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Toxicity of tricyclazole on certain serum biochemical markers of an Indian paddy-field fish, *Channa punctatus* (Bloch)

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Abstract
This maiden study was designed to evaluate acute toxicity of tricyclazole and its responses on certain biomarker in a freshwater fish *Channa punctatus* (Bloch). By Reed-Muench method, mean 96hr-LC_{50} dose of tricyclazole for this fish was calculated 25.0 mg l^{-1}. The LC_{50} dose indicates highly toxic nature of tricyclaozole. Fish were then treated to 0.25 and 1.25 mg l^{-1} sublethal dose of tricyclazole for short term long term exposure and the alterations in activities of glucose, protein, urea and cholesterol were determined in serum. Levels of glucose, urea and cholesterol in fish exposed to tricyclazole increased significantly (p<0.05) at all durations of short-term and long-term exposure. Conversely, protein decreased significantly (p<0.05) in response to tricyclazole at both the concentration and all exposure periods. Therefore, exposure to tricyclazole at sub-lethal concentrations induces severe serum biochemical alterations in *Channa punctatus* that may potentially disrupt their survival in their natural habitat. These findings may be used in the assessment of the potential risk of tricyclazole on food chain and aquatic ecosystems.

Keywords: Tricyclazole, *Channa punctatus*, toxicity, biochemical markers

1. Introduction
Toxicity tests are designed to predict concentration of a toxicant and its duration of treatment that produce an undesirable effect. Acute toxicity test helps in identification of mode of action of a toxicant and information on dose associated with mortality that can be used to set dose levels for repeated-dose studies. Reed & Muench (1938) [21] method is generally opted by researchers in Experimental Biology because of its ease of application.
Fungicides (e.g. tricyclazole and propiconazole) are used to increase yield of crop finally find their way into the aquatic ecosystem. Tricyclazole is one of the fungicides recommended to treat of blast diseases caused by *Pyricularia oryzae* in paddy. Tricyclazole is highly toxic substance and has a high risk of environmental contamination (Loomis & Hayes 1996, Padovani *et al* 2006, Sancho *et al* 2009 and Naik *et al* 2012) [10, 17, 25, 14]. The use of tricyclazole in paddy culture leads to harmful effects on various parameters of aquatic non-target species. Effect of propiconazole on biochemical profile of this fish is studied (Tabassum *et al* 2018) [29].
Channa punctatus (Bloch), a freshwater Indian air breathing fish, is found in paddy fields. However, the toxicity of a fungicide on Indian freshwater fish is lacking in this context. Therefore, the present work was designed to evaluate LC_{50} of tricyclazole and alterations in certain biochemical parameters in intoxicated *Channa punctatus*. The study will be helpful to evaluate variation in health condition of fungicide exposed fish, suitability of environmental conditions for fish and relative sensitivity of fish to tricyclazole.

2. Materials and Methods
2.1. Experimental Layout
Channa punctatus (body weight: 25-30 gm Total length: 12-14 cm) were collected from local fishermen of Arrah during the season of fish from 2018 to 2019. The fishes were disinfected with dilute $KMnO_4$ and then transferred to large aquaria. The fishes were fed with pieces of goat liver and fish food available in the local market.

2.2. Treatments
The experiments were conducted every time in between 11 AM to 1 PM. The physico-chemical features of experimental water were determined following standard method (APHA 2012) [2]. Tricyclazole (75% EC; Molecular formula: $C_9H_7N_3S$; Molecular mass: 189.24g/mol) is a fungicide manufactured by Bayer Crop Science Ltd., Gujarat, India. In each selected concentration of Tricyclazole, ten fishes from fish stock were kept. With the help of the records of dead fishes, 96h-LC_{50} doses were determined by Reed & Muench (1938) [21]. From this value, 1% and 5% of 96h-LC_{50} dose was used for serum biochemical studies.

2.3. Observations
The blood samples were collected by heart puncture method. After coagulation, the uncoagulated and coagulated part were separated and centrifuged at 3000 rpm for 10 minutes. Serum glucose, protein, urea and cholesterol of test fish was measured by methods of Folin & Wu (1926) [8], Lowry *et al* (1951) [11] Patton & Crouch (1977) [18] and Abel *et al* (1952) [1] respectively.

2.4. Data Analysis
Data analysis was performed with the use of Graph Pad Prism 5. Results were presented as mean and standard deviation. The differences between the control and treated groups were compared by using t-test and F-test (p<0.05).

3. Results
The temperature 26.0±2.0 ^{0}C, pH: 7.14±0.08, dissolved oxygen: 6.4±0.4 mg l^{-1}, total alkalinity: 56.0±4.5 mg l^{-1}, hardness: 150.6±5.2 mg l^{-1} and chloride: 16.7±0.2 mg l^{-1} of experimental water was recorded to which fish were exposed.
The data of LC_{50} of tricyclazole on *Channa punctatus* (Bloch) have been summarized in Table 1. The lethal range of tricyclazole from 0.01, 0.1, 1.0, 10.0 and 100.0 mg l^{-1} following Reish & Oshida (1987) [21] method was found. Mortality was lacking in 0.01 to 10.0 mg l^{-1} while total mortalities were found in 100.0 mg l^{-1} of tricyclazole. From Reed & Muench (1938) [21] method, 96hr-LC_{50} dose of 24.0 and 25.02 mg l^{-1} as MLC_{50} and MSC_{50} was calculated. The conformation of 96hr-LC_{50} was done applying following cross checks:
(a) The ideal LC_{50} was calculated from the mean of MLC_{50} and MSC_{50} = $\frac{24.0 + 25.02}{2}$ = 24.51 mg l^{-1}. (b) Sum of doses and sum of mortalities: Its value was $\frac{10+20+30+40}{1+3+6+9} = \frac{100}{19}$ = 5.26. When 5.26 were multiplied with 5 gave the product as 26.30. Therefore, LC_{50} = 26.30 mg l^{-1} and (c) The average 96hr-LC_{50} dose of tricyclazole from this method was calculated to be 24.0+25.02+26.30= 25.107 or 25.0 mg l^{-1} (Table 1).
Mean glucose of 79.13±3.64 mg dl^{-1} of controlled fish was found to increase significantly (p<0.05) from 87.50±1.72 to 127.96±12.9 mg dl^{-1} during short-term while from 86.93±1.89 to 125.86±2.14 mg dl^{-1} during long-term treatment of 0.25 and 1.25 mg l^{-1} tricyclazole. The hyperglycemia shows more dependency on duration compared to the dose of tricyclazole (Table 2). The elevation in glucose was more pronounced in short term treatment compared to long term exposure.
Conversely, mean protein amount of 4.97±0.11 g dl^{-1} in control fish decreased significantly (p<0.05) from 4.52±0.10 to 3.40±0.80 g dl^{-1} during short-term and from 4.48±0.10 to 3.29±0.06 g dl^{-1} during long-term treatment of 0.25 and 1.25 mg l^{-1} tricyclazole respectively. The fall in protein was less in short term treatment compared to long term exposure. The decrease shows equal dependency on both duration and dose of tricyclazole (Table 3).
Mean urea of 15.0±0.37 mg dl^{-1} of control fish increased significantly (p<0.05) from 17.19±0.34 to 23.39±0.19 mg dl^{-1}during short-term and from 16.20±0.12 to 24.87±0.15 mg dl^{-1} during long-term treatment of 0.25 and 1.25 mg l^{-1} tricyclazole respectively. Hyperuremia was found more in long term treatment in comparison to short term exposure. Hyperuremia shows much more dependency on duration than the dose of tricyclazole (Table 4).
Similarly, mean cholesterol level of 80.0±4.0 mg dl^{-1} in control fish increased significantly (p<0.05) from 100.5±2.27 to 133.69±1.92 mg dl^{-1} during short-term and from 98.39±1.25 to 131.68±2.11 mg dl^{-1} during long-term treatment of 0.25 and 1.25 mg l^{-1} tricyclazole respectively. The hypercholesterolemia was more in long term treatment compared to short term exposure. The hypercholesterolemia shows much more dependency on duration than the dose of tricyclazole (Table 5).

Table 1: Reid-Muench (1938) method for 96hr-LC_{50} determination of tricyclazole in *Channa punctatus*

Sl. No.	Dose (mg l^{-1})	Log dose (mg l^{-1})	Experiment		Specific Cumulative			Rate of mortality	% mortality	% survival
			No of mortality	No. of survival	Mortality	Survival	Total			
1	10	1.000	1	9	3	36	39	$\frac{3}{39}$	7.70	66.67
2	20	1.301	3	7	6	12	18	$\frac{6}{18}$	33.33	92.30
3	30	1.477	6	4	12	5	17	$\frac{12}{17}$	70.59	29.41
4	40	1.602	9	1	21	1	22	$\frac{21}{22}$	95.45	4.55

Calculation of median lethal concentration (MLC_{50})	Calculation of median survival concentration (MSC_{50})
$\frac{50.0-33.33}{70.59-33.33} = \frac{16.67}{37.26}$ = **0.45**	$\frac{50.0-29.41}{66.67-29.41} = \frac{20.59}{37.26}$ = 0.553
1.477 – 1.301 = 0.176	1.477 – 1.301 = 0.176
0.45 x 0.176 = 0.0792	0.553 x 0.176 = 0.0973
1.301 + 0.0792 = 1.3802	1.301 + 0.0973 = 1.3983
Antilog of 1.3802 = 24.00 mg l^{-1}	Antilog of 1.3983 = 25.02 mg l^{-1}
MLC_{50} = 24.00 mg l^{-1}	MSC_{50} = 25.02 mg l^{-1}

Table 2: Variations in serum glucose (mg dl^{-1}) of *Channa punctatus* after acute and chronic exposure of two sub-lethal dose of tricyclazole

Sub-lethal Dose of tricyclazole (mg l^{-1})	Normal range of glucose in fish	Controlled value of glucose	Duration of exposure			F value
			a=24 hours b=15 days	a=48 hours b=30 days	a=96 hours b=45 days	
0.25	20.00-100.00	79.13±3.64	87.50±1.72[a] (+10.58%) *	102.76±2.96[a] (+29.86%) ***	104.23±2.28[a] (+31.72%) ***	28.38*** and 11.41** (at 0.5=5.1 0.1=10.9, 0.01=27.0 for n_1=2 and n_2=6) (at 0=4.8, 0.1=9.8, 0.01=23.7 for n_1=3 and n_2=6)
			86.93±1.89[b] (+9.88%) *	100.29±2.03[b] (+26.74%) **	102.46±4.53[b] (+29.48%) ***	
1.25			95.91±1.52[a] (+21.21%) **	120.83±3.01[a] (+52.70%) ***	127.96±1.29[a] (+61.71%) b ***	
			92.76±1.48[b] (+17.22%) **	118.67±3.86[b] (+49.97%) ***	125.86±2.14[b] (+59.05%) ***	
Average			90.78±3.74 (+12.91%) **	110.64±9.19 (+39.82%) ***	115.13±11.82 (+45.49%) ***	

(*= Significant, **= Moderately Significant, ***= Highly Significant)

Table 3: Variations in serum total protein (g dl^{-1}) of *Channa punctatus* after acute and chronic exposure of two sub-lethal dose of tricyclazole

Sub-lethal Dose of tricyclazole (mg l^{-1})	Normal range of total protein in fish	Controlled value of total protein	Duration of exposure			F value
			a=24 hours b=15 days	a=48 hours b=30 days	a=96 hours b=45 days	
0.25	3.00 -10.00	4.97±0.11	4.52±0.10[a] (-9.05%) *	4.48±0.12[a] (-9.86%) *	3.51±0.10[a] (-29.38%) ***	3811.0*** And 57.58*** (at 0.5=5.1, 0.1=10.9, 0.01=27.0 for n_1=2 and n_2=6) (at 0.5=4.8, 0.1=9.8, 0.01=23.7 for n_1=3 and n_2=6)
			4.48±0.10[b] (-9.86%) *	4.40±0.10[b] (-11.47%) **	3.42±0.10[b] (-31.19%) ***	
1.25			4.44±0.08[a] (-10.66%) *	4.38±0.09[a] (-11.87%) **	3.40±0.80[a] (-31.58%) ***	
			4.37±0.10[b] (-12.07%) *	4.25±0.02[b] (-14.49%) **	3.29±0.06[b] (-33.84%) ***	
Average			4.45±0.06 (-10.46%) *	4.38±0.08 (-11.87%) **	3.41±0.08 (-32.72%) ***	

(*= Significant, **= Moderately Significant, ***= Highly Significant)

Table 4: Variations in serum urea (mg dl^{-1}) of *Channa punctatus* after acute and chronic exposure of two sub lethal dose of tricyclazole

Sub-lethal Dose of tricyclazole (mg l^{-1})	Normal range of urea in fish	Controlled value of urea	Duration of exposure			F value
			a=24 hours b=15 days	a=48 hours b=30 days	a=96 hours b=45 days	
0.25	2.50 -20.00	15.0±0.37	17.41±0.84[a] (+16.07%) **	19.39±0.65[a] (+35.93%) ***	23.39±0.17[a] (+55.93%) ***	34.62*** And 7.58* (at 0.5=5.1, 0.1=10.9, 0.01=27.0 for n_1=2 and n_2=6) (at 5=4.8, 0.1=9.8, 0.01=23.7 for n_1=3 and n_2=6)
			16.20±0.12[b] (+8.00%) *	17.10±0.24[b] (+14.0%) **	22.10±0.34[b] (+47.33%) ***	
1.25			17.19±0.34[a] (+14.60%) **	21.67±0.78[a] (+44.47%) ***	22.64±0.92[a] (+50.93%) ***	
			19.26±0.78[b] (+28.40%) ***	22.59±0.19[b] (+50.60%) ***	24.87±0.15[b] (+65.80%) ***	
Average			17.19±0.43 (+21.73%) **	20.19±1.91 (+37.93%) ***	23.25±1.04 (+55.50%) ***	

(NS= Not Significant, *= Significant, **= Moderately Significant, ***= Highly Significant)

Table 4: Variations in serum urea (mg dl^{-1}) of *Channa punctatus* after acute and chronic exposure of two sub lethal dose of tricyclazole

Sub-lethal Dose of tricyclazole (mg l^{-1})	Normal range of urea in fish	Controlled value of urea	Duration of exposure			F value
			a=24 hours b=15 days	a=48 hours b=30 days	a=96 hours b=45 days	
0.25	2.50 -20.00	15.0±0.37	17.41±0.84[a] (+16.07%) **	19.39±0.65[a] (+35.93%) ***	23.39±0.17[a] (+55.93%) ***	34.62*** And 7.58* (at 0.5=5.1, 0.1=10.9, 0.01=27.0 for n_1=2 and n_2=6) (at 5=4.8, 0.1=9.8, 0.01=23.7 for n_1=3 and n_2=6)
			16.20±0.12[b] (+8.00%) *	17.10±0.24[b] (+14.0%) **	22.10±0.34[b](+47.33%) ***	
1.25			17.19±0.34[a] (+14.60%) **	21.67±0.78[a] (+44.47%) ***	22.64±0.92[a] (+50.93%) ***	
			19.26±0.78[b] (+28.40%) ***	22.59±0.19[b] (+50.60%) ***	24.87±0.15[b] (+65.80%) ***	
Average			17.19±0.43 (+21.73%) **	20.19±1.91 (+37.93%) ***	23.25±1.04(+55.50%) ***	

(NS= Not Significant, *= Significant, **= Moderately Significant, ***= Highly Significant)

4. Discussion

The toxicity determination of fungicides seems essential to evaluate the sensitivity and damage to specific organs/systems of an animal (Singh *et al* 2010) [28]. Such observations results as outcome of various diseases in fishes and also reveals underlying physiological conditions of the organs and tissues (Obomanu *et al* 2009) [15].

For this study, experimentation with 10 fishes per dose is necessary for better correlation. The method was modified by Saganuwan (2011) [24] in calculating survival and mortality of percent of test animals to arrive a conclusion. Although, 95% confidence limit cannot be calculated with this method. 96hr-LC_{50} dose of tricyclazole in *Channa punctatus* rates it as a most toxic substance according to toxicant classification (Loomis & Hayes 1996) [10].

The hyperglycemia indicates disrupted carbohydrate metabolism probably after the elevated glycogenolysis. Ferrando & Moliner (1991) [6] saw hyperglycemia in pesticide intoxicated *Channa punctatus*, *Heteropneustes fossilis* and *Cyprinus carpio*. Hyperglycemia was also reported by Firat *et al* (2011) [7] in *Oreochromis niloticus* exposed to copper, lead and cypermethrin. Borges *et al* (2007) [4] suggested that cypermethrin induced hyperglycemia in *Rhamdia quelen* as a sign of stress. The hyperglycemia is a reliable indicator of environmental stress due to general secondary response of intoxicated fish (Sepici-Dincel *et al* 2009) [26]. Hyperglycemia seems to be mediated through ACTH and reduced insulin secretion, increased corticosteroid and glyconeogenesis (Ramesh & Saravanan 1985) [20]. The present finding is in close consortium with the above observations.

The decrease in protein is supported by the findings of Velisek *et al* (2007) [30] who observed its significant decrease in metribuzin treated rainbow trout. Min & Kang (2008) [13] additionally referred decline vogue in *Nile tilapia* afterward benomyl toxicity. A reduction in protein was also reported by Firat *et al* (2011) [7] in *Oreochromis niloticus* exposed to copper, lead and cypermethrin. Conversely, Shaikh *et al* (2014) [27] stated increase in protein of *Channa puncatus* after nuvan treatment. The decrease of protein may be due to the inhibition of transcription disturbing the protein metabolism or liver injury where most translation occurs (Reddy & Vinnela, 2016) [22]. Reduction in protein may also be attributed to intensive proteolysis which contributes after the increase within the free amino acids to be fed in Krebs' cycle. These outcomes may agree with findings of Ram & Singh (1998) [19], when carbofuran triggered biochemical changes in

Channa punctatus treatment resulted in massive decrease in the protein content in various tissues/organs of fish.

Hyperuremia observed in this work was also reported by David *et al* (2004) [5] in *Cyprinus carpio* and Kumar *et al* (2011) [9] exposed to cypermethrin and Balasubramaniam & Kumar (2013) [3] in *Heteropneustes fossilis* induced to sodium arsenate. The air-breathing fish are ureogenic having extra-ordinary level of activity for enzymes of urea cycle. *Channa punctatus* exposed to different concentrations of carbaryl exhibit much biochemical alteration into urea and relates after renal failure in *Cyprinus carpio* (Luskova *et al* 2002) [12].

The hypercholesterolemia following cypermethrin exposure was observed in *Rhamdia quelen* (Borges *et al* 2007) [4]. An elevation in cholesterol was reported by Firat *et al* (2011) [7] in *Oreochromis niloticus* exposed to short- and long-term treatment of copper, lead and cypermethrin. Oner *et al* (2008) [16] found that cholesterol of metal-intoxicated *Oreochromis niloticus* increased compared to corresponding control value. They concluded that the concentration of cholesterol may increase due to hepatic and renal failure causing the release of cholesterol into the blood. Yousef *et al* (2003) [31] reported that changes in cholesterol are related to changes caused by pesticides in the permeability of hepatic cells and that accumulation of pesticides in the liver to disrupt lipid metabolism.

5. Conclusion

The range of serum glucose, protein, urea and cholesterol in control fish was within range of other fishes. Hyperglycemia, decreased serum protein, hyperuremia and hypercholesterolemia are indicators of altered carbohydrate, lipid and protein metabolism in the fish due to tricyclazole exposure. The changes are a reflection of organ dysfunction due to fungicide exposure. This study clearly indicates that the presence of tricyclazole even in small concentration may cause deleterious effects on fish physiology and may potentially disturb their survivability.

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