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Comparative Toxic Effects of Lead, Chlorpyrifos, and Endosulfan on the Male Reproductive System of the Climbing Perch, *Anabas testudineus* as a Biomarker for Environmental Pollution

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Abstract

Environmental pollution from chemicals like pesticides, insecticides, and heavy metals significantly contributes to biodiversity loss. These chemicals, while targeting specific organisms, also harm non-target species and persist in the environment. This study investigated the comparative toxic effects of lead, chlorpyrifos, and endosulfan on the male reproductive system of the climbing perch, *Anabas testudineus*. Fish were exposed for 30 days to sublethal concentrations (one-tenth of the 96h LC50) of lead (30 ppm, T1), chlorpyrifos (0.38 ppm, T2), and endosulfan (1.69 ppb, T3). Several behavioural abnormalities were observed in all treatment groups compared to the control, including irregular opercular movements, loss of equilibrium, retarded swimming, irregular food intake, erratic swimming, and changes in skin colouration. Biochemical analysis revealed a significant decrease in total protein and glycogen concentrations in the testes of all exposed fish compared to the control group. The most substantial reductions in both protein and glycogen were observed in the endosulfan-treated fish. Histopathological examination of testicular tissue showed significant alterations, with prominent testicular histopathology in the endosulfan group (T3), including degenerated germinal epithelium, necrosis, vacuolated testicular lumen, distorted seminiferous tubules, decreased spermatid density, loss of Sertoli cells, and clumping of spermatogonia. Chlorpyrifos (T2) and lead (T1) exposure also showed visible histopathological changes in the testes, though less severe than those observed in the endosulfan group. These findings demonstrate the comparative toxicity of these pollutants on the reproductive system of *A. testudineus*, with endosulfan exhibiting the greatest impact, followed by chlorpyrifos and then lead.

Major Findings: The study highlights the value of integrating behavioural, biochemical, and histopathological evaluations for assessing environmental pollution and emphasizes the potential of using fish reproductive health as a sensitive biomarker for monitoring environmental contamination.

Keywords: Behaviour, Germinal Epithelium, Glycogen, Histopathology, Protein, Sertoli cell, Testes

1. Introduction

Ecosystems maintain a homeostasis essential for biodiversity. Within the freshwater ecosystem male reproductive system of fish has an important role in propagation and ecosystem equilibrium. Anthropogenic pollutants, like lead and pesticides, have infiltrated aquatic habitats and cause significant threats to fish reproductive health[1,2]. Heavy metals and pesticides adversely affect the survival, growth, well-being, and behaviour of

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fish[3-6]. Toxicity of heavy metals and pesticides on the male reproductive system of fish results in disrupted spermatogenesis and reproduction which ultimately leads to the loss of genetic diversity[7-11]. Environmental pollutants[12-14] adversely affect the hormonal regulation of the male reproductive system. Lead, a versatile metal widely used in industries like metal finishing, storage batteries, paints, and electroplating, is also a pervasive environmental pollutant[15]. Despite its industrial importance, lead poses significant toxicity risks, particularly to aquatic life. It disrupts various metabolic processes[16] and triggers energy-draining detoxification mechanisms, hindering fish growth. Additionally, lead acts as an endocrine disruptor and induces oxidative stress, damaging cell membranes[17]. Furthermore, it compromises the immune system of fish, making them more susceptible to diseases[18]. Chlorpyrifos an organophosphate pesticide, poses significant health risks due to its accumulation in fatty tissues or plasma albumin[19]. This accumulation is linked to a range of toxic effects, including hepatic and immunological alterations, genotoxicity, embryotoxicity, teratogenicity, and neurobehavioral problems[20]. CPF's toxicity stems from its ability to disrupt multiple biological processes. These include interfering with neurotransmitter receptors, interacting with various enzymes, obstructing signal transduction pathways, and hampering neuronal differentiation[19,21]. Freshwater and marine ecosystems are essential for the health and survival of a wide range of organisms[22]. However, these ecosystems are increasingly threatened by pollutants, including pesticides like endosulfan. Endosulfan, known to disrupt endocrine function, has been shown to negatively impact the reproductive system of female freshwater teleosts, such as *Anabas testudineus*. Studies have documented significant histological abnormalities in the brain, spleen, and ovaries of *A. testudineus* exposed to endosulfan[23-24], highlighting the potential for this pesticide to cause widespread damage in fish.

While previous studies have investigated the individual effects of these pollutants on fish, this study aims to provide a comparative analysis of their relative toxicity on the male reproductive system of *A. testudineus*, a widely distributed and ecologically important species and it specifically integrates behavioural observations with detailed biochemical and histopathological assessments for understanding of the combined impact of these common pollutants. Furthermore, by directly comparing the effects of these three pollutants under controlled conditions, the study aims to identify the most potent reproductive toxicant among them for this specific fish species. This information is crucial for targeted environmental monitoring and management strategies. Hence the present study aims to evaluate and compare the toxic effects of lead, chlorpyrifos, and endosulfan on the male reproductive system of the freshwater fish *A. testudineus* by assessing behavioural changes, biochemical parameters (protein and glycogen levels), and testicular histopathology.

2. Materials and Methods

Test organism *A. testudineus* was collected from Kayamkulam Lake of Alappuzha District in Kerala, India and carefully brought to the laboratory for acclimation. Prior to acclimation, fish were immersed in a 0.05% potassium permanganate solution for two minutes for the elimination of external pathogens. During the acclimatization period in chlorine-free water, fish were fed two to three times daily with laboratory-prepared food. Every 48 h for removing food debris and metabolic waste, a routine cleaning of the aquaria was conducted. Fish having 14-15 cm in total length and weighing 45-50 g were selected for the experiment.

2.1 Physicochemical Parameters of the Water Used for Rearing

The average water quality parameters during the study were temperature 24.53 ± 1.07°C, pH - 7.6 ± 0.03, dissolved oxygen -t 7.61 ± 0.42 mg/L and carbon dioxide concentration - 6.8 ± 0.3 mg/L.

2.2 Experimental Design

Prior to the experiment, all aquaria (40 L capacity) were disinfected using potassium permanganate ($KMnO_4$) and filled with 40 L seasoned tap water, devoid of chlorine. Three aquaria were designated for each chemical (lead, chlorpyrifos and endosulfan) and one served as control. Sublethal concentrations were determined in this study based on previously established 96-hour LC_{50} values. These values, determined in our laboratory, are 300 ppm for lead, 3.82 ppm for chlorpyrifos, and 16.90 ppb for endosulfan. One-tenth of the 96-h LC_{50} of each chemical – lead 30 ppm (T1), chlorpyrifos 0.38 ppm (T2), endosulfan 1.69 ppb (T3) was selected for study and eight fish in three replicates were exposed to the sublethal concentrations

for 30 days. This experimental approach allowed for the assessment of behavioural, biochemical and histological changes resulting from chronic exposure to different chemical toxicity.

2.3 Observation of Behavioral Changes

A semi-quantitative approach[25,26] was followed for recording and quantifying the behavioural abnormalities linked to feeding, opercular movement, and swimming.

2.4 Biochemical Estimation

After the experimental period of 30 days in sublethal concentrations of lead, chlorpyrifos, and endosulfan, the testes were carefully dissected for the estimation of protein by Folin-phenol reaction method[27] and Glycogen using the Anthrone reagent method[28]. The results have been expressed as milligrams per 100 gram (mg/100g) of wet weight of gonad tissues.

2.5 Preparation of Tissue for Light Microscopy Studies

After the experiment period of 30 days in sublethal concentration of chemicals live fishes were euthanized using Lidocaine hydrochloride (400 mg/L) and testes were removed and fixed in Neutral Buffered Formalin (NBF) for two days. Following fixation, the tissues underwent dehydration through immersion in a series of alcohol solutions with increasing concentrations, culminating in pure, water-free alcohol (70%, 90%, and 100%). Subsequent clearing was accomplished using methyl benzoate. To ensure thorough infiltration, the fixed tissues were subjected to two changes of wax, each lasting 1 hour at 60°C (the melting point of paraffin being 58–60°C). Tissues were embedded in paraffin wax for moulding to blocks and thin sections (5 µm) were cut and stained by using haematoxylin and eosin. Stained sections were observed under a compound research microscope, and photomicrographs were captured using a photomicrography system for documentation of histological structures and alterations.

2.6 Statistical Analysis

The data were analysed using SPSS computer statistical software (version 21). All replicates were used for the calculation of mean values. Differences in biochemical parameters between different chemicals were processed statistically using Analysis of Variance (One-way ANOVA). The biochemical parameters were expressed as means ± standard error.

3. Result

Anabas testudineus exposed to sublethal concentrations (one-tenth of 96 h LC_{50}) of lead, chlorpyrifos, and endosulfan for 30 days displayed various behavioural abnormalities. Histology of the testis of treated fish revealed significant alterations, in addition to the decrease in protein and glycogen content of the male reproductive organ.

3.1 Behavioral Irregularities

The control group exhibited no behavioural abnormalities. The noticeable behavioural changes linked to feeding, opercular movement, and swimming were prominently observed in all treatment groups. The majority of these behavioural irregularities were particularly intense in the T2 and T3 treatments by the end of the 30-day trial period (Table 1).

3.2 Biochemical Parameters

The testis total protein and glycogen concentrations were significantly reduced ($p \leq 0.01$) in fish exposed to chemicals (Figures 1, 2). Endosulfan exposure resulted in the greatest reductions in both parameters, followed by chlorpyrifos and lead. The decrease in total protein concentration in chlorpyrifos-exposed fish surpasses that in lead-exposed fish by 9.07%, and in endosulfan exposure, it's higher by 20.82%.

Similarly, the reduction in total glycogen concentration in fish exposed to chlorpyrifos exceeds that in those exposed to lead by 20.6%, and in endosulfan exposure, it's higher by 32.52%.

3.3 Histopathological Studies

Aquatic ecosystems and their inhabitants are constantly facing contamination by heavy metals and pesticides. Examination of the testicular histology of *A. testudineus* exposed to sublethal concentrations of lead, chlorpyrifos, and endosulfan unveiled distinct changes in histological architecture. Control sections were normal without any histological alterations (Figure 3). Testicular histopathology is more prominent in fish exposed to endosulfan with degenerated germinal Epithelium (DE),

Table 1. Behavioural irregularities of fish exposed to sublethal concentrations of lead, chlorpyrifos and endosulfan for 15 days and 30 days

Chemicals used	Exposure period	Behavioural irregularities					
		Loss of equilibrium control	Irregular food intake	Irregular opercular movements	Retarded swimming	Change in skin colouration	Erratic swimming
T1 (Lead)	15 days	-	*	*	*	*	**
T2 (Chlorpyrifos)		-	*	*	**	**	**
T3 (Endosulfan)		-	*	**	**	**	**
Control	-	-	-	-	-	-	-
T1 (Lead)	30 days	**	**	**	***	**	***
T2 (Chlorpyrifos)		**	***	***	***	***	***
T3 (Endosulfan)		***	***	***	***	***	***

-Normal, * moderate, ** high, ***severe

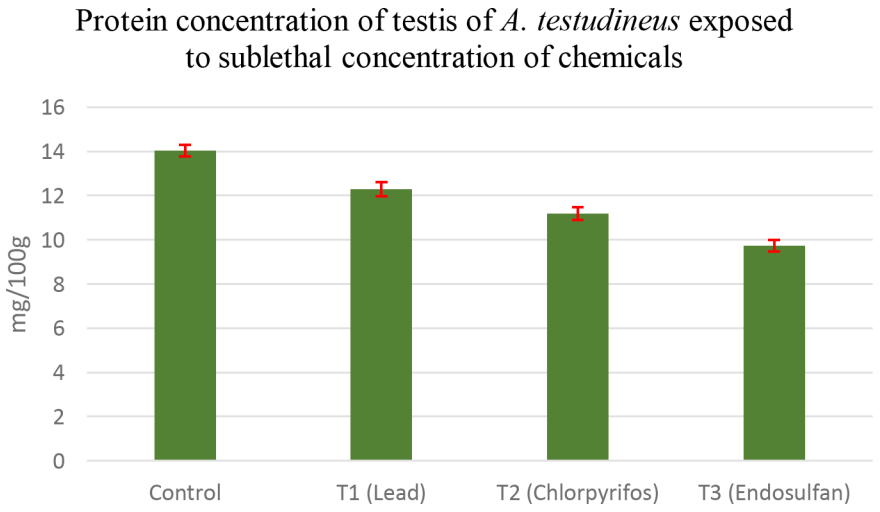


Figure 1. Protein concentration of testis of *A. testudineus* exposed to sublethal concentration of chemicals.

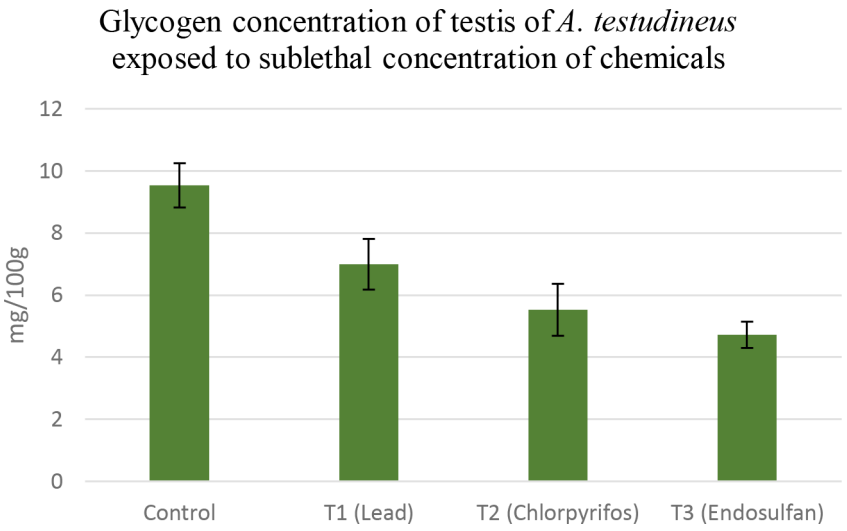


Figure 2. Glycogen concentration of testis of *A. testudineus* exposed to sublethal concentration of chemicals.

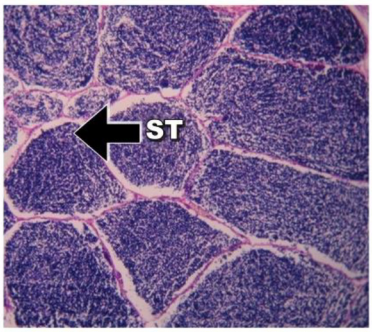


Figure 3. Control – testis *A. testudineus* without any histological alterations.

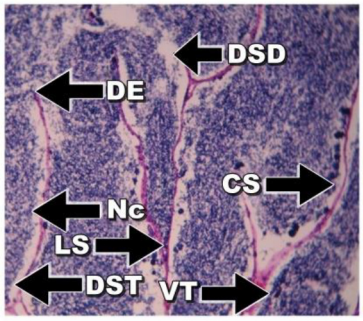


Figure 4. Testicular histopathology of fish exposed to endosulfan with degenerated Germinal Epithelium (DE), Necrosis (Nc), Vacuolated Teticular Lumen (VT), Distorted Seminiferous Tubule (DST), Decrease Spermatid Density (DSD), Loss of Sertoli cells (LS) and Clumping of Spermatogonia (CS).

Necrosis (Nc), Vacuolated Teticular lumen (VT) Distorted Seminiferous Tubule (DST) Decrease Spermatid Density (DSD), Loss of Sertoli cells (LS) and Clumping of Spermatogonia (CS) (Figure 4). In chlorpyrifos exposed fish testis histology shows Vacuolation (V), Necrosis (Nc), distorted seminiferous tubule (DST) and clumping of spermatogonia (CS) (Figure 5). Lead-exposed fish, histology of testes revealed vacuolation (V), necrosis (Nc) and Distorted Seminiferous Tubule (DST) (Figure 6).

4. Discussion

Environmental contamination by heavy metals and pesticides poses a significant threat to aquatic ecosystems and the health of their inhabitants. In this comparative study, *A. testudineus* exposed to sublethal concentrations of lead, chlorpyrifos, and endosulfan exhibited a range of adverse effects, including behavioural abnormalities,

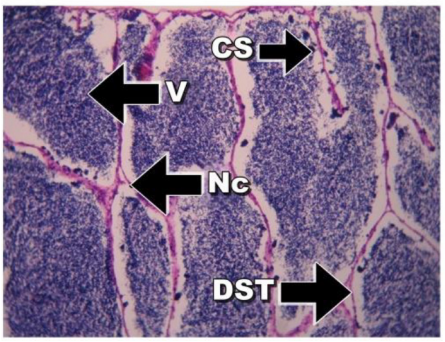


Figure 5. Chlorpyrifos exposed fish testis histology shows Vacuolation (V), necrosis (Nc), Distorted Seminiferous Tubule (DST) and Clumping of Spermatogonia (CS).

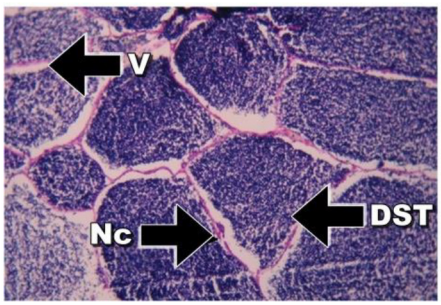


Figure 6. Lead-exposed histology of the testis reveals vacuolation (V), necrosis (Nc) and Distorted Seminiferous Tubule (DST).

reductions in testicular protein and glycogen levels, and pronounced histopathological changes in the testes. These findings emphasize the vulnerability of fish reproductive systems to these common pollutants.

Behavior alterations, unusual movements, and physiological and metabolic disruptions in fish are induced by these toxicants[29]. Behavioural alterations are often sensitive indicators of toxicant exposure in fish[26]. In the present study, *A. testudineus* exposed to all three chemicals displayed noticeable behavioural changes, including irregular opercular movements, loss of equilibrium, retarded swimming, irregular food intake, erratic swimming, and changes in skin colouration. The most severe effects were observed in the chlorpyrifos and endosulfan groups. These observations align with previous research demonstrating that heavy metals and pesticides can disrupt normal fish behaviour, including locomotion, feeding, and social interactions[30-33]. Such behavioural changes can impair a fish's ability to find food, avoid predators, and reproduce, ultimately impacting population dynamics.

In the present study, a remarkable decline was observed in the total protein and glycogen concentration of testis of *A. testudineus* exposed to sublethal concentrations of lead, chlorpyrifos, and endosulfan for 30 days. The significant decrease in testicular protein and glycogen concentrations observed in this study further highlights the disruption of physiological processes by the tested chemicals. Proteins play crucial roles in various cellular functions, including enzyme activity, structural support, and hormone regulation. Reductions in protein levels can indicate impaired protein synthesis or increased protein catabolism, often associated with stress responses and detoxification processes[34,35]. *Channa punctate* exposed to toxicants showed changes and reductions in protein levels due to the inhibition of metabolizing enzymes[36]. The present study aligns with the findings of sub-lethal malathion exposure of *Cirrhinus mrigala* for varying durations[37] which resulted in a significant decrease in total, structural and soluble protein content in various tissues of fish. Our results are also supported by a reduction in protein content in *Cyprinus carpio*[38], *Anabas testudineus*[39], and in *Labeo rohita*[34] upon exposure to malathion.

Glycogen levels of testis of *A. testudineus* exposed to lead and pesticides in the current study showed a decrease in all chemicals which indicates a disruption in the physiological activity of fish. During stress conditions, there is an increased breakdown of glycogen into glucose via glycogenolysis in fish tissues[40] which is controlled by catecholamines and adrenocortical hormones[41]. In stress exposure there is increased utilization of glycogen that provides glucose which undergoes breakdown via the glycolytic pathway to generate ATP, a high-energy compound[34,42,43] to meet energy demands. The observed decrease in glycogen levels in the present study suggests a disruption in energy metabolism, potentially due to increased energy demands for detoxification or reduced energy intake due to behavioural changes[40,41]. These findings correlate with other studies that have reported decreased protein and glycogen levels in fish tissues exposed to various toxicants, including pesticides and heavy metals[37-39]. The observed reductions in protein and glycogen in *A. testudineus* testes suggest a disruption of energy allocation and utilization due to toxicant exposure. This is consistent with the general stress response in fish, where energy reserves are mobilized to cope with the toxic insult[44]. Our findings are also supported by similar toxicity studies with lead and pesticides in the male reproductive system of fish which showed alterations in the production and activity of reproductive hormones such as testosterone that negatively affect the reproductive health and function of male fish[45,46].

Exposure to all three chemicals resulted in varying degrees of testicular histological alteration. Lead exposure induced vacuolation, necrosis, and distorted seminiferous tubules. The observed effects of lead on the testes of *A. testudineus* are consistent with the known toxic mechanisms of this heavy metal. Lead can disrupt spermatogenesis by interfering with germ cell proliferation and differentiation, inducing oxidative stress, and damaging DNA[47-49]. Lead exposure can disrupt the process of spermatogenesis in fish, leading to decreased sperm production and impaired fertility[48]. In the testis of *Channa, punctatus*[50] lead interferes with the proliferation and differentiation of germ cells ultimately reducing sperm count and quality[51,52]. Generation of reactive oxygen species and oxidative damage to sperm cells are induced by lead in male fish[49]. Hence for ensuring the sustainability of aquatic ecosystems, addressing lead pollution and minimizing lead exposure levels are crucial steps.

Chlorpyrifos exposure resulted in similar effects, along with clumping of spermatogonia. Similar to our findings, chlorpyrifos interferes with germ cell development in the testis of *Oreochromis mossambicus*[53] and *Danio rerio*[54]. Chlorpyrifos exposure results in alterations in the production and activity of reproductive hormones such as testosterone that can impair reproductive function, including sperm production, maturation, and motility[55]. Chlorpyrifos, an organophosphate insecticide, can disrupt reproductive function by inhibiting acetylcholinesterase activity, altering hormone levels, and inducing oxidative stress[56].

Endosulfan exposure, however, led to severe histopathology, characterized by degenerated germinal epithelium, vacuolated testicular lumen, decreased spermatid density, loss of Sertoli cells, and clumping of spermatogonia. These histopathological changes provide direct evidence of the toxic effects of these chemicals on the reproductive tissues of fish. Such damage can impair spermatogenesis, reduce sperm quality and quantity, and ultimately compromise reproductive success[12-14]. The severity of the effects observed in this study, particularly with endosulfan, emphasizes the importance of minimizing exposure to these chemicals in aquatic environments. The observed degeneration of the germinal epithelium and loss of Sertoli cells in the

endosulfan-exposed fish are particularly concerning, as these cells are essential for spermatogenesis and sperm maturation. Endosulfan can disrupt the hormonal balance in fish, including alterations in the synthesis and function of reproductive hormones such as testosterone that can affect sperm maturation, motility, and viability[57].

Previous studies have documented similar histopathological changes in fish testes exposed to lead, chlorpyrifos, and endosulfan, further supporting the present findings[53,54,58,59]. The comparative approach employed in this study allows for a direct assessment of the relative toxicity of these three pollutants on the reproductive system of *A. testudineus*. The results indicate that endosulfan exhibits the most potent toxic effects, followed by chlorpyrifos and then lead. This finding highlights the need for careful regulation and monitoring of endosulfan use in areas where it may enter aquatic ecosystems. The use of *A. testudineus* as a bioindicator species in this study highlights its potential for monitoring aquatic pollution and assessing the health of freshwater ecosystems. The observed behavioural, biochemical, and histopathological changes provide a suite of sensitive endpoints for evaluating the impact of pollutants on fish reproductive health.

5. Conclusion

This study demonstrates the comparative toxic effects of lead, chlorpyrifos, and endosulfan on the male reproductive system of *A. testudineus*. Exposure to sublethal concentrations of these common pollutants resulted in adverse effects, including behavioural abnormalities, reduced testicular protein and glycogen levels and significant histopathological damage to the testes. Endosulfan proved to be the most potent reproductive toxicant among the three, followed by chlorpyrifos and then lead. Environmental pollution by heavy metals and pesticides is a serious threat to the sustainable aquatic ecosystem. In the present study exposure of *A. testudineus* to lead, chlorpyrifos and endosulfan reveals disruption of spermatogenesis and alterations in biochemical and histological parameters of testes that can collectively hinder the reproductive health and function of male fish which ultimately results in fish population sustainability. These findings highlight the sensitivity of fish reproductive health to environmental contamination and emphasize the potential for these pollutants to disrupt reproductive processes and threaten fish populations. The integrated approach employed in this study, combining behavioural, biochemical and histopathological assessments, provides a comprehensive framework for evaluating the impact of environmental pollutants on fish health. *A. testudineus* serves as a valuable bioindicator species for monitoring aquatic pollution and the results in this study offer a sensitive means of assessing the health of freshwater ecosystems. These results emphasize the urgent need for stricter regulations on the use and release of these chemicals, as well as the development of alternative pest management strategies to protect aquatic biodiversity and ensure the long-term sustainability of fish populations.

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