

Impact of Kinalax 25 EC on Vital Organ Histomorphology, Blood Cell Structure and Brain Acetylcholinesterase Activity in Silver Barb (*Barbonymus gonionotus*)

Rabeya Akter¹, Arzu Parvin¹, Halima Jahan¹, Zakir Hossain^{1,*} 

¹Department of Fisheries Biology and Genetics, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

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Corresponding Author

Tel.: +8809169116
E-mail: zakir.fbg@bau.edu.bd

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Abstract

The objective of this research was to evaluate the effects of Kinalax 25 EC, an organophosphate pesticide on silver barb (*Barbonymus gonionotus*). Histological bio-assay of gill, liver and kidney of *B. gonionotus*, RBC count and acetylcholinesterase (AChE) activity were performed to evaluate the effects of the Kinalax 25 EC at cellular and enzymatic levels. The LC₅₀ value of Kinalax 25 EC was estimated as 0.071 ppm for *B. gonionotus*. The remarkable changes included missing of gill lamellae, gill clubbing, hyperplasia, nuclear hypertrophy, vacuolation, glomerular expansion, increasing the diameter of renal tubules, hemorrhage, necrosis and pyknosis. Several changes in peripheral nuclear erythrocyte included large lymphocyte, dead cell, fusion of cell, binucleated cell, tear-shaped cell, ghost cell, senile cell and abnormal shape of cell were found. The RBC count was significantly higher ($P>0.01$) in lower doses compared to higher doses of pesticide in *B. gonionotus*. The level of AChE activities in *B. gonionotus* brain significantly decreased ($P>0.01$) in pesticides treated fish compared to control. The result of this study reveals that the synthetic organophosphorus pesticide adversely affects the organ-specific histology, haematology and brain acetylcholinesterase activities of *B. gonionotus*.

Introduction

Environmental pollution is a much talked issue due to its significant impact on aquatic flora and fauna. The used pesticides in crop fields are harmed to fish and other aquatic biota (Helfrich, Weigmann, Hipkins, & Stinson, 1996). Aquatic life can highly be lethal due to pesticides surface runoff into waterbody (Karaca, Varisli, Korkmaz, Özeydin, Perçin, & Orhan, 2014; Toughill, 1999). Physiological and behavioral changes of fish may cause by the pesticides (Helfrich *et al.*, 1996). Zooplankton and insects are the important source of food for young fish that may also kill off by the pesticides

(Hossain, Rahman, & Mollah, 2001). The chlorpyrifos is an organophosphate pesticide which can severely damage the fish organ with very low concentrations (Grue, Gibert, & Seeley, 1997; Cagdas, Kocagoz, Onat, Percin, Ozaydin, & Orhan, 2017).

Changes in histology of different tissues are widely used as biomarkers in examination of the health of fish exposed to pesticides content water (Thophon, Kruatrachue, Upathan, Pokethitiyook, Sahaphong, & Jarikhuan, 2003). It can be used in environmental monitoring and allowing examination of target organs, such as gills, kidneys and livers (Gernhofer, Pawet, Schramm, Müller, & Triebkorn, 2001). Blood

biochemistry profiles that are also adversely affected by pesticides can be used to assess the stress status of fish. The characteristic of blood biochemical parameter is a well-accepted index to determine the physiological and pathological changes in fish (Kori-Siakpere, Ake, & Idoge, 2005; Perçin, & Konyaloğlu, 2008).

The toxicity of organophosphate is related to acetylcholinesterase (AChE) inhibition (Ambali, Akanbi, Igbokwe, Shittu, Kawu, & Ayo, 2007). The AChE is secreted by the brain and other nerve cells. The inhibition of AChE is resulted in deposition of acetylcholine in the central and peripheral synapses, caused changed physiological and neurological processes (Sandahl, Baldwin, Jenkins, & Scholz, 2005). Such physiological modifications can lead to changes in swimming performance, social behaviour, foraging and predation risk. A very few reports are available on the mechanism of fish death due to the effects of chlorpyrifos pesticides. The current study was designed to examine the toxicity caused by chlorpyrifos using the fingerling of silver barb (*Barbonymus gonionotus*) and stinging catfish (*Heteropnesutes fossilis*) as a model system for evaluating LC₅₀ value, and histomorphology, haematology and brain AChE activity.

Materials and Methods

Selection of Pesticides and Test Fish

The organophosphate pesticide i.e. Kinalax 25 EC was selected because it is widely used in agricultural crop management in Bangladesh. Kinalax 25 EC was collected from authorized dealer at Mymensingh, Bangladesh. Silver barb (*B. gonionotus*) was selected because they are important freshwater fish species in Bangladesh.

Determination of Water Quality Parameters

The water quality parameters of aquariums were measured daily. Temperature, pH and dissolved oxygen (DO) were measured using mercury centigrade thermometer, pH meter (Model: pH ep Tester, Romania) and dissolved oxygen meter (Model: HI 9146-DO meter, Romania), respectively.

Experimental Design

This study was conducted in twenty-one aquarium placed in the wet laboratory of the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh. Properly cleaned glass aquaria were contained 25 L of tap water and 10 acclimated *B. gonionotus* having average length and weight of 5.28 ± 1.11 cm and 7.70 ± 0.63 g, respectively were released to each aquarium. The length and weight of the fry were recorded using measuring scale and electric balance (AND GULF, UAE), respectively. Six

different concentrations of pesticides (0.025, 0.05, 0.075, 0.10, 0.125 and 0.150 ppm) were measured by a micropipette, added to water in a glass jar (each with three replications) which gently mixed with water homogenously.

Determination of LC₅₀ Value

The agricultural recommended dose was calculated as 0.05 ppm for Kinalax 25 EC considering a model water depth of 20 cm in rice field. A control was also maintained to compare with pesticide treated fish. Dead or stressed fishes were discarded during the period of acclimatization. All tests were conducted under laboratory conditions at room temperature. The external behaviour changes in fishes were observed and the dead fishes were counted and removed as soon as they stopped their opercular movement. The mortality of fish was recorded at 24, 48, 72, and 96 h of exposure time. The LC₅₀ values for *B. gonionotus* were calculated for 96 h of exposure period by probit analysis of the SPSS.

Organ-Specific Histology of Pesticide Treated Fish

B. gonionotus was exposed at different concentrations of Kinalax 25 EC in glass aquarium for 7 days to observe the histopathological effects. Pesticide was not added in the aquarium of control group. Pesticide exposed fish was dissected to collect the gill, liver and kidney and preserved in 10% neutral buffered formalin. The method of Reza, Rakhi, Hossen, & Hossain, (2017) was followed for histomorphic changes of fish organs.

Morphological Alterations of Blood Cells

Blood smears were prepared on glass slides from fresh blood for the study of morphological alterations of erythrocytes. The blood smeared slides were dried in air, fixed in methanol and Wright's Giemsa was used to stain it. Blood corpuscles were examined by immersion oil microscopy and computer attached microscope under 400× lens (OLYMPUS-CX41) was used for photography.

Estimation of Red Blood Cells

After acclimatization fishes were divided in two groups, 6 fish in each group. Group-1 was served as control where no pesticide was added. Fishes in group-2 were exposed to sublethal concentration of Kinalax 25 EC for 7 days. Blood was collected from the caudal peduncle then taken it into RBC pipette up to 0.5 ppm for counting the red blood cell. Then the pipette was placed on the hayem's solution and the solution was drawn upto 101 marks. The content of the pipette was mixed thoroughly by '8' knot motion. After mixing at least two drop of fluid was expelled and next drop was

placed at the side of the chamber and covered with a cover slip. After 2 min, the cells were counted from the four chambers and one central square, each of which containing 16 small squares. Counting was done using high power objective lens (40×).

The following formula was used to calculate the number of RBC/mm²: No. of cells × dilution factor × depth factor

Total RBC = Number in cubic mm / total No. of small squares

AChE Activity Measurement of Pesticide Treated Fish

H. fossilis was treated with sub-lethal concentration of pesticide for 7 days to analyze the AChE activity and a control was maintained. The whole brain was dissected out after exposure of 10 days and placed in sodium phosphate buffer (pH 8.0) and then weighed and homogenized using homogenizer. The final concentration was 20 mg tissue/ml phosphate buffer. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C and supernatant was collected. An aliquot of supernatant was then collected to measure protein concentration (Lowry, Rosebrough, Farr, & Randall, 1951). A standard curve was used to determine the protein concentration of samples. The AChE activity was determined following the method of Ellman, Courtney, Andres, & Featherstone (1961). Briefly, the tissue homogenate (50 µl) was added to 900 µl of cold sodium phosphate buffer (0.1 M containing 0.1% Triton X-100, pH 8.0) and 50 µl of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB; 6 mM), then vortexed and kept for 10 min at room temperature. Aliquot of 200 µl in triplicate was then placed into 96-well plate and 50 µl of acetylthiocholine chloride (15 mM) was added to start the reaction. The absorbance was measured with a microplate reader (Model: SPECTRA max 340PC384) at 412 nm.

The AChE activity rate was calculated with following formula:

$$R = 5.74 (10^{-4}) \Delta A / C_0$$

Where, R = rate in moles substrate hydrolyzed per min per g of tissue;

ΔA = change in absorbance per min;

C₀ = original concentration of tissue.

AChE activity was calculated as nmol/min/mg protein.

Statistical Analysis

The statistical analysis was performed considering at P<0.01 significance by using SPSS ver. 17.0 computer software program. Duncan multiple range test was performed to determine the significant difference of different groups.

Results

Physico-Chemical Parameters

Temperature, DO and pH were recorded regularly during the experimental period. The average temperature, DO, and pH were recorded as 27.0±3.0°C, 7.5±1.0 ppm and, 9.25±2.1, respectively. Kinalax 25 EC was dissolved in water and it did not have any effects on water quality parameters.

LC₅₀ Value of Kinalax 25 EC and Morphometric and Behaviour Changes of *B. gonionotus*

The LC₅₀ value of Kinalax 25 EC was found to be 0.071 ppm for *B. gonionotus*. Some morphometric changes were observed after exposure to 96 h of *B. gonionotus*. Their vertebral column on the caudal region was bent and showed abnormal swimming (data not shown). The behaviors of tested fish were observed during the experimental period. Several abnormal behaviors such as restlessness, arena movements, loss of balance, hyper operculum activities, strong spasm, and paralysis were observed due to the effects of pesticides. Lastly, the fish was settled down to the bottom of the aquarium. After settling down, slow operculum movement was observed and then gradual fish death was observed.

Histopathological Observation of Fish Exposed to Pesticides

Normal structure of gill arch, primary and secondary gill lamellae with no pathology were found. At the dose of 0.025 ppm Kinalax 25 EC blood congestion, clubbing, hyperplasia, missing of secondary lamellae, vacuolation, hemorrhage, pyknosis were found (Figure 1). Normal and systematic arrangement of hepatocytes and no structural alteration was assessed in the control group. Mild alterations including nuclear hypertrophy, cytoplasmic vacuolation, vacuole, pyknotic area, hemorrhage, necrotic area were found at the dose of 0.025 ppm Kinalax 25 EC (Figure 2). In the control group normal regular and systematic arrangement of kidney tubules and hematopoietic cells were found. At the dose of .025 ppm Kinalax 25 EC glomerular expansion, dilation of Bowman's space, cellular degeneration, increasing the diameter of renal tubule, pyknotic area, melanomacrophage were found (Figure 3).

Morphological Alterations of Erythrocytes of Blood Smears Exposed to Kinalax 25 EC

Uniform blood smears from normal healthy unpolluted fish samples revealed that each erythrocyte is an oval shaped cell with a concentric nucleus with the outer edge of the cell. At the dose of 0.025 ppm Kinalax 25 EC binucleated cell, tear-shaped cell, senile cell,

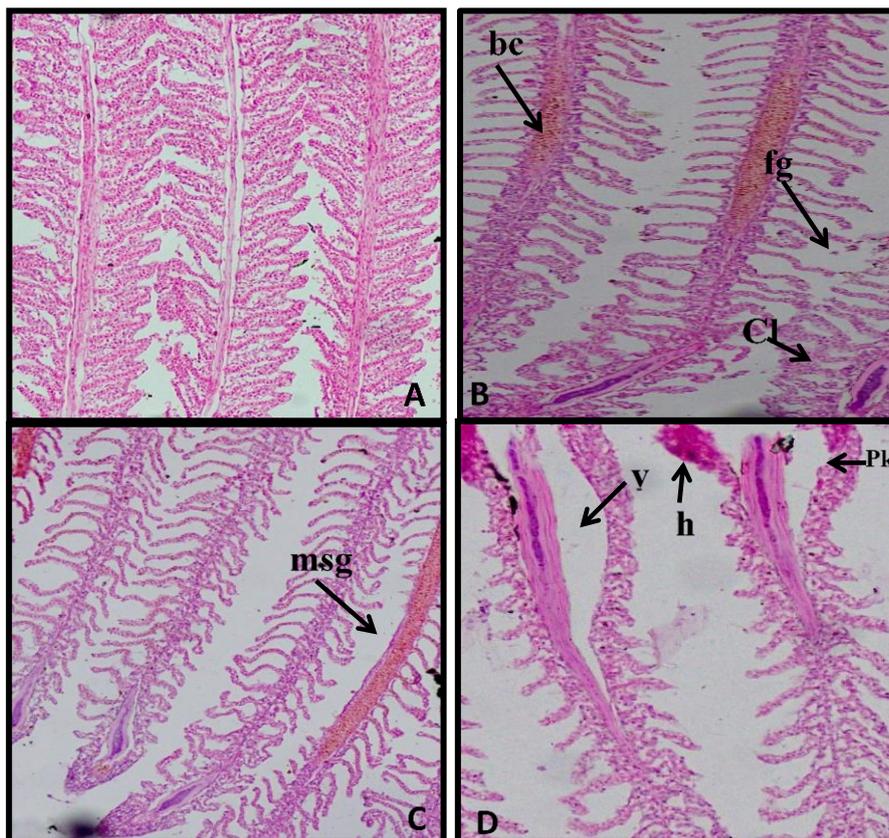


Figure 1. Photograph of gill of *B. gonionotus* after 7 days exposure to 0.025 ppm Kinalax 25 EC. (A)- control – normal epithelial cell and secondary lamellae were found; (B)- bc-blood congestion, cl-clubbing, fg-fungal granuloma; (C)- msg-missing of secondary lamellae; (D)-v-vacuolation, h-hemorrhage, pk-pyknosis were observed.

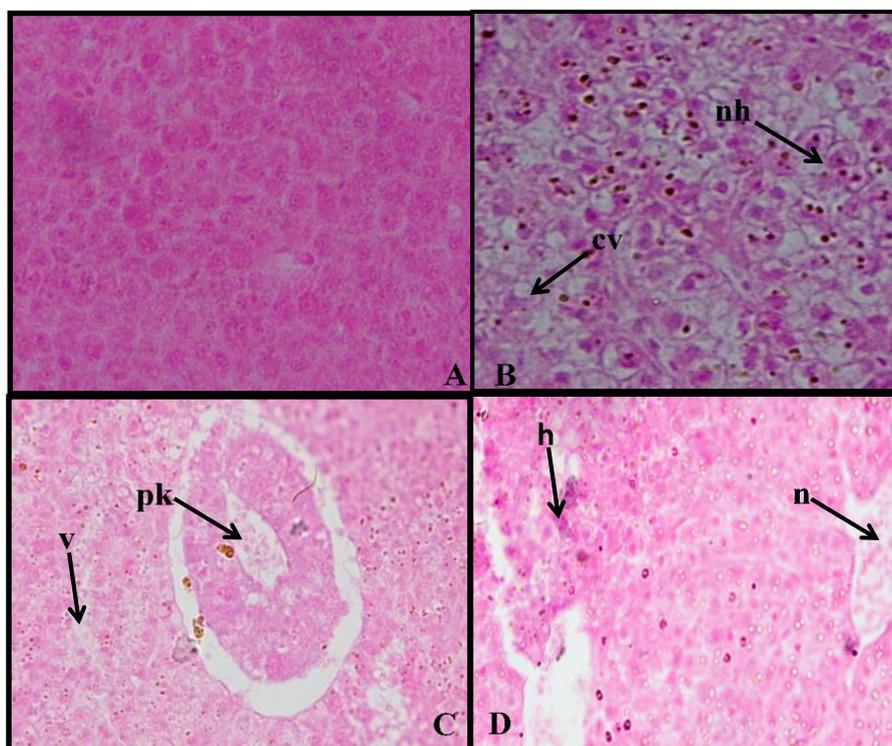


Figure 2. Photograph of liver of *B. gonionotus* after 7 days exposure to 0.025 ppm Kinalax 25 EC. (A)-control – normal regular and systematic arrangement of hepatocytes were found; (B)-nh-nuclear hypertrophy, cv-cytoplasmic vacuolation; (C)-v-vacuole, pk-pyknotic area; (D)- h-hemorrhage, n-necrotic area were observed.

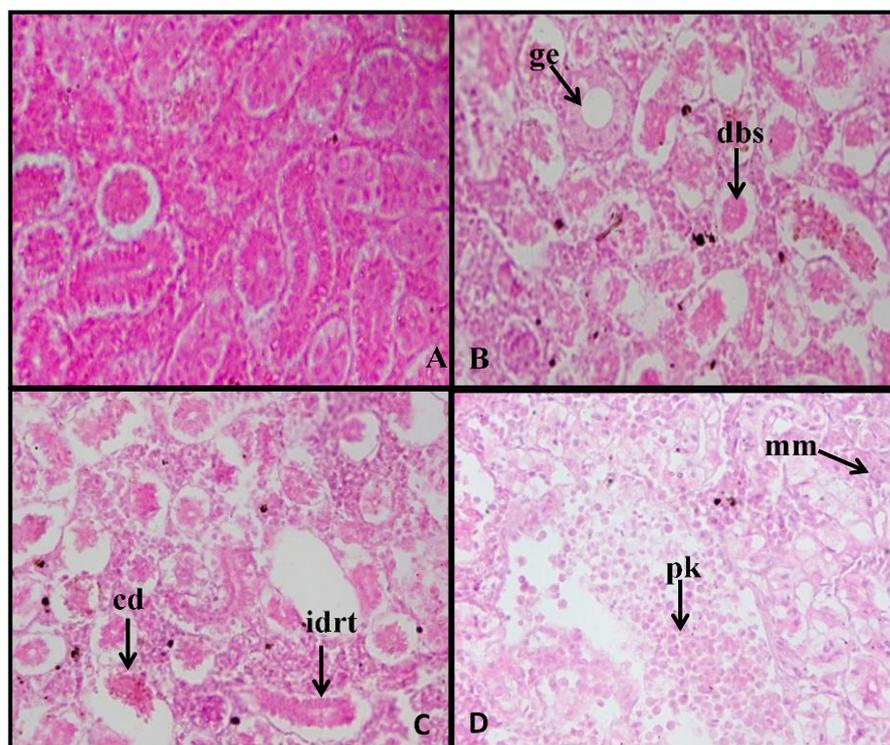


Figure 3. Photograph of kidney of *B. gonionotus* after 7 days exposure to 0.025 ppm Kinalax 25 EC. (A)-control – normal regular and systematic arrangement of kidney tubules and hematopoietic cells were found; (B)- ge-glomerular expansion, dbs-dilation of Bowman's space; (C)-cd-cellular degeneration, idrt-increasing the diameter of renal tubule; (D)- pk-pyknotic area, mm-melanomacrophage were observed.

absence of nucleus, abnormal shape of cell, ghost cell and large lymphocyte were found (Figure 4).

Determination of Red Blood Cell Count of Fish Exposed to Kinalax 25 EC

After 10 days exposure, red blood cell was counted of two sublethal concentrations of Kinalax 25 EC. The mean of red blood cell count was significantly ($P < 0.01$) lower when fish was treated with Kinalax 25 EC (Table 1).

AChE Activity of Fish Brain Exposed to Kinalax 25 EC

The AChE activity in the brain of *H. fossilis* exposed to Kinalax at the dose of 0.0002 ppm was calculated as 52.8 ± 6.6 nmol/min/mg protein. Significant decrease of enzymatic activity was found in ($P < 0.01$) of that enzyme at agricultural recommended dose (Figure 5).

Discussion

LC₅₀ Values of Kinalax 25 EC for *B. gonionotus*

The lethal effects of pesticides on test animals can be expressed as LC₅₀ value. In the present study, the LC₅₀ value of Kinalax 25 EC was 0.071 ppm for *B. gonionotus*. Deka and Mahanta (2012) found that the LC₅₀ value of

Malathion was 0.98 ppm for *Heteropneustes fossilis* at 96 h exposure where as Hossain, Haldar, and Mollah (2000) estimated the LC₅₀ value of Diazinon as 2.97 ppm for *L. rohita* at 96 h exposure. Hossain *et al.* (2001) found the LC₅₀ values were 0.3530 and 1.2809 ppm for Diazinon 60 EC and Dimecron 100 SCW, respectively at 48 h exposure on a zooplankton, *Diaptomus*. Sharbidre, Metkari, and Patode (2011) recorded the LC₅₀ values of methyl parathion and chlorpyrifos to guppy fish, *Poecilia reticulata* were 8.48 ppm and 0.176 ppm, respectively. In addition, the LC₅₀ values were 6.75, 22.95, and 375.26 ppm for *Anabas testudineus*, *Channa punctatus* and *Barbodes gonionotus*, respectively on Dimecron 100 SCW at 96 h (Hossain, Rahman, and Mollah, 2002). Furthermore, the LC₅₀ value of Darsban 20 EC was 0.005 ppm for *Clarias gariepinus* (Lovely, Rahman, Hossain, and Mollah, 2003). These indicated that the LC₅₀ value is species specific and different pesticides have different LC₅₀ value.

Histopathological Observation in Pesticide Treated Fish

Histopathology is an important biomarker to assess the fish health in stressed environment. Histology can identify the early signs of disease and injury in cells, tissues, or organs. Marchand, Van Dyk, Pieterse, Barnhoorn, and Bornman (2009) reported the

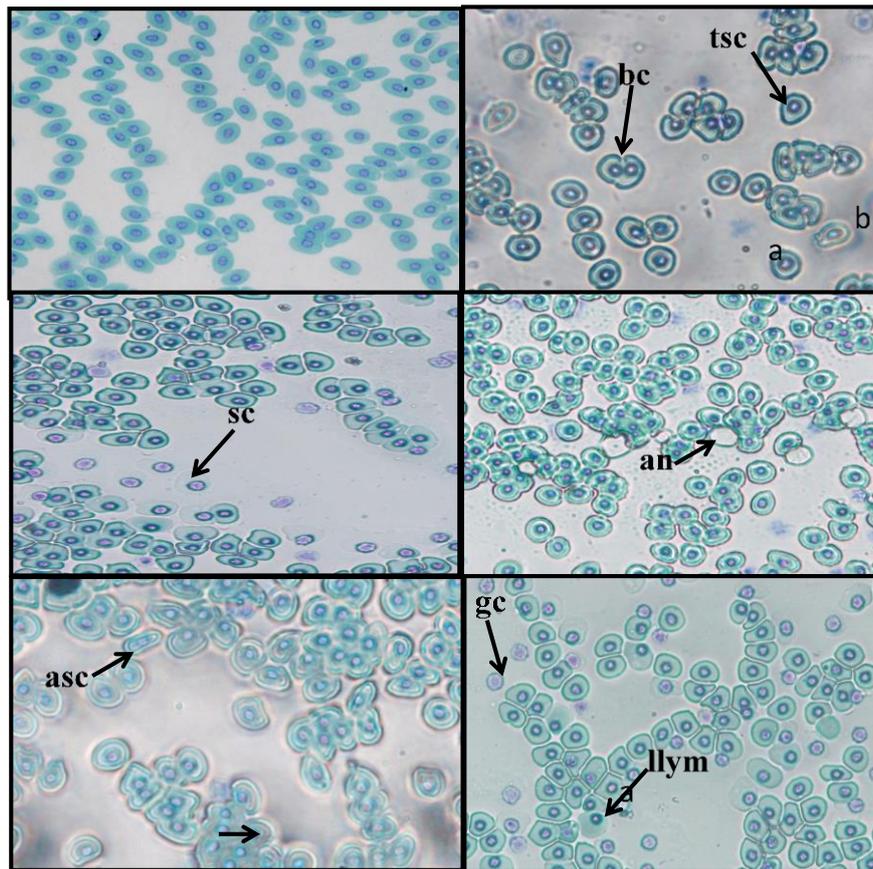


Figure 4. Photomicrograph of blood smears of *B. gonionotus* after 7 days exposure to 0.025 ppm Kinalax 25 EC. **(A)**- control – normal regular and systematic arrangement of nucleus of erythrocytes were found; **(B)**- bc-binucleated cell, tsc-tear-shaped cell; **(C)**- sc-senile cell; **(D)**- an-absence of nucleus; **(E)**- asc-abnormal shape of cell ; **(F)** gc-ghost cell, llym-large lymphocyte were observed.

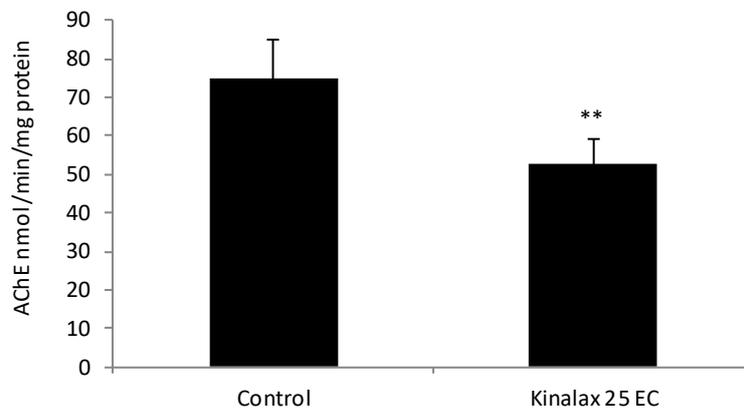


Figure 5. AChE activity (nmol/min/mg protein) measured in *H. fossilis* exposed to Kinalax 25 EC for 7 days. Data presented as mean \pm SD. ** P<0.01 vs. Control.

Table 1. Mean RBC count of *H. fossilis* at two different concentrations of Kinalax 25 EC

Treatment	Concentration (ppm)	Cell count (Million RBC)
Control	-	7.82 \pm 0.58
Kinalax 25 EC	0.002	5.10 \pm 0.45**
	0.004	2.91 \pm 0.37**

**P<0.01 vs control

alterations in various tissues of fish organ into the polluted ecosystem.

Mild to severe alteration in gills were recorded in *B. gonionotus* treated with Kinalax 25 EC pesticide. In this case, the observed pathological symptoms were prominent at higher dose of pesticides. The result is similar to Zodrow, Stegemanb, and Tanguay (2004) who recorded hypertrophy and fusion in gill lamellae of zebrafish. Additionally, Çağlan, Benli, and Ozkul (2010) found telangiectasis at the tip of secondary gill lamellae of Nile tilapia exposed with an organophosphate pesticide. Moreover, Reza *et al.*, (2017) was also found moderate gill clubbing, hemorrhage, pyknosis in gill at 0.058 ppm of an organophosphate pesticide in *Labeo rohita* whereas the gills were found in almost normal condition in *B. gonionotus* except some missing of secondary gill lamellae.

In the present study, the tissues of liver showed ultrastructural damage in comparison with control including glomerular expansion dilation of Bowman's space, cellular degeneration, swelling the diameter of renal tubule, pyknotic area and melanomacrophage for *B. gonionotus*. Similar results were found by Hossain *et al.* (2002) and Rahman, Hossain, Mollah, and Ahmed (2002) from the liver of three fish species exposed to organophosphate pesticide. Zodrow *et al.* (2004) found the lipidosis and hepatocyte hypertrophy. Necrotic foci and lipid droplets in liver of *Cyprinus carpio* and interactions of antioxidant enzyme activity and lipid peroxidation effect on pesticides were recorded by Oropesa, Cambero, Gómez, Roncero and Soler (2009); Karaca *et al.*, (2014) and Cagdas *et al.*, (2017). Reza *et al.* (2017) additionally found mild changes in vacuole, hemorrhage, and fatty degeneration in the liver tissues of *L. rohita* treated with 0.058 ppm Envoy 50 SC, whereas moderate hemorrhage, fatty degeneration, lipid droplets were observed for the same fish species at 0.108 ppm.

In the current study, the kidney tissues exhibited slight to severe damages compared to control. The result partially agrees with Hossain *et al.* (2002) and Rahman *et al.* (2002) as they found comparatively more pathologies in *B. gonionotus*. Yang, Zha, Li, Li and Wang (2010) found lesions in kidney tissues including an extensive expansion in the lumen, degenerative and necrotic changes of tubular epithelia, and shrinkage of the glomeruli at 10 µg^{-L} of atrazine exposed to rare minnow (*Gobiocypris rarus*) for 28 days. Reza *et al.* (2017) found mild vacuole, degenerating kidney tubule and hemorrhage at 0.058 ppm in *Labeo rohita* and at the same dose, the kidney tissues of *B. gonionotus* appeared normal but some melanin pigment and vacuoles were also observed in these tissue samples. Pathology included moderate hemorrhage, pyknosis, hyaline for *L. rohita* were found in the kidney of fishes treated at 0.108 ppm Envoy 50 SC, and moderate vacuole, pyknosis and necrosis for *B. gonionotus* were found. However, 2,3,7,8-Tetrachlorodibenzo-p-dioxin exposed zebrafish

did not show any changes in kidney tissue (Zodrow *et al.*, 2004). More structural damages in *L. rohita* compared to *B. gonionotus* showed that *L. rohita* was much more vulnerable to pesticides exposure. Sogut and Percin (2011) studied the toxicant effects on the target organ kidney of Bluefin Tuna.

Haematological Alteration of Pesticide Treated Fish

Blood parameters such as haematological and biochemical indices can be used as important markers of identifying the structural and functional status of fish exposed to pesticides (Adhikari, Sarkar, Chatterjee, Mahapatra, and Ayyappan, 2004; Evans and Claiborne, 2005). Satyanarayan, Bejankiwar, Chaudhari, Kotangale and Satyanarayan (2004) studied the blood characteristics and histological changes in erythrocytes of the fish species *Cyprinus carpio* and *Puntius ticto* due to pesticides exposure. In the present study, several alterations in peripheral erythrocyte i.e. binucleated cell, tear-shaped cell, senile cell, absence of nucleus, abnormal shape of cell, ghost cell and large lymphocyte were found due to sublethal exposure of Kinalax 25 EC. Saravanan, Kumar, and Ramesh (2011) also found that the RBC count was decreased in the pesticide treated fish. In this study, sublethal exposure to Kinalax 25 EC resulted in significant lower RBC count in the higher dose than the lower.

AChE Activity in Pesticide Treated Fish

The AChE activity is sensitive for organophosphates and carbamate pesticides than other contaminants, but the inhibition of this enzyme has also been used to indicate the stress of fish to contaminants. In the present study, a significant ($P < 0.01$) AChE inhibition was found in pesticides exposed fish. Inhibition of brain and serum AChE by DDVP (an organophosphate pesticide) in 11 freshwater teleost species was found by Chuiko (2000). Similar inhibition of AChE activities with organophosphates has also been reported (Valbonesi, Brunelli, Mattioli, Rossi, and Fabbri, 2011; Rodrigues, Caldeira, Castro, Gonçalves, Nunes, and Antunes, 2011 and Colovic, Krstic, Uscumlic, and Vasic, 2011). Moreover, pesticide mediated enzymatic inhibition affects in *O. niloticus* was showed by Pessoa, Luchmannb, Ribera, Verasa, Correac, Nogueirab, and Carvalho, (2011). Reza *et al.* (2017) showed significant inhibition of AChE activity on *L. rohita* when exposed to organophosphate pesticide.

Conclusion

The results showed that the presence of Kinalax 25 EC in freshwater ecosystem could cause deleterious effects on fish inhibiting normal physiological functions.

Conflict of Interest

The authors declare that there is no conflict of interest of academic or financial nature with any individual or organization.

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