectively.

4. Discussion

Assessing the toxicological impact to the aquatic ecosystems becomes difficult due to several factors viz, the short persistence of these insecticides in the water column and due to low solubility and rapid degradation [4, 17]. Fishes have long been used and considered ideal sentinels in toxicology [18]. The impact of environmental stressors occurs on the entire body surface, their ecological relevance and ease of culture have made them suitable for determining the effect and influence of chemical pesticides in aquatic ecosystems [4].

Reports on 96h LC_{50} of a pyrethroid to fish Bluegill and lake trout which was less than 1.0µg/ L [19]. The 96h LC_{50} value of mosquito fish, *Gambusia affinis* was estimated as 1.107 µg/L [20]. By this it is clear that pyrethroid insecticides are toxic to fish species. An EC_{50}, the concentration at which the effect occurs in 50% of the test population, for eastern oyster is 0.59 ng/L. The new class of insecticide phenylpyrazole however has not been reported for its toxicity against the selected fish species. The present investigation revealed LC_{50} value of 3.0 mg/L for the selected fish *O. mossambicus*. Evaluating the previous reports and comparing with present one helps to derive the conclusion that either pyrethroids are more toxic as in comparison to FPN, or the test species *O. mossambicus* remains to be fairly resistant to toxic concentrations of the selected insecticide. The fate of FPN in aquatic ecosystems depends on the nature of system components such as suspended solids (mineral and organic particulates) and aquatic organisms (algae, macrophytes, or aquatic animals). Various symptoms of poisoning can be observed from studies involving the determination of LC_{50}.

In the present study the control group fish maintained a compact schooling and moved in a coordinated manner covering about 1/3rd of the bottom during the initial 9 days of the 14 days experiment and from the 10th day onwards the schooling became less compact covering up to 2/3rd of the tank area. The fish were observed to scrub the bottom surface of the tank. Similar observations were done by Sobha *et al*. [21]. The fishes showed upward swim and normal buccal, opercular and fin beats. They were active for feeding and maintained well-synchronized movements, our outcome is in agreement with the previous reports of Halappa and David [4]. In the sub-lethal concentrations of FPN, the fish exhibited loss of coordination including abnormal behavioural patterns viz dashing movement, backward swim, downward swim, upward swim, lateral swimming, loss of equilibrium, whirling cork like moment, forward and backward jerks accompanied by burst swimming. One of the mechanism which explains the changes in behavioural and morphological abnormalities could be due to the inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine in cholinergic synapses ensuing hyper stimulation and has been previously reported [22, 23, 24, 25].

Biochemical profile in fish has proved to be a sensitive index for evaluation of the fish metabolism [26]. In various fish species, proteins are of importance as structural compounds, biocatalysts and hormones for control of growth and differentiations. So variation in fish proteins could be used as bio indicator for monitoring physiological status of the tested fish [27]. Measurement of total protein in serum or plasma is of considerable diagnostic value in fish as it relates to general nutritional status [28, 29]. In case of the control fish, the total protein showed no significant variation. As reported by [30], muscle proteins comes first in the sequence of the organs containing major protein levels, the second being liver and the third being kidney. During stress, the fish is in need of more energy to detoxify the toxicant and overcome the stress; the protein is used to meet this increased energy demand [6]. The decrement in the total, structural and soluble proteins suggests the possibility of decrease in the protein biosynthesis by sub-lethal concentrations of FPN in the present study. Similar observations were made by David and Kartheek [31] on sub-lethal exposure of sodium cyanide in freshwater fish *C. carpio*. To understand the variation among the total, structural and soluble protein, estimation was carried out. The ratios namely Sop/Tp (soluble protein/ Total protein), Stp/Tp (structural protein/ Total protein) and Sop/Stp (soluble protein/ Structural protein) were calculated. The decrease in

the protein content as observed in the present study in the fish may be due to metabolic utilization of the ketoacids to gluconeogenesis pathway for the synthesis of glucose, or due to directing the free amino acids for the synthesis of proteins, or for the maintenance of osmo and ionic regulation [32]. It could also be due to the production of heat shock proteins or destructive free radicals or could be a part of heavy metal induced apoptosis [30]. The findings are in agreement with Yacoub and Gad [26], who reported the decrease in protein levels in *O. niloticus*. This may be due to fact that toxicants react with the cell nucleoproteins and nucleic acids and consequently affect the protein biosynthesis and cellular integrity.

5. Conclusion

Based on the outcome of the current study it can be concluded that the FPN is highly toxic to freshwater fish *Oreochromis mossambicus* as it has an acute toxicity value of 3.0 mg/L. Additionally, the toxicant is found to modulate the biochemical and behavioural aspects following sublethal exposure. It is hence suggested that care must be taken when FPN is used or disposed under the aquatic vicinity.

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