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Research Article

Hematological and Histopathological Changes of Juvenile Grass Carp (*Ctenopharyngodon idella*) Exposed to Lethal and Sublethal Concentrations of Roundup (Glyphosate 41% SL)

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The presence of pesticides and their potential toxic effects on fish can pose a threat to aquatic ecosystems and human health. The present study aimed to evaluate the effect of commercial formulations of glyphosate (Roundup) on the survival rate, hematological parameters, and tissues (gills and liver) of juvenile grass carp (*Ctenopharyngodon idella*). For these purposes, we exposed the fish to 0, 50, 100, and 150 $mL\,L^{-1}$ of Roundup for 96 hr. Results showed a significant correlation between the fish's mortality rate and pesticide concentrations ($p < 0.01$); the LC_{50} 96 hr of Roundup was 75.838 $mL\,L^{-1}$ in the present study. The concentrations above 50 $mL\,L^{-1}$ induced significant tissue lesions seen as lamellar aneurism, leukocyte infiltration, distal hyperplasia, cloudy swelling, macrophage aggregates, and necrosis in the gills and liver. We also reported a significant correlation between the severity of tissue damage and Roundup concentration. The hematocrit, hemoglobin, white, and red blood cell count significantly reduced after 96 hr of exposure to 100 and 150 $mL\,L^{-1}$. The opposite trend was reported for concentrations of glucose, albumin, cholesterol, total protein, and triglycerides. Finally, the fish exposed to 100 and 150 $mL\,L^{-1}$ of Roundup displayed some clinical signs, such as increasing operculum movement, darkening the skin, and swimming near the surface during the movement test. Overall, our results showed that concentrations of Roundup higher than 50 $mL\,L^{-1}$ can induce various toxic effects and significantly reduce the survival chance of juvenile grass carp. Roundup altered fish behavior, tissue functioning, and biochemical processes. In this study, we provided some basic knowledge about the effects of a glyphosate-based herbicide on aquatic organisms and possible environmental management.

1. Introduction

In the era of industrial agriculture, agrochemicals have become unavoidable for the increase in crops and field production [1, 2]. As a result of this, we are now facing a global problem of pollution from chemicals used in the agricultural industry [3, 4]. Herbicides as an effective solution to control weeds have become a dominant feature of agriculture. However, there is a serious concern regarding the expansion of their use due to their side effects on the biodiversity of aquatic environments, terrestrial ecosystems, and human health [5, 6].

Glyphosate (*N*-phosphonomethyl glycine) certainly stands as one of the most used herbicides in recent decades [7, 8]. It is a broad-spectrum nonselective organophosphorus herbicide. Glyphosate's chemical characteristics allow it to distribute throughout the plant and damage meristems after a few days, making this herbicide extremely effective in controlling perennial weeds [9]. To increase glyphosate efficiency, it is always blended with different surfactants. The most widely commercially used glyphosate-based herbicide today is Roundup® (glyphosate 41% SL, Bayer AG, Leverkusen, Germania), which contains polyoxyethylene amine, a surfactant that improves the absorption and translocation of the active ingredient in the plant [10]. Today, it is possible to state that a close dependence between high-yielding orchards and herbicides has been established, as they are an essential component for plant survival and weed control. However, the choice of herbicides in Iran is very narrow and is limited to two types of herbicides: glyphosate and paraquat [11, 12].

The ability of Roundup to easily reach aquatic ecosystems by runoff, drainage, and leaching makes it a widely distributed environmental contaminant [13]. The toxic effects of this xenobiotic involve different biological aspects and are not limited only to survival but extend to alterations in metabolism [14]. Fiorino et al. [15] reported acute concentration of glyphosate as an active substance of many herbicides addition to the hatching retardation of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), led to a significant reduction in the survival rate and growth. Also, there were significant correlations between acute concentrations of glyphosate and their physical structure changes, including eye diameter and heartbeat in early life stages. Cavaş and Könen [16] studied the genotoxic effects of Roundup (glyphosate 41%) on the DNA structure of red blood cells of goldfish (*Carassius auratus*). Their study results showed that 5, 10, and 15 ppm of Roundup led to different levels of anemia by inducing nuclear abnormalities and DNA damage at interval times 48, 72, and 144 hr posttreatment; the highest anemia was related to the treatment of 15 ppm. Also, Maurya et al. [17] reported residual concentrations of organochlorine pesticides (OCPs) in industry west water significantly changed blood parameters (including red blood cell (RBC), white blood cell (WBC), and mean corpuscular volume (MCV)) of stinging catfish (*Heteropneustes fossilis*) on 1st, 5th, 10th, and 20th days after exposure. Sublethal concentrations of OCPs (10%, 20%, 30%, 40%, and 50%) led to significant tissue damage on gills, liver, intestine, and muscles at the 1st, 5th, 10th, and 20th days after exposure. Their study results clearly showed that the accumulation pattern of pesticides and the spread of tissue lesions had a significant correlation. The massive increase in the use of glyphosate since the 1970s has led governments of several countries to take action and try to reduce or ban its use. Spain, Italy, Germany, Canada, Portugal, and the Netherlands have already banned glyphosate. The majority of glyphosate restrictions worldwide were introduced after 2015. The International Agency for Research on Cancer reports on glyphosate's ability to induce cancerogenesis in humans [18]. For years, numerous studies have been conducted to assess the effects of this herbicide on different animal populations [15–22]. The toxic effects of glyphosate were studied on different classes of vertebrates. The majority of past ecotoxicology studies have confirmed that fish species are a good model for assessing the toxicity of substances in the aquatic system, due to their efficiency in metabolizing xenobiotics, sensitivity to contaminants, and position in the aquatic food chain [23, 24].

Glyphosate and its common formulations have shown toxic effects on both aquatic invertebrate and vertebrate species [25, 26]. In *Cherax quadricarinatus*, the freshwater crayfish, exposure to 22.5 mg L^{-1} of this herbicide led to reduced somatic cell growth, glycogen, lipid, and protein levels in the muscle after 50 days [27]. Negative effects of glyphosate were also observed for various freshwater fish. For example, Nešković et al. [28] reported exposure to 10 mg L^{-1} glyphosate led to increased alkaline phosphatase activity in the liver of freshwater carp (*C. carpio* L.) after 14 days; also, histopathological changes were seen as epithelial hyperplasia and subepithelial edema at this concentration and above than it. In addition, there is leukocyte infiltration, hypertrophy of chloride cells, and lifting and rupture of the respiratory epithelium. Cattaneo et al. [29] found that 10 mg L^{-1} of Roundup in Europian carp (*C. carpio*) can decrease acetylcholinesterase activity in the brain and muscle and induce oxidative damage after 96 hr. The evaluated toxicity effect of 120 g L^{-1} of glyphosate and 10 mg L^{-1} Roundup revealed that both pollutants increase cell proliferation and cell turnover and lead to an upregulation of metabolic processes in brown trout (*Salmo trutta*) after 14 days [30]. The results obtained from toxicological studies are not definitive and are highly dependent on environmental conditions, age, gender, and species [31]. The continuation of such studies, in addition to increasing the reliability of the results, can broaden the conversation on glyphosate toxicity and its impact on human and environmental health [32].

Despite the results of previous studies about the toxicity effect of glyphosate on nontarget organisms, commercial formulations of glyphosate, such as Roundup, are still widely used worldwide [33]. There is no single approach to its consumption [34]. For example, according to the ENDURE network reports [35], "Despite the decision of some European countries such as Spain and Italy to ban the use of Roundup and other Glyphosate derivatives, sales of glyphosate represent 33% of total herbicide sales in the EU 28 + 3."

The present study aimed to evaluate the toxic effects of the commercial formulations of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) on juvenile grass carp (*Ctenopharyngodon idella*) as an important commercial species in the north of Iran [36]. Grass carp farms in the north of Iran are usually located near rice and cotton farms and have joint water resources with them [37]; in addition, grass carp is an herbivorous freshwater fish [38]. The north of Iran (southern areas of the Caspian Sea) is one of the centers of consumption of pesticides, including glyphosate, due to the high volume of agricultural production and its subtropical climates [39].

2. Materials and Methods

2.1. Preparation. Juvenile grass carp, *C. idella* ($N = 150$), were purchased from local farms and transported to the laboratory (Faculty of Natural Resources, University of Guilan, Guilan province, Iran). Fish were divided into five tanks

(300 L—30 fish in each tank). Individuals were maintained for 2 weeks in tanks for acclimatization to laboratory conditions [31]. The fish were fed by carp commercial feed (SFC, Faradaneh Co., Shahrekord, Iran), equivalent to 2% of biomass weight twice a day during the adaption period [23]. Each experimental unit had independent aeration, and 40% of the water in the tank was replaced daily [40]. Water physicochemical parameters, the pH, temperature, dissolved oxygen (DO), NH_3 concentration, and total hardness of water ($CaCO_3$ concentration) were measured. Used equipment was: digital soil and substrate pH meter (S500 pro, Aqua Masters, Burbank, California, US), a DO meter for aquaculture (HI9147, HANNA Instruments, Bertoki, Slovenia), and multiparameter photometers (7100, Palintest Co., Gateshead, UK). All measurements were performed twice a day [31]. Water parameters were as follows: pH 7.8–8.2, DO 7.9–8.6 mg L^{-1}, $NH_3 < 0.02$ mg L^{-1}, temperature 24 ± 2°C and $CaCo_3$ 210 mg L^{-1} during the adaption period; finally, photoperiod of 16 : 8 hr (light–dark cycle).

2.2. Acute Toxicity Test (LC_{50} 96 hr Test). After the acclimatization period, 84 fingerling fish were divided into four groups (average weight 14.4 ± 1.7 g and total length 12.3 ± 0.82) with three replicates (12 aquarium—$150 \times 60 \times 65$ cm) and exposed to four concentrations of commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany). According to the manufacturer of Roundup, the solvent used was distilled water, and the concentration of glyphosate was 410 mg L^{-1} [26]. Nominal concentrations of Roundup were 0, 50, 100, and 150 mL L^{-1}, and the test time was 96 hr; nominal concentrations of Roundup were selected according to the Deivasigamani [40] study and laboratory facilities. The fish mortality rate was calculated at 24, 48, 72, and 96 hr after exposure [41]. Fish were transferred into the aquarium or the test tank 24 hr before the beginning of the test to adapt fish to the test tank condition and were not fed during the toxicity test to keep static condition. Water physicochemical parameters were kept the same for one of the acclimatization periods (pH 7.9–8.6, DO 7.9–8.6 mg L^{-1}, $NH_3 < 0.02$ mg L^{-1}, temperature 24 ± 2°C and total hardness 210 mg L^{-1} $CaCO_3$). The LC_{50} 96 hr test was a static system [42]. Finally, glyphosate concentrations were added manually, and the pesticide was distributed by water circulation inside the test tank [43]. Also, fish swimming was recorded for 15 min at intervals of 12 hr by the camera (Canon, SX230 Hs, 5.0–70 mm) during the LC_{50} 96 hr test; the camera was in front of the aquarium [31].

2.3. Histopathological Observation. The samples of gills and liver were collected at 96 hr of fish exposure to Roundup (three samples per treatment), and they were fixed with diluted Formalin solution (Formaldehyde 10% v/v, Sigma®, Missouri, USA). Formalin of the gill and liver samples was replaced 24 hr after sampling. The middle parts of the liver and second-gill arch from the fish's left side were selected for sampling, respectively [44]. All samples were taken from fish that survived exposure to the pesticide, and three slides were prepared for each treatment [45]. The samples were placed in a series of alcohols (50%, 70%, 80%, and 96%) for half an hour. Immediately after that, they were washed with 1-butanol alcohol (for 2 hr) and then placed into chloroform to clarify for 1 hr [46]. After this step, the samples were placed into an incubator at 37°C for paraffinization and softening by the solution of chloroform and paraffin (1 : 1). After that, samples were incubated in pure paraffin at 54°C and were prepared for tissue incisions after cooling. The tissue incisions were obtained by the automatic tissue processor machine (TP1020, Leica Microsystems Inc., Buffalo Grove, IL, USA), and their thickness was 6 μm. The tissue incisions were stained with hematoxylin-eosin [47]. Tissue damages were observed and evaluated by light microscopy (Model RH-85 UXL, UNILAB®, India). The tissue damages were classified as none, mild, moderate, and severe according to the described method in Abalaka's [48] study and modified with the laboratory facilities. Organs with numerical values ranging from 0 to 10 were classified as normal tissue (none), 11 to 20 as mildly damaged tissue (mild), 21 to 50 as moderately damaged tissue (moderate), and 51 to 100 as severely damaged (severe).

2.4. Hematological Biomarkers. Blood samples were collected through the caudal vein technique. According to this technique, the sample was taken from the midline just posteriorly of the anal fin. Blood samples were centrifuged for 10 min at 2,500 rpm and transferred to the laboratory for hematological analysis. Blood samples of all fish in each tank were polled, and finally, 12 blood samples were analyzed (three samples for each treatment). Total protein, albumin, globulin, triglyceride, cholesterol, and glucose in serum were measured from each sample using an automatic biochemical analyzer (Roche Hitachi 911 Chemistry Analyzer, Tokyo, Japan) and their corresponding kits (Pars Azmoon Inc., Tehran, Iran). The samples were taken from live fish in sublethal concentrations treatments of Roundup at 96 hr after exposure. Hematocrit (Hct) was determined by centrifuging whole blood in heparinized micro-Hct capillary tubes at $3{,}500 \times g$ for 10 min (Osterode, Germany) at 24°C. The mean RBC count, mean WBC count, and mean concentration of hemoglobin (Hb) were calculated according to Houston [49].

2.5. Data Analyses. The lethal concentration of glyphosate in intervals of 24, 48, 72, and 96 hr (LC_{50} 24, 48, 72, and 96 hr of Roundup) was estimated through a probit test with a 95% confidence [50]. We used the Spearman test to find the correlation between different nominal concentrations of commercial formulations of glyphosate (Roundup 41% SL) and mortality (2-tail). Finally, the correlation between the mortality rate of fish and the concentration of Roundup and the significant difference between average measured indicators of treatments were appraised with the two-tailed significance Spearman tests and the Analysis of Variance (ANOVA) test with a 95% confidence by SPSS software (IBM SPSS Statistics 20), respectively.

The video data were analyzed by Adobe After Effects software (AAE CS6) on the Windows platform (Windows 7 Ultimate, Microsoft corporation©). The clinical signs of fish were reported by direct observation of recorded videos, counting average movement of the gill operculum in 1 min, and comparison color of the object (fish) during a period by Adobe After Effects CS6 software (AAE CS6) [31].

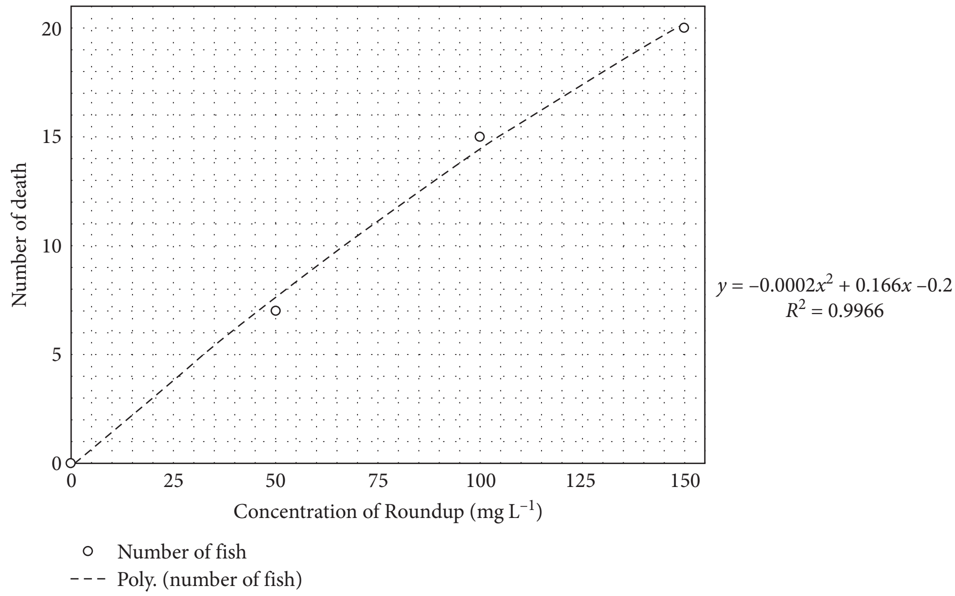


FIGURE 1: The polynomial regression between nominal concentrations of commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) with mortality rate (number of deaths) of the grass carp (*Ctenopharyngodon idella*) 96 hr after exposure ($p < 0.01$). The average weight of fish was 14.4 ± 1.7 g.

3. Results and Discussion

3.1. Results

3.1.1. Acute Toxicity Test (LC_{50} 96 hr). The analysis of the present study showed there was a significant correlation between increasing the concentration of Roundup and increasing the grass carp juvenile number of deaths ($p < 0.01$); it is shown in Figure 1.

The results of the toxicity test showed mortality in all treatment phases (except for the control group) (Table 1). The LC_{50} 96 hr of the commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) was 75.838 mL L^{-1}, while LC_{50} for 24, 48, and 72 hr were 120.343, 110.495, and 85.424 mL L^{-1}, respectively (Table 2).

Exposure to different concentrations of Roundup leads to some clinical signs in fish, such as increasing operculum movement, fast swimming, anxiety, darkening of the skin, swimming near the water surface, and death with an open mouth.

3.1.2. Histopathological Observation. Tissue damage was observed in different degrees in all individuals exposed to the herbicide (50, 100, and 150 mL L^{-1}). Histopathological changes started to be observed at the treatment with the lowest pollutant concentration: mild lamellar fusion, basal, and distal hyperplasia. In addition, moderate epithelial hyperplasia was evident. At a concentration of 100 mL L^{-1}, the damage increased in severity (Table 3).

We reported a mild lamellar aneurysm, leucocyte infiltration, and mild necrosis. The greatest damage to the gills was found, as expected, at the highest concentration (150 mL L^{-1}) of Roundup. The samples were characterized by severe necrosis and leukocyte infiltration, as well as

TABLE 1: The mortality rate of grass carp (*Ctenopharyngodon idella*) after exposure to different concentrations of commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany).

Concentration (mL L^{-1})	Number of fish	No. of mortality			
		24 hr	48 hr	72 hr	96 hr
0	21	0	0	0	0
50	21	3	5	6	7
100	21	7	9	13	15
150	21	11	15	19	20

Note. All concentrations are nominal. The average weight of fish was 14.4 ± 1.7 g.

TABLE 2: Lethal concentration of commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) for grass carp (*Ctenopharyngodon idella*).

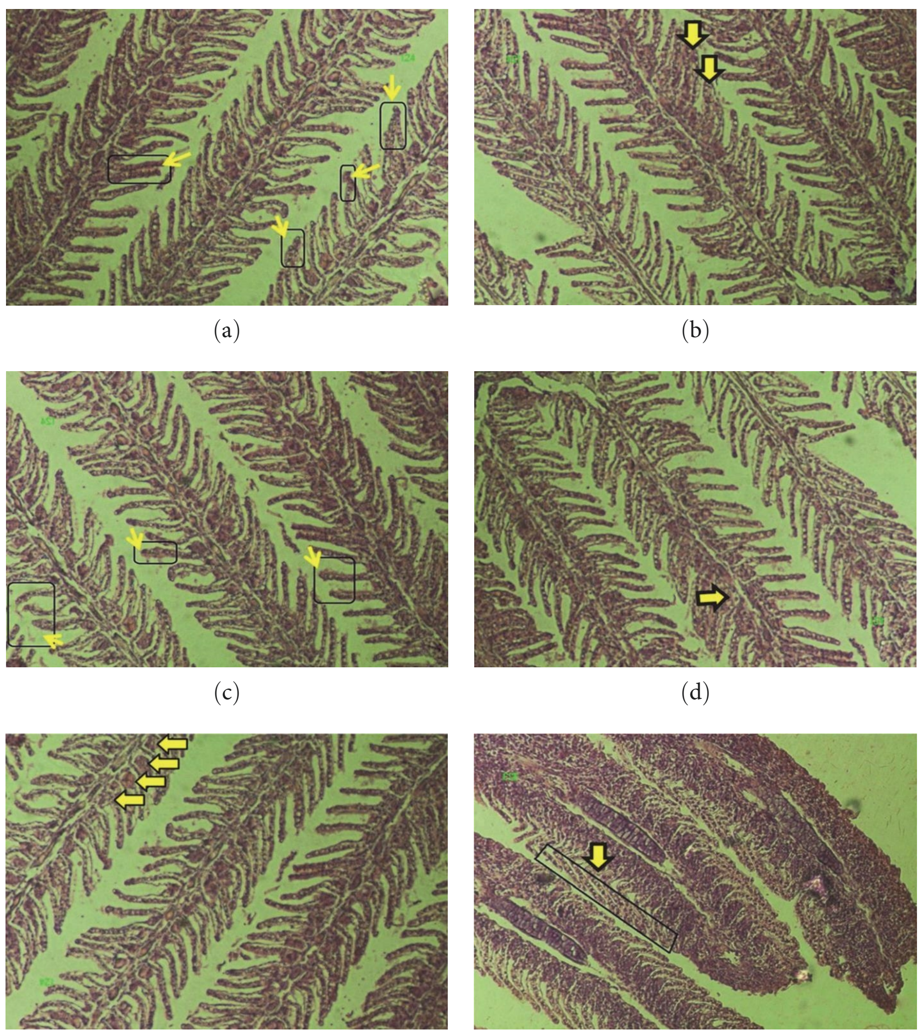
Point	Concentration (mL L^{-1})			
	24 hr	48 hr	72 hr	96 hr
LC_{10}	46.408	30.863	26.655	23.489
LC_{20}	64.034	58.199	46.829	41.459
LC_{30}	78.042	77.910	61.376	54.417
LC_{40}	100.853	94.753	73.806	65.489
LC_{50}	120.343	110.495	85.424	75.838
LC_{60}	156.778	126.237	97.042	86.187
LC_{70}	176.269	143.080	109.471	97.258
LC_{80}	199.079	162.791	124.018	110.216
LC_{90}	230.714	190.127	144.192	128.186
LC_{95}	266.838	212.701	160.853	143.027

Note. All concentrations are nominal. The average weight of fish was 14.4 ± 1.7 g.

Table 3: The correlation between the commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) and damages of grass carp gills (*Ctenopharyngodon idella*).

Tissue damages	Concentration (mL L^{-1})			
	0	50	100	150
Lamellar fusion	–	++	+++	+++
Lamellar aneurism	–	–	++	+++
Epithelial hypertrophy	–	+++	++++	+++
Leukocyte infiltration	–	–	++	++++
Basal Hyperplasia	–	++	++	+++
Distal hyperplasia	–	++	+++	+++
Necrosis	–	–	++	++++

Note. All concentrations are nominal. Also, none (–), mild (++), moderate (+++), and severe (++++) of tissue damage.



(a) (b) (c) (d) (e) (f)

Figure 2: The grass carp (*Ctenopharyngodon idella*) histopathological changes of the gills by commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany): (a) lamellar fusion (100 mL L^{-1} of Roundup); (b) lamellar aneurism (150 mL L^{-1} of Roundup); (c) distal hyperplasia (100 mL L^{-1} of Roundup); (d) leukocyte infiltration (150 mL L^{-1} of Roundup); (e) basal hyperplasia (100 mL L^{-1} of Roundup); (f) epithelial hypertrophy (50 mL L^{-1} of Roundup). All pictures are magnified × 10.

moderate lamellar fusion, lamellar aneurysm, epithelial hypertrophy, and basal and distal hyperplasia (Figure 2).

Regarding tissue damage in the liver of *C. idella*, they ranged from mild at the lowest Roundup concentration, moderate at the intermediate concentration, and severe in the highest herbicide concentration treatment. There was observed a high degree of necrosis, cloudy swelling, and numerous macrophage aggregates at 100 mL L^{-1} of Roundup.

Table 4: The correlation between the commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany) and the damages to the liver of grass carp (*Ctenopharyngodon idella*).

Tissue damages	Concentration ($mL\,L^{-1}$)			
	0	50	100	150
Necrosis	–	++	++++	++++
Hydropic swelling	–	++	+++	++++
Cloudy swelling	–	++	++++	++++
Lipidosis	–	++	+++	+++
Macrophage aggregates	–	++	++++	+++
Dilation of sinusoid	–	++	+++	+++

Note. All concentrations are nominal. Also, none (–), mild (++), moderate (+++), and severe (++++) of tissue damage.

As the Roundup concentration increased, the hydropic swelling also increased. All tissue damage data are shown in Table 4 and Figure 3.

3.1.3. Hematological Parameters. Values for hematological and biochemical parameters are shown in Table 5. The level of total protein at the 50 $mL\,L^{-1}$ concentration increased compared to the control group. The trend of increase in total protein concentrations followed the increase of glyphosate concentration, with the highest values in individuals treated with 150 $mL\,L^{-1}$ of Roundup in comparison to other groups. The albumin level was lowest in the control group, with a significant difference compared to the other groups. The albumin level did not differ significantly among fish individuals exposed to glyphosate. As for the cholesterol level, it increases significantly in the intermediate (100 $mL\,L^{-1}$) and maximum (150 $mL\,L^{-1}$) concentration groups when compared to control and individuals exposed to 50 $mL\,L^{-1}$.

Exposure of fish for 96 hr to this herbicide induced a significant increase in triglyceride concentrations. However, we did not observe any significant difference between the treated groups. The glucose level was lower in the 100 and 150 $mL\,L^{-1}$ glyphosate concentration group as compared to the control group and the group with the lowest glyphosate concentration. Comparison between fish that experienced the highest and intermediary concentrations showed a significant decrease in individuals from the 150 $mL\,L^{-1}$ glyphosate group.

The Hb values and the WBC count in fish from the 100 and 150 $mL\,L^{-1}$ groups were significantly lower than the ones in the control and 50 $mL\,L^{-1}$ groups. The values for Hct and the red blood cell count were lowest in fish exposed to the highest concentration of Roundup, followed by ones facing intermediate concentrations, while no differences were reported between the control group and the group exposed to 50 $mL\,L^{-1}$ Roundup.

3.2. Discussion. Growing public concern about the consequences of the massive use of glyphosate herbicide in industrial agriculture raised many questions about its use and effects on nontarget organisms [51]. This study aimed to assess the potentially toxic effects of the commercial formulation of glyphosate, Roundup, on juvenile grass carp as a nontarget aquatic organism. Glyphosate displayed a lethal and sublethal impact on grass carp. We found a dose-dependent correlation between glyphosate concentrations mortality rate and tissue damage. Glyphosate-induced profound changes in liver and gill tissues, which underwent necrosis, hyperplasia, hypertrophy, and leukocyte infiltration.

Standard ranges of the blood parameter values were significantly dependent on species, age, gender, and environmental conditions [16, 52]; however, the Hb and the RBC count were significantly reduced during the treatment with higher doses of glyphosate. The reduced number of RBCs can be the result of hemorrhage, hemolysis, and reduced erythropoiesis [53]. White blood cell counts also declined during the exposure period, reaching their lowest point in the group with the highest glyphosate concentration. Changes in WBC are usually seen in an aquatic organism exposed to herbicides [52]. WBC is directly related to the stress level and health of each organism [17]. Roundup's ability to affect tissues can reflect on hemopoiesis and block the maturation and release of white blood cells [54].

In glyphosate-treated fish, we reported an increase in cholesterol levels. A study conducted by Stoyanova [55] on common carp suggested that the accumulated glyphosate in the fish's liver can change lipid metabolism and increase cholesterol values. In addition, cholesterol is the base molecule for steroid synthesis, including the stress hormone. Therefore, an increase in cortisol under stressful conditions also may contribute to an increase in cholesterol levels [56]. Putative liver dysfunction caused by Roundup can also alter other blood biochemical parameters (i.e., total protein, triglyceride, and albumin) of fish individuals. We reported liver lesions, severe necrosis, cloudy swelling, and numerous macrophage aggregates.

The glucose level was significantly reduced after acute exposure to this herbicide. Loro et al. [57] also reported decreased plasmatic glucose levels and hypoglycemia after exposure to glyphosate-based herbicides. Hypoglycemia can be caused by stress, irritation, and rapid movements of the fish. Our result indicated that the treated fish showed behavior changes: anxiety, fast swimming, and increasing operculum movement. The similar result was reported in *Rhamdia quelen* and *Leporinus obtusidens* [58]. Histopathological changes on the gills (i.e., lamellar fusion, lamellar aneurism, epithelial hypertrophy, leukocyte infiltration, and distal hyperplasia) induced by Roundup can affect the

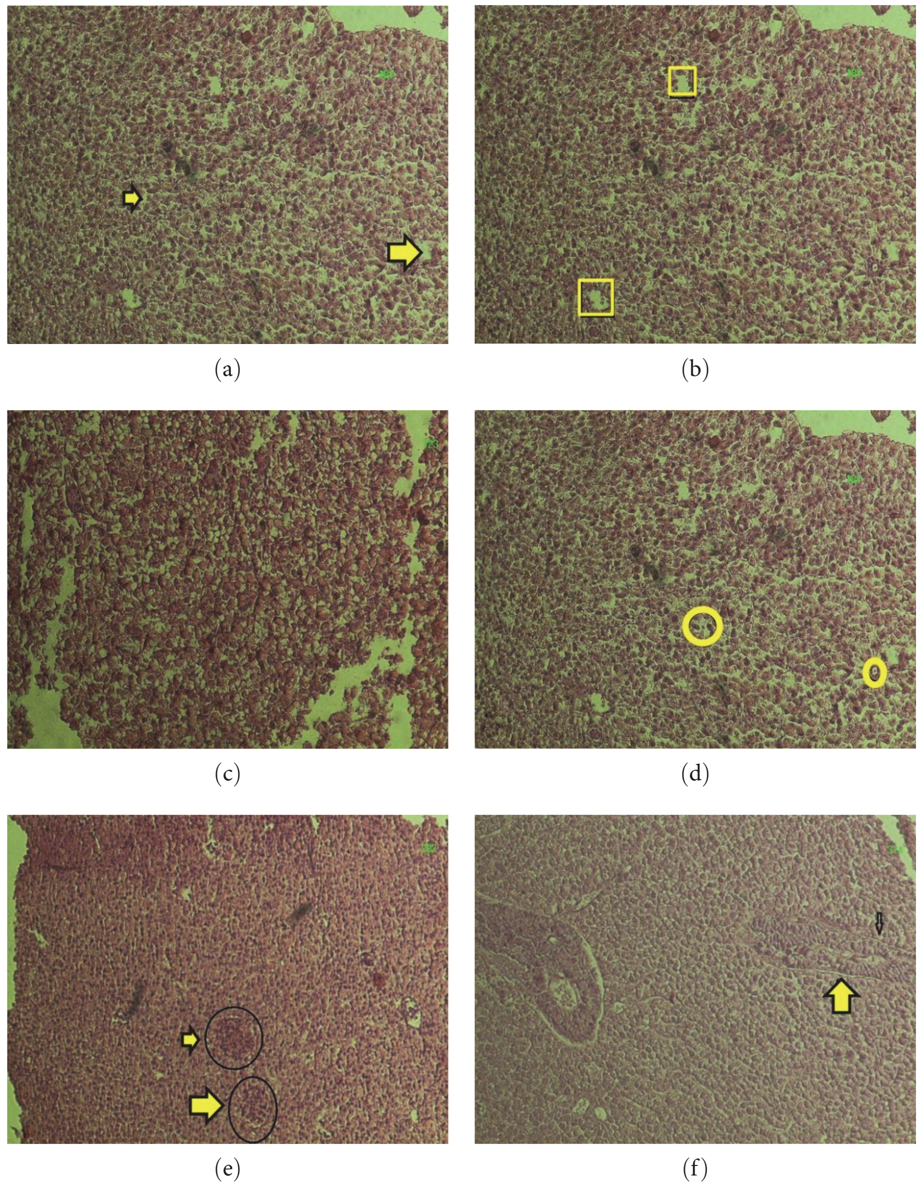


Figure 3: The grass carp (*Ctenopharyngodon idella*) histopathological changes of the liver by commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany); (a) necrosis (50 mL L^{-1} of Roundup); (b) hydropic swelling (50 mL L^{-1} of Roundup); (c) lipidosis (100 mL L^{-1} of Roundup); (d) cloudy swelling (50 mL L^{-1} of Roundup); (e) macrophage aggregates (150 mL L^{-1} of Roundup); (f) dilation of the sinusoid (100 mL L^{-1} of Roundup). All pictures are magnified × 10.

Table 5: Results of blood biochemical tests (mean ± SD) of adult grass carp (*Ctenopharyngodon idella*) at 96 hr after exposure to different levels of commercial formulation of glyphosate (Roundup 41% SL, Bayer AG, Leverkusen, Germany).

Blood parameters	Control (0 mL L^{-1})	50 mL L^{-1}	100 mL L^{-1}	150 mL L^{-1}
RBC (10^6 μL)	2.31 ± 0.06^a	2.30 ± 0.8^a	1.56 ± 0.09^b	1.29 ± 0.72^c
WBC (10^4 μL)	$2.27 \pm 0.53\ ^a$	2.23 ± 0.69^a	1.81 ± 0.12^b	1.79 ± 0.32^b
Hematocrit (%)	45.33 ± 0.6^a	44.2 ± 0.7^a	37.4 ± 0.02^b	32.9 ± 0.13^c
Hemoglobin (g/dL)	8.04 ± 0.31^a	7.91 ± 0.76^a	5.25 ± 0.46^b	5.24 ± 0.63^b
Glucose (mg/dL)	4.14 ± 0.07^a	4.12 ± 0.11^a	3.24 ± 0.03^b	$2.04 \pm 0.\ 13^c$
Total protein (mg/dL)	1.88 ± 0.31^d	2.94 ± 0.12^b	2.96 ± 0.13^b	3.68 ± 0.21^a
Triglyceride (mg/dL)	1.89 ± 0.16^d	2.33 ± 0.04^a	2.31 ± 0.09^a	2.33 ± 0.07^a
Cholesterol (mg/dL)	3.87 ± 0.22^c	3.89 ± 0.31^c	4.16 ± 0.04^a	4.15 ± 0.04^a
Albumin (mg/dL)	0.52 ± 0.05^b	0.67 ± 0.12^a	0.65 ± 0.41^a	0.66 ± 0.03^a

Note: Different letters (a–d) in the same row indicate significant differences ($p < 0.05$).

respiration process and reduce the survival chance of intoxicated fish [44].

4. Conclusion

Based on the results from this study, we can conclude that glyphosate, its commercial formulation—Roundup, causes toxic effects on grass carp, altering biochemical parameters of the blood and inducing serious damage to fish tissue. This is one of the studies confirming the toxic effects of herbicide's massive use in industrial agriculture on nontarget organisms.

Data Availability

The datasets used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Institutional guidelines for the ethical issue were followed. The experimental protocol (no. 19861e) was authorized by the Institutional Animal Care and Ethics Committee of Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Iran.

Disclosure

This manuscript has been presented as a preprint on Research Square (https://doi.org/10.21203/rs.3.rs-1435323/v1) and has not been peer-reviewed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Dariush Azadikhah, Matin Varcheh, and Ahmad Mohamadi Yalsuyi; the work and data analysis was written by Mohammad Mansouri Chorehi; the work was supervised by Caterina Faggio and Mohammad Forouhar Vajargah.

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References

[1] Y. Shang, M. K. Hasan, G. J. Ahammed, M. Li, H. Yin, and J. Zhou, "Applications of nanotechnology in plant growth and crop protection: a review," *Molecules*, vol. 24, no. 14, Article ID 2558, 2019.

[2] M. F. Vajargah, J. I. Namin, R. Mohsenpour, A. M. Yalsuyi, M. D. Prokić, and C. Faggio, "Histological effects of sublethal concentrations of insecticide Lindane on intestinal tissue of grass carp (*Ctenopharyngodon idella*)," *Veterinary Research Communications*, vol. 45, no. 4, pp. 373–380, 2021.

[3] O. I. Dar, R. Aslam, D. Pan et al., "Source, bioaccumulation, degradability and toxicity of triclosan in aquatic environments: a review," *Environmental Technology & Innovation*, vol. 25, Article ID 102122, 2022.

[4] A.-S. Curpan, F. Impellitteri, G. Plavan, A. Ciobica, and C. Faggio, "*Mytilus galloprovincialis*: an essential, low-cost model organism for the impact of xenobiotics on oxidative stress and public health," *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 256, Article ID 109302, 2022.

[5] P. Sehonova, Z. Svobodova, P. Dolezelova, P. Vosmerova, and C. Faggio, "Effects of waterborne antidepressants on non-target animals living in the aquatic environment: a review," *The Science of the Total Environment*, vol. 631–632, pp. 789–794, 2018.

[6] A. Stara, R. Bellinvia, J. Velisek, A. Strouhova, A. Kouba, and C. Faggio, "Acute exposure of common yabby (*Cherax destructor*) to the neonicotinoid pesticide," *Science of the total environment*, vol. 665, pp. 718–723, 2019.

[7] J. Blahova, C. Cocilovo, L. Plhalova, Z. Svobodova, and C. Faggio, "Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (*Danio rerio*)," *Environmental Toxicology and Pharmacology*, vol. 77, Article ID 103370, 2020.

[8] World Health Organization, *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification, 2019*, World Health Organization, 2020.

[9] S. O. Duke, "The history and current status of glyphosate," *Pest Management Science*, vol. 74, no. 5, pp. 1027–1034, 2018.

[10] B. Van, H. C. Ariena, M. M. He et al., "Environmental and health effects of the herbicide glyphosate," *Science of the Total Environment*, vol. 616, pp. 255–268, 2018.

[11] F. L. Meza-Joya, M. P. Ramírez-Pinilla, and J. L. Fuentes-Lorenzo, "Toxic, cytotoxic, and genotoxic effects of a glyphosate formulation (Roundup® SL-Cosmoflux® 411F) in the direct-developing frog *Eleutherodactylus johnstonei*," *Environmental and Molecular Mutagenesis*, vol. 54, no. 5, pp. 362–373, 2013.

[12] K. Lugowska, "The effects of Roundup on gametes and early development of common carp (*Cyprinus carpio* L.)," *Fish Physiology and Biochemistry*, vol. 44, no. 4, pp. 1109–1117, 2018.

[13] R. Kanissery, B. Gairhe, D. Kadyampakeni, O. Batuman, and F. Alferez, "Glyphosate: its environmental persistence and impact on crop health and nutrition," *Plants*, vol. 8, no. 11, Article ID 499, 2019.

[14] M. J. Costa, D. A. Monteiro, A. L. Oliveira-Neto, F. T. Rantin, and A. L. Kalinin, "Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to roundup original®," *Ecotoxicology*, vol. 17, no. 3, pp. 153–163, 2008.

[15] E. Fiorino, P. Sehonova, L. Plhalova, J. Blahova, Z. Svobodova, and C. Faggio, "Effects of glyphosate on early life stages: comparison between *Cyprinus carpio* and *Danio rerio*," *Environmental Science and Pollution Research International*, vol. 25, no. 9, pp. 8542–8549, 2018.

[16] T. Çavaş and S. Könen, "Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay," *Mutagenesis*, vol. 22, no. 4, pp. 263–268, 2007.

[17] P. K. Maurya, D. S. Malik, K. K. Yadav, N. Gupta, and S. Kumar, "Haematological and histological changes in fish *Heteropneustes fossilis* exposed to pesticides from industrial waste water," *Human and Ecological Risk Assessment: An International Journal*, 2019.

[18] M. Braun, F. Koger, D. Klingelhöfer, R. Müller, and D. A. Groneberg, "Particulate matter emissions of four different cigarette types of one popular brand: influence of

tobacco strength and additives," *International Journal of Environmental Research and Public Health*, vol. 16, no. 2, Article ID 144271, 2019.

[19] J. P. Giesy, S. Dobson, and K. R. Solomon, *Ecotoxicological Risk Assessment for Roundup® Herbicide*, Springer New York, 2000.

[20] F. Peixoto, "Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation," *Chemosphere*, vol. 61, no. 8, pp. 1115–1122, 2005.

[21] D. W. Kelly, R. Poulin, D. M. Tompkins, and C. R. Townsend, "Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival," *Journal of Applied Ecology*, vol. 47, no. 2, pp. 498–504, 2010.

[22] M. A. Romano, R. M. Romano, L. D. Santos et al., "Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression," *Archives of Toxicology*, vol. 86, no. 4, pp. 663–673, 2012.

[23] F. Fazio, G. Piccione, K. Tribulato et al., "Bioaccumulation of heavy metals in blood and tissue of striped mullet in two Italian lakes," *Journal of Aquatic Animal Health*, vol. 26, no. 4, pp. 278–284, 2014.

[24] E. R. Lauriano, G. C. Simona Pergolizzi, M. Kuciel, A. Alesci, and C. Faggio, "Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish *Carassius auratus* fed with a high-cholesterol diet," *Fish & Shellfish Immunology*, vol. 59, pp. 250–255, 2016.

[25] M. Cuhra, T. Traavik, and T. Bøhn, "Clone- and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in Daphnia magna." *Ecotoxicology*, vol. 22, no. 2, pp. 251–262, 2013.

[26] J. P. K. Gill, N. Sethi, A. Mohan, S. Datta, and M. Girdhar, "Glyphosate toxicity for animals," *Environmental Chemistry Letters*, vol. 16, pp. 401–426, 2018.

[27] J. L. Frontera, I. Vatnick, A. Chaulet, and E. M. Rodríguez, "Effects of glyphosate and polyoxyethylenamine on growth and energetic reserves in the freshwater crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae)," *Archives of Environmental Contamination and Toxicology*, vol. 61, no. 4, pp. 590–598, 2011.

[28] N. K. Neškovic, V. Poleksic, I. Elezovic, V. Karan, and M. Budimir, "Biochemical and histopathological effects of glyphosate on carp, *Cyprinus carpio* L," *Bulletin of Environmental Contamination and Toxicology*, vol. 56, no. 2, pp. 295–302, 1996.

[29] R. Cattaneo, B. Clasen, V. L. Loro et al., "Toxicological responses of *Cyprinus carpio* exposed to a commercial formulation containing glyphosate." *Bulletin of Environmental Contamination and Toxicology*, vol. 87, no. 6, pp. 597–602, 2011.

[30] U. Webster, M. Tamsyn, and E. M. Santos, "Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup," *BMC Genomics*, vol. 16, no. 1, pp. 1–14, 2015.

[31] A. M. Yalsuyi, A. Hajimoradloo, R. Ghorbani, V.-A. Jafari, M. D. Prokić, and C. Faggio, "Behavior evaluation of rainbow trout (*Oncorhynchus mykiss*) following temperature and ammonia alterations." *Environmental Toxicology and Pharmacology*, vol. 86, Article ID 103648, 2021.

[32] R. Lacroix and D. M. Kurrasch, "Glyphosate toxicity: in vivo, in vitro, and epidemiological evidence," *Toxicological Sciences*, vol. 192, no. 2, pp. 131–140, 2023.

[33] C. Martins-Gomes, T. L. Silva, T. Andreani, and A. M. Silva, "Glyphosate vs. glyphosate-based herbicides exposure: a review on their toxicity," *Journal of Xenobiotics*, vol. 12, no. 1, pp. 21–40, 2022.

[34] R. Ofosu, E. D. Agyemang, A. Márton, G. Pásztor, J. Taller, and G. Kazinczi, "Herbicide resistance: managing weeds in a changing world," *Agronomy*, vol. 13, no. 6, Article ID 1595, 2023.

[35] C. Antier, P. Kudsk, X. Reboud, L. Ulber, P. V. Baret, and A. Messéan, "Glyphosate use in the European agricultural sector and a framework for its further monitoring," *Sustainability*, vol. 12, no. 14, Article ID 5682, 2020.

[36] M. M. Dehshiri, "Invasive alien species of Iran," in *Invasive Alien Species: Observations and Issues from Around the World 2*, pp. 103–125, 2021.

[37] H. Salehi, "Carp culture in Iran," *Aquaculture Asia*, vol. 9, pp. 8–11, 2004.

[38] S. Zou, L. Gong, T. A. Khan et al., "Comparative analysis and gut bacterial community assemblages of grass carp and crucian carp in new lineages from the Dongting Lake area," *MicrobiologyOpen*, vol. 9, no. 5, Article ID e996, 2020.

[39] S. H. Nikookar, M. Fazeli-Dinan, S. P. Ziapour et al., "First report of biochemical mechanisms of insecticide resistance in the field population of *Culex pipiens* (Diptera: Culicidae) from Sari, Mazandaran, north of Iran," *Journal of Arthropod-Borne Diseases*, vol. 13, no. 4, pp. 378–390, 2019.

[40] S. Deivasigamani, "Effect of herbicides on fish and histological evaluation of common carp (*Cyprinus carpio*)," *International Journal of Applied Research*, vol. 1, no. 7, pp. 437–440, 2015.

[41] H. Naz, S. Abdullah, K. Abbas et al., "Toxic effect of insecticides mixtures on antioxidant enzymes in different organs of fish, *Labeo rohita*," *Pakistan Journal of Zoology*, vol. 51, no. 4, Article ID 1355, 2019.

[42] S. Vali, N. Majidiyan, A. M. Yalsuyi, M. F. Vajargah, M. D. Prokić, and C. Faggio, "Ecotoxicological effects of silver nanoparticles (Ag-NPs) on parturition time, survival rate, reproductive success and blood parameters of adult common molly (*Poecilia sphenops*) and their larvae," *Water*, vol. 14, no. 2, p. 144, 2022.

[43] L. Du-Carrée, R. B. Jessy, J. Cachot et al., "Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*," *Science of the Total Environment*, vol. 801, Article ID 149462, 2021.

[44] M. Dang, K. Pittman, C. Sonne et al., "Histological mucous cell quantification and mucosal mapping reveal different aspects of mucous cell responses in gills and skin of shorthorn sculpins (*Myoxocephalus scorpius*)," *Fish & shellfish immunology*, vol. 100, pp. 334–344, 2020.

[45] L. Glusczak, V. L. Loro, A. Pretto et al., "Acute exposure to glyphosate herbicide affects oxidative parameters in piava (*Leporinus obtusidens*)," *Archives of environmental contamination and toxicology*, vol. 61, no. 4, pp. 624–630, 2011.

[46] S. S. Jeng, T. Y. Lin, M. S. Wang, Y. Y. Chang, C. Y. Chen, and C. C. Chang, "Anoxia survival in common carp and crucian carp is related to high zinc concentration in tissues," *Fisheries science*, vol. 74, pp. 627–634, 2008.

[47] A. Shagan, W. Zhang, M. Mehta, S. Levi, D. S. Kohane, and B. Mizrahi, "Hot glue gun releasing biocompatible tissue adhesive," *Advanced Functional Materials*, vol. 30, no. 18, Article ID 1900998, 2020.

[48] S. E. Abalaka, "Heavy metals bioaccumulation and histopathological changes in *Auchenoglanis occidentalis* fish from Tiga dam, Nigeria," *Journal of Environmental Health Science and Engineering*, vol. 13, pp. 1–8, 2015.

[49] A. H. Houston, "Blood and circulation," *Methods for fish biology*, pp. 415–488, 1990.
[50] K. Rana and P. Gautam, "*Estimation of Median Lethal Concentration* (*LC50*) *Value and Its Confidence Interval for the Effect of Carbamate Pesticide* (*Methiocarb*) *on Caenorhabditis Elegans*," No. 8937, EasyChair, 2020.
[51] H. M. Nagib, *Herbicides: Properties, Synthesis and Control of Weeds*, BoD-Books on Demand, 2012.
[52] S. J. Gholami-Seyedkolaei, A. Mirvaghefi, H. Farahmand, and A. A. Kosari, "Effect of a glyphosate-based herbicide in *Cyprinus carpio*: assessment of acetylcholinesterase activity, hematological responses and serum biochemical parameters," *Ecotoxicology and Environmental Safety*, vol. 98, pp. 135–141, 2013.
[53] A. K. Khan, N. Shah, A. Gul et al., "Comparative study of toxicological impinge of glyphosate and atrazine (herbicide) on stress biomarkers; blood biochemical and hematological parameters of the freshwater common carp (*Cyprinus carpio*)," *Polish Journal of Environmental Studies*, vol. 25, no. 5, pp. 1995–2001, 2016.
[54] C. Kavitha, A. Malarvizhi, S. S. Kumaran, and M. Ramesh, "Toxicological effects of arsenate exposure on hematological, biochemical and liver transaminases activity in an Indian major carp, *Catla catla*," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2848–2854, 2010.
[55] S. Stoyanova, V. Yancheva, I. Iliev et al., "Glyphosate induces morphological and enzymatic changes in common carp (*Cyprinus carpio* L.) liver," *Bulgarian the Journal of Agricultural Science*, vol. 21, Article ID 409, 2015.
[56] R. Kazemi, M. Pourdehghani, A. Y Jourdehi, M. Yarmohammadi, and M. N Tajan, "Cardiovascular system physiology of aquatic animals and applied techniques of fish hematology," *Shabak Published Book*, 2010.
[57] V. L. Loro, L. Glusczak, B. S. Moraes et al., "Glyphosate-based herbicide affects biochemical parameters in *Rhamdia quelen* (Quoy & Gaimard, 1824) and *Leporinus obtusidens* (Valenciennes, 1837)," *Neotropical Ichthyology*, vol. 13, pp. 229–236, 2015.
[58] M. B. da Fonseca, L. Glusczak, B. S. Moraes et al., "The 2, 4-D herbicide effects on acetylcholinesterase activity and metabolic parameters of piava freshwater fish (*Leporinus obtusidens*)," *Ecotoxicology and Environmental Safety*, vol. 69, no. 3, pp. 416–420, 2008.