Toxicology International, *31(3) : 417-428; 2024. DOI: 10.18311/ti/2024/v31i3/42152*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Azoxystrobin Induced Changes in the Gill Histoarchitecture, Brain Acetylcholinesterase Activity and the Behavior of the Fish *Pethia conchonius* from the River Teesta

Arpita Ray, Debojit Dutta, Bappaditya Ghosh and Min Bahadur*

Genetics and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, P.O. NBU, District, Darjeeling - 734013, West Bengal, India; min.b@rediffmail.com

Abstract

Azoxystrobin is a globally used strobilurin fungicide, which contaminates waterbodies through surface run-off. Its bioaccumulation in aquatic animals via food chains can induce serious pathophysiological disturbances. Therefore, histopathological and neuronal effects of azoxystrobin have been assessed in the fish, *Pethia conchonius* in the laboratory condition. Azoxystrobin-treated fish showed slow movement, crowding at the bottom, loss of equilibrium, and excess mucus secretion at all concentrations (0.025mg/L, 0.0514 mg/L, and 0.103mg/L) at 48 hours of exposure compared to the control. A significant dose and time-dependent inhibition in acetylcholinesterase activity was observed ($p<0.05$). The highest acetylcholinesterase inhibition (45.45 ± 1.07) was noted for the highest concentration at 96 hours of exposure than the control groups (88.35 ± 0.71). In contrast to the control, different histopathological changes in gill tissues have been observed like, epithelial lifting, lamellar fusion, epithelial hyperplasia, and the curling of secondary lamellae in the azoxystrobin-exposed groups after 24 hours of treatment. The results of this study indicated that azoxystrobin is neurotoxic as well as damaging to gills.

Keywords: Acetylcholinesterase, Azoxystrobin, Histology, *Pethia conchonius*

1. Introduction

Due to increasing tolerance/resistance to pesticides in agricultural pests and the subsequent insurgence of new pests, the use of pesticides increased exponentially[1,2]. Synthetic chemical pesticides like insecticides, fungicides, herbicides, rodenticides, etc. are widely used to control insect pests, fungal pathogens, weeds, and other small rodents[3]. Regardless of their contribution to crop productivity and the economy, when used indiscriminately, pesticides can contaminate soil and water. The majority of pesticides used in agriculture are not pest-specific, which can disrupt the biological function of both the aquatic and terrestrial non-target organisms, including human[4,5]. Chemical fungicides are used to prevent the growth of fungal pathogens. There are several classes of fungicides, such as benzimidazoles, strobilurins, triazoles, dithiocarbamides, carboxylic amides, and

*Author for correspondence

Article Received on: 21.03.2024 Revised on: 20.05.2024 Accepted on: 24.05.2024

chlorothalonils[6]. Among these main fungicide classes, strobilurins are one of the most commonly used fungicide groups, accounting for 22.9% of the total fungicide market share[7-9]. The global Azoxystrobin market was valued at USD 794.5 million in 2020, which will reach USD 1771.5 million by the end of 2027, growing at a compound annual growth rate of 14.3%. India is the 4th largest market of azoxystrobin in Asia[10]. Azoxystrobin (AZX), a broad-spectrum systemic fungicide belonging to the β-methoxyacrylate strobilurins, is one of the globally used fungicides around the world, and as of 2014, AZX was the first best-selling fungicide[11], which was used to control a wide range of fungal pathogenesis[12-14]. AZX prevents the electron transfer between cytochrome b and cytochrome c1, resulting in the disruption of the mitochondrial respiratory system of the fungal pathogens[15]. AZX has water solubility of 6 mg/L at 20°C, an octanol-water coefficient (log Kow) of 2.5[12,] and a half-life of 15–28 days in aquatic environments[16].

AZX is one of the most commonly detected fungicides in the aquatic ecosystem. It was reported from the streams of the United States, 20 streams in Germany, surface water in Demark, and in the river in China at the mean concentration of 0.16 µg/L[17], 1.43 µg/L[18], 0.15 µg/L[19] and 34 µg/L[20], respectively. AZX has been shown to be less toxic to birds than the freshwater fishes and the other invertebrates[16]. In agricultural regions of Sweden, Han *et al.*[21] detected AZX at concentrations of 0.3 µg/L in streams and greater than 1.0 µg/L in water samples. Corcoran *et al.*[23] documented the presence of AZX in freshwater ecosystems in Argentina at concentrations ranging from 0.01 to 0.06µg/L. AZX residues detected in water and soil bioaccumulate in aquatic animals, especially fish[22] through the food chain. Fungicide accumulation may affect the physiology and the survival[23] of the aquatic animals.

Biomarkers facilitate the early identification of detrimental impacts of environmental pollutants on aquatic organisms[24,25]. Histopathological changes in various tissues can be used as an important biomarker to assess the fungicide toxicity[26]. Hussain *et al.*[27] reported significant damages in the gill tissue of *Wallago attu* and *Cirrhinus mrigala*. Histopathological changes may cause altered physiological functions leading to changes in fish behavior or external phenotype[28]. Despite their lack of specificity, histological modifications have been employed for several decades in diverse fish tissues and organs to evaluate the impacts of various chemical pollutants, both in field and laboratory investigations[29-33].

The gills serve as the principal organ responsible for oxygen absorption in fish, constantly interacting with hazardous substances in water. As a result, the stress experienced by the gills is higher[34]. Different studies have shown that changes in gill morphology are indicator of the toxic effects of insecticides[26,35,36]. Another important biomarker to assess the toxicity of chemicals is to evaluate the activity of the cholinesterase. Research conducted on fish has revealed that various pesticides have the potential to influence neurotransmission systems, such as the cholinergic system[37-40]. The cholinergic system plays an important role in the nervous system, with Acetylcholine (ACh) serving as its principal neurotransmitter[41-43]. ACh is involved in both the central and peripheral nervous systems and regulates neuronal response and behavior[44]. Cholinesterases break down the acetylcholine present in the synaptic cleft into choline and acetate and thus inhibit the actions triggered by the ACh molecules[45]. Inhibition of the AChE activity leads to an over-accumulation of ACh resulting in prolonged excitatory postsynaptic potential. This ultimately results in paralysis and mortality due to the hyperstimulation of nerves or muscle fibers induced by the repetitive and uncontrolled firing of neurons[46], hence AChE is the key target known as the esteric site, for binding of some toxic compounds resulting in detrimental effects[47].

Behavioral alterations are also a sensitive marker of environmental stress. Changes in fish behavior can serve as valuable indicators for assessing ecological changes in the physicochemical properties of the habitat[48]. Numerous pesticides have shown neurotoxic effects on the CNS, leading to behavioral changes[37-39,49].

Several studies have reported the presence of pesticides in the rivers of North Bengal, India[50,51]. Teesta is one of the major rivers in the northern part of West Bengal, which originates in the Himalayas in Sikkim and flows through Darjeeling, Kalimpong, Jalpaiguri, and Coochbehar in North Bengal. Teesta is likely to be contaminated with pesticides due to the presence of agricultural fields on its bank and anthropogenic activities. LC-MS analysis of the surface water sampled from Teesta has showed the presence of several fungicides along with AZX (unpublished). Although fungicides are used extensively, their impacts have not been studied as intensively as compared to insecticides and herbicides. There are no studies on the contamination and detrimental effects of fungicides on the organisms of Teesta. Hence, a resident fish, *Pethia conchonius* from Teesta has been used as the

experimental organism, which is widely used in the fields of toxicology[52-55].

Therefore, the present study encompasses the effects of AZX on the gill histopathology, brain AChE activity, and the behavioral changes in the fish *Pethia conchonius* from Teesta.

2. Materials and Methods

2.1 Reagents

Amistar® (Syngenta India Limited, India) (CAS No. 131860-33-8), a commercial formulation of Azoxystrobin (AZX) was used for bioassay. The chemicals and reagents were obtained from Sigma Aldrich® and SRL, and they were of analytical grade. For the stock solution, a concentration of 100mg/ml of AZX was prepared in distilled water.

2.2 Test Species

The specimens of *Pethia conchonius*, commonly referred to as Rosy barb, belonging to the Actinopterygii Class, Cypriniformes Order, and Cyprinidae Family, were obtained from the river Teesta at the Gajoldoba Teesta barrage in Jalpaiguri, with coordinates of 26.7515°N 88.5856°E. The specimens had an average length of 6.33 ± 0.51 cm and a weight of 3.92 ± 0.40 g. Fish specimens were brought to the laboratory under well-aerated conditions and thereafter transferred to 20 L glass aquaria measuring 40cmX25cmX25cm. The fish specimens were subjected to external decontamination by dipping in 0.05% potassium permanganate ($KMnO_4$) solution for 2 minutes. The fish were acclimatized for 15 days at room temperature (25 ± 2 °C) according to the criteria set by the Organization for Economic Co-operation and Development (OECD), 2019 (Test Guideline no. 203)[56]. The fish were fed with commercial fish food twice daily till the commencement of the experiments. The aquaria were cleaned daily by siphoning the remnants of the food and fecal/excretory substances.

2.3 Experimental Design

The 96-h LC_{50} (with 95% confidence limits) of Azoxystrobin for *Pethia conchonius* was estimated to be 0.514 mg/L. Based on the 96-h LC_{50} value, three sub-lethal concentrations of AZX, CI, CII and CIII comprising 20^{th}, 10^{th} and 5^{th} part of 0.514 mg/L (0.025mg/L, 0.0514 mg/L and 0.103mg/L, respectively) have been used to treat *Pethia conchonius* in a 20L aquaria to evaluate its adverse effects. A total of sixteen fish specimens (n=16) were starved for 24-hours before the start of experiment. The fish were treated with the sub-lethal concentrations of AZX for 96 hours. Fish kept in de-chlorinated pesticide free tap water was taken as the control. Three replicates were set up for each concentration. Four fish specimens (n=4) were randomly sampled from each group at different time intervals: 24 hours, 48 hours, 72 hours, and 96 hours. The gill and brain tissues were dissected for histological analysis and brain acetylcholinesterase assay, respectively.

For the behavioral study, a similar set was prepared. A graphical illustration of the study is given in Figure 1. There was no recorded mortality in the course of this investigation. The experiments and procedures were approved by the Institutional Animal Ethics Committee of the University of North Bengal, reference number IAEC/NBU/2022/31 dated 23.09.2022 (Proposal No. 13).

2.4 Behavioral Study

For the study of behavioral response, three groups of fish, each consisting of four individuals (n=4), were exposed to three sub-lethal concentrations of AZX. Their responses were compared with the control group kept in untreated test water. The same tap water was utilized for both the control as well as treated groups. The behavior was

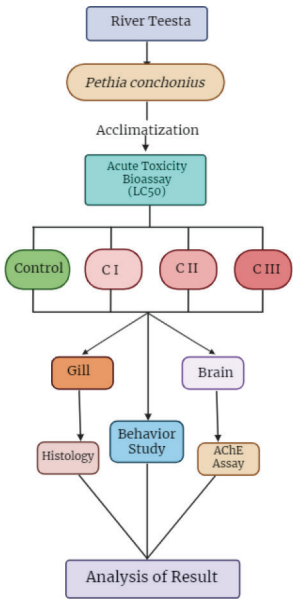


Figure 1. Schematic representation of the experimental setup.

observed continuously for 1-2 hours at a 24-hours interval across four days for both the control and treated groups. Specific behaviors such as quick opercular movement, mucus secretion, slow movement, bottom settling, and loss of equilibrium for both the treated and the control groups were recorded by photographing using a Camera (Nikon COOLPIX L830).

2.5 AChE Assay

Brain tissue was dissected out and homogenized in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4). Homogenate was centrifuged at 8000xg for 10 minutes and the supernatant was used as an enzyme source. AChE activity was measured following the protocol of Ellman *et al.*[57] with minor modifications. 400µl of the supernatant was mixed with 100µl of 0.01 M DTNB, 20µl of acetylthiocholine iodide (0.075 M), and 2.6 ml of phosphate buffer (0.1 M, pH 8.0) in a glass cuvette. The final volume was adjusted to 3.12 ml. Absorbance was recorded spectrophotometrically (Shimadzu, UV-1900i) at 412 nm wavelength for 3 minutes. Enzyme activity was expressed as µM/min/mg tissue. The supernatant was substituted with distilled water for the blank.

2.6 Gill Histology

2.6.1 Fixation and Slide Preparation

Histoarchitectural study was carried out according to Bernet *et al.*[58] with slight modifications. Briefly, gill tissues were treated in Bouin's fixative (75 ml of saturated aqueous picric acid, 25ml of glacial acetic acid, 5ml of formaldehyde) for 24 h, thoroughly washed in running water for 2-2½ hours, dehydrated passing through 50%, 70%, 90%, and absolute ethanol. The tissues were cleared in xylene for 10 minutes and finally incubated in 1:1 xylene and paraffin mixture for 30 minutes at 60°C. The tissues were subsequently immersed in molten paraffin and a paraffin block was prepared.

Sections of 6µm thickness were obtained from the blocks using a Rotary Microtome (Spencer Microtome 820, India). The serial sections were placed on clean, grease-free slides coated with Mayer's albumin and stretched on a hot plate (60°C).

2.6.2 Staining

The slides were deparaffinized in xylene for 5-10 minutes, rehydrated passing through 100%, 90%, 80%, 70%, 50%, 30% ethanol each for 5-10 minutes. The slides were washed in distilled water, stained in Harris Haematoxylin (20g ammonium/potassium alum, 0.5g mercuric oxide, 10ml glacial acetic acid, 190 ml distilled water) for 10 minutes, and dehydrated passing through graded alcohol (30%, 50%, and 70%) for 5-10 minutes each. Slides were stained with alcoholic eosin (90% ethanol) for 2-5 minutes, differentiated in 90%, and dehydrated in absolute ethanol for 5 minutes each. Slides were cleared in xylene for 10 minutes and mounted in DPX (Ranbaxy Laboratories Ltd). The slides were examined at 40X and 100X magnifications using Nikon Eclipse E200 microscope equipped with a photographic adapter.

2.7 Statistical Analysis

The current study aimed to compare the concentration of AZX and the duration of exposure as independent factors with the dependent variable, AChE activity, to observe any changes.

The results of the Shapiro-Wilk normality test revealed that the AChE activity in all groups exhibited a normal distribution (p>0.05). The homogeneity of variances was assessed by conducting Levene's Test of Equality of Variances. Given the normal distribution of the data, One-Way Analysis of Variance (ANOVA) was performed on the concentration and duration of exposure variables individually followed by a Tukey HSDa test for homoscedastic data and a Dunnett T3 test for heteroscedastic data.

3. Results

3.1 Behavioral Changes

The overall behavioral pattern was monitored in the control and the AZX-treated groups of *Pethia conchonius* after the exposure of three sub-lethal concentrations (CI, CII, and CIII) of AZX for 96 hours. The behavioral changes exhibited by *Pethia conchonius* exposed to AZX have been shown in Table 1.

Initially, fishes under treatment showed rapid opercular movement during the first 24 hours of exposure to AZX, but the control group showed no change. Additionally, an alteration in swimming patterns was noted. Fish became sluggish after exposure to AZX in all sub-lethal concentrations. At lower concentrations, the sluggish behavior was noticed after 48 hours, however at highest concentrations of AZX (0.103 mg/L), it was

Table 1. Behavioral changes in the control and the AZX-exposed *Pethia conchonius* for different period.

Concentration	Control				CI				CII				CIII			
Duration of Exposure (hours)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Quick opercular Movement	-	-	-	-	+	-	-	-	+	-	-	-	++	-	-	-
Mucus Secretion	-	-	-	-	-	+	+	++	-	+	++	++	-	++	++	+++
Slow Movement	-	-	-	-	-	+	+	++	-	++	++	++	+	++	++	++
Loss of equilibrium	-	-	-	-	-	-	+	+	-	-	+	++	-	++	++	+++
Bottom settling	-	-	-	-	-	+	+	+	-	+	++	++	+	+	++	++

Note: The negative sign (-) represents no change. The positive symbol: + represents a low or slight change, ++ represents a mild or moderate change, and +++ represents a high or maximum change.

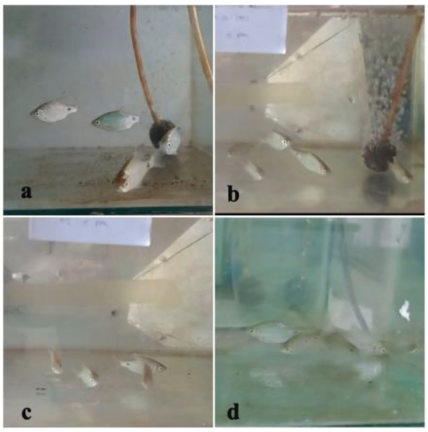


Figure 2. Behavioral changes in the control and AZX-exposed *Pethia conchonius*: **(a)** normal swimming in control; **(b)** downward swimming; **(c)** loss of equilibrium; **(d)** bottom aggregation in AZX-exposed fish.

observed from the first hour to the completion of the trial. Loss of equilibrium was noticed after 72 hours in CI, and CII but was very prominent at 48 hours in the highest concentration (CIII). The exposed fish also exhibited a loss of equilibrium and turned to the side during swimming. After 72 hours of exposure, an aggregation behavior of the treated fish was noticed where the fish aggregated at the bottom corner of the aquaria. AZX-treated fish secreted a higher amount of mucus than the control group after 48 hours of exposure. Similar behaviors were noted during the early hours of exposure for all concentrations. However, subsequent observations revealed stronger impacts at higher concentrations compared to lower concentrations.

3.2 AChE Activity

The results of the spectrophotometric analysis showed a concentration-dependent decrease in AChE activity in the treated groups compared to the control. Values that exhibit homogeneous subgroups characterized by a shared alphabet or numeric value suggest the absence of a statistically significant difference ($p>0.05$). The control groups did not exhibit any statistically significant differences at various time intervals, leading to the formation of a single homogeneous subset referred to as 'a1' ($p>0.05$). However, the groups exposed to AZX demonstrated a significant reduction in the activity of AChE as the period of exposure increased ($p<0.001$). The highest AChE activities were 80.71 ± 0.72, 73.65 ± 0.64, and 71.03 ± 0.33 for CI, CII, and CIII, respectively at 24 hours of exposure. Maximum inhibition of AChE activity was observed at 96 hours for all the sub-lethal concentrations with the mean AChE activity of 63.79 ± 1.27, 55.02 ± 1.47, and 45.45 ± 1.07 for CI, CII, and CIII, respectively.

3.3 Histology

The gill of the control fish did not exhibit any histological alterations. Figure 4 illustrates the structural characteristics of the gill in the control *Pethia conchonius*. The transverse section of the gill tissues of control fish showed normal histoarchitecture with intact lamellar gill epithelium, primary (Pl) and secondary gill lamellae (Sl). The control fish had well-structured gill filaments and lamellae, characterized by the presence of fat epithelial cells and pillar cells that lined the blood sinusoids.

The most common changes in the AZX-treated groups were epithelial lifting (El), hyperplasia (Hy), lamellar fusion (Fu), collapsed secondary lamellae (Co), and the curling of secondary lamellae (Cu). After 24 hours of exposure to AZX, structural changes, including the curling of secondary lamellae, fusing of adjacent secondary lamellae, and hyperplasia of main lamella

Table 2. Effects of the sub-lethal concentrations of AZX on AChE activity (Mean±SD) in *Pethia conchonius*.

		Concentration			
		Control	CI	CII	CII
		Mean AChE Activity			
Duration (hours)	24	87.54 ± 0.48^{a1}	80.71 ± 0.72^{b1***}	73.65 ± 0.64^{c1***}	71.03 ± 0.33^{d1***}
	48	87.29 ± 0.29^{a1}	76.53 ± 1.17^{b2***}	73.67 ± 0.89^{c1***}	65.59 ± 1.25^{d2***}
	72	87.84 ± 1.18^{a1}	69.28 ± 0.65^{b3***}	64.85 ± 0.89^{c2***}	61.04 ± 0.29^{d3***}
	96	88.35 ± 0.71^{a1}	63.79 ± 1.27^{b4***}	55.02 ± 1.47^{c3***}	45.45 ± 1.07^{d4***}

• Superscript lowercase alphabets denote values with different homogenous subsets which significantly differ at (p<0.05). ANOVA test followed by Post hoc Dunn's Test.
• Numeric indicates a stepwise significant difference in values between concentrations within exposure duration (2 = p<0.05, 3 = p<0.01, 4 = p<0.001).
• Asterisks (***) indicate that the mean difference is significant at the p<0.001 level.

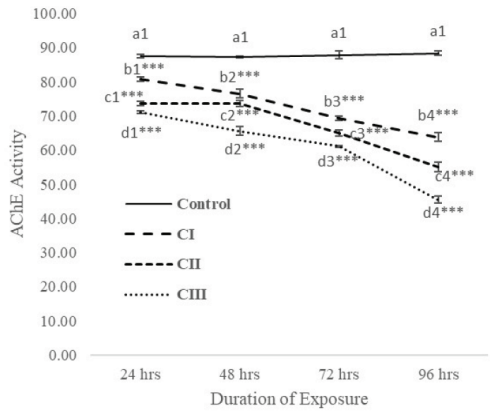


Figure 3. Effects of the three sub-lethal concentrations of AZX on brain AChE activity in *Pethia conchonius*.
• Superscript lowercase alphabets denotes values with different homogenous subsets which significantly differ at (p<0.05). ANOVA test followed by Post hoc Dunn's Test.
• Numeric indicates stepwise significant difference in values between concentrations within exposure duration (2= p<0.05, 3= p<0.01, 4= p<0.001).
Asterisks (***) indicate that the mean difference is significant at the p<0.001 level.

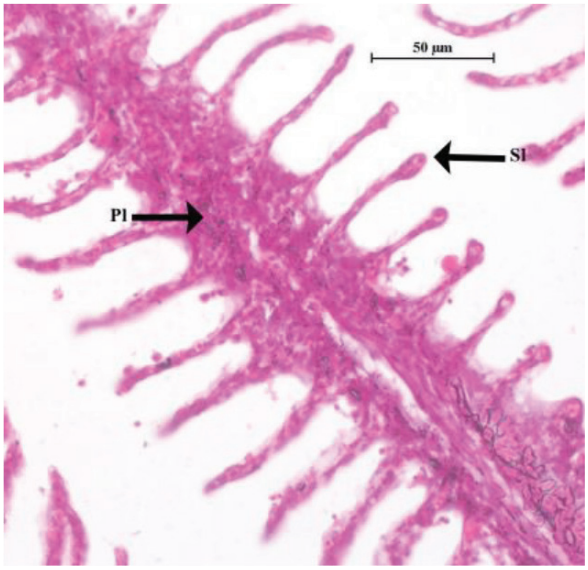


Figure 4. Normal gill histoarchitecture with compact primary and secondary gill lamellae. 'PI' indicates primary gill lamellae and 'SI' indicates secondary gill lamellae. Bar=50µm (H-E staining, magnification 40X).

Table 3. Histological alterations in the control and the AZX-exposed *Pethia conchonius* for different period.

Concentration	Control				C I				C II				C III			
Duration of Exposure (hours)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Epithelial hyperplasia	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+
Epithelial lifting	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Collapsed secondary lamellae	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+
Degeneration of secondary lamellae	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Lamellar fusion	-	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+
Curling of secondary lamellae	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+

Note: Negative symbol (-) indicates No change, Positive symbol (+) indicates the presence of the abnormality.

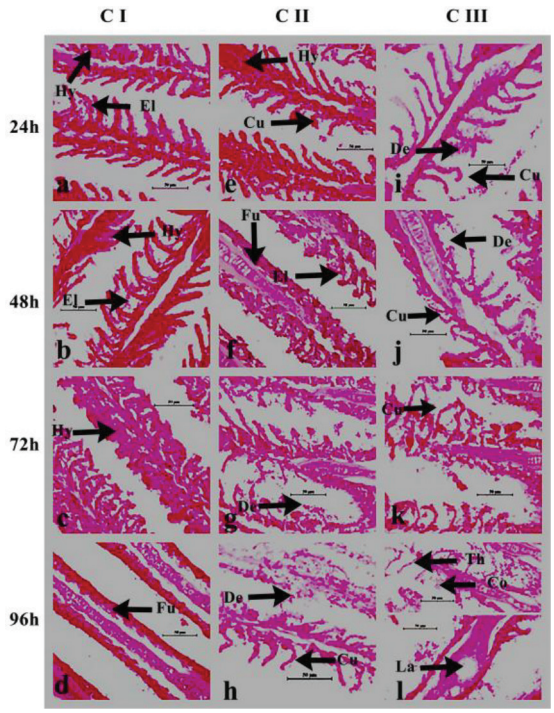


Figure 5. Histological alteration in the AZX-exposed *Pethia conchonius:* CI **(a-d)** , CII **(e-h)** and CIII **(i-l)**. Abbreviations used denote: Hy=epithelial hyperplasia, El=epithelial lifting, Fu=fusion of secondary lamellae, De=degeneration of secondary lamellae, Cu=curling of secondary lamellae, Th=thinning of secondary lamellae, La=denotes lacunae formation. Bar=50µm. (H-E staining, magnification 40X).

(Figures 5(a), (e), and (i)) were detected. Degeneration of secondary lamellae (De) was observed after 48 hours in all the exposed groups (Figures 5 (b), (f) and (j)). After 96 hours, at the highest SLC of AZX, the gills of treated fish showed collapsed secondary lamellae (Co) with lacunae formation (La) (Figure 5 (l)). Moreover, there was a complete detachment of the epithelial layer from the middle region of each lamella (Table 3).

4. Discussion

AZX has been considered as one of the highly toxic fungicides to both marine and freshwater organisms[59]. Several workers reported the toxic effects of AZX related to oxidative stress, endocrine disruption, developmental deformities as well as genotoxic and mutagenic effects in various aquatic organisms[60-64], but lacunae are there regarding behavioral, histological, and neurological changes along with altered acetylcholinesterase activity.

The current investigation was conducted to assess the histopathological changes in the gills as well as the probable neurotoxic impact on *Pethia conchonius*. Histological study is often regarded as the most sensitive approach for examining the cellular changes that may manifest in essential organs[65]. Fish when exposed to sub-lethal doses of different fungicides or other chemical pollutants in the environment, may exhibit histopathological abnormalities in their tissues based on the extent of toxicity[66]. Gills are the primary target site for water-borne toxins/contaminants due to their direct and constant interaction with water[67-69]. Gills not only absorb oxygen but also regulate ions, maintain acid-base balance, and eliminate nitrogenous wastes from the organism. So, the ecological toxicants have a severe impact on these vital organs and indirectly impair the health of fish[68]. Mallatt[67] reported an array of histoarchitechtural changes in the gill tissues of fish, which are the manifestation of defense mechanisms against the toxicants.

The observed deformities in the gills of *Pethia conchonius* exposed to sub-lethal concentrations of AZX, such as curling of secondary lamellae, fusion of adjacent secondary lamellae, hyperplasia of lamellar epithelium, and collapsed secondary lamellae, corroborate the findings reported by Kazmi *et al.*[70], who showed various microscopic changes in the gills of treated fish due to exposure to AZX (100 µg/L). These alterations included necrosis of lamellar cells in the secondary lamellae, degeneration of both primary and secondary lamellae, as well as the presence of aneurysms and disorganization of the cartilaginous core in the gills of the fish.

Our findings indicated that AZX is toxic even at a concentration of 25 µg/L. Nataraj *et al.*[71] reported histopathological changes such as epithelial lifting, lamellar fusion, hypertrophy, and epithelial necrosis in the gills of *Labeo rohita* exposed to difenoconazole. Similar results have also been reported in *Cyprinus carpio*[72] and Rainbow trout[73] exposed to the fungicides fenamidone and captan, respectively. Raibeemol and Chitra[74] have also reported similar histopathological alterations in *Pseudetroplus maculatus* exposed to 0.661 µg/ L of chlorpyrifos. Histopathological alterations in *Cyprinus carpio* and *Cirrhinus mrigala* exposed to organophosphates like quinalphos and dichlorvos have also been reported[75,76]. Gill tissues serve as the primary locations for the interaction of dissolved pollutants, making them a valuable indicator in the field of environmental monitoring. The findings of our investigation provide compelling evidence that acute

exposure to AZX at sub-lethal concentrations can lead to alterations in the gill tissues of the experimental organism *Pethia conchonius*. In addition, it can be inferred that AZX infiltrated the fish's body via the outer layer of skin and gill, or by directly ingesting water, leading to the deterioration of essential organs.

Behavioral changes serve as an early indicator of potential toxic effects in fish. Our observations of sluggish movement, loss of equilibrium, aggregation at the bottom, rapid opercular movement, and excess mucus secretion, are comparable with other studies showing[49,77-81] irregular, erratic and darting swimming movements, hyperexcitability, loss of equilibrium and sinking to the bottom in chlorpyrifos exposed *Cyprinus carpio*[77] and *Gambusia affinis*[78]. Similar results have also been reported in *Tor putitora* exposed to cypermethrin[80]. Our laboratory has also shown behavioral alterations in *Pethia conchonius* exposed to imidacloprid[49]. The changes in the behavior increased with increasing concentration and duration of exposure to AZX. Exposure to all sub-lethal concentrations resulted in changes in all parameters that were examined. The observed decrease in speed, loss of equilibrium, and lethargic bottom-crowding behavior indicated that the AZX-treated fish has impaired movements. The locomotory capacity of *Pethia conchonius* was highly disorganized and reduced. Different studies have shown significantly reduced locomotory and swimming activity in carbendazim and tebuconazole exposed *Danio rerio*[82,83].

Mucus secretion is an innate immune response in fish[77,81]. Excessive mucus secretion with respect to AZX exposure indicated initial defense against the fungicide. Rapid opercular movement and gulping of air observed in *Pethia conchonius* after AZX exposure could be a response to respiratory distress[77]. In the present study, AZX reduced the locomotion activity, making the treated fish slower than the control group. Propiconazole has been reported[84] to alter the aforementioned behavioral patterns, which is consistent with the present study.

In this study, the observed behavioral changes can be correlated with the inhibition of AChE activity, causing an elevated level of acetylcholine (ACh) at cholinergic synapses and subsequent overstimulation[77]. AChE activity was measured to determine if there is any correlation between the AChE activity and behavior of fish exposed to AZX that interrupts the cholinergic system, as ACh is associated with movement[45]. In the present study, *Pethia conchonius* showing decreased activity or inhibition of AChE (Table 2 and Figure 3) at all the sub-lethal concentrations of AZX (CI, CII, and CIII) had altered behavior. Results also showed a dose and time-dependent inhibition of AChE activity compared to the control group. The highest AChE inhibition i.e., almost 2 times compared to the control in the SLC III group after 96 hours of exposure suggests inhibitory impact of AZX on the AChE activity and potentially induce behavioral modifications in the fish indicating the involvement of cholinergic signaling. The application of AZX, bifenthrin, and cyproconazole in a rice field, resulted in a notable decrease in AChE activity in the brain and muscle tissues of *A. lacustris*, as well as in the brain of *M. nigripinnis*[22]. Propiconazole was able to reduce AChE activity in *Clarias batrachus* too[84]. Similarly, Altenhofen *et al.*[83] reported AChE inhibition in zebrafish exposed to 4 and 6 mg/L tebuconazole after 96 hours. AChE serves as a reliable biomarker for assessing cholinergic function since its level is regulated by the interaction between ACh and its receptors[85]. Therefore, our results indicate that AChE inhibition can lead to the accumulation of the neurotransmitter, ACh in the synaptic clefts that can overstimulate the neural transmission. An alternative explanation for the result could be that exposure to AZX may be inducing damage to cholinergic neurons, leading to a decrease in AChE activity[83].

Therefore, it can be proposed that the behavioral modifications seen in *Pethia conchonius* may be related to the inhibition of acetylcholinesterase (AChE) induced by AZX. AZX also induced alterations in morphological characteristics and cholinergic signaling by AChE inhibition and these modifications may potentially be associated with the observed behavioral impairment in the fish species *Pethia conchonius*.

This study has potential implementation in aquatic ecosystems and human health. Pesticides, due to agricultural run-off or direct applications, can contaminate water bodies and bioaccumulate in the aquatic organisms. Ultimately, such pesticides may reach to the human through contaminated water or consumption of affected fish and pose severe health risks, particularly in the agricultural workers who handle pesticides directly. Therefore, this research will contribute to the advancement of scientific knowledge regarding the toxicity of fungicide, promote appropriate usage, and aid in the development of science-based policies and practices that safeguard human health and the environment.

5. Conclusion

In conclusion, our results strongly suggest that AZX is highly toxic to both the somatic and neural cells/tissues. It adversely affects the cholinergic signaling by inhibiting AChE, which can be correlated with the behavioral changes observed in the fish *Pethia conchonius*. Histopathological damages of the respiratory tissues (gills) can lead to respiratory problems. This study will help monitoring the presence and harmful effects of strobilurin fungicides in aquatic ecosystems.

6. Acknowledgements

The Head, Department of Zoology, University of North Bengal is sincerely acknowledged for providing departmental instrument facility under the Fund for Improvement of Science and Technology Infrastructure Programme (FIST), Department of Science and Technology, New Delhi, India. The Council of Scientific and Industrial Research (CSIR), New Delhi is also sincerely acknowledged for granting SRF to the first author. The authors express their gratitude to all those who provided assistance in the execution of the study, particularly the fisherman.

7. References

1. Tudi M, Daniel Ruan H, Wang L, Lyu J, Sadler R, Connell D, Chu C, Phung DT. Agriculture development, pesticide application and its impact on the environment. Int J Environ Res Public Health. 2021; 18(3):1112. https://doi.org/10.3390/ijerph18031112
2. Avenot HF, Michailides TJ. Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. Plant Disease. 2007; 91(10):1345-50. https://doi.org/10.1094/PDIS-91-10-1345
3. Bernardes MF, Pazin M, Pereira LC, Dorta DJ. Impact of pesticides on environmental and human health. Toxicology Studies Cells, Drugs, and the Environment. 2015; 195-233. https://doi.org/10.5772/59710
4. Sarkar S, Gil JD, Keeley J, Jansen K. The use of pesticides in developing countries and their impact on health and the right to food. European Union; 2021.
5. Seltenrich N. More pieces of the puzzle: New insights into azoxystrobin exposures and neurotoxicity. EHP. 2022; 130(4):044002. https://doi.org/10.1289/EHP11166
6. Zubrod JP, Bundschuh M, Arts G, Brühl CA, Imfeld G, Knäbel A, Payraudeau S, Rasmussen JJ, Rohr J, Scharmüller A, Smalling K. Fungicides: An overlooked pesticide class? Environ Sci Technol. 2019; 53(7):3347-65. https://doi.org/10.1021/acs.est.8b04392
7. McDougall P. AgriService: Products Section, 2014 Market. Phillips McDougall-AgriService; 2015.
8. Li H, Cao F, Zhao F, Yang Y, Teng M, Wang C, Qiu L. Developmental toxicity, oxidative stress and immunotoxicity induced by three strobilurins (pyraclostrobin, trifloxystrobin and picoxystrobin) in zebrafish embryos. Chemosphere. 2018; 207:781-90. https://doi.org/10.1016/j.chemosphere.2018.05.146
9. Lu B. Azoxystrobin and pyraclostrobin dominate the market of Strobilurin fungicides. Shangdong Pesticide News. 2018; 4:24-30.
10. Azoxystrobin Market Size, Industry Changing Aspect and Forecast to 2032 with the Top key players, Global Industry Forecast [Internet], 2024, March, cited 2024, May 11; available from: https://www.maximizemarketresearch.com/market-report/global-azoxystrobin-market/107455/
11. Wang X, Li X, Wang Y, Qin Y, Yan B, Martyniuk CJ. A comprehensive review of strobilurin fungicide toxicity in aquatic species: Emphasis on mode of action from the zebrafish model. Environ Pollut. 2021; 275:116671. https://doi.org/10.1016/j.envpol.2021.116671
12. Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B. The strobilurin fungicides. Pest Manag Sci. 2002; 58(7):649-62. https://doi.org/10.1002/ps.520
13. Wang H, Huang Y, Wang J, Chen X, Wei K, Wang M, Shang S. Activities of azoxystrobin and difenoconazole against *Alternaria alternata* and their control efficacy. Crop Protection. 2016; 90:54-8. https://doi.org/10.1016/j.cropro.2016.08.022
14. Hu W, Liu CW, Jiménez JA, McCoy ES, Hsiao YC, Lin W, Engel SM, Lu K, Zylka MJ. Detection of azoxystrobin fungicide and metabolite azoxystrobin-acid in pregnant women and children, estimation of daily intake, and evaluation of placental and lactational transfer in mice. EHP. 2022; 130(2):027013. https://doi.org/10.1289/EHP9808
15. Wang R, Huang N, Ji J, Chen C. An integrated approach for evaluating the interactive effects between azoxystrobin and ochratoxin A: Pathway-based *in vivo* analyses. Pestic Biochem Phys. 2023; 195:105556. https://doi.org/10.1016/j.pestbp.2023.105556
16. Tomlin CD. The pesticide manual; 2000. (No. Ed. 12, 1250).
17. Reilly TJ, Smalling KL, Orlando JL, Kuivila KM. Occurrence of boscalid and other selected fungicides in surface water and groundwater in three targeted use areas in the United States. Chemosphere. 2012; 89(3):228-34. https://doi.org/10.1016/j.chemosphere.2012.04.023
18. Schriever CA, von der Ohe PC, Liess M. Estimating pesticide runoff in small streams. Chemosphere. 2007; 68(11):2161-71. https://doi.org/10.1016/j.chemosphere.2007.01.086

19. Jørgensen LF, Kjær J, Olsen P, Rosenbom AE. Leaching of azoxystrobin and its degradation product R234886 from Danish agricultural field sites. Chemosphere. 2012; 88(5):554-62. https://doi.org/10.1016/j.chemosphere.2012.03.027
20. Wang Q, Zhong WY, Huang S. Determination of azoxystrobin residues in surface water by HPLC with solid-phase extraction. Anhui Med Pharm J. 2009; 13:611-2.
21. Han Y, Liu T, Wang J, Wang J, Zhang C, Zhu L. Genotoxicity and oxidative stress induced by the fungicide azoxystrobin in zebrafish (*Danio rerio*) livers. Pestic Biochem Phys. 2016; 133:13-9. https://doi.org/10.1016/j.pestbp.2016.03.011
22. Rossi AS, Fantón N, Michlig MP, Repetti MR, Cazenave J. Fish inhabiting rice fields: Bioaccumulation, oxidative stress and neurotoxic effects after pesticides application. Ecol Indic. 2020; 113:106186. https://doi.org/10.1016/j.ecolind.2020.106186
23. Corcoran S, Metcalfe CD, Sultana T, Amé MV, Menone ML. Pesticides in surface waters in Argentina monitored using polar organic chemical integrative samplers. Bull Environ Contam Toxicol. 2020; 104:21-6. https://doi.org/10.1007/s00128-019-02758-z
24. Van der Oost R, Beyer J, Vermeulen NP. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol. 2003; 13(2):57-149. https://doi.org/10.1016/S1382-6689(02)00126-6
25. De la Torre FR, Ferrari L, Salibián A. Biomarkers of a native fish species (*Cnesterodon decemmaculatus*) application to the water toxicity assessment of a peri-urban polluted river of Argentina. Chemosphere. 2005; 59(4):577-83. https://doi.org/10.1016/j.chemosphere.2004.12.039
26. Thophon S, Kruatrachue M, Upatham ES, Pokethitiyook P, Sahaphong S, Jaritkhuan S. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure. Environ Pollut. 2003; 121(3):307-20. https://doi.org/10.1016/S0269-7491(02)00270-1
27. Hussain B, Fatima M, Al-Ghanim KA, Mahboob S. Environmentally induced nephrotoxicity and histopathological alternations in *Wallago attu* and *Cirrhinus mrigla*. Saudi J Biol Sci. 2019; 26(4):752-7. https://doi.org/10.1016/j.sjbs.2019.02.003
28. Van Dyk JC, Marchand MJ, Pieterse GM, Barnhoorn IE, Bornman MS. Histological changes in the gills of *Clarias gariepinus* (Teleostei: Clariidae) from a polluted South African urban aquatic system. Afr J Aquat Sci. 2009; 34(3):283-91. https://doi.org/10.2989/AJAS.2009.34.3.10.986
29. Hinton DE, Lauren DJ, McCuskey RS, McCuskey PA, Lantz RC. *In vivo* microscopy of liver microvasculature in rainbow trout (*Oncorhynchus mykiss*). Mar Environ Res. 1989; 28(1-4):407-10. https://doi.org/10.1016/0141-1136(89)90270-5
30. Manera M, Dezfuli BS, DePasquale JA, Giari L. Multivariate approach to gill pathology in European sea bass after experimental exposure to cadmium and terbuthylazine. Ecotoxicol Environ Saf. 2016; 129:282-90. https://doi.org/10.1016/j.ecoenv.2016.03.039
31. Ramírez-Duarte WF, Rondón-Barragán IS, Eslava-Mocha PR. Acute toxicity and histopathological alterations of Roundup® herbicide on" cachama blanca"(*Piaractus brachypomus*). Pesq Vet Bras. 2008; 28:547-54. https://doi.org/10.1590/S0100-736X2008001100002
32. Figueiredo-Fernandes A, Ferreira-Cardoso JV, Garcia-Santos S, Monteiro SM, Carrola J, Matos P, Fontaínhas-Fernandes A. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesq Vet Bras. 2007; 27:103-9. https://doi.org/10.1590/S0100-736X2007000300004
33. Fontaínhas-Fernandes A, Luzio A, Garcia-Santos S, Carrola J, Monteiro S. Gill histopathological alterations in Nile tilapia, *Oreochromis niloticus* exposed to treated sewage water. BABT. 2008; 51:1057-63. https://doi.org/10.1590/S1516-89132008000500023
34. Shah ZU, Parveen S. Oxidative, biochemical and histopathological alterations in fishes from pesticide-contaminated river Ganga, India. Sci Rep. 2022; 12(1):3628. https://doi.org/10.1038/s41598-022-07506-8
35. de Oliveira Ribeiro CA, Belger L, Pelletier E, Rouleau C. Histopathological evidence of inorganic mercury and methyl mercury toxicity in the arctic charr (*Salvelinus alpinus*). Environ Res. 2002; 90(3):217-25. https://doi.org/10.1016/S0013-9351(02)00025-7
36. Maurya PK, Malik DS. Bioaccumulation of heavy metals in tissues of selected fish species from Ganga river, India, and risk assessment for human health. HERA. 2018. https://doi.org/10.1080/10807039.2018.1456897
37. Jin Y, Zhu Z, Wang Y, Yang E, Feng X, Fu Z. The fungicide imazalil induces developmental abnormalities and alters locomotor activity during early developmental stages in zebrafish. Chemosphere. 2016; 153:455-61. https://doi.org/10.1016/j.chemosphere.2016.03.085
38. Pereira VM, Bortolotto JW, Kist LW, de Azevedo MB, Fritsch RS, da Luz Oliveira R, Pereira TC, Bonan CD, Vianna MR, Bogo MR. Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (*Danio rerio*). Neurotoxicology. 2012; 33(3):469-75. https://doi.org/10.1016/j.neuro.2012.03.005
39. Schmidel AJ, Assmann KL, Werlang CC, Bertoncello KT, Francescon F, Rambo CL, Beltrame GM, Calegari D, Batista CB, Blaser RE, Júnior WA. Subchronic atrazine exposure changes the defensive behavior profile and disrupts the brain acetylcholinesterase activity of zebrafish. NT and T. 2014; 44:62-9. https://doi.org/10.1016/j.ntt.2014.05.006

40. Senger MR, Rosemberg DB, Rico EP, de Bem Arizi M, Dias RD, Bogo MR, Bonan CD. *In vitro* effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain. Toxicol *In Vitro*. 2006; 20(6):954-8. https://doi.org/10.1016/j.tiv.2005.12.002
41. Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol. 1997; 53(5):603-25. https://doi.org/10.1016/S0301-0082(97)00050-6
42. Geffard M, Vieillemaringe J, Heinrich-Rock AM, Duris P. Anti-acetylcholine antibodies and first immunocytochemical application in the insect brain. Neurosci Lett. 1985; 57(1):1-6. https://doi.org/10.1016/0304-3940(85)90031-X
43. Mesulam M, Guillozet A, Shaw P, Quinn B. Widely spread butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer's brain. Neurobiol Dis. 2002; 9(1):88-93. https://doi.org/10.1006/nbdi.2001.0462
44. Murphy PC, Sillito AM. Cholinergic enhancement of direction selectivity in the visual cortex of the cat. Neurosci. 1991; 40(1):13-20. https://doi.org/10.1016/0306-4522(91)90170-S
45. Sam C, Bordoni B. Physiol Acetylcholine; 2020. https://www.ncbi.nlm.nih.gov/books/NBK557825/#__NBK557825_ai__
46. Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol. 2013; 11(3):315-35. https://doi.org/10.2174/1570159X11311030006
47. Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T. Acetylcholinesterase as a biomarker in environmental and occupational medicine: New insights and future perspectives. Biomed Res Int. 2013; 1-8 https://doi.org/10.1155/2013/321213
48. Kumar R, Kumari R, Mishra BK. Behavioural and morphological changes induced in the freshwater fish, *Clarias batrachus* exposed to chlorpyrifos 50%+ cypermethrin 5% EC.
49. Dutta D, Ray A, Bhattacharya E, Ghosh B, Bahadur M. Neurotoxic Effects of Imidacloprid on *Pethia conchonius* (Rosy Barb), a Common Freshwater Fish of India. Toxicol Int. 2024; 31(1):43-54. https://doi.org/10.18311/ti/2024/v31i1/35473
50. Singh S, Bhutia D, Sarkar S, Rai BK, Pal J, Bhattacharjee S, Bahadur M. Analyses of pesticide residues in water, sediment and fish tissue from river Deomoni flowing through the tea gardens of Terai Region of West Bengal, India. Int J Fish Aquat. 2015; 3(2):17-23.
51. Das A. Comparative study of pollution status of two main rivers: Karola and Tista of Jalpaiguri, West Bengal. India J Chem Pharm Res. 2017; 9(7):76-81.
52. Gill TS, Tewari H, Pande J. Use of the fish enzyme system in monitoring water quality: Effects of mercury on tissue enzymes. CBPC. 1990; 97(2):287-92. https://doi.org/10.1016/0742-8413(90)90143-W
53. Varadi L, Hidas A, Varkonyi E, Horvath L. Interesting phenomena in the hybridization of carp (*Cyprinus carpio*) and rosy barb (*Barbus conchonius*). Aquac. 1995; 129(1-4):211-4. https://doi.org/10.1016/0044-8486(94)00251-I
54. Kirankumar S, Anathy V, Pandian TJ. Hormonal induction of supermale golden rosy barb and isolation of Y-chromosome specific markers. Gen Comp Endocrinol. 2003; 134(1):62-71. https://doi.org/10.1016/S0016-6480(03)00218-1
55. Bhattacharya H, Lun L, Gomez GDR Biochemical effects to the toxicity of CCl_4 on rosy barbs *(Puntius conchonius*). Our Nature. 2005; 3(1):20-25. https://doi.org/10.3126/on.v3i1.330
56. Organisation for Economic Co-operation and Development, OECD Test No. 203: fish, acute toxicity test. France: OECD Publishing; 2019
57. Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7(2):88-95. https://doi.org/10.1016/0006-2952(61)90145-9
58. Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. J Fish Dis. 1999; 22(1):25-34. https://doi.org/10.1046/j.1365-2761.1999.00134.x
59. Rodrigues ET, Lopes I, Pardal MÂ. Occurrence, fate, and effects of azoxystrobin in aquatic ecosystems: a review. Environ Int. 2013; 53:18-28. https://doi.org/10.1016/j.envint.2012.12.005
60. Ali D, Ibrahim KE, Hussain SA, Abdel-Daim MM. Role of ROS generation in acute genotoxicity of azoxystrobin fungicide on freshwater snail *Lymnaea luteola* L. ESPR. 2021; 28:5566-74. https://doi.org/10.1007/s11356-020-10895-w
61. Mu XY, Huang Y, Luo JB, Shen G, Li X, Lei Y, Li Y, Wang C. Evaluation of the acute and developmental toxicity of azoxystrobin on zebrafish via multiple life stage assays. Acta Sci Circum. 2017; 37(3):1122-32.
62. Jiang J, Shi Y, Yu R, Chen L, Zhao X. Biological response of zebrafish after short-term exposure to azoxystrobin. Chemosphere. 2018; 202:56-64. https://doi.org/10.1016/j.chemosphere.2018.03.055
63. Bony S, Gaillard I, Devaux A. Genotoxicity assessment of two vineyard pesticides in zebrafish. J Environ Anal Chem. 2010; 90(3-6):421-8. https://doi.org/10.1080/03067310903033659
64. Crupkin AC, Fulvi AB, Iturburu FG, Medici S, Mendieta J, Panzeri AM, Menone ML. Evaluation of hematological parameters, oxidative stress, and DNA damage in the cichlid *Australoheros facetus* exposed to the fungicide azoxystrobin. Ecotoxicol Environ Saf. 2021; 207:111286. https://doi.org/10.1016/j.ecoenv.2020.111286
65. Dutta HM, Munshi JS, Roy PK, Singh NK, Adhikari S, Killius J. Ultrastructural changes in the respiratory lamellae of the catfish, *Heteropneustes fossilis* after sublethal exposure

to malathion. Environ Pollut. 1996; 92(3):329-41. https://doi.org/10.1016/0269-7491(95)00101-8

66. Altinok I, Capkin E. Histopathology of rainbow trout exposed to sublethal concentrations of methiocarb or endosulfan. Toxicol Pathol. 2007; 35(3):405-10. https://doi.org/10.1080/01926230701230353
67. Mallatt J. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J Fish Aquat Sci. 1985; 42(4):630-48. https://doi.org/10.1139/f85-083
68. Qureshi IZ, Bibi A, Shahid S, Ghazanfar M. Exposure to sub-acute doses of fipronil and buprofezin in combination or alone induces biochemical, hematological, histopathological and genotoxic damage in common carp (*Cyprinus carpio* L.). Aquat Toxicol. 2016; 179:103-14. https://doi.org/10.1016/j.aquatox.2016.08.012
69. Mahboob S, Al-Ghanim KA, Al-Balawi HF, Al-Misned F, Ahmed Z. Toxicological effects of heavy metals on histological alterations in various organs in Nile tilapia (*Oreochromis niloticus*) from a freshwater reservoir. J King Saud Univ Sci. 2020; 32(1):970-3. https://doi.org/10.1016/j.jksus.2019.07.004
70. Kazmi SA, Iqbal R, Al-Doaiss AA, Ali M, Hussain R, Latif F, Raza GA. Azoxystrobin-induced oxidative stress in gills, hematological biomarkers and histopathological ailments in freshwater fish. Pak Vet J. 2023; 43(2). https://doi.org/10.29261/pakvetj/2023.025
71. Nataraj B, Hemalatha D, Malafaia G, Maharajan K, Ramesh M. "Fishcide" effect of the fungicide difenoconazole in freshwater fish (*Labeo rohita*): A multi-endpoint approach. Sci Total Environ. 2023; 857:159425. https://doi.org/10.1016/j.scitotenv.2022.159425
72. Stoyanova S, Yancheva VS, Velcheva I, Uchikova E, Georgieva E. Histological alterations in common carp *(Cyprinus carpio* Linnaeus, 1758) gills as potential biomarkers for fungicide contamination. BABT. 2015; 58:757-64. https://doi.org/10.1590/S1516-89132015050151
73. Boran H, Capkin E, Altinok I, Terzi E. Assessment of acute toxicity and histopathology of the fungicide captan in rainbow trout. Exp Toxicol Pathol. 2012; 64(3):175-9. https://doi.org/10.1016/j.etp.2010.08.003
74. Raibeemol KP, Chitra KC. Histopathological alteration in the gill of the freshwater fish *Pseudetroplus maculatus* (Bloch, 1795) under chlorpyrifos toxicity. Int J Bio Biomed Res. 2016; 3:141-6. https://doi.org/10.22192/ijarbs.2016.03.12.018
75. Chamarthi RR, Bangeppagari M, Gooty JM, Mandala S, Tirado JO, Marigoudar SR. Histopathological alterations in the gill, liver, and brain of *Cyprinus carpio* on exposure to quinalphos. Am J Life Sci. 2014; 2(4):211-6. https://doi.org/10.11648/j.ajls.20140204.14
76. Velmurugan B, Selvanayagam M, Cengiz EI, Unlu E. Histopathological changes in the gill and liver tissues of freshwater fish, *Cirrhinus mrigala* exposed to dichlorvos. BABT. 2009; 52:1291-6. https://doi.org/10.1590/S1516-89132009000500029
77. Halappa R, David M. Behavioral responses of the freshwater fish, *Cyprinus carpio* (Linnaeus) following sublethal exposure to chlorpyrifos. TrJFAS. 2009; 9(2).
78. Kavitha P, Rao JV. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, *Gambusia affinis*. Environ Toxicol Pharmacol. 2008; 26(2):192-8. https://doi.org/10.1016/j.etap.2008.03.010
79. Padmanabha H, Correa F, Rubio C, Baeza A, Osorio S, Mendez J, Jones JH, Diuk-Wasser MA. Human social behavior and demography drive patterns of fine-scale dengue transmission in endemic areas of colombia. PloS one. 2015; 10(12):e0144451. https://doi.org/10.1371/journal.pone.0144451
80. Ullah R, Zuberi A, Ullah S, Ullah I, Dawar FU. Cypermethrin induced behavioral and biochemical changes in mahseer, *Tor putitora*. J Toxicol Sci. 2014; 39(6):829-36. https://doi.org/10.2131/jts.39.829
81. Patil VK, David M. Behaviour and respiratory dysfunction as an index of malathion toxicity in the freshwater fish, *Labeo rohita* (Hamilton). TrJFAS. 2008; 8(2). https://doi.org/10.1515/JBCPP.2008.19.2.167
82. de Oliveira MR, Profeta IV, Saraiva Raimondi Lopes JV, Costa RM, Matos e Chaib VR, Domingues AG, Beirão MV, Santos Rubio KT, Martucci ME, Eskinazi-Sant'Anna EM, de Azevedo CS. Effects of the fungicide carbendazim on the behavior of the zebrafish *Danio rerio* (Cypriniformes, Cyprinidae). Acta Ethol. 2024; 1-1. https://doi.org/10.1007/s10211-024-00438-8
83. Altenhofen S, Nabinger DD, Wiprich MT, Pereira TC, Bogo MR, Bonan CD. Tebuconazole alters morphological, behavioral, and neurochemical parameters in larvae and adult zebrafish (*Danio rerio*). Chemosphere. 2017; 180:483-90. https://doi.org/10.1016/j.chemosphere.2017.04.029
84. Srivastava P, Singh A. Evidence of micronuclei in fish blood as a biomarker of genotoxicity due to surface runoff agricultural fungicide (Propiconazole). JTEHS. 2015; 7(1):4-8. https://doi.org/10.5897/JTEHS2015.0325
85. Fernandez HL, Hodges-Savola CA. Trophic regulation of acetylcholinesterase isoenzymes in adult mammalian skeletal muscles. Neurochem Res. 1992; 17:115-24. https://doi.org/10.1007/BF00966872