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Potential of Antioxidant Enzymes as Biochemical Biomarkers for Glyphosate-Based Herbicide in *Oreochromis niloticus*

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ABSTRACT

The current study examined the toxicity of Roundup, which is a herbicide containing glyphosate that is commonly used in agriculture, on *Oreochromis niloticus*, a type of freshwater fish. The researchers investigated the effectiveness of using antioxidant enzymes as biochemical biomarkers to assess the potentially toxic impact of Roundup on the fish. The sub-lethal concentration of Roundup in tilapia is 1.05 mg/l for 96 hours of exposure. The fish were exposed to sub-lethal concentration (36.8 to 46.9 mg/L) of Roundup for a period of 24, 48, 72, and 96 hours, and their antioxidant enzyme activities were measured, respectively. According to the findings, Roundup exposure led to a noticeable rise in the levels of superoxide dismutase, catalase, and peroxidase activities in both the liver and gill tissues of the fish, which suggested the occurrence of oxidative stress. These changes in antioxidant enzyme activities were concentration-dependent, suggesting that Roundup exposure can induce oxidative damage in fish. The study concluded that Roundup affects the enzymatic activity of both organs in a concentration- and time-dependent manner. Catalase and peroxidase activities were generally inhibited, while superoxide dismutase activity was affected differently depending on the organ, concentration, and time of exposure.

Keywords: biochemical, biomarkers, enzyme, fish, gills, liver

1. INTRODUCTION

Due to the fact that approximately 70% of Pakistan's population relies on the agricultural sector, there has been a significant increase in the use of

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pesticides over the last 30 years to meet the growing demands of the population [1]. The accessibility and affordability of these pesticides have led to their indiscriminate use, resulting in significant ecological changes in major freshwater resources such as water, sediment, and fish [2]. Glyphosate, an organophosphorus herbicide, is currently the most commonly used herbicide on a global scale [3]. It is marketed under numerous brands and commercialized in over 100 countries worldwide [4]. The primary toxic component of formulated glyphosate-based products for aquatic organisms are surfactants like POEA found in Roundup. Several previous studies have suggested that pesticides can cause alterations in the water present in the ponds surrounding the cultivation regions, as well as rainwater and groundwater [5] through mechanisms such as volatilization, spray drift, surface runoff, chemical-biological degradation, leaching, and sorption [6].

Antioxidant enzymes, including CAT, POD, and SOD, are important in fish because they protect the fish from oxidative stress caused by factors such as pollution and diseases. These enzymes help to neutralize harmful free radicals and reactive oxygen species, which can damage cells and tissues. Additionally, these enzymes play a crucial role in the overall health and survival of fish, as oxidative stress can lead to a range of negative effects, including reduced growth rates, decreased immune function, and increased mortality. Therefore, understanding the role of antioxidant enzymes in fish can help to inform efforts to protect and maintain healthy fish populations [7].

There is limited information available in the previous literature regarding the harmful effects of glyphosate on organisms exposed to it in their natural environment. However, certain synthetic agrochemical compounds like glyphosate may become more toxic to non-target species due to differences in their bioactive properties. These compounds often cause sub-lethal damage to tissues, disrupt endocrine function, induce immunotoxicity and neurotoxicity, and alter the metabolism of the affected animals [8]. According to Naz et al. [9], industrial and agricultural water sources release a range of toxins into both rivers and canals, which then accumulate in sand. This accumulation of toxins in aquatic environments has emerged as a global issue, leading to health issues for both humans and aquatic animals [10]. Nile tilapia (*Oreochromis niloticus*) is a popular commercial fish species and is often used as a bio indicator to assess the

quality of aquatic ecosystems, particularly for pollutants such as herbicides [11]. It is also considered a suitable biological model for toxicological studies due to its sensitivity to diazinon, an organophosphate pesticide herbicides [12]. Aquatic organisms exposed to pesticides often experience oxidative stress, which is caused by the production of ROS in response to toxicants [13]. The effects of lufenuron exposure on *Oreochromis niloticus* were examined through histopathological analysis of various organs, including the gills, kidneys, livers, brain, and hearts. The findings revealed severe alterations and indicated that lufenuron caused DNA damage and oxidative stress in the fish. The study aimed to investigate the mechanisms behind lufenuron's toxicity, as well as to raise concerns about the use and disposal of lufenuron in terms of its potential to cause environmental pollution. The study also proposed measures to mitigate contamination in aquatic populations [14]. The comet assay, also known as Single Cell Gel Electrophoresis (SCGE), as a genotoxicity technique that detects DNA damage caused by alkylating chemicals, intercalating agents, and oxidizing agents. The comet assay is a useful method for monitoring genotoxicity in aquatic environments, where fish are often used as test organisms to detect DNA damage resulting from the ingestion of mutagens and pro-mutagens in freshwater and saltwater. This technique has also been employed to determine the genotoxic potential of water resources such as rivers and lakes. The purpose of the current study was to investigate the adverse effects of lufenuron exposure on various health biomarkers of *Oreochromis niloticus* [14].

The objective of the current study was to examine the Roundup exposure affects oxidative stress in fish by assessing variations in the activities of antioxidant enzymes. The aim wato obtain an understanding of the possible adverse impacts of Roundup exposure on the well-being of freshwater fish and the general health of aquatic ecosystems. The aim of the research is to increase our knowledge about the dangers related to the usage of herbicides that contain glyphosate in freshwater ecosystems. Additionally, it seeks to lay the groundwork for more extensive investigations into the lasting consequences of Roundup exposure on aquatic creatures such as fish.

2. MATERIAL AND METHODOLOGY

At the laboratory of Government Sadiq College Women University in Bahawalpur, Pakistan, an experimental study was conducted, adhering to

the ethical principles for laboratory animal usage set by the Ethical Committee of Government Sadiq College Women University Bahawalpur.

2.1. Sample Collection and Management

Thirty freshwater fish of the species *Oreochromis niloticus*, with a weight range of 75-80g, were procured from the nearby Fish Seed Hatchery in Bahawalpur. The fish were fed with the commercial feed palettes. These fish were transferred to the Zoology department testing laboratory of Government Sadiq College Women University Bahawalpur in plastic bags equipped with enough oxygen. The fish were subsequently relocated to glass tanks filled with 100 liters of water. The experiment employed five 100L tanks for each concentration, with each tank measuring 36 inches in length and 12 inches in width, housing six fish in each tank. Prior to commencing the experiment, the fish were given a period of 15 days to acclimate. To ensure that the fish had enough oxygen, continuous air pumps were utilized. The temperature of the aquarium water was kept constant, and measurements of the water's physical properties were taken before and after the study, as outlined in Table 1. The study was carried out in compliance with the ethical standards set forth by the Ethical Committee of Government Sadiq College Women University Bahawalpur.

Table 1. Physical Parameters of Water

Parameters	Values
Dissolved oxygen (mg/L)	6.70±0.1
Electrical conductivity at 25°(µmhos/cm)	393.10±2.2
PH	6.89±0.05
Total dissolved solid (mg/L)	153.89±3.9
Total hardness (CaCO3, mg/L)	174.59±2.1
Water temperature (°C)	23.80±0.3

2.2. Experimental Design

During the acclimation period, 30 fish [14] were distributed randomly into the control group (without exposure) and four experimental groups, which were subjected to different concentrations of Roundup original (46.9, 44.4, 40.0, and 36.8 mg L−1). The selection of these concentrations was informed by previous research, which examined the impact of acute

exposure to Roundup® at comparable concentrations in other fish species [15].

2.3. Evaluation of Antioxidant Enzymes

After exposure, fish from each group were euthanized at different time points (24, 48, 72, and 96 hours), and their gills and liver were extracted. Briefly, for removal of erythrocytes, all the organs were washed using phosphate buffer solution having 6.5 PH. After that all the organs were triturated using 0.2 mL normal saline and then homogenate was prepared (1:4 w/v) by adding cold buffer. The homogenate from each tissue was centrifuged (10000 rpm) at 40C for 15 minutes. After centrifugation, the supernatants were removed and placed at -800C for enzyme profiling. By using UV-visible spectrophotometer, the amount of (reactive oxygen species) ROS in the gills and liver of each fish was measured by different antioxidant enzymes, which were such as peroxidase [16], catalase [17], and superoxide dismutase (SOD) were measured according to previous protocols [16]. Additionally, the gill tissue homogenates from individual fish, which were used to quantify the level of antioxidant enzymes, such as catalase, superoxide dismutase (SOD), and peroxidase described by [16, 17].

At the Zoology Lab of the Department of Zoology, Government Sadiq College Women University, Bahawalpur, 36 fish samples were carefully dissected for the laddering assay. The dissection process was performed with great care to avoid contamination and the fish muscles were preserved in plastic bottles containing 0.9% saline solution. The bottles were properly labeled and stored at a temperature of -20^{o}C, with plans to process the samples within two weeks. It is advisable to store muscle tissues at a temperature of -20^{o}C or lower for future use, as this can enhance their usability [18].

2.4. DNA Extraction and Laddering assay

The Phenol Chloroform Isoamyl method was used to extract DNA from muscle tissue samples weighing 100-200 milligrams [18]. The samples were homogenized and mixed with 1 ml of lysis buffer containing 1 M Tris, 5 M NaCl, 0.5 M EDTA, 10% SDS, and 20 µl of Proteinase K. The mixture was then incubated overnight in a water bath at 50 degrees Celsius. On the following day, 1 ml of PCI (phenol chloroform isoamyl alcohol) was added in the prepared solution and the contents were centrifuged at 15,000 rpm for

10 minutes. The supernatant was transferred to fresh tubes and 20 µl of glycogen blue and 1 ml of chilled isopropyl alcohol were added in the mixture. The mixture was frozen overnight at 30 degrees Celsius and centrifuged at 15,000 rpm for 10 minutes. The resulting DNA pellet was washed three times with 70% ethanol and later dried for 15 minutes at the room temperature. Finally, 100 µl of injection water was added to the dried DNA pellet and the extracted DNA was stored at -8 degrees Celsius. To identify any genomic DNA damage, the extracted DNA was separated at 1% agarose gels and visualized with ethidium bromide (6 µg/ml) under 300 nm UV light.

2.5. Statistical analysis

SPSS software (version 15) was utilized for statistical analysis and ANOVA was employed to evaluate the significance of differences between the Roundup-treated and untreated fish groups. The results were expressed as mean ± SE, and a significance level of $P<0.05$ was adopted.

3. RESULTS

3.1. Antioxidant enzyme activities

The study investigated the impact of various pesticide doses on the antioxidant enzymes in different organs of fish, such as the liver and gills. The results revealed that the levels of catalase [17] and superoxide dismutase (SOD) were significantly lower in the heart, gills, liver, and brain tissues of fish exposed to different concentrations of insecticides as compared to untreated fish. Conversely, fish, which were exposed to the same concentrations of insecticides had higher levels of peroxidase [16] in their gills, liver, and brain tissues than those, which were untreated. According to the results of the study, there was a significant decrease in the levels of antioxidant enzymes (POD, CAT, and SOD) in the gills of fish exposed to Roundup GBH at concentrations of 0.80, 1.30, 1.90, 2.50 µg/L mg L^{-1} for 24, 48, 72, and 96 hours as compared to the control group. The fish in group-E exhibited a substantial reduction in SOD and CAT levels in their liver tissue after 24 hours of exposure. Meanwhile, groups D-E demonstrated lower SOD and CAT levels in their liver tissue after 48 and 72 hours of exposure as compared to the control group. Furthermore, the concentration of peroxidase in the liver, kidneys, and gills tissues of treated fish in groups D-E was significantly lower ($p < 0.05$) than in the non-treated fish. All results were presented as mean ± SE, and statistical significance

was assessed using one-way ANOVA analysis with a significance threshold of $p<0.05$. (Table 2, 3).

Table 2. Different Antioxidant Enzymes in Liver of Fish *Oreochromis niloticus*

Parameter/hour	Groups				
	A (control)	B (0.80ppm)	C (1.30 ppm)	D (1.90ppm)	E (2.50)
Catalase (units/min)					
24	4.92 ± 0.02	6.67 ± 0.02	4.72 ± 0.02	6.54 ± 0.01	5.67 ± 0.06*
48	4.83 ± 0.03	5.64 ± 0.03	4.46 ± 0.04	5.61 ± 0.08*	5.38 ± 0.07*
72	4.56 ± 0.06*	5.78 ± 0.01	6.47 ± 0.03	4.91 ± 0.04	4.62 ± 0.06*
96	5.61 ± 0.05*	5.64 ± 0.04	5.78 ± 0.06*	4.56 ± 0.07*	4.90 ± 0.03
Superoxide dismutase (units/mg)					
24	12.98 ± 0.11	12.91 ± 0.01	12.86 ± 0.01	11.67 ± 0.05	9.11 ± 0.03*
48	13.04 ± 0.03	12.75 ± 0.06	12.50 ± 0.02	9.20 ± 0.49*	9.00± 0.05*
72	13.07 ± 0.05	12.80 ± 0.04	12.29 ± 0.05	8.22 ± 0.29*	8.37 ± 0.01*
96	11.91 ± 0.07*	13.45 ± 0.02	11.77 ± 0.05	9.41 ± 0.07*	9.45 ± 0.01
Peroxidase (units/min)					
24	5.21 ± 0.01	6.11 ± 0.02	5.07 ± 0.02	4.79 ± 0.02*	4.23 ± 0.01*
48	5.20 ± 0.05	6.12 ± 0.02	2.96 ± 0.02	2.61 ± 0.03*	4.11 ± 0.01*
72	5.23 ± 0.02	6.09 ± 0.08	2.85 ± 0.04	2.24 ± 0.04*	4.02 ± 0.01*
96	3.94 ± 0.02	6.07 ± 0.05	5.19 ± 0.02	5.71 ± 0.01	3.10 ± 0.03

Table 3. Enzymes in Gills of Fish *Oreochromis niloticus*

Parameter/hour	Groups				
Catalase (units/min)	A (control)	B (0.80)	C (1.30 ppm)	D (1.90 ppm)	E (2.50)
24	3.23 ± 0.00	3.25 ± 0.01	3.18 ± 0.03	3.68 ± 0.01	3.34 ± 0.04*
48	3.42 ± 0.00	3.11 ± 0.02	3.17 ± 0.02	2.83 ± 0.06*	3.27 ± 0.03*
72	3.06 ± 0.00	2.87 ± 0.01	3.47 ± 0.06	3.23 ± 0.04*	3.01 ± 0.01
96	3.11 ± 0.00	3.36 ± 0.02	3.12 ± 0.02	3.45 ± 0.01	2.26 ± 0.01
Superoxide dismutase (units/mg protein)					
24	5.85 ± 0.04	5.73 ± 0.04	5.63 ± 0.01	4.89 ± 0.10	5.64 ± 0.04*
48	5.79 ± 0.03	5.56 ± 0.01	5.88 ± 0.03	4.16 ± 0.03*	5.29 ± 0.01*
72	5.93 ± 0.04	5.94 ± 0.02	6.26 ± 0.01	4.99 ± 0.04*	5.48 ± 0.11*

Parameter/hour	Groups				
Catalase (units/min)	A (control)	B (0.80)	C (1.30 ppm)	D (1.90 ppm)	E (2.50)
96	5.97 ± 0.03	6.45 ± 0.02	5.35 ± 0.02	5.02 ± 0.05*	4.96± 0.09*
Peroxidase (units/min)					
24	1.42 ± 0.01	1.29 ± 0.01	1.22 ± 0.01	1.31 ± 0.04*	0134 ± 0.02
48	1.34 ± 0.01	1.31 ± 0.03	1.28 ± 0.02	1.29 ± 0.05*	1.32 ± 0.01
72	1.37 ± 0.01	1.28 ± 0.02	1.26 ± 0.01	1.25 ± 0.03*	1.29 ± 0.02
96	1.45± 0.03	1.31± 0.01	1.31± 0.02	1.34± 0.01	1.41± 0.01

3.2. DNA Quantification

Nano Drop technology measures the absorbance of the light by a DNA sample to determine its concentration and purity. A quick and easy method, which requires only a small amount of the sample. The technique is commonly used in research and diagnostic laboratories for DNA quantification [19]. The Nanodrop technique was employed to measure both the quantity and quality of genomic DNA using a Spectrophotometer (Agilent 8453, California, USA). The absorbance values of the DNA sample, measured at 260/280 nm, were recorded for 5 seconds (Figure 1) to determine the quantity and purity of the sample**.**

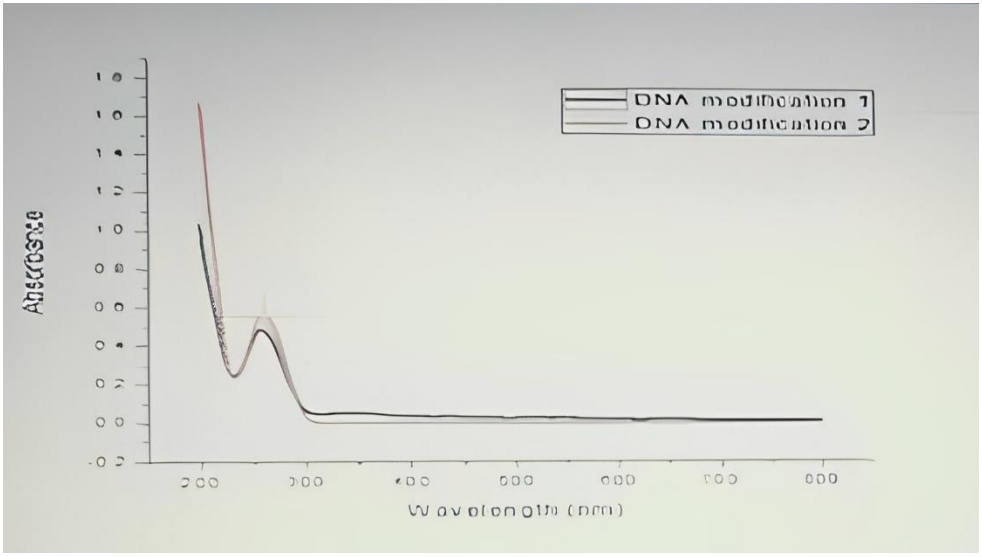

Figure 1. Absorbance Peaks Showing Good Quality of DNA

The quality and quantity of genomic DNA were assessed using the Nanodrop technique and a spectrophotometer (Agilent 8453, California, USA). The absorbance values at 260-280 nm wavelength were used to evaluate the DNA quality and DNA concentration values (ng/µl) and the ratio of 260/280, which were noted for purification purposes. The following equation was utilized to calculate the DNA concentration: volume (g/ml) = OD 260 × 100 (dilution factor) × 50g/ml/1000. The results were acceptable only if the ratio of OD 260/OD 280 was 1.8-2.0, indicating good DNA quality. Table 4 summarizes the obtained data.

Table 4. DNA Quantification using Spectrophotometer (Agilent 8453, California, USA) DNA damage

24 days	Insecticide concentration	260/280 ratio	DNA concentration
Gills	0.80µg/L	1.49	44.9µg/ml
Liver		1.28	44.65µg/ml
Gills	1.30µg/ml	1.28	41µg/ml
Liver		1.95	24.1µg/ml
Gills	1.90µg/ml	1.67	24.25µg/ml
Liver		1.65	23.45µg/ml
Gills	2.50g/ml	1.67	24.25µg/ml
Liver		1.65	23.45µg/ml
48 days	Insecticide concentration	260/280 ratio	DNA concentration
Gills	0.80µg/L	1.58	22.7µg/ml
Liver		1.47	21µg/ml
Gills	1.30µg/ml	1.41	20.5µg/ml
Liver		1.71	18.75µg/ml
Gills	1.90µg/ml	1.24	17.65µg/ml
Liver		1.13	16.35µg/ml
Gills	2.50g/ml	1.41	20.5µg/ml
Liver		1.58	22.7µg/ml
72 days	Insecticide concentration	260/280 ratio	DNA concentration
Gills	0.80µg/L	1.54	16.25µg/ml

72 days	Insecticide concentration	260/280 ratio	DNA concentration
Liver		1.47	14.65µg/ml
Gills	1.30µg/ml	1.60	14.35µg/ml
Liver		1.40	13.3µg/ml
Gills	1.90µg/ml	1.37	13µg/ml
Liver		1.26	12.3µg/ml
Gill	2.50g/ml	1.44	16.26µg/ml
Liver		1.46	14.66µg/ml
96 days	Insecticide concentration	260/280 ratio	DNA concentration
		1.54	16.24µg/ml
Gill	0.80µg/L	1.46	14.63µg/ml
Liver		1.68	14.34µg/ml
Gill	1.30µg/ml	1.47	13.3µg/ml
Liver		1.36	13.00µg/ml
Gill	1.90µg/ml	1.25	12.10µg/ml
Liver		1.45	16.21µg/ml
Gill	2.50g/ml	1.47	14.61µg/ml
Liver		1.46	14.66µg/ml

4. DISCUSSION

Glyphosate, a primary component in numerous herbicides, is extensively employed in agriculture industry to eliminate unwanted weeds and in non-agricultural areas for vegetation clearance. Its usage can lead to contamination of nearby water bodies through irrigated food crops, which can cause various abnormalities in the organisms inhabiting the polluted water. Due to its frequent use and specificity, glyphosate has been found to induce several toxicological problems in exposed species, including humans. Organisms' antioxidant systems play a crucial role in responding to oxidative stress by generating antioxidant metabolites and enzymes. Catalase is a significant enzyme among these enzymes, as it helps in preventing the harmful impacts of ROS on cells [20]. In the current investigation, the liver tissue levels of lipid peroxidation, which is the primary immune organ, exhibited a significant decrease ($P < 0.05$) in groups

exposed to ≥20 mg/L of GBH. Moreover, the catalase activities in liver tissues also increased. Previous research has reported significant alterations in oxidative stress in fish upon exposure to GBH [21]. The current study found that fish exposed to glyphosate had increased levels of ROS and TBARS, while their levels of GSH and total proteins decreased, respectively. The liver tissues of treated fish showed these effects throughout the experiment. Additionally, the kidneys of fish in groups D-E showed significantly higher levels of oxidative stress and ROS contents after the 4th day of exposure as compared to unexposed fish [22]. The levels of lipid peroxidation in liver tissues of fish exposed to GBH exhibited diverse outcomes, either increasing, decreasing, or remaining stable, depending mainly on the fish weight (or age) and exposure time, similar to the levels of catalase. Such oxidative conditions have been observed in various fish species upon exposure to different pollutants, including GBH, as per earlier studies. In this study, the DNA fragments displayed irregular and stepped smears due to the inaccurate process of DNA cleavage that occurs during apoptosis. This result is consistent with earlier findings [23] conjecture that more than one mechanism could be responsible for the fragmentation patterns leading to cell death and the cumulative effect of these events can trigger apoptosis and necrotic cell death. The ends were employed to assess the intricate genotoxic effects of the insecticide at the DNA level of many fish species under study. Various tissues are commonly used to determine DNA damage, namely , gills and liver [24]. In this study, the liver -detoxification organ- and the gills were selected as organs that are directly exposed to environmental contaminants. Gills and liver, being metabolically active organs that have higher metal accumulation than other organs, share this characteristic. Metal ion complexes formation can cause DNA damage. During apoptosis, DNA cleavage occurs at sites between nucleosomes, leading to DNA damage, which is a typical feature [25]. Moreover, chromatin is broken down by proteases and endonucleases into a smearing pattern, which is an indication of necrosis when proteases break down the histones and expose the entire length of the nucleases.

4.1. Conclusion

By analyzing the activities of antioxidant enzymes as biochemical biomarkers in freshwater fish *Oreochromis niloticus* (glyphosate-based herbicide) exposed to Roundup, it was concluded that the exposure to Roundup resulted in oxidative stress as well as DNA damage in the fish.

Therefore, the current conclusion was supported by the changes noticed in the activities of antioxidant enzymes. The study found a significant increase in oxidative stress by the activities of superoxide dismutase (SOD), catalasend peroxidase. The active ingredient in Roundup and glyphosate, can generate reactive oxygen species, which can damage the DNA molecules. This damage can lead to mutations and genetic instability, potentially increasing the risk of cancer and other diseases. Moreover, this sort of damage can lead to reduced growth, reproduction, and survival of fish populations. Additionally, glyphosate can also affect the aquatic food chain, potentially affecting the health of other species, which rely on fish as a prime food source. These findings emphasized the need for proper regulation and management of herbicide usage in aquatic ecosystems to minimize the impact on fish and other aquatic organisms. Thus, it is crucial to monitor the use of glyphosate-based herbicides in freshwater environments and take measures to reduce their potential negative impacts on aquatic organisms. Exposure to Roundup herbicide led to changes in the DNA concentration and 260/280 ratio in fish organs such as the liver and gills. The gills showed higher vulnerability to Roundup herbicide exposure than the liver. This suggested that Roundup herbicide exposure could cause DNA damage in fish organs. Hence, further research is required to evaluate the long-term effects of Roundup herbicide exposure on fish populations and the broader ecosystem. For this purpose, measures should be taken to reduce Roundup herbicide usage and minimize its potential negative impacts on aquatic life. Further research can also comprehensively understand the long-term effects of Roundup exposure on fish and other aquatic organisms.

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