

Histology, toxicity tests, and mortality of zebrafish (*Danio rerio*) following exposure to Butachlor

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ABSTRACT:

This study aims to develop an environmental management model for the effects of the agricultural pesticide Butachlor based on an experimental toxicity model using zebrafish. The research is divided into two stages: Stage 1 involves performing laboratory studies, while Stage 2 focuses on developing environmental management strategies to mitigate the effects of Butachlor. The LC₅₀96h level was first determined in a pilot test. Then, in the main experiment, 600 zebrafish were randomly divided into four groups, each with three replications, for 30 days: T1 was exposed to 40% of the LC₅₀96h concentration of Butachlor, T2 to 60%, T3 to 80%, and there was a control group (C). Sampling occurred on days 0, 15, and 30 of the experiment, and histopathological examinations of the fish liver and gills were conducted. The histological results for the gills revealed several issues: hyperemia, swollen epithelium in the secondary lamellae, concentrated chloride cells at the base of the secondary lamellae, vasodilation, hyperemia at the tip of the secondary lamellae, and clubbed tips in the secondary lamellae of the groups exposed to Butachlor. Additionally, liver histology demonstrated changes in liver tissue, including hyperemia and fatty changes due to Butachlor exposure. These results indicate that Butachlor induced an oxidative stress response in the fish in a dose-dependent manner. Simultaneously, tissue damage increased with higher concentrations and longer exposure durations to Butachlor. Given the relevance of using zebrafish as a model for studying human diseases, the findings of this study are likely generalizable to humans and underscore the potential risk that Butachlor, sometimes used in rice fields, poses to human health.

Keywords: Butachlor, zebrafish, oxidative stress, histology.

INTRODUCTION:

Butachlor (N-(Butoxymethyl)-2-chloro-N-(2,6-diethylphenyl)acetamide) is a key member of the chloro-acetamide herbicide family, widely used in modern agriculture across Asia, especially in countries such as India, Pakistan and Iran, where it is often applied in rice fields in the northern regions (Ateeq et al. 2006; Berger et al. 2012; Ahmadiwand et al. 2015). Zebrafish (*Danio rerio*) serve as a significant experimental model for studying human diseases due to their substantial genetic, physiological, and pharmacological similarities to humans (Maxwell 2005). Belonging to the minnow family (Cyprinidae) within the order Cypriniformes (Rieger 2011), zebrafish are one of the ornamental freshwater fish found in tropical regions and hold considerable economic value in Iran. This species' scientific name is *Danio rerio*, and its commercial name is zebra danio. Other factors that make zebrafish an ideal model for human disease research include their small size at the larval and embryonic stages, rapid external fertilization, high fecundity, optical transparency (which allows direct observation of internal organs),

and resilience to adverse temperature conditions and environmental (Streisinger 1981).

Fang et al. (2012) studied zebrafish as a model organism to investigate the adverse effects of phosphorus toxins, finding that the toxins suppressed antioxidant activity in these fish. Xiang et al. (2018), in their study entitled "Oxidative stress response induced by butachlor in zebrafish embryo/larvae: the protective effect of vitamin C," reported that butachlor significantly increased mortality and malformation rates in zebrafish, which were dependent on the dosage and duration of exposure. Furthermore, they elevated reactive oxygen species (ROS) and malondialdehyde (MDA) after 72 hours. Saravanan et al. (2017) examined the hematological and biochemical parameters in the freshwater fish *Cyprinus carpio* exposed to varying concentrations of butachlor and oxadiazon (0.5, 5, and 50 mg/L) for 96 hours. They observed increased levels of Na⁺, WBC, Cl⁻, cholesterol, and triglyceride, along with decreases in haemoglobin (Hb), hematocrit (Hct), and red blood cells (RBC). No significant changes were noted in mean cellular haemoglobin concentration (MCHC).

Tataji and Kumar (2016) studied the toxicity of butachlor in *Channa punctata* (Bloch). They examined the effects of the LC₅₀96h concentration of Butachlor (160 ppb) on fish enzymes. Their findings revealed reductions in total glycogen and proteins of tissues, including nucleic acids, deoxyribonucleic acid (DNA), and ribose nucleic acid (RNA), along with increased activity of lactate dehydrogenase (LDH).

The application of herbicides can disrupt biodiversity due to their effects on the local flora and fauna. Herbicides also influence the diversity and density of soil microorganisms. Some herbicides, such as Butachlor (trademarked as Machete), damage aquatic life, while some others, such as paraquat, are as toxic as insecticides. These herbicides can disrupt food chains in ecosystems, resulting in the decline of certain species and the proliferation or invasion of others. Additionally, the destruction of plant species induced by herbicides affects the animal species that depend on those plants. Therefore, the present study aims to investigate the histological, toxicological, and mortality effects of the agricultural pesticide Butachlor on zebrafish.

MATERIALS AND METHODS:

The present study is applied descriptive-survey research. The required data were collected through both field and desk studies. The research was conducted at the Razef Knowledge-Based Research Complex, Islamic Azad University (Science and Research Branch), Shahriar, Tehran. The experiments occurred in separate aquariums, each equipped with an aeration system and 67 L of chloride-free water (dimensions: 50×27×50). An open-circuit system was used to prevent the spread of contamination between aquariums. To allow the fish to adapt to the laboratory conditions, apparently healthy zebrafish were introduced to the holding area 14 days before the main experiment. During this adaptation period, they were subjected to a light/dark cycle of 16:8 hours and were fed a commercial diet (without supplements) at a rate of 5% of their body weight, twice a day. Water temperature was measured daily, while other physical and chemical parameters were assessed biweekly during the experiments. The water temperature was maintained at 27.5±1°C, with dissolved oxygen levels at 6.12±1.08 mg/L, pH at 7.6±0.6, and ammonia levels at 0.0023±0.0007 mg/L. Overall, the physicochemical conditions were kept consistent across all aquariums.

After determining the LC₅₀96h, 600 zebrafish were randomly divided into four groups, each with three replications, for 30 days: T1 was exposed to 40% of the LC₅₀96h concentration of Butachlor, T2 to 60%, T3 to 80%, and there was a control group (C). Sampling occurred on days 0, 15, and 30 of the experiment, and histopathological examinations of the fish liver and gills were conducted. The study assessed the following parameters: activity of superoxide dismutase (SOD), total glutathione (GSH), and the amount of malondialdehyde (MDA) in the liver. Table

1 provides the characteristics of the herbicide Butachlor used in the experiments.

Table 1: Characteristics of the herbicide Butachlor employed in the experiments

Testing Item		Index	Results
1	Content	95% Min	95.2%
2	Acidity (As H ₂ SO ₄)	2g/kg Max	0.7g/kg
3	Water content	3g/kg Max	1.5g/kg
4	Acetone-Insolubles	0.2g/kg Max	0.09g/kg
5	Appearance	Yellowish Transparent Liquid	Yellowish Transparent Liquid
Conclusion		Qualifies	

Since there was no information available regarding the toxicity of Butachlor in adult zebrafish, a pilot test was first conducted to estimate the LC₅₀ concentration. In this test, 80 adult zebrafish were exposed to various concentrations of Butachlor (0.1, 0.05, 0.15, and 0.2 ml in 67 L of water) across four groups (20 fish per group) in 67-liter aquariums for 96 hours. To maintain stable herbicide concentrations in the aquariums throughout the testing period, a solution containing the desired doses was prepared daily, and the water in the aquariums was replaced. The LC₅₀ was determined to be 0.05 ml by examining the mortality rate.

Treatment after determining the LC₅₀: After determining the LC₅₀, a main experiment was conducted in 120-liter aquariums (dimensions: 28×86×50 cm) with 600 zebrafish acclimatized to the holding area. The fish were divided into four groups (150 zebrafish per aquarium) with two replications for 30 days. Throughout the experiment, the water in all aquariums was replaced daily with water containing the specified concentration of Butachlor. The treatments were as follows:

- 1- Control group (C): No presence of the herbicide Butachlor
- 2- Treatment 1 (T1): 40% of (LC₅₀ 96h= 0.05/67L) = 0.03 ml in 120 L of water
- 3- Treatment 2 (T2): 60% of (LC₅₀ 96h= 0.05/67L) = 0.05 ml in 120 L of water
- 4- Treatment 3 (T3): 80% of (LC₅₀ 96h= 0.05/67L) = 0.07 ml in 120 L of water

Sampling occurred on days 0, 15, and 30 to investigate the effects of herbicide Butachlor on biochemical factors SOD, GSH, MDA, as well as to assess pathological damage to liver and gill tissues, measure the absorption of toxicant in zebrafish tissue, and evaluate the persistence of the toxin as an environmental pollutant in water. All experiments were performed in triplicate.

Fish tissue samples were thawed and homogenized on ice with a cold buffer consisting of 250 mmolL⁻¹ sucrose, 5 mmolL⁻¹ Tris-HCl, and 1 mmolL⁻¹ Edetic acid-2Na (pH=7.5) at a volume ratio of 1:10. The prepared homogenate was centrifuged for 15 minutes, and the supernatant was used to measure enzymatic

activity (Jin et al. 2010). The levels of MDA, GSH, and SOD activity in zebrafish tissue were determined per the manufacturer's kit instructions (Navand Salamat) (Liu et al. 2008).

After fixation in Bouin's solution, the samples were dehydrated in ascending ethanol solutions (50, 70, 80 and 96%), purified with chloroform, and embedded with paraffin in a mixture of pure paraffin and chloroform at 56°C. After molding, sections were cut at 5 microns in thickness, and slides were prepared and stained with hematoxylin-eosin (H&E). Finally, they were mounted on slides using Entellan adhesive, and histopathological examinations were performed using a light microscope (Nikon E600, Japan) (Paulsen 2000).

Findings:

After 96 hours, the LC₅₀ concentration of Butachlor was determined to be 0.05 in 67 L of water, based on an examination of the recorded mortality rate at 24-hour intervals. Toxicity tests were then conducted using different percentages of the LC₅₀ concentration of Butachlor (Table 2).

Table 2: Data on mortality rate at different concentrations and sampling intervals in zebrafish.

Exposure concentration (ml)	Total number of fish	Number of live fish at different intervals				Mortality rate (%)
		24	48	72	96	
0.05	20	18±1.41 ^a	20±2.82 ^a	18±1.41 ^a	8±1.41 ^a	50
0.1	20	20±1.41 ^a	13±0.00 ^b	11±1.41 ^b	8±0.00 ^a	75
0.15	20	17±0.00 ^b	13±1.41 ^b	8±0.00 ^c	6±0.00 ^a	70
0.02	20	15±0.00 ^c	8±0.00 ^c	7±0.00 ^c	1±0.00 ^b	90

Letters a, b, and c indicate significant differences between groups.

The results of the pathological studies conducted on Day 15 were as follows: group C exhibited normal gill tissue (Figs. 1 and 2). In group T1, mild hyperemia was observed (Fig. 3). Group T2 showed hyperemia, swollen epithelium in the secondary lamellae, concentrated chloride cells at the base of the secondary lamellae, vasodilation, and hyperemia at the tips of the secondary lamellae (Fig. 4). In group T3, hyperemia, concentrated chloride cells at the base of the secondary lamellae, and clubbed tips in the secondary lamellae were noted (Figs. 5 and 6).

On Day 30, histological assessments showed normal gill tissue in group C (Fig. 7) and mild hyperemia in group T1 (Fig. 8). Group T2 exhibited hyperemia and clubbed tips in the secondary lamellae (Fig. 9). In group T3, changes included hyperemia, concentrated

chloride cells at the base of the secondary lamellae, and clubbed tips in the secondary lamellae (Fig. 10).

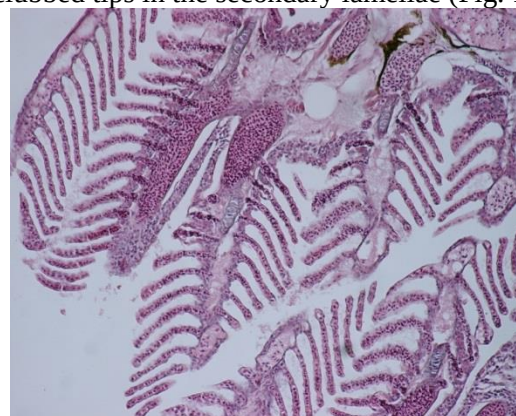


Figure 1: Gill histology for group C on Day 15 (HE, X200).

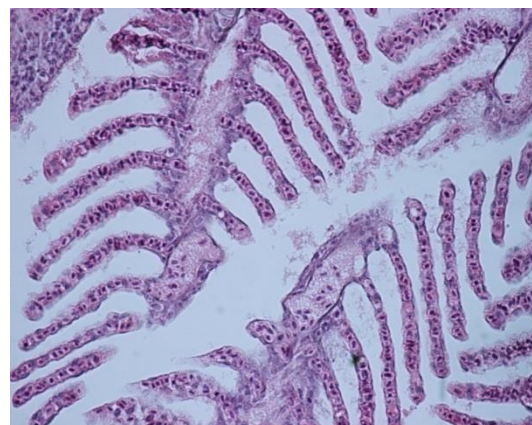


Figure 2: Gill histology for group C on Day 15 (HE, X400).

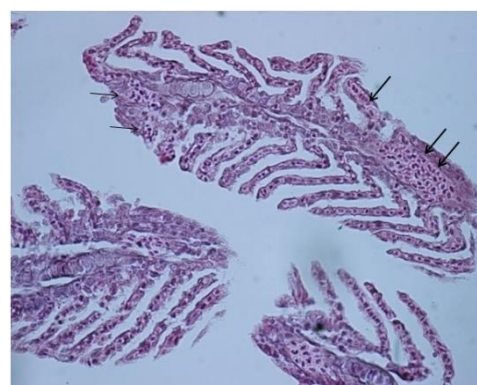


Figure 3: Gill histology for group T1 on Day 15. Arrows show mild hyperemia (HE, X400).

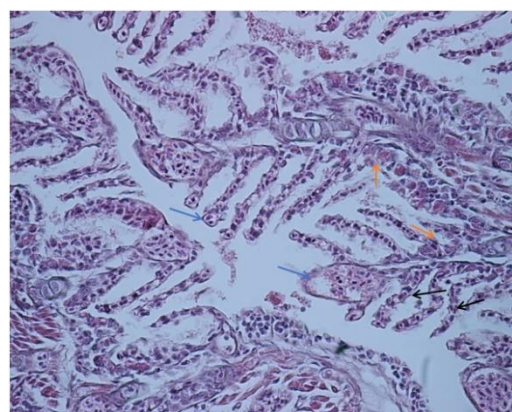


Figure 4: Gill histology for group T2 on Day 15. Black arrow: hyperemia, swollen epithelium in the secondary lamellae. Orange arrow: concentrated chloride cells at the base of the secondary lamellae. Blue arrow: Vasodilation and hyperemia at the tip of the secondary lamellae (HE, X400).

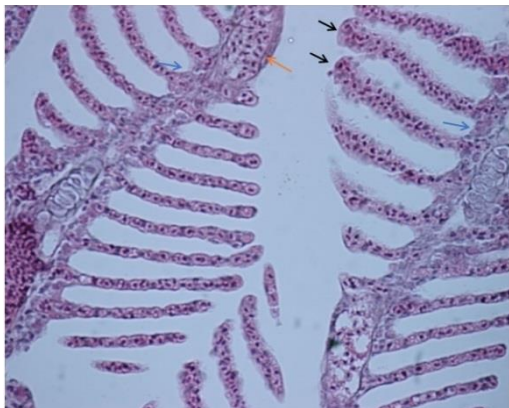


Figure 5: Gill histology for group T3 on Day 15. Orange arrow: hyperemia. Blue arrow: relatively concentrated chloride cells at the base of the secondary lamellae. Black arrow: clubbed tip in the secondary lamellae (HE, X400).

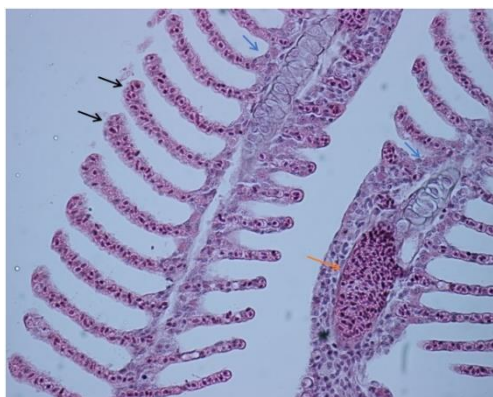


Figure 6: Gill histology for group T3 on Day 15. Orange arrow: hyperemia. Blue arrow: relatively concentrated chloride cells at the base of the secondary lamellae. Black arrow: clubbed tip in the secondary lamellae (HE, X400).

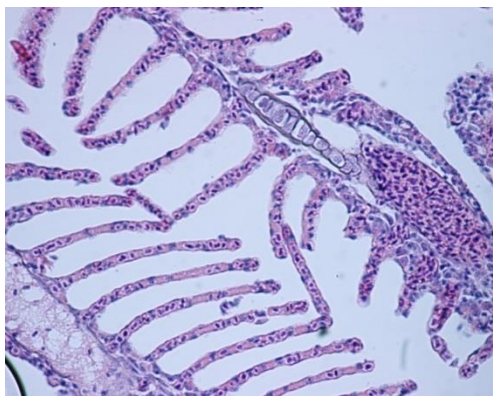


Figure 7: Histopathology for group C on Day 30 (HE, X400).

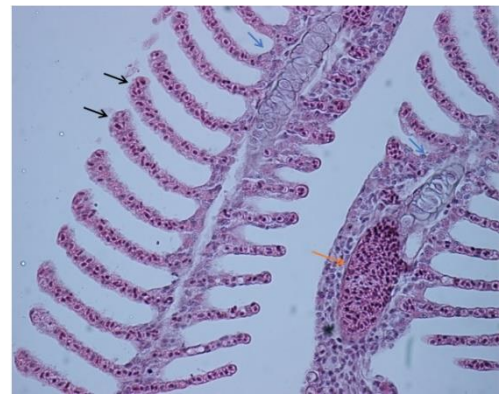


Figure 8: Histopathology for group T1 on Day 30. Red arrow: mild hyperemia. Black arrow: clubbed lamellae (HE, X400).

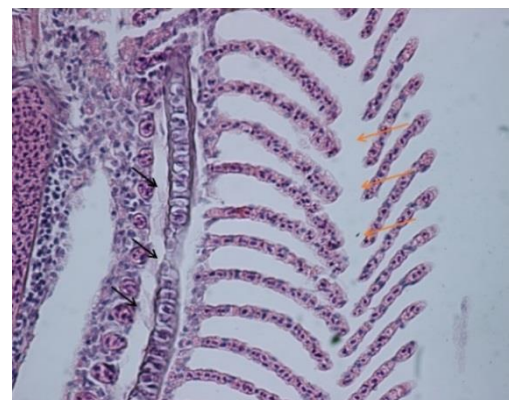


Figure 9: Histopathology for group T2 on Day 30. Black arrow: hyperemia. Orange arrow: clubbed tip in the secondary lamellae (HE, X400).

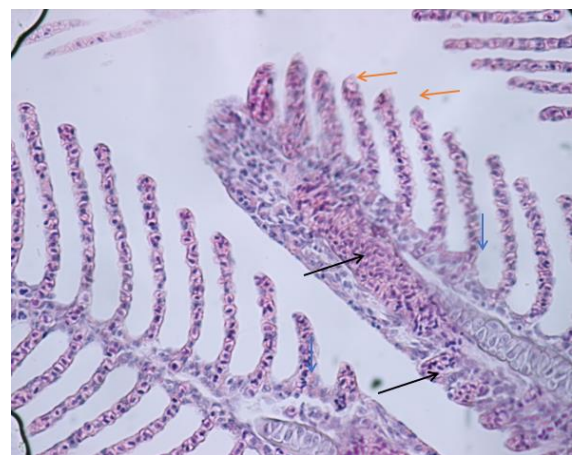


Figure 10: Histopathology for group T3 on Day 30. Black arrow: hyperemia. Blue arrow: concentrated chloride cells at the base of secondary lamellae. Orange arrow: clubbed tip in the secondary lamellae (HE, X400).

On Day 15, the histopathological results indicated normal liver tissue in group C (Figs. 11 and 12). Group T1 displayed hyperemia (Fig. 13), while group T2 exhibited hyperemia and fatty changes (indicated by the appearance of empty vacuoles in the hepatocyte cytoplasm) (Fig. 14). Group T3 showed both hyperemia and edema (Fig. 15).

On Day 30, the histopathological results indicated normal liver tissue in group C, mild hyperemia and

slight fatty changes in group T1, significant hyperemia and fatty changes in group T2, and hyperemia and fatty changes in group T3.

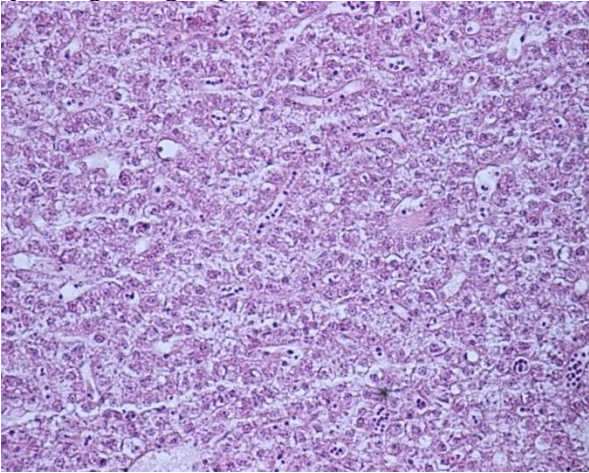


Figure 11: Liver histopathology for group C on Day 15. Mild hyperemia and slight fatty change (overview of the tissue and the appearance of empty vacuoles within the hepatocyte cytoplasm) (HE, X400).

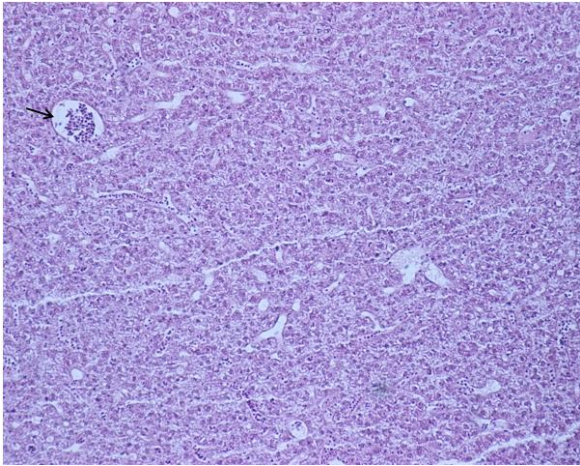


Figure 12: Liver histopathology for group C on Day 15. Black arrow: mild hyperemia and slight fatty change (HE, X400).

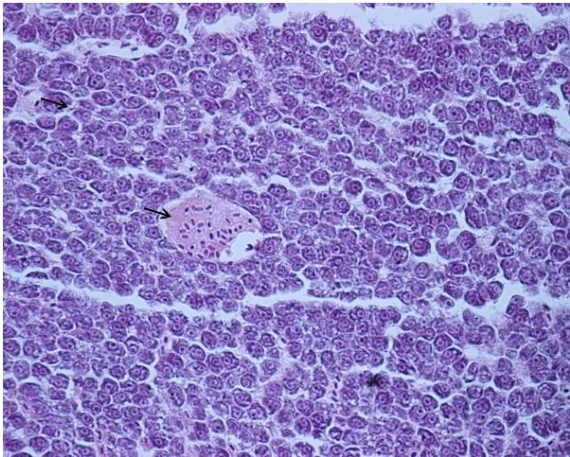


Figure 13: Liver histopathology for group T1 on Day 15. Black arrow: hyperemia (HE, X400).

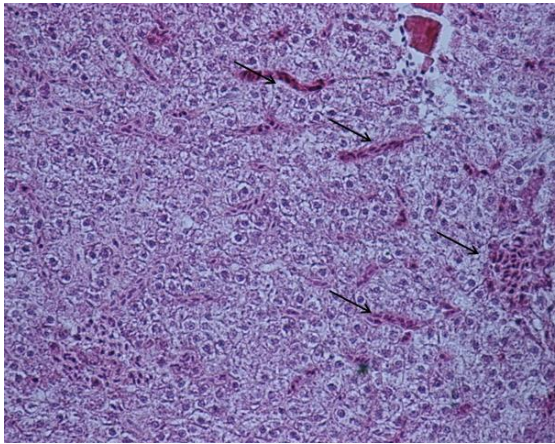


Figure 14: Liver histopathology for group T2 on Day 15. Black arrow: hyperemia and fatty changes (HE, X400).

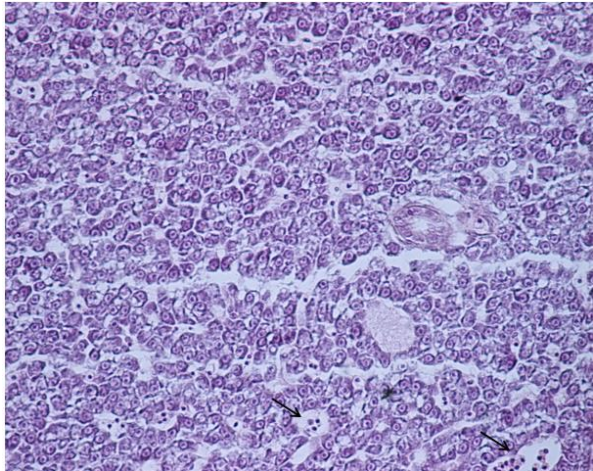


Figure 15: Liver histopathology for group T3 on Day 15. Black arrow: hyperemia and edema (increased intercellular space) (HE, X400).

Mortality rate:

Figure 16 illustrates the number of fish deaths on days 15 and 30. The results indicate that the mortality rate was directly related to the duration of exposure to the toxin, rather than its concentration. The highest mortality rate was observed in group T2 ($P < 0.05$).

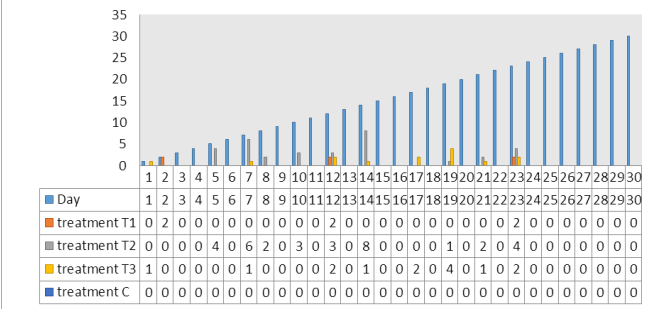


Figure 16: Number of fish deaths in the groups on days 15 and 30

Toxicity test for water:

Table 3 displays the average concentration of toxin in water on days 0, 15, and 30 for the four groups (T1, T2, T3, and CONTRL). An ANOVA test was conducted to compare the mean concentration of the toxin in water among the groups. The null hypothesis

of equal means was rejected for days 15 and 30, with a P-value of $0.000 < 0.05$, indicating a statistically significant difference among the groups on these days. To identify specific differences, means were further analyzed using the LSD test.

Table 3: ANOVA test results for comparing the mean concentration of the toxin in water among the groups.

Variable	Group	Mean	Sd.	F-value	Sig.
Day 0	C	.0000	.00000	-	-
	T1	.0000	.00000		
	T2	.0000	.00000		
	T3	.0000	.00000		
Day 15	C	.0000	.00000	1927.900	.000
	T1	103.0000	1.41421		
	T2	309.0000	1.41421		
	T3	551.0000	15.55635		
Day 30	C	.0000	.00000	3377.008	.000
	T1	47.0000	4.24264		
	T2	805.5000	6.36396		
	T3	1199.5000	27.57716		

Table 4 shows the significant differences among the groups (T1, T2, T3, and CONTRL) on days 15 and 30.

Table 4: LSD test results for comparing the mean concentration of the toxin in water among the groups.

Day	Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
15.00	C	T1	-103.0000*	7.84219	.000	-124.7734	-81.2266
		T2	-309.0000*	7.84219	.000	-330.7734	-287.2266
		T3	-551.0000*	7.84219	.000	-572.7734	-529.2266
	T1	C	103.0000*	7.84219	.000	81.2266	124.7734
		T2	-206.0000*	7.84219	.000	-227.7734	-184.2266
	T2	C	309.0000*	7.84219	.000	287.2266	330.7734
		T1	103.0000*	7.84219	.000	81.2266	124.7734
		T3	-551.0000*	7.84219	.000	-572.7734	-529.2266

Day	Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
30.00	T2	T3	-448.0000*	7.84219	.000	-469.7734	-426.2266
		C	309.0000*	7.84219	.000	287.2266	330.7734
		T1	206.0000*	7.84219	.000	184.2266	227.7734
		T3	-242.0000*	7.84219	.000	-263.7734	-220.2266
	T3	C	551.0000*	7.84219	.000	529.2266	572.7734
		T1	448.0000*	7.84219	.000	426.2266	469.7734
		T2	242.0000*	7.84219	.000	220.2266	263.7734
	C	T1	-47.0000*	14.30909	.030	-86.7284	-7.2716
		T2	-805.5000*	14.30909	.000	-845.2284	-765.7716
		T3	-1199.5000*	14.30909	.000	-1239.2284	-1159.7716
	T1	C	47.0000*	14.30909	.030	7.2716	86.7284
		T2	-758.5000*	14.30909	.000	-798.2284	-718.7716
		T3	-1152.5000*	14.30909	.000	-1192.2284	-1112.7716
30.00	T2	C	805.5000*	14.30909	.000	765.7716	845.2284
		T1	758.5000*	14.30909	.000	718.7716	798.2284
		T3	-394.0000*	14.30909	.000	-433.7284	-354.2716
	T3	C	1199.5000*	14.30909	.000	1159.7716	1239.2284
		T1	1152.5	14.3	.0	1112.	1192.

Day	Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
			0000*	0909	0.0	7716	2284
		T2	394.0000*	14.30909	.000	354.2716	433.7284

Toxicity test for tissue:

Table 5 presents the average concentration of the toxin in tissue on days 0, 15, and 30 for the four groups (T1, T2, T3, and CONTRL). An ANOVA test was performed to compare the mean concentration of the toxin in tissue among the groups. The null hypothesis of equal means was rejected for days 15 and 30, with a P-value of $0.000 < 0.05$, indicating a statistically significant difference among the groups on these days. To pinpoint the specific differences, means were analyzed using the LSD test.

Table 5: ANOVA test results for comparing the mean concentration of the toxin in tissue among the groups.

Variable	Group	Mean	Sd.	F-value	Sig.
Day 0	C	.0000	.00000	-	-
	T1	.0000	.00000		
	T2	.0000	.00000		
	T3	.0000	.00000		
Day 15	C	.0000	.00000	1738.567	.000
	T1	1.4500	.07071		
	T2	440.0000	14.14214		
	T3	94.5000	.70711		
Day 30	C	.0000	.00000	78024.954	.000
	T1	458.5000	2.12132		
	T2	918.0000	2.82843		
	T3	521.0000	1.41421		

Table 6 indicates a significant difference between the groups (except for T1 and CONTRL) on day 15. Furthermore, there is a statistically significant difference among all groups (T1, T2, T3, and CONTRL) on day 30.

Table 6: LSD test results for comparing the mean concentration of the toxin in tissue among the groups.

Day	Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
15.00	C	T1	-1.45000	7.07999	.848	-21.1072	18.2072
		T2	-440.0000*	7.07999	.000	-459.6572	-420.3428
		T3	-94.5000*	7.07999	.000	-114.1572	-74.8428
	T1	C	1.45000	7.07999	.848	-18.2072	21.1072
		T2	-438.5500*	7.07999	.000	-458.2072	-418.8928
		T3	-93.0500*	7.07999	.000	-112.7072	-73.3928
	T2	C	440.0000*	7.07999	.000	420.3428	459.6572
		T1	438.5500*	7.07999	.000	418.8928	458.2072
		T3	345.5000*	7.07999	.000	325.8428	365.1572
	T3	C	94.5000*	7.07999	.000	74.8428	114.1572
		T1	93.0500*	7.07999	.000	73.3928	112.7072
		T2	-345.5000*	7.07999	.000	-365.1572	-325.8428
30.00	C	T1	-458.5000*	1.90394	.000	-463.7862	-453.2138
		T2	-918.0000*	1.90394	.000	-923.2862	-912.7138
		T3	-521.0000*	1.90394	.000	-526.2862	-515.7138
	T1	C	458.5000*	1.90394	.000	453.2138	463.7862
		T2	-459.5000*	1.90394	.000	-464.7862	-454.2138
		T3	-62.5000*	1.90394	.000	-67.7862	-57.2138
	T2	C	918.0	1.90	.0	912.	923.

Da y	Gr oup (I)	Gr oup (J)	Mean Differ ence (I-J)	Std. Err or	Si g.	95% Confidence Interval	
						Low er Bou nd	Upp er Bou nd
			0000*	394	00	7138	2862
		T1	459.5 0000*	1.90 394	.0 00	454. 2138	464. 7862
		T3	397.0 0000*	1.90 394	.0 00	391. 7138	402. 2862
	T3	C	521.0 0000*	1.90 394	.0 00	515. 7138	526. 2862
		T1	62.50 000*	1.90 394	.0 00	57.2 138	67.7 862
		T2	- 397.0 0000*	1.90 394	.0 00	- 402. 2862	- 391. 7138

DISCUSSION:

The results indicated that butachlor poisoning caused several damages to the gills, including hyperemia, swollen epithelium in the secondary lamellae, concentrated chloride cells at the base of the secondary lamellae, vasodilation, hyperemia at the tip of the secondary lamellae, and clubbed tips in the secondary lamellae. These damages worsened with increasing toxin concentration and longer exposure duration. Histopathological studies showed that butachlor poisoning also resulted in hyperemia, fatty changes, and edema in the liver tissue, which worsened with increasing toxin concentration and longer exposure duration. Notably, during this study, no abnormal behaviors such as irregular swimming, breathing difficulties, unusual movements, or lethargy were observed in the test subjects. This contrasts with findings from various previous studies on different herbicides (Elmdoust et al. 2017). This difference could be explained by the subacute herbicide dosage used in this experiment.

Oxidative stress is known to contribute to the development of various diseases, including cancer, immune disorders, and neurodegenerative diseases (Almeida et al. 2011; Cao et al. 2020; Hu et al. 2019; Olivari et al. 2008; Zeng et al. 2009). It results from an imbalance between oxidation and antioxidant systems, leading to an accumulation of reactive oxygen species (ROS) that can cause cell apoptosis (Chowdhury et al. 2009).

Pesticides can induce oxidative stress, leading to the production of free radicals and lipid peroxidation, which are key mechanisms involved in pesticide toxicity (Wang et al. 2013; Valavanidis et al. 2006; Liu et al. 2008). Environmental pollutants can trigger oxidative stress by increasing ROS production or disrupting antioxidant enzymes and molecules (Andersson-Sj 2016; Cao et al. 2019; Ren et al. 2020; Rossaro and Cortesi 2013; Stainier 2001), although each organism has a unique antioxidant defense

mechanism, which primarily consists of both enzymatic and non-enzymatic components (Venancio et al. 2013). Therefore, measuring biochemical factors such as SOD, GSH, and MDA, has been recognized as crucial indices of the physiological status and primary immunity of fish. These factors are heavily influenced by diet, pollutants, and environmental stressors (Ansari & Ansari, 2014; Zhou et al., 2010). Butachlor is one such pollutant. Recent studies suggest that its toxic side effects may be linked to heightened ROS levels, which could account for the varied responses to poisoning (Farombi et al. 2008). In this context, numerous genes associated with the oxidative stress response have been identified and confirmed in zebrafish (Craig et al. 2007; Liu et al. 2008), underscoring the significance of this study, especially given reported changes in antioxidant enzymes in fish experiencing oxidative stress (Oruc and Usta 2007).

Another standard method used for determining water pollution level includes histopathological examinations of fish (Roncarati et al. 2006). Histological studies play a critical role in understanding the effects of environmental pollutants on aquatic animals (Soldatov 2005). Agricultural and industrial pollutants can cause histopathological changes in aquatic animals at sublethal concentrations (Velisek et al. 2009; Jayachandran & Pugazhendy 2009). These damages can serve as indices for evaluating how pollutants affect aquatic life and reflect the overall health of the aquatic ecosystem (Cengiz & Unlu 2006).

Among the studies on the effects of butachlor on aquatic animals, one can mention the studies by Piri et al. (1997) on *Daphnia magna*, investigations by Pazhand et al. (2005) regarding the lethal concentration (LC50/96H) of butachlor for *Acipenser Persicus* and *Acipenser Stellatus* Fingerlings, research by Kumari et al. (2009) on the physiological and biochemical effects of butachlor on *Aulosira feritissima*, and an examination by Farokhriz Lasheydani et al. (2009) of its impact on hematological factors of *Rutilus frisii kutum*.

Pathological studies have shown that butachlor poisoning can result in hyperemia, fatty changes, and edema in liver tissue, with damage increasing in severity as toxin concentration and exposure duration rise. The liver is a crucial organ that plays a vital role in detoxification, intermediary metabolism of proteins, fats, and carbohydrates, plasma protein synthesis, and bile secretion. Additionally, it detoxifies external compounds and toxins, such as herbicides; therefore, the structural integrity of the liver is essential for assessing fish health (Gholamian 2014). Although the liver helps to reduce toxic compounds, increased exposure to harmful substances can lead to significant structural damage to this organ (Cengiz & Unlu 2006; Velmurugan et al. 2009). This was also verified in the current study.

Contrary to this study, previous reports found that metabolites induced by butachlor can lead to severe liver necrosis (Ateeq et al. 2006). Necrotic tissue is a

clear indicator of tissue damage from pollutant exposure (Maxwell and Dutta 2005), which may result from the host's inability to regenerate liver cells after prolonged exposure or high doses of pollutants (Far et al. 2012; Forouhar Vajargah et al. 2018). However, necrosis was not observed in the liver and gill tissues in this study, likely due to the use of subacute toxicity concentrations, indicating that the zebrafish liver functions appropriately when exposed to sublethal concentrations of butachlor. Additionally, some studies have reported the accumulation of hemosiderin pigment in liver tissue due to hemoglobin catabolism caused by liver hemorrhage induced by butachlor (Gholamian 2014). This accumulation was not observed in our tissue sections, further suggesting reduced damage in the liver at subacute doses.

The gill tissue was also examined pathologically in this study. Butachlor poisoning resulted in swollen epithelium in the secondary lamellae, concentrated chloride cells at the base of these lamellae, vasodilation, hyperemia at the tip of the secondary lamellae, and clubbed tips in the secondary lamellae. These injuries intensified with higher toxin concentrations and longer exposure durations. In fish, gills are among the first organs, after the skin, to encounter toxins and water-soluble pollutants. Thus, studying the structural changes in gills is critical in toxicity discussions under stress conditions (Bais and Lokhande 2012). Given that gills are external organs, they are prime targets for pollutants, and damage can lead to widespread effects, causing respiratory disorders and osmotic and ionic imbalances (Smith et al. 2007). Belicheva and Sharova (2015) have noted that compensatory mechanisms in response to stress, including hyperplasia and edema of the gills, can function as a barrier that increases the distance between the pollutant and the bloodstream.

In light of these findings, it is essential for governments to enact regulations that restrict the production and use of chemicals recognized by the Convention, as well as their import and export by individuals and governmental bodies. Furthermore, many farmers struggle to comprehend the information provided on herbicide labels, a challenge attributed to factors such as the complexity of technical jargon and unfamiliarity with foreign languages. Illiteracy is another significant factor contributing to this issue. To address this problem, educational packages and briefings can be developed to enhance understanding.

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