

Toxicity testing, hematological alterations, and acetylcholinesterase expression on *Barbonymus gonionotus* (Bleeker, 1849) exposed to glyphosate-based herbicide

¹Chutima Thanomsit, ²Sugunya Kumla, ²Patcharee Mongkolvai, ²Boonthiwa Chartchumni, ³Amnuay Wattanakornsiri, ⁴Khobkul Nongnutch, ⁵Jakkaphun Nanuam, ⁶Witchuda Prasatkaew, ⁵Phochit Nanthanawat

¹ Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, 32000 Surin, Thailand; ² Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, 47160 Sakon Nakhon, Thailand; ³ Department of Agriculture and Environment, Faculty of Science and Technology, Surindra Rajabhat University, 32000 Surin, Thailand; ⁴ Faculty of Industry and Technology, Rajamangala University of Technology Isan, 47160 Sakon Nakhon, Thailand; ⁵ Office of Educational Affairs, Faculty of Science, Burapha University, 20131 Chonburi, Thailand; ⁶ Department of Environmental Management, Faculty of Science and Technology, Dhonburi Rajabhat University, 10540 Samutprakan, Thailand.
Corresponding author: P. Nanthanawat, phochit@go.buu.ac.th

Abstract. This study investigated the toxicity of 2,4-D dimethylammonium (2,4-D DMA), a herbicide commonly used in developing countries like Thailand, and its potential risks to aquatic organisms and human health. Specifically, the research focused on assessing the toxic effects of the herbicide on silver barb, *Barbonymus gonionotus* (Bleeker, 1849), examining biochemical markers, antioxidant responses, hematological changes, and histological alterations. Silver barbs were exposed to varying concentrations of the herbicide, and the cumulative mortality was monitored over different exposure times (24, 48, 72, and 96 hours). Toxicity was assessed by calculating LC50 values, and the expression of acetylcholinesterase (AChE) and antioxidant enzyme activities, including catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST), as well as hematological profiles, were evaluated. Histopathological changes in the gills, liver, intestines, and stomach were also examined. The mortality rate was both concentration- and time-dependent, with LC50 values decreasing over time: 140.76 mg L⁻¹ at 24 hours, 112.72 mg L⁻¹ at 48 hours, 81.87 mg L⁻¹ at 72 hours, and 70.653 mg L⁻¹ at 96 hours. Exposure to the herbicide increased the activities of CAT, SOD, and GST, with a tendency for these levels to rise over time. Hematological analysis showed significant alterations in blood indices, and AChE expression varied with exposure duration, with a molecular weight of 71 kDa observed. Histologically, gills exhibited hyperplasia, lamellar fusion, epithelial lifting, edema, and blood congestion. The liver showed vacuolation, while the intestines and stomach displayed lesions and epithelial damage, including erosion and fusion of mucous-secreting cells. These findings highlighted the significant toxic effects of the herbicide on silver barb, both at the biochemical and cellular levels. The study underscores the potential environmental and health risks associated with this herbicide, providing important insights for future monitoring and regulation of its use in aquatic ecosystems.

Key Words: acetylcholinesterase, 2,4-D dimethylammonium, histology, herbicide, silver barb.

Introduction. Herbicides are agrochemicals regularly used to control weeds in crops and aquaculture ponds (Tudi et al 2021). They account for approximately 50% of all agrochemicals used globally (Dey & Saha 2014). Even at low concentrations, herbicides can induce behavioral changes in fish species. Their residues have been found to accumulate in fish. As a result, they must adapt to both external and internal stimuli to cope with the challenges of surviving in an ever-changing environment and various environmental factors (Ramesh & Munshawi 2009; Hamisu et al 2024).

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide in developing countries, valued for its versatility, cost-effectiveness, and regulatory approval (Zuanazzi 2020; Casimero et al 2022). First introduced in 1946, 2,4-D has quickly gained global popularity for controlling weeds in crops such as wheat, rice, maize, and aquatic plants. However, its excessive use presents environmental risks and can harm non-target species, including those within the food chain (Kumla et al 2025).

Several studies have investigated the toxicity of 2,4-D on aquatic organisms, revealing its adverse effects on fish, crustaceans, and other aquatic species. For example, Khanchanasal et al (2022) examined the impact of 2,4-D on Nile tilapia, noting increased cumulative mortality and significant changes in biomarker expression, such as AChE. Additionally, Thanomsit et al (2024) explored the effects of 2,4-D on aquatic invertebrates, including prawns and golden apple snails, resulting in both physiological and histological alterations. These studies highlight existing research on the toxicity of 2,4-D in Thai aquatic species, but further investigation into the long-term ecological consequences is still needed (Kumla et al 2025).

Silver barb, *Barbonymus gonionotus* (Bleeker, 1849) is a native freshwater species that holds significant commercial value in Thailand (Sribanjam et al 2018). To our best knowledge, studies on the effects of 2,4-D dimethylammonium (2,4-D DMA) herbicide on silver barb, both in laboratory and field conditions, remain limited and unanswered. In this study, we investigated the toxicity of the herbicide 2,4-D DMA in silver barb with examination of its toxic effects, AChE expression, antioxidant changes, hematological profiles, and histological alterations. This research would help to contribute to monitoring the contamination of the herbicide in silver barbs and other fish species.

Material and Method

Chemical. The herbicide used in this study was 2,4-D DMA acetate 84% W/V soluble liquid (SL) in commercial form. General chemicals used were of analytical grade, and the chemicals used for protein studies and acetylcholinesterase analysis were from Bio-Rad.

Experimental animals and the study of cumulative mortality rate. In this study, adult silver barbs with an average weight of 150 ± 5.1 grams and a length of 23.02 ± 2.5 cm were acclimatized in a 500 L fiber tank for 14 days. Fish were divided into 18 tanks (6 different concentrations, with 3 replicates per concentration), with 20 fish placed in each tank. The herbicide 2,4-D DMA was added to the tanks at 5 different concentrations: 33.6, 42, 50.4, 58.8, and 67.2 mg L⁻¹, compared to a control group. Mortality was recorded at 0, 24, 48, 72, and 96 hours to determine median lethal concentration (LC50) values using the probit analysis method based on the OECD (2019) guideline. Next, concentrations that did not cause mortality were selected for further studies, including examining changes in tissue cell morphology, protein patterns, and AChE expression using western blot techniques. Additionally, blood cell changes and acetylcholinesterase enzyme activity were assessed. Non-lethal concentrations determined from the LC50 test were subsequently used for subchronic exposure experiments and further biochemical and histological analyses.

Protein extraction from silver barb for protein profiling and protein quantification

Protein extraction. The gill samples of the silver barb, which were exposed to concentrations that did not cause mortality, as well as the control group, were collected at 0, 7, 14, 21, and 28 days. The samples were then homogenized in a 0.02 M Tris-HCl buffer (pH 7.2) containing Phenylmethylsulfonyl Fluoride at a ratio of 1 gram of tissue to 1.5 mL of buffer. Afterward, the mixture was centrifuged at 4°C at 3,500 RPM for 1 hour. The supernatant was collected and used for protein quantification, which was compared with Bovine Serum Albumin (BSA) as a standard (Nanthanawat et al 2022).

Protein quantification. The BSA standard protein was diluted to concentrations of 0.03125, 0.0625, 0.25, 0.5, and 1 mg mL⁻¹, with 0.5 mL of each concentration. Then, protein samples extracted from the brain, muscle, gill, and blood were diluted to 1:10 with

distilled water. For each protein sample (10 μL of brain, gill, muscle, and blood), two replicates were used. To each sample, 200 μL of diluted Dye Reagent was added and mixed thoroughly, then allowed to settle at room temperature for 5 minutes. The absorbance was measured at a wavelength of 595 nm. The absorbance values of the BSA solutions at different concentrations were recorded to create a standard curve relating BSA concentration to absorbance. The absorbance of the samples was then compared with the protein concentration based on the standard curve (Khanchanasal et al 2022).

Oxidative stress analysis. Antioxidant enzyme activities, CAT, SOD, and GST, were measured in gill tissue extracts following standard spectrophotometric protocols.

CAT activity. CAT activity was measured following the method of Beers & Sizer (1952). In this procedure, H_2O_2 was used as the substrate, and the decomposition of H_2O_2 by CAT was monitored using a UV-Vis spectrophotometer at 240 nm for 3 minutes. The reaction was carried out with 20 μL of sample supernatant and 980 μL of 10 mM H_2O_2 , as described by Hamisu et al (2024). Enzyme activity was expressed as units per milligram of total protein.

SOD activity. The enzymatic activity of SOD was measured using a commercial kit (Cayman Chemicals). This assay employed a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase, with absorbance readings taken at 540 nm. One unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% dismutation of the superoxide radical (Hamisu et al 2024). Enzyme activity was expressed as units per milligram of total protein.

GST activity. GST activity was measured by the conjugation of reduced glutathione (GSH) with 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate. The change in absorbance was monitored at 340 nm, and the enzymatic activity was calculated as nmol of CDNB conjugate formed per minute per milligram of protein, using a molar extinction coefficient of 9.6 $\text{mM}^{-1}\text{cm}^{-1}$. This method followed the procedures described by Habig et al. (1974) and Sinaei and Rahmanpour (2013). Enzyme activity was expressed as units per milligram of total protein.

Measurement of AChE enzyme activity. AChE activity and expression were analyzed to assess the neurotoxic effects of 2,4-D DMA exposure. Gill samples from silver barb exposed to non-lethal concentrations of the substance, as well as control groups, were collected at 0, 7, 14, 21, and 28 days to analyze the activity of the AChE enzyme. This was done by applying the method of Ellman et al (1961), which measures the rate of thiocholine production from the hydrolysis of acetylthiocholine (ACh). The reaction product then reacts continuously with dithiobisnitrobenzoate to form 2-nitro-5-mercaptobenzoate, resulting in a yellow color. The reaction was carried out in a 96-well microplate, and the intensity of the yellow color was measured by absorbance at 412 nm using a microplate reader. Absorbance was measured at 412 nm, and AChE activity was expressed as $\mu\text{mol thiocholine}^{-1}\text{min}^{-1}\text{mg}^{-1}\text{protein}$ (Nittayachit et al 2009).

Detection of AChE protein using SDS-PAGE and Western blot techniques. Gill samples from silver barb exposed to sub-lethal concentrations of the substance, as well as control groups, were collected at 0, 7, 14, 21, and 28 days for protein extraction using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then stained with 0.1% Coomassie Brilliant Blue R-250 for protein visualization. AChE was detected using the Western blot technique, where proteins were transferred from the gel to a nitrocellulose membrane. The membrane was then incubated in a blocking solution (5% skim milk in Phosphate-Buffered Saline (PBS)) for 1 hour, followed by washing with 0.1% Tween 20 in PBS. The membrane was then incubated with a rabbit anti-fish acetylcholinesterase antibody for 2 hours. After washing off excess antibody, the membrane was incubated with an enzyme-conjugated anti-rabbit immunoglobulin (GAR-HRP) antibody for 1 hour. The membrane was washed again and then incubated in

substrate solution. The appearance of a dark brown band indicated the reaction of the antibody with the sample (Thanomsit et al 2024). Protein bands corresponding to AChE (~71 kDa) were visualized using a DAB substrate solution.

Hematological analysis. Hematological parameters were analyzed to evaluate physiological stress responses. For hematological analysis, blood was initially collected from three fish at the start of the experiment. Subsequently, blood was sampled from three fish per concentration group at intervals of 0, 7, 14, 21, and 28 days of exposure. Blood samples were collected from the caudal vein using heparinized syringes and analyzed for red blood cell (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and leukocyte differential counts following Natt & Herrick (1952); Hrubec & Smith (2000); Sebastião et al (2011).

Study of tissue changes in silver barb. The study of tissue changes in silver barb was adapted from the method of Kumla et al (2025). Silver barb samples were randomly collected from the control and experimental groups exposed to non-lethal concentrations of the substance at 0, 7, 14, 21, and 28 days, with 3 fish per group. Tissues (gill, liver, intestines, and stomach) were fixed in 10% neutral buffered formalin, processed using standard paraffin embedding, and sectioned at 5 μm thickness. Next, the sections were transferred onto slides and dried at 45°C to ensure the tissue adhered properly to the slides. Afterward, the slides were washed to remove paraffin and stained with hematoxylin and eosin. Histopathological changes were evaluated under a light microscope and photographed for documentation.

Statistical data analysis. Data were analyzed using a Completely Randomized Design (CRD). Differences among treatment means were assessed using Duncan's Multiple Range Test (DMRT) at a 95% confidence level ($p < 0.05$) with SAS® OnDemand for Academics software.

Results

Toxicity testing. The results present the effects of 2,4-D DMA exposure on mortality, oxidative stress, protein content, AChE activity, hematological parameters, and tissue histopathology in silver barb. Cumulative mortality increases proportionally with both concentration and exposure duration, as shown in Figure 1. The calculated LC50 values at 24, 48, 72, and 96 hours are 140.76, 112.72, 81.87, and 70.56 mg L^{-1} , respectively, indicating a time-dependent increase in toxicity.

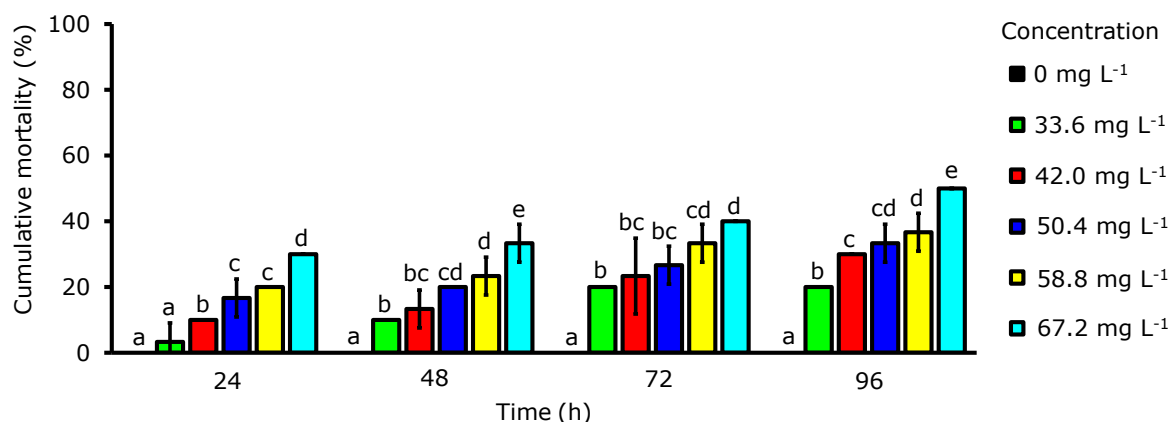


Figure 1. Cumulative mortality percentage of silver barb exposed to 2,4-D DMA at concentrations of 0, 33.6, 42.0, 50.4, 58.8, and 67.2 mg L^{-1} . (Note: Different letters above bars indicate statistically significant differences between groups at the time and concentration point ($p < 0.05$)).

Oxidative stress enzyme activity. The activities of antioxidant enzymes (CAT, SOD, and GST) in the liver of silver barb increase progressively with longer exposure periods as shown in Figure 2. CAT activity shows a significant, time-dependent increase throughout the exposure period ($p < 0.05$). SOD activity followed a similar pattern, showing significantly higher levels after 14, 21, and 28 days compared to the control ($p < 0.05$). GST activity also increases gradually, reaching its maximum after 28 days, indicating enhanced detoxification activity in response to prolonged herbicide exposure. The elevated antioxidant enzyme activities suggest that oxidative stress was induced by 2,4-D DMA exposure in silver barb.

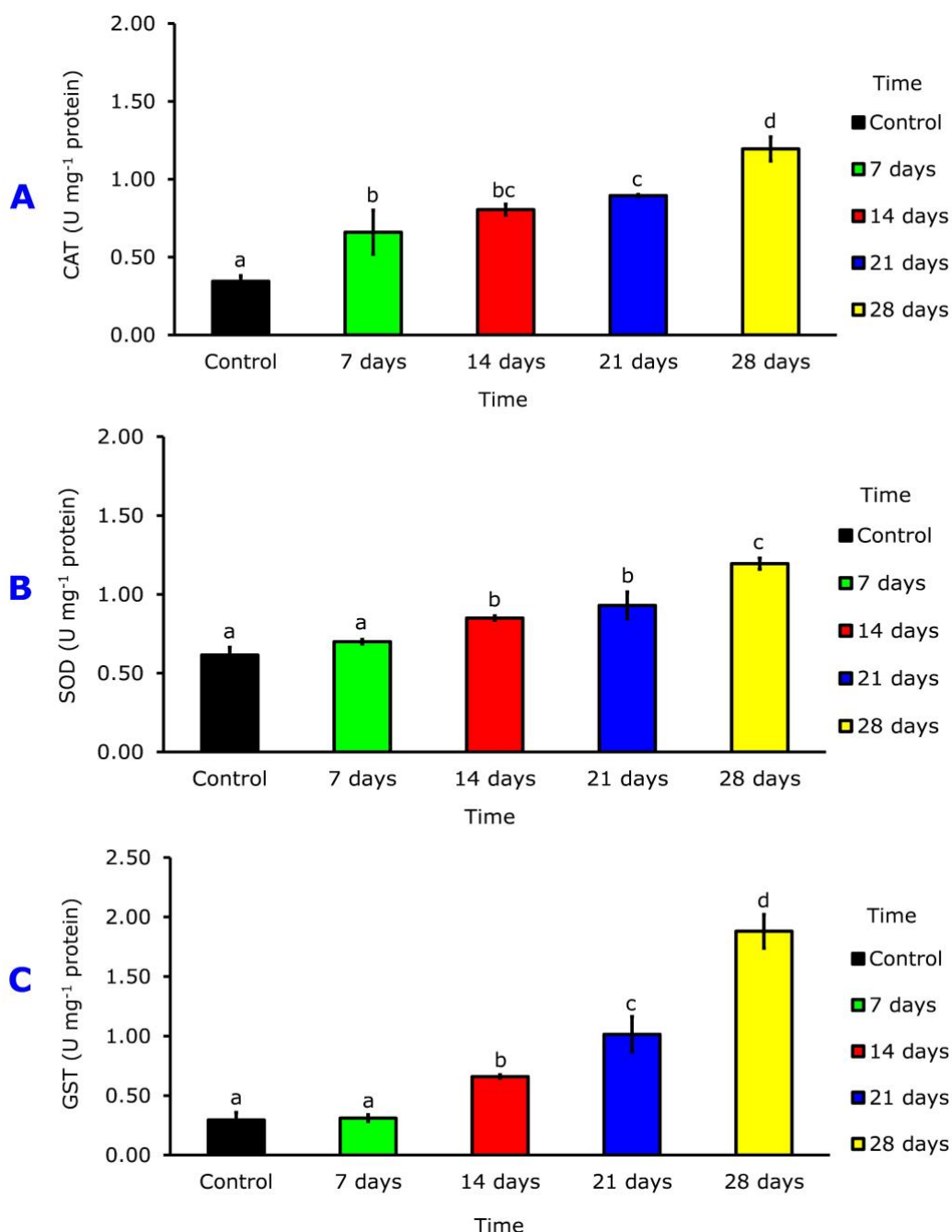


Figure 2. Oxidative stress analysis on silver barb exposed to sub-lethal concentration of 2,4-D DMA for 0, 7, 14, 21, and 28 days: (a) CAT activity, (B) SOD activity, (C) GST activity. (Note: Different letters above bars indicate statistically significant differences between groups at each time point ($p < 0.05$)).

Total protein concentration and AChE activity. Total protein content and AChE activity in gill tissues were evaluated to assess the biochemical effects of 2,4-D DMA exposure. Total protein levels show a gradual decline over time, with values significantly lower than the control from day 14 onward ($p < 0.05$) as shown in Figure 3. Conversely, AChE activity decreases steadily with increasing exposure duration, reflecting possible neurotoxic effects of the herbicide.

The reduction in both total protein and AChE activity suggests a disruption in normal metabolic and neural functions in fish exposed to sub-lethal concentrations of the herbicide.

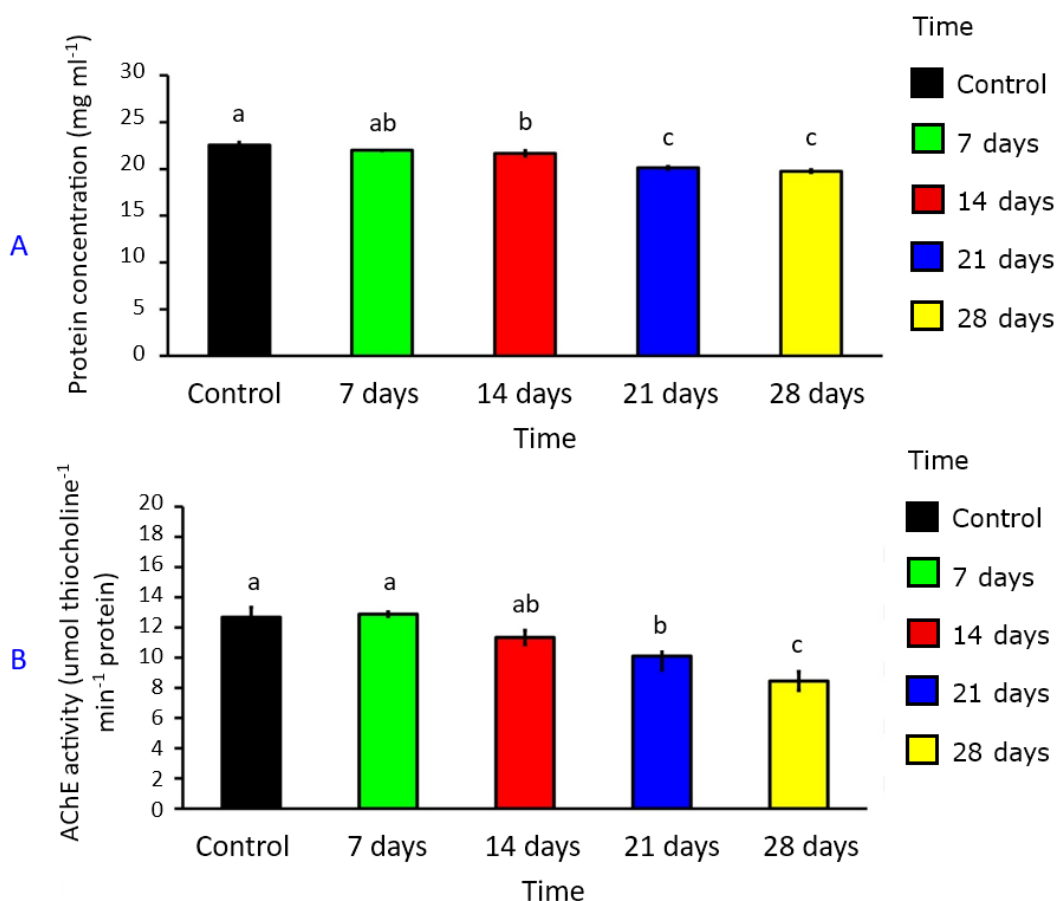


Figure 3. (A) Total protein concentration, (B) AChE activity of the silver barb exposed to sub-lethal concentration of 2, 4-D dimethylammonium for 0, 7, 14, 21, and 28 days. (Note: Different letters above bars indicate statistically significant differences between groups at each time point ($p < 0.05$)).

Protein profiling by SDS-PAGE and Western blot confirmed the presence of AChE with an approximate molecular weight of 71 kDa. In Figure 4, positive immunoreactivity with PAb-AChE antibody is observed across protein concentrations between 40-90 μg , with the optimal reaction obtained at 40 μg and a dilution of 1:50. The detected AChE had a molecular weight of 71 kDa. Since the protein amount used for the study yielded a positive result at all concentration levels, the concentration of 40 μg and the antibody concentration were selected for further development (Figures 4A and 4B).

The band intensity corresponding to AChE decreases progressively with exposure time, supporting the observed reduction in enzymatic activity, as observed from Figures 4C and 4D.

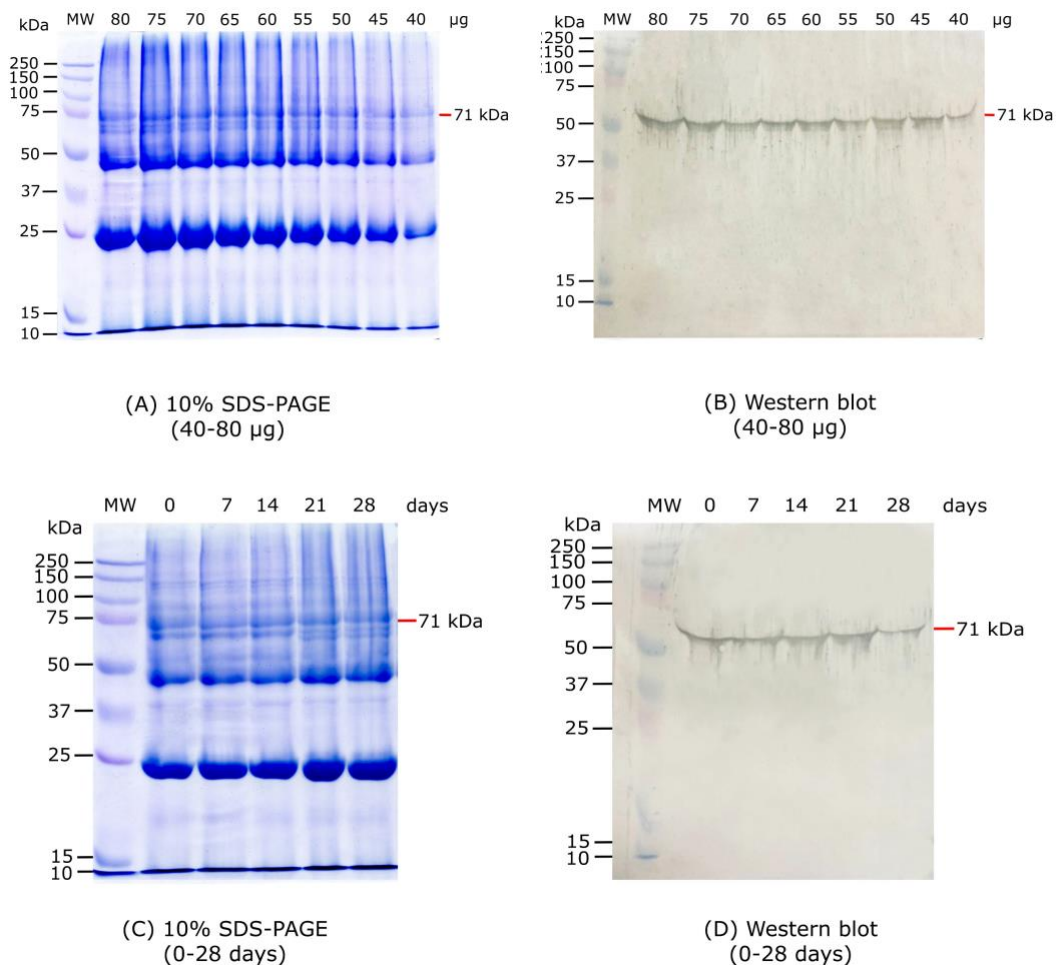


Figure 4. 10% SDS-PAGE and Western blot analysis (A) 10% SDS-PAGE of protein extraction from gill at concentrations of 40-80 µg, (B) Western blot analysis of the specificity of PAb-AChE antibody at a dilution of 1:50, (C) 10% SDS-PAGE of protein extraction from gill of silver barb exposed to 2,4-D DMA at sub-lethal concentration at 0,7,14,21, and 28 days, (D) Western blot analysis AChE extraction from the gill of silver barb exposed to the herbicide at a sub-lethal concentration at 0, 7, 14, 21, and 28 days.

Hematological parameters and abnormality percentage. Hematological profiles revealed significant, time-dependent alterations in all measured parameters (Table 1). RBC count, Hematocrit, Hemoglobin, MCV, MCH, and MCHC values declined with longer exposure durations, indicating potential anemia and reduced oxygen-carrying capacity. The study found that exposure duration affects all the parameters studied, with a tendency for these values to decrease as the exposure period increases. Microscopic examination of blood smears shows morphological abnormalities in erythrocytes, neutrophils, monocytes, lymphocytes, and thrombocytes (Figure 5). The proportion of abnormal cells increases with exposure time, reaching its peak on day 28 (Table 2). These hematological alterations reflect physiological stress and immune disturbances associated with herbicide exposure.

Table 1
 Mean values of erythrocyte parameters of silver barb exposed to 2,4-D DMA at sub-lethal concentration at 0, 7, 14, 21, and 28 days

Mean values of erythrocytes parameter	Times				
	Control	7 days	14 days	21 days	28 days
RBC ($10^6 \mu\text{L}^{-1}$)	250.62±7.94 ^a	242.62±0.47 ^{ab}	240.05±0.05 ^b	238.78±0.95 ^b	236.01±1.09 ^b
Hematocrit (%)	22.65±0.66 ^a	21.90±0.23 ^a	20.66±0.56 ^b	19.66±0.45 ^{bc}	19.06±0.07 ^c
Hemoglobin (g dL ⁻¹)	18.95±0.06 ^a	18.19±0.21 ^b	17.95±0.10 ^{bc}	17.50±0.22 ^c	16.79±0.32 ^d
MCV (fL)	244.20±1.44 ^a	241.17±1.03 ^b	238.01±1.25 ^c	236.34±1.41 ^c	233.17±0.40 ^d
MCH (g dL ⁻¹)	24.51±0.39 ^a	24.01±0.16 ^{ab}	23.50±0.08 ^{bc}	22.95±0.08 ^{cd}	22.51±0.23 ^d
MCHC (g dL ⁻¹)	44.95±1.17 ^a	43.84±0.24 ^{ab}	43.03±0.03 ^{bc}	42.22±0.33 ^{cd}	40.89±0.31 ^d

Note: Data are presented as means ± S.E.M., n = 3. Different letters indicate the significant difference from the control group with $p < 0.05$ according to ANOVA followed by Duncan's test.

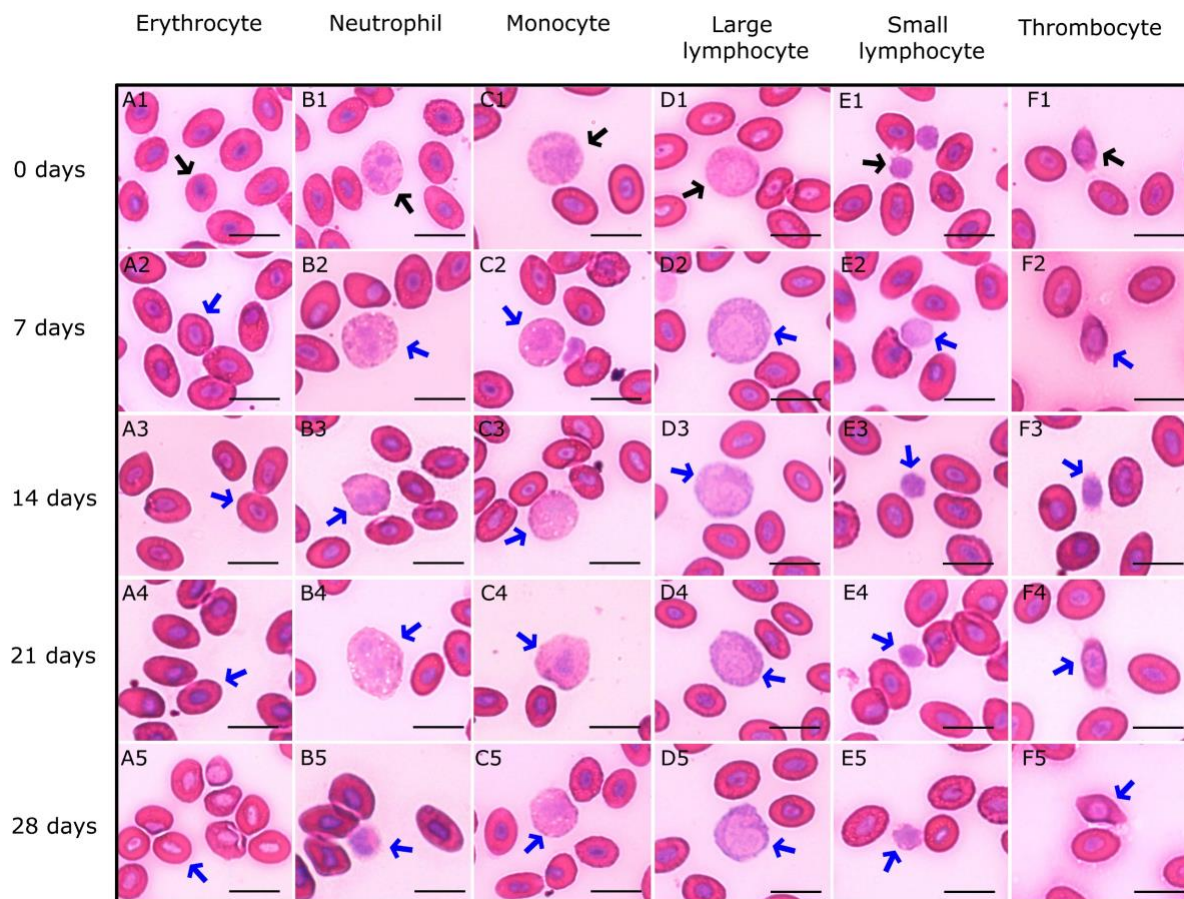


Figure 5. Abnormalities characteristics of white blood cells in silver barb exposed to 2,4-D DMA dimethylammonium at sub-lethal concentration at 0,7,14,21 and 28 days.

Table 2
Abnormalities percentage of the blood cells in silver barb exposed to 2,4-D DMA at sub-lethal concentration at 0,7,14,21, and 28 days

Abnormalities percentage (%)	Times				
	Control	7 days	14 days	21 days	28 days
Erythrocytes	0.00±0.00 ^a	2.50±0.71 ^b	3.00±0.00 ^b	4.50±0.71 ^c	5.00±0.00 ^c
Neutrophils	0.00±0.00 ^a	1.00±0.00 ^b	2.00±0.00 ^c	2.50±0.71 ^{cd}	3.00±0.00 ^d
Monocytes	0.00±0.00 ^a	1.50±0.71 ^{ab}	2.00±0.00 ^{ab}	2.00±1.41 ^{ab}	3.50±0.71 ^b
Larges lymphocytes	0.00±0.00 ^a	0.00±0.00 ^{ab}	1.00±0.00 ^b	2.00±0.00 ^c	3.00±0.00 ^d
Small lymphocytes	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.50±0.71 ^{ab}	1.00±0.00 ^b
Thrombocytes	0.00±0.00 ^a	0.00±0.00 ^a	0.50±0.71 ^{ab}	1.00±0.00 ^{ab}	1.50±0.71 ^a

Note: Data are presented as means ± S.E.M., n = 3. Different letters indicate the significant difference from the control group with p<0.05 according to ANOVA followed by Duncan's test.

In Figure 6, gill tissues exhibit hyperplasia, lamellar fusion, epithelial lifting, and edema, along with blood congestion and pillar cell deformation. The liver shows cytoplasmic vacuolation, while intestinal sections display epithelial wounds and microvilli lesions. Stomach tissues exhibit epithelial erosion, fusion of mucus-secreting cells, and mucosal lesions. These histological changes indicate that 2,4-D DMA caused multi-organ toxicity affecting both respiratory and digestive functions in silver barb.



Figure 6. Histological alterations of the gill, liver, intestine, and stomach of silver barb exposed to 2,4-D DMA at sub-lethal concentration for 0,7, 14, 21, and 28 days.

(Note: hyperplasia (HP), fusion of lamellae (FL), epithelial lifting (EL), clubbed tips (CT), edema (E), blood congestion (BG), deformed pillar (PS), vacuolation (V), wounds and lesions of microvilli (WV), Epithelial erosion (eEP), fusion of mucous secreting cells (FM), and wounds and lesions of mucus (WM)).

Discussion. The herbicide 2,4-D DMA accumulates in aquatic animals, posing risks to both their populations and human consumers. Currently, in Thailand, there is a high usage of the herbicide (Thanomsit et al 2025). According to Cantawon et al (2024), 2,4-D DMA is ranked as the third most widely used herbicide in the country. Therefore, it is utmost important to investigate the effects of this chemical on silver barb, an economically important fish found in natural water sources. Overall, the results demonstrated that the herbicide induces oxidative stress, hematological disruption, and tissue damage in silver barb, suggesting that even sub-lethal concentrations can significantly impair fish physiology.

Cumulative mortality rate and LC50. The study on cumulative mortality rates found that it depended on both the exposure duration and concentration levels, which is consistent with the study by Seiyaboh et al (2020) on *Clarias gariepinus* fingerlings exposed to 2,4-D dimethylamine salt. Furthermore, the findings align with the results of Khanchanasal et al (2022), who studied the effects of 2,4-D DMA on Nile tilapia. In this study, the silver barb, an herbivorous fish classified in the same group as the tilapia, was used (Tsfahun & Sale Alebachew 2023). However, the study on *Clarias gariepinus*, a carnivorous fish (Kawamura et al 2017), was included for comparison. The authors aimed to demonstrate that fish species may be influenced by the cumulative mortality rate and LC50 values. However, when considering the LC50, differences were observed, possibly due to the type of fish and the chemical formulation. Walker et al (2006) reported that the toxicity of a substance is related to the chemical type, exposure pathways, concentration levels, and animal species involved.

Oxidative stress. Antioxidants are crucial biosensor parameters because they provide valuable insights into the toxicity mechanisms of pollutants on organisms. Antioxidants act as defensive biomarkers, with changes in the activity of key enzymes, including SOD, CAT, and glutathione peroxidase, reflecting oxidative stress (Chen et al 2016). Hamisu et al (2024) studied the effects of 2,4-D DMA on African catfish, assessing oxidative stress expression. Their study found that acute toxicity caused significant changes in liver enzyme activity, particularly for SOD and CAT at concentrations of 1.36, 1.42, 1.48, 1.54, and 1.6 ml L⁻¹, while sub-lethal concentrations showed no significant changes. In contrast, the current study observed a tendency for the activities of CAT, SOD, and GST to be increased over time with prolonged exposure. CAT activity showed significantly higher levels after 7, 14, 21, and 28 days compared to the control ($p < 0.05$). However, SOD and GST activity demonstrated significantly higher levels after 14, 21, and 28 days compared to the control ($p < 0.05$).

Protein extraction and AChE expression. AChE activity has been used to assess exposure to the herbicide 2,4-D DMA. Nanthanawat et al (2022) and Walker et al (2006) suggested that AChE is a specific biomarker for exposure to herbicides. In this study, when silver barb was exposed to 2,4-D DMA, it was found that AChE activity tended to decrease as the exposure duration increased. This is consistent with the study of Nualkaw et al (2022), which investigated the effects of the herbicide paraquat on Nile tilapia. Besides, the herbicide affected AChE activity, and the measured values tended to decrease as the exposure time increased.

This study also examined the protein patterns in the gill tissue of silver barb using SDS-PAGE and Western blot techniques. It was found that AChE had a molecular weight of 71 kDa, which is consistent with the study of Nanthanawat et al (2022) in Nile tilapia exposed to the herbicide glyphosate. Furthermore, this finding is also in line with the study of Nualkaw et al (2022), which reported that AChE in Nile tilapia exposed to paraquat had a molecular weight of 71 kDa. Moreover, it aligns with the studies of Khanchanasal et al (2022) and Thanomsit et al (2024), which reported that AChE isolated from Nile tilapia exposed to the herbicide 2,4-D DMA also had a molecular weight of 71 kDa.

Sinha et al (2022) suggested that blood can provide accurate information on the effects of pesticides when exposed to the environment. In most cases, hematological parameters serve as early warning indicators of altered physiological conditions in fish due

to stress, pollution, pesticide exposure, or infection. Key hematological parameters of fish include total erythrocyte count, hemoglobin content, packed cell volume, erythrocyte sedimentation rate, absolute values, total leucocyte count, coagulation time, and thrombocyte count (Singh & Srivastava 2010). Changes in the aquatic environment quality can be easily assessed by studying alterations in these parameters in fish (Sinha et al 2022).

Hematological profiles. Given the importance of these parameters, the present study investigated hematological parameters and the percentage of abnormalities in silver barb exposed to the herbicide. The findings revealed that the exposure duration affected all studied parameters, including RBC, hematocrit, hemoglobin, MCV, MCH, and MCHC, with a general trend of decline as exposure time increased. Furthermore, abnormalities in various blood cells, such as erythrocytes, neutrophils, monocytes, large lymphocytes, small lymphocytes, and thrombocytes, were observed. These abnormalities were primarily in the nucleus, and cell morphology deviated from the control group ($p < 0.05$). This result aligns with the study by Alarape et al (2024), which reported the effects of glyphosate-based herbicide (Force Up®) on the hematological parameters and biochemical indices of African catfish (*Clarias gariepinus*) over 96 hours. Their study found that exposure duration influenced the hematological profile, with prolonged exposure resulting in both changes in the number and shape of blood cells.

Tissue changes. Histopathology is often the most straightforward method for assessing both short- and long-term toxic effects in field studies. Histopathological analysis is considered a highly sensitive parameter, essential for identifying cellular changes in target organs such as the gills, liver, kidney, brain, spleen, gonads, and muscles (Hamisu et al 2024). In this study, the tissue changes in the gills, liver, intestine, and stomach of silver barb exposed to the herbicide for 0, 7, 14, 21, and 28 days were examined.

The results showed that in the control group (0 days), no changes were observed in any of the tissues studied. However, after exposure to the herbicide for 7 to 28 days, notable changes were observed. In the gills, there were signs of hyperplasia, fusion of lamellae, epithelial lifting, clubbed tips, edema, blood congestion, and deformed pillar cells. In the liver, vacuolation was observed. In the intestine, there were wounds and lesions of the microvilli, while in the stomach, epithelial erosion, fusion of mucous-secreting cells, and wounds and lesions of mucous were noted. These findings are consistent with the study by Khanchanasal et al (2022), which reported that exposure to 2,4-D DMA at sub-lethal concentrations led to histopathological changes in Nile tilapia. In the gills, they found hyperplasia, epithelial lifting, partial fusion of lamellae, edema, and lamellae disorganization, along with blood congestion. In muscle tissue, dilation of muscle fibers, splitting of muscle, and blood congestion were also noted. Furthermore, this study aligns with the work of Thanomsit et al (2024), which reported that exposure to 2,4-D DMA affected the gills, liver, and muscle tissue in Nile tilapia.

Conclusions. This study showed the significant toxic effects of herbicide 2,4-D DMA in silver barb, comprising increased mortality, altered antioxidant enzyme and AChE activity, altered hematological profile, and caused histopathological lesions in the main organs of silver barb. Agricultural management in using the herbicide 2,4-D DMA is very important for protecting against potential environmental and health risks.

Acknowledgements. This work was supported by the Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology, Isan Surin Campus.

Conflict of interest. The authors declare that there is no conflict of interest.

References

- Alarape S. A., Adeoye D. D., Amusa A. O., Adeyemo O. K., 2024 Haematological parameters and biochemical indices of African catfish (*Clarias gariepinus*) exposed to glyphosate-based herbicide (Force up®) for 96 hours. *Frontier in Toxicology* 6:1448861.
- Beers R. F., Sizer I. W., 1952 A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* 195(1):133-140.
- Cantawon N., Kiatprasert P., Prasatkaew W., Mongkolvai P., Nanthanawat P., Thanomsit C., 2024 Effects of the herbicide 2, 4-D dimethylammonium on induction of Vitellogenin synthesis in the plasma of Nile tilapia (*Oreochromis niloticus*) and field study. *Agriculture and Technology RMUTI Journal* 3:83-98.
- Casimero M., Abit M. J., Ramirez A. H., Dimaano N. G., Mendoza J., 2022 Herbicide use history and weed management in Southeast Asia. *Advances in Weed Science* 40:e020220054.
- Chen M., Yin, J. Lian Y., Yuan S., Wang F., Song M., Wang, H., 2016 Oxidative stress and immunotoxicity induced by graphene oxide in zebrafish. *Aquatic Toxicology* 174:54-60.
- Dey C., Saha S. K., 2014 Comparative study on acute toxicity bioassay of dimethoate and lambda-cyhalothrin and effects on thyroid hormones of freshwater teleost fish *Labeo rohita* (Hamilton). *International Journal of Environmental Research* 8(4):1085-1092.
- Ellman G. L., Courtney K. D., Andres V., Featherstone R. M., 1961 A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7(2):88-95.
- Habig W. H., Pabst M. J., Jokoby W. B., 1974 Glutathione S-transferase: The first enzyme step in mercapturic acid formation. *Journal of Biological Chemistry* 249:7130-7139.
- Hamisu Y., Au J., Bawa B. S., 2024 Toxicity of the herbicides 2,4-D dimethyl amine salt on behavioral, histological and oxidative stress biomarker of African catfish *Clarias gariepinus* (Burchell, 1822) fingerlings. *FUDMA Journal of Science* 8(6):177-185.
- Hrubec T. C., Smith S. A., 2000 Hematology of fish. In: Schalm's Veterinary Hematology. Feldman B. F., Zinkl J. G., Jain N. C. (eds.), Lippincott Williams & Wilkins, Canada. pp. 1120-1125.
- Kawamura G., Bagarinao T., Yong A. S. K., Sao P. W., Lim L. S., Senoo S., 2017 Optimum low salinity to reduce cannibalism and improve survival of the larvae of freshwater African catfish *Clarias gariepinus*. *Fisheries Science* 83(4):597-605.
- Khanchanasal P., Nuankaew C., Saowakoon S., Nanuam J., Nanthanawat P., Thanomsit C., 2022 Toxicity level of 2, 4-D dimethylammonium in Nile tilapia and Acetylcholinesterase (AChE) expression (Biomarker) to identify exposure in sub-lethal concentration. *Burapha Science Journal* 1278-1299.
- Kumla S., Chartchumni B., Nanthanawat P., Nanuam J., Ruttanakorn S., Meemon P., Thanomsit C., 2025 Morphological, biochemical, and histological alterations in juvenile giant freshwater prawns (*Macrobrachium rosenbergii*) exposed to sub-lethal concentrations of 2, 4-D dimethylammonium. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 110212.
- Nanthanawat P., Kiatprasert P., Wattanakornsiri A., Nanuam J., Prasatkaew, W., Ruttanakorn S., Thanomsit C., 2022 Development of an antibody technique for acetylcholinesterase expression detection in the gill of Nile tilapia (*Oreochromis niloticus*) as a glyphosate-based herbicide biomarker. *Toxicology Report* 9:1548-1556.
- Natt M. P., Herrickand C. A., 1952 A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poultry Science* 31(4):735-738.
- Nittayachit P., Chanthai S., Petkam, R., 2009 [Effects of chlorpyrifos on acetylcholinesterase activity in the brain, plasma and red blood cell of Nile tilapia (*Oreochromis niloticus*)]. *KKU Research Journal* 14(1):55-67. [in Thai]
- Nualkaw C., Wattanakornsiri A., Nanuam J., Nanthanawat P., Prasatkaew W., Meeprom C., Saowakoon S., Thanomsit C., 2022 Effects of paraquat-based herbicide on

- acetylcholinesterase (AChE), antioxidant status and histological alterations in freshwater fish: Nile tilapia (*Oreochromis niloticus*). *AAFL Bioflux* 15(6):3197-3211.
- Ramesh H., Muniswamy D., 2009 Behavioral responses of the freshwater fish, *Cyprinus carpio* (Linnaeus) following sublethal exposure to Chlorpyrifos. *Turkish Journal of Fisheries and Aquatic Sciences* 9(2):233-238.
- Sebastião F. A., Nomura D., Sakabe R., Pilarski F., 2011 Hematology and productive performance of Nile tilapia (*Oreochromis niloticus*) naturally infected with *Flavobacterium columnare*. *Brazilian Journal of Microbiology* 42:282-289.
- Seiyaboh E. I., Enaregha E. B., Izah S. C., 2020 Acute toxicity of *Clarias gariepinus* fingerlings exposed to 2,4-D dimethylamine salt. *Journal of Public Health International* 2(4):1-7.
- Sinaei M. F., Rahmanpour S., 2013 Evaluation of glutathione S-transferase activity as a biomarker of PAH pollution in Mudskipper, *Boleophthalmus dussumieri*, Persian Gulf. *Bulletin of Environmental Contamination and Toxicology* 90(3):369-374.
- Singh N. N., Srivastava A. K., 2010 Haematological parameters as bioindicators of insecticide exposure in teleosts. *Ecotoxicology* 19(5):838-854.
- Sinha D. K., Gour J. K., Singh M. K., Nigam A. K., 2022 Effect of pesticides on haematological parameters of fish: recent updates. *Journal of Scientific Research* 66(01):269-283.
- Sribanjam S., Charoenwattanasak S., Champasri T., Champasri C., Yuangsoi B., 2018 Toxic effects of herbicide glyphosate on enzymes activities and histopathological changes in gill and liver tissue of freshwater fish, Silver barb (*Barbonymus gonionotus*). *Bioscience Research* 15(2):1251-1260.
- Tesfahun A., Alebachew, S., 2023 Food and feeding habits of the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) from Ribb reservoir, Lake Tana sub-basin, Ethiopia. *Food & Agriculture* 9(1):2212457.
- Thanomsit C., Khanchanasal P., Prasatkaew W., Nanuam J., Meemon P., Wattanakornsiri A., Nanthanawat P., 2024 Adverse effects of 2,4-D dimethylammonium based herbicide on acetylcholinesterase expression in Nile tilapia (*Oreochromis niloticus*). *Environmental Toxicology and Pharmacology* 106:104383.
- Thanomsit C., Kumla S., Saetiew J., Saenjae J., Nanthanawat P., Nanuam J., Meemon P., 2025 Application of optical coherence tomography (OCT) to evaluate the adverse effects of 2, 4-D dimethylammonium on morphological changes in Riceland Prawn (*Macrobrachium lanchesteri*). *Environmental Toxicology and Pharmacology* 113:104608.
- Tudi M. Ruan H. D., Wang L., Lyu J., Sadler R., Connell D., Chu C., Phung D. T., 2021 Agriculture development, pesticide application and its impact on the environment. *International Journal of Environmental Research and Public Health* 18(3):1112.
- Walker C. H., Hopkin S. P., Peakall D. B., 2006 Principle of ecotoxicology. Taylor & Francis. New York, USA. pp. 1-329.
- Zuanazzi N. R., Ghisi N. C., Oliveira E. C., 2020 Analysis of global trends and gaps for studies about 2,4-D herbicide toxicity: a scientometric review. *Chemosphere* 241:125016.
- ***OECD (The Organization for Economic Cooperation and Development), 2019 Test guidelines for the chemicals - OECD 2019. Test guideline No.203 Fish, acute toxicity testing. Available at: https://www.oecd.org/en/publications/test-no-203-fish-acute-toxicity-test_9789264069961-en.html. Accessed at: September 2025.

Received: 16 October 2025. Accepted: 17 November 2025. Published online: 30 December 2025.

Authors:

Chutima Thanomsit, Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin Campus, 32000 Surin, Thailand, e-mail: Chutima.th@rmuti.ac.th

Sugunya Kumla, Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, 47160 Sakon Nakhon, Thailand, e-mail: suganyakumla@gmail.com

Patcharee Mongkolvai, Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, 47160 Sakon Nakhon, Thailand, e-mail: patcharee.mo@hotmail.com

Boonthiwa Chartchumni, Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, 47160 Sakon Nakhon, Thailand, e-mail: boonthiwa.Ch@rmuti.ac.th

Amnuay Wattanakornsiri, Department of Agriculture and Environment, Faculty of Science and Technology, Surindra Rajabhat University, 32000 Surin, Thailand; e-mail: amnuaywatanakornsiri@hotmail.co.th

Khobkul Nongnutch, Faculty of Industry and Technology, Rajamangala University of Technology Isan Sakon Nakhon Campus, 47160 Sakon Nakhon, Thailand, e-mail: khopkul25@gmail.com

Jakkaphun Nanuam, Office of Educational Affairs, Faculty of Science, Burapha University, 20131 Chonburi, Thailand, e-mail: jakkaphu@buu.ac.th

Witchuda Prasatkaew, Department of Environmental Management, Faculty of Science and Technology, Dhonburi Rajabhat University, 59/1 Moo 14, Thetsaban Bang Pu 119 Alley, Sukhumvit Road, Bang Pla, Bang Phli, 10540 Samutprakan, Thailand, e-mail: witchuda.p@dru.ac.th

Phochit Nanthanawat, Office of Educational Affairs, Faculty of Science, Burapha University, 169 Longhard Bangsaen Rd., Saensuk, Muang, 20131 Chonburi, Thailand, e-mail: phochit@go.buu.ac.th

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

How to cite this article:

Thanomsit C., Kumla S., Mongkolvai P., Chartchumni B., Wattanakornsiri A., Nongnuch K., Nanuam J., Prasatkaew W., Nanthanawat P., 2025 Toxicity testing, hematological alterations, and Acetylcholinesterase expression on *Barbonymus gonionotus* (Bleeker, 1849) exposed to glyphosate-based herbicide. *AAFL Bioflux* 18(6):2952-2965.