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Neurotoxic Effects of Imidacloprid on *Pethia conchonius* (Rosy Barb), a Common Freshwater Fish of India

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Abstract

Insecticides are essential to control arthropod pests in agriculture. However, due to their stability and extended half-lives, they contaminate freshwater aquatic systems like lakes, ponds, and rivers by surface run-offs and leaching. Neonicotinoids are a globally used agricultural pesticides that act as an agonist to the nicotinic acetylcholine receptor (nAChRs) and are known to have harmful effects on non-target organisms like fish. This study aimed to determine the neurotoxic, behavioural, and histopathological effect of three sub-lethal concentrations (SLC I, SLC II, and SLC III) of Imidacloprid (IMI), a neonicotinoid, on the freshwater fish *Pethia conchonius*. Fish were exposed to IMI for 96 hr, during which their behaviour was recorded, and the brain tissues were collected at 24 hr intervals. Compared to the control group, the IMI-exposed fish showed changes in behaviour, such as jerky, erratic swimming, disequilibrium, and mucus secretion. A significant decrease in Acetylcholinesterase (AChE) activity and histopathological damage were recorded in the brain tissues. The severity of damage and decline in activity was both concentration and time-dependent. The AChE inhibition was observed for SLC III after 96 hr (33.70±2.52) compared to control at 96 hr (84.63±4.25). The optic tectum showed detachment in its layers along with necrosis, and vacuolation. The results indicate that IMI is highly neurotoxic which not only inhibits AChE activity but also causes neural damage in the brain leading to a wide range of behavioural alterations.

Keywords: Acetylcholinesterase, Histology, Imidacloprid, Neurotoxicity, Optic Tectum, *Pethia conchonius*

1. Introduction

Synthetic pesticides are widely used throughout the world to increase crop production by controlling pest problems. India is one of the largest pesticide consumers, accounting for almost 58720 tonnes from 2021 to 2022[1]. Cotton (46%), followed by paddy and wheat, are the crops that require maximum pesticide use in India[2]. Organophosphates, neonicotinoids, and carbamates are the most widely used pesticide classes in India[3]. The most common crops grown in West Bengal are rice, wheat, tea, and various kinds of vegetables and fruits[2]. In West Bengal, alpha-cypermethrin (46%), methyl parathion (25.6%), imidacloprid (16.4%), dichlorvos (7.8%), and phorate (4%) are the pesticides most frequently used in order of their amount of consumption[4].

Chemical pesticides control the arthropod pest and are hazardous to the environment and non-target organisms, including humans, and cause severe environmental and health problems. Many pesticides have extended

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half-life properties that make them biologically stable in the soil as well as in water, polluting ecosystems[5-7]. Increasing use of pesticides to improve crop productivity has resulted in contamination of both the terrestrial and aquatic environment, leading to negative impact and bioaccumulation in non-target organisms, including humans[8,9].

Neonicotinoids constitute a widely used class of insecticides comprising synthetic compounds that show structural similarity with nicotine and bind to the nicotinic acetylcholine receptor (nAChRs)[10,11]. Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) (IMI) is a neonicotinoid systemic pesticide with an agonistic effect on the post-synaptic nAChRs that impairs the central nervous system by continuous activation of the receptor[12]. IMI is highly toxic to their target pests, however, once in the soil or aquatic ecosystems, it may cause undesirable, hazardous effects on non-target organisms like cattle, birds, bees, rodents, beneficial micro-organisms, and humans[2,13,14]. IMI can reach aquatic ecosystems through aerial pathways like unjudicial application near riverbeds or waterborne paths like leaching through the soil and surface run-off from agricultural fields[15,16]. Due to the accumulation of IMI in the water bodies, aquatic organisms, particularly fishes, are in direct contact with IMI, they are at high risk of a wide range of genotoxic effects. The gills, liver, heart, and brain of fish are major organs highly susceptible to changes in water quality or exposure to toxic chemicals[17]. Numerous studies have shown that IMI alters physiology and behaviour in fishes and has genotoxic and cytotoxic effects even in sub-lethal doses[6,12,15,18,19]. Various histopathological effects were seen in the gills, liver, kidney, and brain tissues of fish, like gill hyperplasia, degeneration of hepatocytes, necrosis, infiltration of inflammatory cells, etc[6,20]. Salunke *et al.,* have shown histopathological alterations like necrosis, vacuolization, lesions, and neurodegeneration in the brain tissues of *Oreocromis mossambicus*[21]. Histopathological alteration like neuronal vacuolation and neuronophagia due to IMI exposure was also recorded in brain tissues of *Clarias gariepinus* along with a marked decrease in acetylcholine esterase activity by Rahman *et al*[22]. Studies by Gunal *et al.,* revealed histopathological changes like a fusion of secondary lamellae, hyperaemia of primary lamellae, and telangiectasia in gills of Nile Tilapia along with vacuolisation, accumulation of fat in the cytoplasm and mononuclear cell infiltration in hepatocytes indicating inflammation and cell death after exposure to sub-lethal concentration of IMI[23].

The adverse impact of IMI has also been correlated with an overall reduction in acetylcholinesterase (AChE) activity. The enzyme AChE hydrolyzes acetylcholine into choline and acetic acid. Acetylcholine is an excitatory mediator, which, unless broken down into constituent components after successful neurotransmission, will cause hyperstimulation of neurons of the central nervous system. Neonicotinoids have been shown to reduce AChE activity by acting as an agonist to their post-synaptic nicotinic acetylcholine receptor, leading to an accumulation of acetylcholine in the cholinergic synapses and hyperstimulation[24,25]. This hyperstimulation leads to changes in the behaviour and physiology of fish. Numerous studies have reported changes in AChE activity in the brain that can be correlated with altered behaviour in fishes[21,26]. Reduction in brain AChE activity and behavioural changes like erratic swimming, reduced exploratory behaviour and momentary freezing have been observed in Zebrafishes exposed to IMI for 96 hr[26]. Similar responses along with loss of equilibrium and surfacing behaviour have also been recorded in *Prochilodus lineatus* exposed to IMI[27]. Thus, AChE activity can be treated as a specific biomarker for pesticide contamination in fish, including other vertebrate species.

Several studies have indicated pesticide contamination in different rivers of West Bengal, including Ganga and Hoogly in South Bengal and Deomani, Changa, Manza, Tepu, and Karala in the Terai region of North Bengal[28-32]. Teesta is one of the major rivers in North Bengal, draining the Darjeeling, Kalimpong, Jalpaiguri, and Coochbehar districts. The river Teesta flows through areas mainly involved in monoculture cultivation of tea, rice, and cash crops like nuts and vegetables. Recently, there have been reports of IMI contamination in the Teesta[19], but a thorough study of its effect on aquatic organisms in this area still needs to be completed. Therefore, this study contemplates the effect of IMI on *Pethia conchonius,* a common freshwater fish of river Teesta, to assess the AChE activity, histoarchitecture of the brain and behavioural alterations.

2. Materials and Methods

2.1 Chemical used for Study

A commercially available Imidacloprid (IMI) (CAS No. 138261-42-3) formulation called Victor Plus (70%

W/W) manufactured by Insecticides (India) Limited was purchased from the local pesticide shop. For experimental purposes, a stock solution of the chemical (IMI) was prepared by dissolving 100 mg/ml of distilled water.

2.2 Experimental Fish

Pethia conchonius (Class: Actinopterygii, Order: Cypriniformes, Family: Cyprinidae) has been used for experimental purposes as it is an abundantly found and consumed fish in the area. Additionally, these fishes are easy to maintain and handle under controlled laboratory conditions.

The adult *Pethia conchonius* specimens were collected from two different sites of river Teesta adjoining agricultural patches: Site I: Gajoldoba Teesta barrage Jalpaiguri, West Bengal (Coordinates: 26.7515°N 88.5856°E) and Site II: Domohoni, Jalpaiguri, West Bengal (Coordinates: 26.3430°N 88.4542°E). The fish were then carefully transported to the laboratory under well-aerated conditions. The mean length and weight of the fish were 6.19 ± 0.78 cm and 3.90 ± 0.60 g, respectively. To prevent dermal infections, the live fish specimens received a prophylactic treatment of 0.05% Potassium Permanganate ($KMnO_4$) for 2 minutes.

The fish were acclimatized at a temperature of 25°C±2°C in well-ventilated glass aquaria (23 x 23 x 30 cm) for 15 days and were fed commercial fish food twice daily[33]. To lower the ammonia level in the water, faecal matter and other waste substances were siphoned daily.

2.3 Determination of Sub-lethal Concentration

An acute toxicity test for IMI was conducted previously to determine the lethal concentration of 50% (LC_{50}) of IMI in the laboratory for *Pethia conchonius* according to the OECD guidelines[33] for fish toxicity studies, and it was found to be 227.33 mg L^{-1} [19]. Three sub-lethal concentrations (SLC I, SLC II, and SLC III) at $1/12^{th}$, $1/8^{th}$, and $1/4^{th}$ (18.94 mg L^{-1}, 28.41 mg L^{-1}, and 56.83 mg L^{-1}, respectively) of LC_{50} diluted in distilled water were selected for further toxicity studies[34].

2.4 Physiochemical Characteristics of Test Water

Standard techniques were used to analyze the physiochemical characteristics of the water used in aquaria, including temperature, pH, Dissolved Oxygen (DO), total alkalinity, and total hardness[35].

2.5 Analytical Quantification of Imidacloprid in Test Water

The analytical concentration of imidacloprid in the test water was measured by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The pesticide was extracted from the test water sample using the AOAC official method 990.06.

2.6 Ethics approval

The present investigation has been approved by the Institutional Animal Ethics Committee of the University of North Bengal vide ref No. IAEC/NBU/2022/30 dated 23.09.2022.

2.7 Experimental Setup

Experimental fish *Pethia conchonius* was collected from River Teesta and acclimatized in dechlorinated tap water for 15 days according to OECD (2019) guidelines in well-aerated conditions[33]. Water from River Teesta previously screened by LC-MS/MS for the presence of pesticides showed the presence of Imidacloprid[19].

Based on acute toxicity bioassay, three sublethal concentrations, namely SLC I (1/12th of LC_{50}= 18.94 mg L^{-1}), SLC II (1/8th of LC_{50}= 28.41 mg L^{-1}), and SLC III (1/4th of LC_{50}= 56.83 mg L^{-1}) of IMI were used for experimental purposes. Three groups of 16 each (n=16) were exposed to each of the three sub-lethal concentrations of IMI for 96 hr. One group (n=16) was maintained in dechlorinated tap water without IMI exposure as a control. The behaviour of the fish was recorded for 1-2 hr continuously at an interval of 24 hr, 48 hr, 72 hr, and 96 hr. At each of these intervals, 4 specimens out of the 16 were dissected to collect the brain tissues to study the histopathological as well as acetylcholinesterase activity effect of the pesticide. The experimental design of the present study has been represented as a flowchart (Figure 1).

2.8 Behavioural Study

For behavioural analysis, previously acclimatized fish were divided into 4 groups, each containing four fish, subjected to the three sub-lethal concentrations of Imidacloprid and compared to a control group, which was kept in dechlorinated and untreated test water. Tap water from the

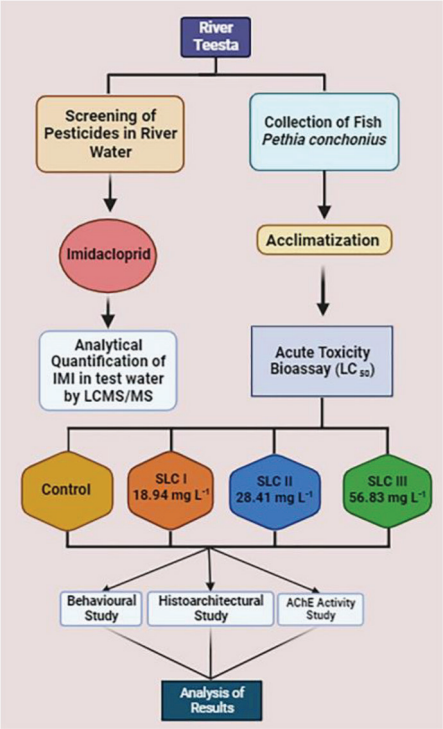


Figure 1. Schematic representation of the experimental design.

same source was used for all groups. The behavioural pattern of the fish was noted continuously for 1-2 hr at an interval of 24 hr for four days for both the control and treated groups.

2.9 Histopathological Study

After exposure to sub-lethal doses of IMI for 96 hr, the brain tissues from both the exposed and control groups of fish were dissected for histopathological study following the method of Bernet *et al.*, with some minor modifications[36].

The brain tissues were fixed in Bouin's fluid (Aqueous picric acid 75ml, glacial acetic acid 25ml, and formaldehyde 5ml) for 24 hr, followed by dehydration in graded ethanol series (50%, 70%, 90% and absolute ethanol). Then, the tissues were cleared in xylene for 10 mins and transferred into a 1:1 xylene and paraffin mixture for infiltration for at least 30 minutes at 60°C. The tissues were then moved into molten paraffin for embedding and block preparation. Using a Microtome (Spencer Microtome 820, India), the blocks were then cut into sections of 6 µm thicknesses. The sections were affixed on clean, grease-free slides using Mayer's albumin and stretched on a hot plate. For staining, the slides with sections were deparaffinized in xylene for 5-10 mins, rehydrated in descending grades of ethanol (100%, 90%, 70%, 50%, 30% for 5-10 mins each), washed in distilled water and stained with Haematoxylin for 10 mins. The slides were then dehydrated in ascending grades of ethanol (30%, 50%, and 70%, each for 5-10 mins) and stained in Alcoholic Eosin (in 90% ethanol) for 2-5 mins, differentiated in 90% for 5 min. Finally, the slides were cleared in xylene for 10 mins, removed excess xylene by dabbing on blotting paper, and mounted with DPX. The slides were finally observed under a bright-filled compound microscope (Nikon Eclipse E200, Nikon) for histopathological analysis at 1000X magnification.

2.10 Determination of Acetylcholine Esterase Activity

Acetylcholinesterase activity was detected in the brain tissue using the spectrophotometric method following Ellman *et al.*, with slight modifications[37]. Tissues were homogenized and centrifuged in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) to collect the supernatant as the test sample. For spectrophotometric analysis, 50µl of supernatant, 100 µl of 0.01 M DTNB, 20 µl of acetylthiocholine iodide (0.075 M), and 3 ml of phosphate buffer (0.1 M, pH 8.0) were taken in a standard cuvette, and absorbance was measured at 412 nm. For blank, the supernatant was replaced with distilled water. The activity was determined in terms of µmol/min/mg tissue.

2.11 Statistical Analysis

In the present study, the concentration of the imidacloprid and duration of exposure were considered independent variables to see the changes in AChE activity (dependent variables). Shapiro-Wilk normality test revealed that the AChE activity among all the groups was normally distributed ($p>0.05$). Levene's Test of Equality of Variances was also conducted to evaluate the homogeneity of variances. As the data were normally distributed, One-Way Analysis of Variance (ANOVA) was performed separately concentration-wise and duration-wise, followed by Tukey HSD[a] (for homoscedastic data) and Dunnett T3 (for heteroscedastic data) post hoc test. The Welch correction was also applied in ANOVA for the heteroscedastic data. The AChE activity was not homoscedastic ($p<0.05$) in all the concentration groups of IMI. Therefore, a non-parametric Quade's ANCOVA was performed to see the effect of the concentration of IMI on AChE activity after adjusting the dependent variables with the duration of exposure.

3. Results

3.1 Analytical Concentration of Imidacloprid

The result showed that out of 56.83 mg L^{-1} IMI, 20.89 mg L^{-1} residual IMI was present in the test water. It indicated a degradation of 50.59% of IMI after 96 hr of exposure.

3.2 Behavioural Study

An overall change in the behavioural pattern was monitored in the experimental groups of *Pethia conchonius* that were under acute exposure for 96 hr to three sub-lethal concentrations (SLC I, SLC II, and SLC III) of IMI in comparison to the control group (Figure 2). Activities of the fish were observed continuously for 1-2 hr at an interval of 24 hr for 96 hr. Initially, a change in swimming patterns was observed. Fish under treatment showed hyperactivity during the first 48 hr of exposure to IMI with erratic and jerky swimming movement, while the control group remained unaffected. The IMI-exposed fish also showed a significant loss of equilibrium and tilted to the side while swimming. During the last two days of the study period, the experimental fish showed a loss of activity and aggregating behaviour in the corner at the bottom of the aquaria.

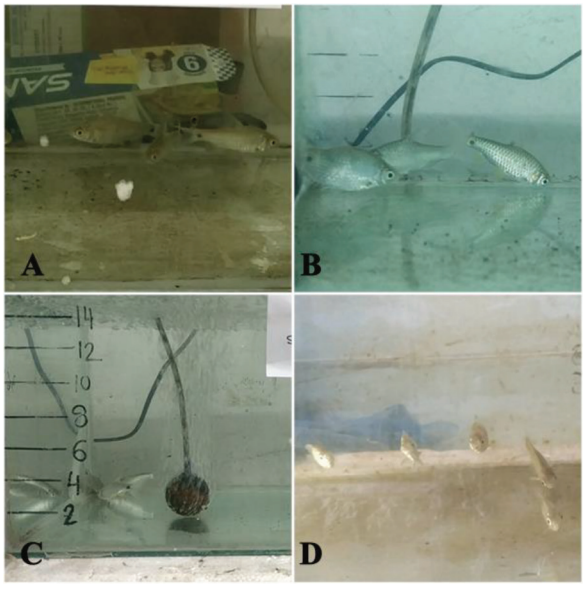


Figure 2. IMI-induced behavioural alterations in fish *Pethia conchonius*. **(A)** Represents normal swimming behaviour (Control). **(B)** Represents a down swimming pattern. **(C)** Represents bottom aggregation, and **(D)** Represents Loss of equilibrium (B, C, D IMI exposed).

The experimental groups also had a higher mucus secretion than the control. The observations initially were similar for all concentrations of IMI; however, later, more pronounced effects were observed at higher concentrations (SLC III: 56.83 mg L^{-1}) than lower ones (SLC II: 28.41 mg L^{-1}, SLC I: 18.94 mg L^{-1}). The observations revealed that exposure to different concentrations of IMI induces alterations in behaviour and can be correlated to the changes in acetylcholine esterase activity in the brain. The observation has been recorded in Table 1.

3.3 AChE Assay

To correlate the behavioural changes, the acetylcholinesterase (AChE) activity in the brain tissue of *Pethia conchonius* exposed to IMI as well as in the control fish, was estimated (Table 2). The effect of different sub-lethal concentrations (SLC I, SLC II and SLC III) of IMI on the activity of AChE was recorded for 96 hr (Figure 3) at an interval of 24 hr. A time-dependent decrease in AChE activity was observed in all concentrations of exposed groups, whereas the activity of AChE in the control group remained the same.

The results also showed a concentration-dependent decrease in AChE activity in a decreasing order compared to the control group. Values with homogenous subsets having a common alphabet or numeric indicate no significant difference ($p>0.05$). There were no significant differences between the control groups at different intervals, resulting in a single homogenous subset 'a1' ($p>0.05$). Decrease in the brain AChE activity was higher in SLC III exposed fish with the activities 72.50±3.31, 62.14±4.20, 48.48±3.89 and 33.70±2.52 for 24 hr to 96 hr, respectively. In SLC II, the AChE activities at 24 hr (69.57±2.92) and 48 hr (64.86±1.40) belong to the same homogeneous subset "b1" showing no significant difference between them, while, for SLC I the activities were 76.68±2.03, 69.16±1.14, 64.37±4.72 and 54.56±3.73 for 24 hr to 96 hr of exposure, the decrease was lowest among the three treated groups (Table 3).

3.3.1 Non-Parametric Quade's ANCOVA

ANCOVA analyses after adjusting AChE activity with duration of exposure revealed that concentration of IMI has a significant impact on the acetylcholinesterase activity in the exposed groups (F value - 69.783, df - 3, *P* value <0.001, R^2 - 0.826) corresponding 82.6% variation of AChE activity, which different sub-lethal concentrations of IMI can explain in the present study.

Table 1. Behavioural effects of IMI on *Pethia conchonius* subjected to three sub-lethal concentrations of the pesticide for 96 hours

Concentrations	Control				SLC I				SLC II				SLC III			
Exposure Time (hrs)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Erratic Swimming	-	-	-	-	++	++	+	+	++	++	+	+	+++	+++	++	+
Quick Opercular Movement	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Surfacing	-	-	-	-	+	+	+	+	++	++	+	-	+++	+++	++	-
Loss of Equilibrium	-	-	-	-	+	++	+++	+++	+	+	++	+	++	++	++	++
Bottom Settling	-	-	-	-	-	-	+	+	-	-	+	++	-	+	++	+++
Initial Hyperactivity	-	-	-	-	++	+	-	-	++	++	+	+	+++	+++	++	+
Mucus Secretion	-	-	-	-	-	+	+	+	-	+	+	+	-	-	++	+++
Aggregation at Bottom	-	-	-	-	-	-	+	++	-	-	+	++	-	-	++	+++

Note: Negative symbol (-) denotes No change/Normal, Single Positive symbol (+) denotes Low/Slight change, Double Positive symbol (++) denotes Mild/ Moderate change and Triple Positive symbol (+++) denotes High/Maximum change).

Table 2. Analysis of results according to concentration and duration by ANOVA

Category	Analysis	F statistic	df1	df2	P value
Concentration-wise					
24 hrs	ANOVA	14.984	3	8	0.001
48 hrs	Welch-ANOVA	99.098	3	4.297	<0.001
72 hrs	Welch-ANOVA	76.225	3	3.359	0.001
96 hrs	ANOVA	109.842	3	8	<0.001
Duration-wise					
Control	ANOVA	0.298	3	8	0.826
SLC I	ANOVA	24.786	3	8	<0.001
SLC II	ANOVA	17.801	3	8	<0.001
SLC III	ANOVA	67.972	3	8	<0.001

Note: df= degree of freedom

3.4 Histoarchitectural Alterations

In the present study, the brain tissues revealed histopathological alterations in the different layers of the optic tectum of *Pethia conchonius* exposed to IMI. The extent of tectal abnormalities was found to be more severe in fish treated with higher concentrations of IMI than the lower concentrations. The optic tectum is a bilobed structure present in the dorsal part of the mesencephalon. It is the primary visual centre consisting of six different layers i.e., Stratum Periventricular (SPV) present on the inner-most side, Stratum Album Centrale (SAC), followed by Stratum Griseum Centrale (SGC), Stratum Fibro-Sum Et Grisium Superficiale (SFGS), Stratum Opticum (SO), and Stratum Marginale (SM) (Figure 4).

In the control group, there were no alterations or detachment of the six layers in the optic tectum representing the typical architecture of the brain of *Pethia conchonius*. The treated group showed slight detachment in the SO, SM, and SFGC layers. Mild necrosis was also observed in SGC, SAC, and SPV layers at SLC I for 24

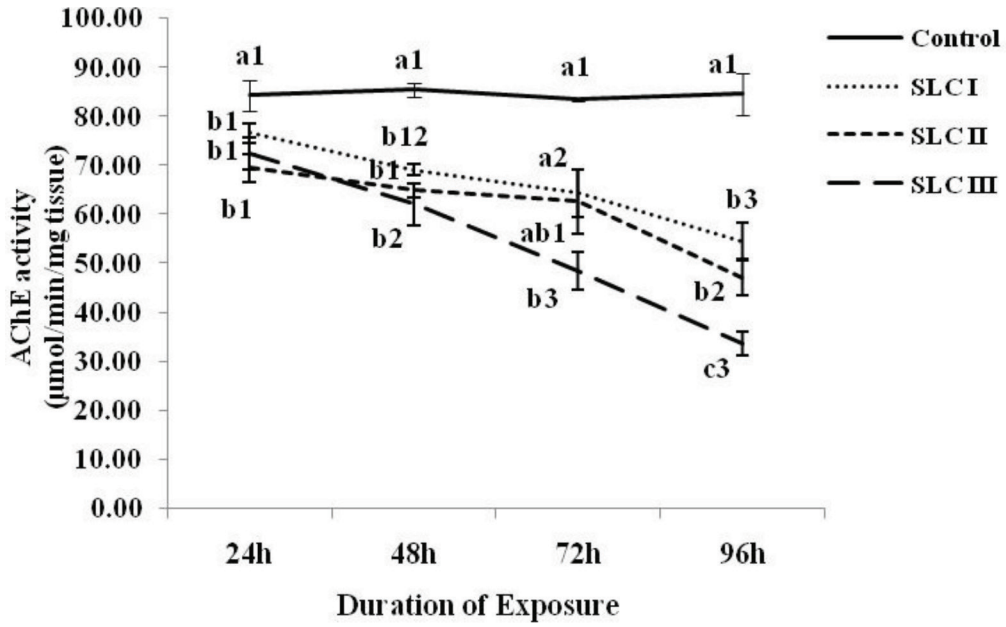


Figure 3. Data from IMI Exposed and control groups representing the AChE activity in *Pethia conchonius*.

Table 3. Effect of 96 hr exposure of the three sub-lethal concentrations of IMI on brain AChE activity (Mean±SD) in *Pethia conchonius*

Duration of exposure	Concentrations of IMI			
	Control	SLC I	SLC II	SLC III
24 hrs	84.43±3.13^{a1}	76.68±2.03^{b1}	69.57±2.92^{b1}	72.50±3.31^{b1}
48 hrs	85.56±1.41^{a1}	69.16±1.14^{b12}	64.86±1.40^{b1}	62.14±4.20^{b2}
72 hrs	83.46±0.29^{a1}	64.37±4.72^{a2}	62.67±6.43^{ab1}	48.48±3.89^{b3}
96 hrs	84.63±4.25^{a1}	54.56±3.73^{b3}	47.02±3.54^{b2}	33.70±2.52^{c4}

Different alphabet (lowercase) indicates mean AChE activity with different homogeneous subsets (p<0.05) along the concentration of IMI within the same duration of exposure.

Different numeric indicate mean AChE activity with different homogeneous subsets (p<0.05) along the exposure duration within the same IMI concentration.

to 96 hours (Figure 5). These changes in the brain were more pronounced in SLC II and SLC III IMI-exposed groups (Figure 5). In SLC III, the layers of SM, SO and SFGC showed complete detachment with degeneration of neurons, as evidenced by a reduction in size. In the areas of SGC and SAC, there was congestion, spongiosis, and clear spaces that denote necrosis around the nucleus of mononuclear cells. In the inner-most layer of SPV, the detachment and necrosis were very prominent in the fish exposed to SLC III of IMI for 96 hr.

At the highest concentration (SLC III), the SPV layer was entirely detached from the SAC, and the rest of the layers were fused, forming a very narrow structure compared to the control group. Vacuolization around the mononuclear cell is prominent in the fish *Pethia conchonius* exposed to SLC II and III of IMI.

The histoarchitectural alterations in the optic tectum of the brain of *Pethia conchonius* showed the adverse impact of IMI on the fish brain, ultimately leading to death.

4. Discussion

Synthetic pesticides are known for both their efficacy and persistence in the environment. Along with their biotransformation products, they may remain in and interact with the environment and non-target organisms[38]. Neonicotinoids like IMI are agonists to the

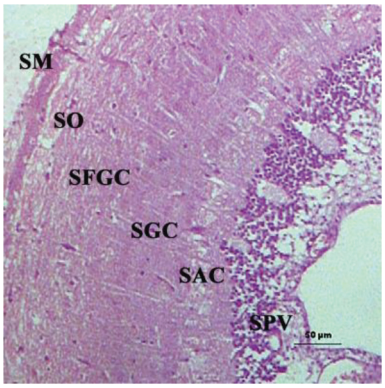


Figure 4. Optic tectum of control fish *Pethia conchonius*. Microphotograph shows Stratum Periventricular (SPV), Str. Album Central (SAC), Str. Griseum Centrale (SGC), Str. Fibrosum Et Grisiumsuperfi Ciale (SFGS), Str. Opticum (SO), and Str. Marginale (SM). No lesion was noted in any of the six layers. Bar=50µm.

organism's innate (nicotinic acetylcholine receptors) nAChRs which cause a disruption in proper neuronal activity by accumulation of acetylcholine in postsynaptic nAChRs. Neuronal hyperstimulation by AChE is related to impaired motor skills and behaviour changes in fish[24].

Exposure to synthetic pesticides like IMI and their subsequent bioaccumulation has been reported to cause abnormal developmental abilities[39], haematological changes[40], oxidative stress as reported in the case of antioxidant enzyme disruption like SOD[41], CAT[42], and MDA[43], histopathological damages[44], as well as genotoxic effects like micronuclei and comet formation[19,45] in various freshwater fishes in natural as well as controlled laboratory environments suggesting the potential threat these pesticides pose to aquatic organisms like fishes and through bioaccumulation to higher organisms in the food chain as well[46].

In our study, we have used fish species (*Pethia conchonius*) as a model to monitor the effect of synthetic pesticide (IMI) on the behaviour, acetylcholinesterase activity, and histoarchitectural changes in the brain upon acute (96 hours) exposure to IMI.

Behaviour is not a random process, it is a consequence of a highly structured and anticipated sequence of signals relayed in the brain that ensures the fitness and survival of the species[24]. Thus, behavioural alterations serve as a valuable biomarker to evaluate the effects of toxicants/pollutants manifested as environmental stress. In the present study, *Pethia conchonius*, exposed to different sub-lethal concentrations of IMI, showed detectable behavioural changes in the exposed groups compared to the control group. Behavioural alterations were mild to severe with increasing concentration (SLC I, SLC II, and SLC III) and duration of exposure (24 hr, 48 hr, 72 hr and 96 hr). Our findings of behavioural changes and decline in the AChE in the brain tissue with histopathological changes in IMI-treated groups indicate a correlation between them. Erratic and jerky swimming movement was initially observed in all test groups, followed by lethargic bottom settling towards the end of the study period, and loss of equilibrium became more severe in the test groups with increasing concentration of IMI, being most severe at SLC III. This behavioural change can be correlated with the change in AChE activity in the brain of the exposed fish, as locomotion is a function of proper synaptic transmission in the brain catalyzed by AChE. Low activity of AChE in the IMI exposed fishes impairs synaptic transmission. A concentration-dependent increase of mucus secretion was also exhibited by the fishes all over their body after 48 hr of IMI exposure, suggesting an effect of the agrochemical. Neurotoxic and behavioural studies on *Labeo rohita* exposed to IMI and Curzate have been conducted by Desai and Parikh with similar results[47]. Recently, behavioural responses like erratic movement and "freezing" or bottom inactivity have been reported in Zebra fish exposed to IMI[26]. Salunke *et al.*, recorded the effect of IMI on behavioural responses like jerky movements, hyperactivity, loss of equilibrium, and AChE activity in the brain of *Oreochromis mossambicus*[24]. The findings of behavioural changes, AChE activities, and brain histopathological manifestations in the IMI-treated *Pethia conchonius* corroborate the above studies.

Studies have also shown that sub-lethal exposure to other pesticides, like Chlorpyrifos, an organophosphate, induces darting swimming movements, hyperexcitability, loss of equilibrium, and sinking behaviour in *Cyprinus carpio*[48]. Neurotoxicity and behavioural impairment due to IMI exposure have also been studied by Crosby *et al.,* in *Danio rerio* fingerlings that showed decreased activity, decreased sensory motor response to stimuli, and decreased novel tank exploration behaviour[39]. Cypermethrin-induced changes in behaviour, including jumping, hyperactivity, aggressiveness, erratic swimming, and fast opercular beating have also been reported within 48 hours of exposure to the pesticide in *Tor putitora*[40]. A previous study in our laboratory to determine acute toxicity of Chlorpyrifos in *Danio rerio* also showed surfacing to gulp air, loss of balance, resting at the bottom

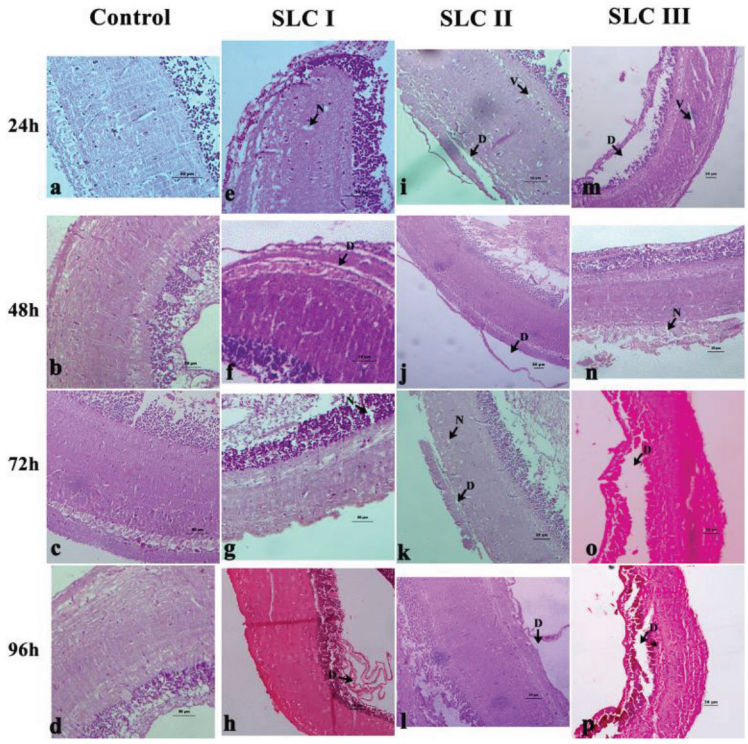


Figure 5. Histological architecture of the control **(a-d)** and the IMI exposed **(e-p)** brain (optic tectum) of *Pethia conchonius*. 'D' denotes detachment of tectum layers, 'V' denotes vacuolization, 'N' denotes Necrosis. Bar=50µm.

in a lateral position and erratic swimming behaviour before death upon exposure to Chlorpyrifos[28]. These findings further support the effect of synthetic pesticides on the behaviour of fish as revealed in our study.

Neonicotinoids are potent agonists to nicotinic acetylcholine receptors (nAChRs). IMI competes with acetylcholinesterase to bind to post-synaptic nAChRs, causing continuous cholinergic neuronal transmission that results in neurotoxicity[49]. Our findings showed both a time and dose-dependent decrease in AChE activity upon exposure to three sub-lethal concentrations of IMI (SLC I, SLC II, and SLC III) compared to the control group of *Pethia conchonius* that indicate the neurotoxic nature of IMI and support the findings of Li *et al*[49]. In the control group, the concentration of AChE remained constant throughout the study period. In the treated groups, the concentration of AChE in the brain tissues of *Pethia conchonius* decreased by almost 1.4 times in SLC I and SLC II and 2 times in SLC III in 96 hr. Compared to the control group (84.63 ± 4.25^{a1}), the AChE inhibition was maximum for SLC III at 96 hr of treatment (33.70 ± 2.52^{c4}). A decrease in AChE activity leading to acetylcholine accumulation in synapses causes neurological impairment affecting many functions, including feeding, swimming, and behaviour[50]. Impairment of behavioural responses and swimming patterns recorded in our study in *Pethia conchonius* can be correlated with the decrease in AChE activity, indicating AChE inhibition in the brain tissues of *Pethia conchonius*. Several studies on other freshwater fishes like *Oncorhynchus mykiss*[51], *Labeo rohita*[52], *Danio rerio*[26], and marine fishes like *Mullus barbatus* have reported similar results of AChE inhibition upon exposure to IMI accompanied with oxidative stress and behavioural alterations[47,53]. A concentration and time-dependent decreasing trend of IMI on brain AChE activity in *Pethia conchonius* was observed in the present study.

Topographic mapping of the retina and other sensory information (auditory, electro-sensory etc) is facilitated by the optic tectum. This shows the position of objects and events in body-centred space and regulates eye movements. The optic tectum is one of the major neuronal processors whose functioning is imperative for the survival and reproduction of the fish. In the present study, histopathological changes in the brain of *Pethia conchonius* were progressive over the periods of exposure and concentration of the IMI exposure. This suggests that histopathological responses depend not only on the concentration of pesticide but also on the duration of

the exposure to the pesticide. Damage in the superficial layers (SM, SO and SFGC) in the form of mild necrosis and detachment of layers were seen most prominently at higher concentrations and longer duration (96 hr) of IMI exposure. This region is mainly concerned with visual input and these damages are likely to be caused due to damage in AChE synapses and apoptosis of nerve cells. The complete detachment of the layers was observed along with necrosis of mononuclear cells that gave rise to clear spaces in the deeper layers of SGC, SAC and SPV. This region of the brain is also said to be related to motor functioning and the damage of which can be correlated to the changes in swimming patterns in the fish indicating the neurotoxic effect of IMI. Histopathological studies by Devi and Mishra on *Channa punctacus* upon exposure to Chlorpyrifos showed similar results[43]. Lakshmaiah reported brain damage in *Cyprinus carpio* when exposed to phorate for 96 hr[53].

5. Conclusion

An acute (96 hours) exposure of IMI to *Pethia conchonius* showed a significant change in swimming behaviour, body equilibrium, and hyperactivity that can be correlated to the decrease in AChE activity in the brain of these fishes. Histopathological changes in the structure of the optic tectum of the brain, recorded as necrosis, vacuolation, and detachment of brain tissue layers indicate the neurotoxic nature of IMI. The present findings strongly indicate the inhibitory effect of IMI on AChE activity, severe damage on the optic tectum of the brain leading to a wide range of behavioural changes. Consumption of IMI-contaminated fish may affect humans, the highest consumer at the trophic level.

6. References

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