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Ionic Regulation and Gill Na^+/k^+ -ATPase Activities in Freshwater Fish Grass Carp *Ctenopharyngodon idella* during Glyphosate Toxic Exposure

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ABSTRACT: The Impact of Glyphosate on grass carp gill Na^+/K^+-ATPase interaction and electrolyte concentration (Na^+, K^+ and Cl^-). The effects of glyphosate on grass carp electrolyte concentrations (Na^+, K^+, and Cl^-) even gills Na+/K+-ATPase activity were studied using *Ctenopharyngodon idella*. It was found that glyphosate, with a 24-hour LC_{50} of 1.36 ppm for grass carp, had adverse effects on gill Na^+/K^+-ATPase function and led to hyponatremia, hypokalaemia, also hypo chloremia upon acute exposure. 35 days of sublethal exposure to 1.36 ppm of glyphosate resulted in a reduction in plasma Na^+ level during the absorption period and blood plasma K^+ content up to day 28, while plasma Cl^- levels also dropped. With increasing exposure time, gills Na^+/K^+-ATPase activity was found to decrease. The studies indicated that glyphosate is extremely hazardous to freshwater fish, and changes in blood ion levels and gill Na^+/K^+-ATPase action could be utilized as responsive glyphosate indicators and its effects.

Keywords: Grass carp, *Ctenopharyngodon idella*, Glyphosate, Acute and Sublethal toxicity, Electrolytes.

INTRODUCTION

Pesticides are frequently employed to enhance the quality and production of agriculture products. Synthetic chemical pesticides contain undesirable side effects that can have an influence on users directly or indirectly. These side effects include unpleasant water tastes and smells, lethal effects on a range of aquatic non-target creatures, and unpleasant side effects on users (Sathyamoorthi *et al.,* 2019; Kumaresan *et al.,* 2019).

Glyphosate are widely used during pesticides in farming and are extensively applied an initial technique to herbicide application (Sharma *et al.,* 2019). Glyphosate, also known as N-(Phosphonomethyl) glycine, has been a popular cultural, post-emergence organophosphorus herbicide worldwide now been established to the market in the 1970s. This herbicide, first marketed the Roundup, is efficient in eliminating both terrestrial and aquatic plant and is widely used in horticulture, farming, silviculture, and garden maintenance. Although glyphosate is considered to be non-persistent in soils, it is persistent in water due to its high-water solubility (PPDB, 2012). One of the most widely used herbicides in the world due to its great potency, excellent weed-controlling abilities, and broad-spectrum herbicidal activity is glyphosate (N-phosphonomethyl glycine) (Liu *et al.,* 2019).

Harmful impacts on aquatic organisms that were not among the targets of the study have been recorded (Gandhi *et al.*, 2021).

To increase its effectiveness, surfactants like polyethoxylated tallow amine (POEA) are added (De Moura *et al.,* 2017). The use of herbicides can negatively impact aquatic organisms and non-target species, such as invertebrates, finfish, and shellfish, which can be highly sensitive to these chemicals (Schaaf, 2017; Vera *et al.,* 2012). Pesticides can enter aquatic ecosystems through spray drift and surface runoff, leading to their accumulation in some species, potentially affecting various levels of the food chain (Abrantes *et al.*, 2010; Rimayi *et al.,* 2018; Ojemaye *et al.*, 2020a). Because of its high-water solubility and widespread use, particularly in shallow water systems, glyphosate can provide a serious risk to non-target species of an aquatic ecosystems. Roundup glyphosate acid equivalent concentrations in natural water can range from 0.01 to 0.07 mg/L, with extreme cases reaching concentrations as high as 1.7 mg/L following the application of the herbicide (Guilherme *et al.,* 2010). The use of aquatic animal health biomarkers and the evaluation of polluted water bodies are commonly employed as early diagnostic methods (Cajaraville *et al.,* 2000). Based on 73 studies from 21 countries, Brovini *et al.* (2021) analyzed glyphosate concentrations in freshwater ecosystems all over the world. In 95% of the nations, they looked into, they found that there was a moderate to high danger to aquatic creatures and that the majority of countries (90%) did not have limiting laws for aquatic glyphosate concentrations. Due to land run-off, glyphosate has recently been found in surface waters and sediments (the primary sink for glyphosate in aquatic bodies) (Stachowski-Haberkorn *et al.,* 2008).

Fish can be analyzed to determine pesticide exposure or its effects on aquatic ecosystems. Environmental toxicological risk assessments often use the inhibition and induction of enzymatic and biochemical indicators as early warning signs (Osten *et al.,* 2005). Fish have the

ability to absorb and concentrate toxins from water directly or through secondary sources such as small fish, crustaceans, and aquatic vegetation (Polat *et al.,* 2016). Therefore, it is important to examine the potential exposure of key organisms to represent the effects of contaminants in aquatic ecosystems. A multi-assessment approach using biological indicators at different levels of biological organization is necessary to gain a comprehensive understanding of harmful impacts (Bonifacio *et al.,* 2016). The chronic uses of Roundup® has been found to potentially pose a hazard to non-target organisms and fish in shallow water, according to toxicological studies by Giesy *et al.* (2000); Relyea (2005). To evaluate the contamination of environmental pollutants in aquatic ecosystems, various biological indicators, such as hematological, biochemical, ion regulatory, and enzyme variables, are often used (Li *et al*., 2010). Fish tend to accumulate pollutants in their fat tissue, including the liver, and when the content of these tissues reach a threshold, adverse effects can occur (Omar *et al.,* 2014). Roundup® usage was already reported to cause genotoxic harm to the blood serum and gills cell of the neotropical fish species *Prochilodus lineatus* (Cavalcante *et al*., 2008). The role of blood parameters in determining the impact of toxins on an organism is crucial as blood serves as a physiological reflector of the entire body. Electrolytes play a critical role in maintaining the body's functions, such as contributing to the majority of osmotically active particles, providing buffer systems, regulating pH, and maintaining ionic equilibrium for normal neuromuscular and tissue operations. Electrolytes ions play an important function in regulating the osmolarity a system in aquatic fish, which have a particularly sensitive physiological regulation of their primary electrolytes and often change in response to contaminants such as pesticides (McDonald *et al.,* 1989). Plasma glucose and protein levels, along with other biochemical markers, are frequently used as sensitive biomarkers of exposure to environmental chemicals. The plasma ion concentrations, determined on osmotic or particular ion concentrations, including those of sodium (Na+), potassium (K+), and chloride (Cl-), are used an indicator. However, these ions are extremely hypersensitive to environmental stress (McDonald *et al.,* 1989; Lavanya *et al.,* 2011). At the cellular and organismal levels, ion homeostasis depends on the membrane enzyme Na+/K+-ATPase, which is critical to maintain stability (Dang *et al.,* 2000). The Na+/K+-ATPase enzyme are commonly displayed in the chloride cell's tubular structure, particularly in fish gills, and is crucial for preserving ion regulated (Metz *et al.,* 2003; Grosell *et al.,* 2004; Kalay, 2006). The present investigation is focused on studying the hematological, biochemical, and ion regulatory parameters in the freshwater fish, grass carp (*Ctenopharyngodon idella*), and the impact of glyphosate exposure in short- and long-term exposure scenarios (Langiano and Martinez 2008).

MATERIAL AND METHODS

A. Toxicant

Glyphosate, also known as N-(Phosphonomethyl) glycine, was used in a commercial formulation called Roundup, produced by Monsanto Company in St. Louis, Missouri, USA. The active ingredient in Roundup was 480 g/L of the isopropyl ammonium salt of glyphosate, with 360 g of glyphosate per litre, and POEA as a surfactant. The remaining chemicals were supplied by Merck Chemical Co. in Darmstadt, Germany (Source not mentioned).

B. Collection of fish and maintenance

Healthy grass carp *Ctenopharyngodon idella* specimens were obtained from the Siraco Fish Farm in Nerunjipettai, Erode district of Tamilnadu, India. The fish were 10.2 cm ± 2.02 cm in length and weighed 7.9 g ± 2.1 g. The specimens were acclimated for 3 weeks in a controlled laboratory environment in a 1000 L cement tank. During the acclimation period, the fish were given free access to rice bran and groundnut oil cake, and water changed daily to remove excretory debris. The trial began 24 hours after the last feeding. Tap water that was not chlorinated was used, and its physicochemical properties were as follows: temperature 27.2 ± 2.01°C, pH 6.8 ± 7.1, salinity 0.15 ± 0.05 ppt, dissolved oxygen 4.25 ± 0.01 mgl^{-1}, alkalinity 130 ± 0.5 mgl^{-1}, calcium 25.2 ± 0.6 mgl^{-1}, and magnesium 8.5 ± 0.4 mgl^{-1}. These parameters were determined using the APHA (1998) technique.

C. Estimation of 24 h LC_{50} Value

To estimate the toxic effects of glyphosate, a 24-hour acute toxicity test was evaluated within the limitations of laboratory equipment. Five different doses of glyphosate (0.20, 0.40, 0.60, 0.80, and 0.100 ppm) were obtained from the stock and utilized to determine 24-hour of LC_{50} value. Ten randomly selected fish were placed in divide glass tanks (120 cm × 80 cm × 40 cm) for each dose, with three replicates and isolated duplicates for each dose. The tanks were filled with toxic-free water, and the experimental aquarium was filled with an equal amount of acetone. The mortality and survival of the carp were recorded 24 hours later, and deceased fish being removed immediately from the tank. Food was discontinued during in the bioassay. Finney (1978) probit analysis method was used to calculate the 24-hour LC_{50} concentration, which was determined to be 1.36 ppm, representing the level at which 50% of the fish died after 24 hours. The sub-lethal concentration was determined to be $1/10^{th}$ of the 24-hour LC_{50} value of glyphosate, or 1.36 ppm (Sprague, 1971).

D. Acute toxicity test

60 fingerlings (20 in each group) were selected for the acute test for toxic effects and found to the toxicant glyphosate (1.36ppm). The test was conducted concurrently with the control group that was not exposed to the toxicant.

At the end of the 24-hour period, fish from both control and study aquariums were removed for the analysis of plasma electrolytes (Na^+, K^+, and Cl^-) and Na^+/K^+-ATPase activity.

E. Sublethal toxicity test

For the sublethal toxicity experiments, 200 fingerling fish were selected and divided into two groups of 100 fish each. Each group was exposed to glyphosate herbicide at a concentration below the lethal level (1.36 ppm). A related to control setup was also kept. Before the sublethal experiments, the fish were given unlimited access to food. Fresh water was added to the aquarium every 24 hours, and to maintain consistency of the glyphosate concentration in the herbicide group, 1.36 ppm was added daily. No mortalities were observed during the 35-day experiment period. After 7th, 14th, 21st, 28th, and 35th periods of exposure, grass carp from both the control and experiment aquariums were randomly chosen for analysis. Blood was drawn from each group for plasma electrolyte analysis, and at the same time, gills were collected to measure the activity of Na^+/K^+-ATPase enzyme.

F. Estimation of plasma electrolytes

Blood serum was taken from the fish's cardiac region when using plastic syringe with a 26-gauge syringe that had been pre-soaked with anticoagulant and stored on refrigeration. To test the ions Na+, K+, and Cl^- in the blood plasma, the plasma from the blood samples was extracted in accordance with the techniques described by Maruna (1958); Sunderman and Sunderman (1959); Schoenfeld and Lewellan (1964).

(i) Sodium and potassium Analyzer. The concentrations of sodium and potassium was determined using the techniques described by Maruna (1958) with the use of a Biomarker Reagent kit from Monozyme Laboratories in Secunderabad, Hyderabad, India.

(ii) Chloride Analyzer. The concentration of chloride was determined using a changed version of the procedures from Tietz (1990); Young *et al.* (1975) with the help of a laboratory test solution kit provided by Prism Scientific Pvt Ltd., Mumbai, India.

G. Determination of gills Na^+/K^+-ATPase technique

Following the extraction of serum, the fish become cleaned the consumption of double-distilled water and dried using filter sheets. Afterwards, 100mg of gill tissue was taken from each of the control and treatment group fish, weighed, and equilibrated in 1.0ml of ice-cold pH 7.4 tris-HCl buffer using a Teflon homogenizer. The homogenate was then homogenised for 15 minutes at 1000 rpm and 4°C, and the suspension was transferred to measure the Na^+/K^+-ATPase activity as per the techniques described by Shiosaka *et al.* (1971).

H. Statistical assessment

The statistical data was concluded with a significance level of $P<0.05$, used the student's t-test to calculate the t values and assess their significance.

RESULT

A. LC_{50} value

In this study, the exposure level of the freshwater fish grass carp (*Ctenopharyngodon idella*) to the herbicide glyphosate was set at 1.36 ppm, which is 1/10th of the acute 24-hour LC_{50}.

B. Plasma electrolytes for Na^+/K^+-ATPase

The blood plasma electrolyte profiles (Na+, K+ and Cl^-) in glyphosate-contaminated fish up to an acute dosage of 1.36 ppm are shown in Table 1 of this study. The results indicated that, after 24 hours of exposure, fish treated with herbicides showed decreased plasma electrolyte levels than control group, with the highest reductions in Na+ (40.57%), Cl^- (36.04%), and K+ (3.38%). Although the plasma K+ level never was changed significantly ($p<0.05$), the alterations in ionic concentration are determined to be statistically significant ($p<0.05$) for the blood serum Na+ and Cl^- levels. In conclusion, glyphosate treatment significantly reduced the reaction of the gill Na+/K+ -ATPase in comparison to control fish ($p<0.05$).

C. Sublethal toxicity on plasma electrolytes

The exposure concentration of glyphosate in freshwater fish grass carp *Ctenopharyngodon idella* was 1.36 ppm, which was 1/10th of the acute 24-hour LC_{50}. Table 1 lists the plasma levels of Na^+, K^+, and Cl^- electrolytes in glyphosate-effected fish after a 24-hour exposure period. When compared to the control group, the plasma electrolyte levels of glyphosate-treated fish were lower, with the highest percentages of Na^+ (40.57%), Cl^- (36.04%), and K^+ (3.38). The variations in ionic content were statistical significantly changed ($p<0.05$) Furthermore, plasma Na^+ and Cl^- concentration, but not significantly ($p<0.05$) changed for plasma K^+. The repression of gill Na^+/K^+-ATPase interaction in glyphosate-treated fish was also statistically significantly from fish with in control group. Over the course of the 35-day exposure period, the serum Na^+ concentration in the control fish decreased (Fig. 1a). By the end of the seventh day, the serum Na^+ levels in the glyphosate-treated fish had increased. However, the plasma Na^+ concentration was considerably lower ($p<0.05$) in the control group at days 14, 21, and 28. The serum K^+ level in glyphosate-treated fish was reduced at days 7, 21, and 28, but not at days 14 and 35 (Fig. 1b). Throughout the exhibition duration, the plasma Cl^- levels in the glyphosate-experimental fish were significantly lower compared to those of both the control and treated fish (Fig. 1c).

D. Sublethal toxicity in Na^+/K^+-ATPase

A correlation between the duration of exposure to glyphosate and the suppression of Na^+/K^+-ATPase action in gills of fish was observed during the experiments process. The gills of the treated group of fish displayed a reduced in Na^+/K^+-ATPase activity, as shown in Fig. 1d.

Table 1: Changes in plasma electrolytes (Na^+/K^+ -ATPase activity in a freshwater fish grass carp *Ctenopharyngodon idella*) experiment with acute levels of glyphosate 1.36 ppm in 24 h.

Parameters	Control	Treated	Percentage
Sodium (mmol/L)	87.5± 2.35	52±1.075	-40.57
Potassium (mmol/L)	6.5±0.188	4.3±0.116	-3.38
Chloride (mEq/L)	86±2.403	55±1.178	-36.04
Na+/K+ -ATPase (µg/h/g)	25.3±0.12	8.4±0.21	-6.67

Value is mean ± S.D of five individual observation, (+) denotes per cent increase over control, (-) denotes per cent decrease over control * Values are significant at ($p<0.05$)

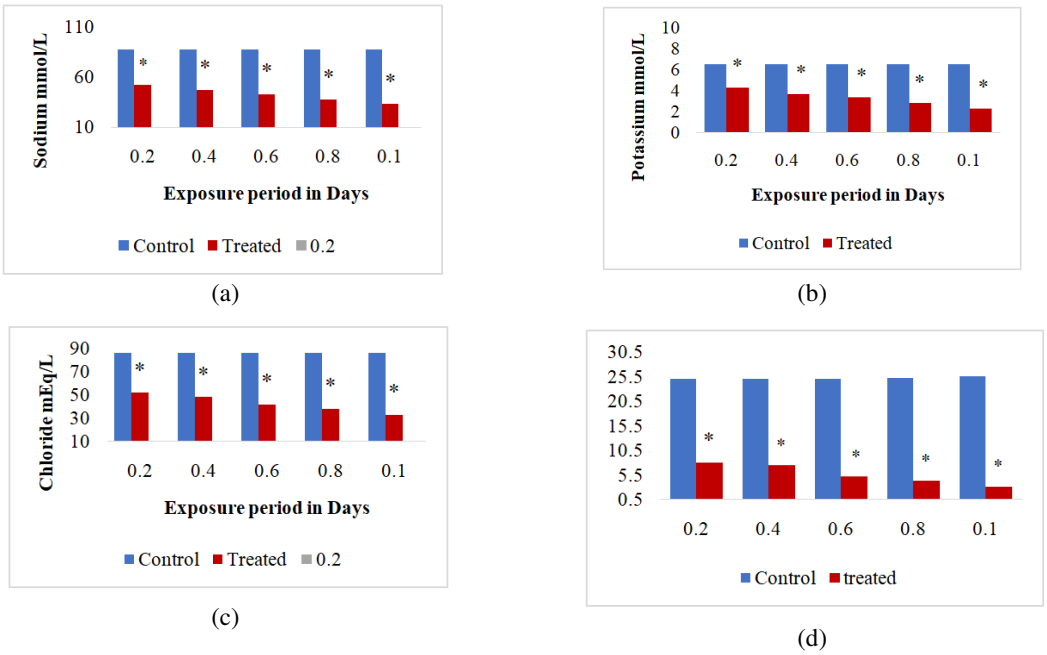

Fig. 1. a – d. Plasma sodium (Na+), potassium (K+), and chloride (Cl^-) and gills Na^+/K^+ -ATPase concentration of experimental and glyphosate treated fish 1.36 ppm.

DISCUSSION

The use of surfactants is a significant factor contributing to the overall risk associated with the commercial formulations of glyphosate (Amarante *et al.,* 2002; Diamond and Durkin 1997; Peixoto, 2005). In particular, POEA, which is a major component in commercial glyphosate-based herbicide formulations such as Roundup®, is considered to being harmful fish than herbicides itself (Giesy *et al.,* 2000). Herbicide contamination in aquatic environments poses a significant threat to various aquatic species. Glyphosate was found to have a 24-hour LC_{50} value of 1.36 ppm for grass carp fingerlings, indicating the chemical's toxicity to fish. The contamination levels of glyphosate vary based on the type of fish (Borges *et al.,* 2007). Herbicide contamination in aquatic environments actions a significant endangered freshwater habitat. Glyphosate has been shown to have a 24-hour LC_{50} value of 1.36 ppm for grass carp fingerlings, indicating its toxicity to fish. The exposure of glyphosate changes depending on the type of fish, with some species being more sensitive to its effects than others. According to Neskovic *et al.* (1996), common carp have an estimated LC_{50} value of 645 mgl^{-1} after 48 hours and 620 mgl^{-1} after 96 hours when exposed to the Rodeo® formulation. Jiraungkoorskul *et al.* (2002) found that Nile tilapia had an LC_{50} value of 16.8mgl^{-1} after 96 hours. Albinati *et al.* (2007) discovered that piaucu had an LC_{50} value of 15.2 mgl^{-1} after 96 hours when exposed to Rodeo®. Langiano and Martinez (2008) found that curimbatá had an LC_{50} value of 13.7 mgl^{-1} after 96 hours when exposed to Roundup®. In contrast, Shiogiri *et al.* (2010) found that the LC_{50} value for *P. mesopotamicus* exposed to the RR formulations was greater than the values discovered for other species when exposed to Rodeo® or Roundup®. The bioaccumulation of the RR formulation of glyphosate to *P. mesopotamicus* is still uncertain, as the amounts of POEA surfactant and other ingredients are not known. However, the calculated LC_{50} 48h value of glyphosate in this preparation is close to the ecologically explored found in typical crop treatments (3.7 mg L^{-1}), which raises concerns about the potential impact on aquatic systems where this fish species is prevalent (Giesy *et al*., 2000). Responses to harmful chemicals was already shown to be effective in morphological alterations in fish gills and liver (Cerqueira and Fernandes 2002; Crestani *et al.,* 2007; Mela *et al.,* 2007). Studies on glyphosate exposure in fish have reported enlarged gills, oedema, infiltration of leukocytes in the

epithelium, and hyperplasia in carp (Neskovic *et al.,* 1996) and aneurysms, fusion of the lamellae, hypertrophy of cells, and filamentous growth in Nile tilapia gills (Jiraungkoorskul *et al.,* 2002). Despite this, the RR formulation has been officially approved for use in field crops in Brazil, increasing the risk of contamination in aquatic environments. In general, physiological and biochemical data can provide more insight into identifying substances that warrant further hazard analysis. During acute exposure to pesticides, fish may exhibit behavioural abnormalities, such as significant changes in opercular beats rate, loss of balance, sluggishness, and settling at the base. These symptoms can cause to breathing difficulty and immobility. Additionally, dead fish may have a film of mucus in the surface. The high response to fish central nervous system to toxic chemical may be responsible for the increased sensitivity of fish to synthetic toxic chemical, which could be due to variations between different kinds of pesticides inhalation (Begum, 2005).

The assessment of physiological and biochemical changes in fish effected to environmental toxins has become a crucial method for evaluating aquatic ecosystems. Blood is a crucial indicator of an animal's physiological state, as it reflects its health and is in direct contact with numerous body systems and tissues. Freshwater teleost fish use ion and osmoregulatory systems to maintain their body fluid equilibrium and physiological functions. According to McCarty and Houston (1976), decreased amounts of blood plasma electrolytes are often associated with increased tissue concentration of key extracellular electrolytes like sodium and chloride. When exposed to fenvelerate, studies on the fish species *L. rohita* showed a decrease in the gills, muscle, and liver Na^+, K^+, and Ca^{2+} ions, suggesting that fenvelerate may have disrupted the cell membrane's permeability and caused a disturbance in the Na^+/K^+ and Ca^{2+} ionic pump due to tissue injury (Reddy and Philip 1994). The study of the impact of ecological toxic effects of fish has become an important tool for evaluating water ecosystems. *Tilapia mossambicus* exposed to atrazine showed an increase in blood serum sodium, potassium, and chloride ions, leading to a decrease in these ions in the muscle of the fish, indicating abnormalities in the osmoregulatory process (Prasad and Reddy 1994). The induction or depression of enzymes in fish or other species have been reported as a tool for observing pollution. Na^+/K^+-ATPase, an enzyme found in the basolateral membrane of gill epithelial cells in freshwater creatures, is actively involved in the transfer of electrolytes through the gills (Parvez *et al.,* 2006). At the cellular and organismal levels, Na^+/K^+-ATPase is an evolutionarily conserved transmembrane enzyme is required for osmoregulation (Dang *et al.,* 2000). Biological contaminants can affect the activity of Na^+/K^+-ATPase by interfering with energy production or by directly interacting using the enzyme (Watson and Beamish 1980). Aquatic species need to maintain balance in osmotic pressure, acid-base and water and ion flow by transporting ions such as Na^+, K^+, and Cl^- through the gills (Mayer *et al.,* 1992). Measuring Na^+/K^+-ATPase interaction can be a critical indicator for acceptable levels of environmental pollutants, as ATPase plays a direct role in electrolyte balance (Mathan *et al.,* 2010; Oruc *et al*., 2002). According to recent studies, glyphosate has a significant impact on ATPase function, leading to a significantly decrease in Na^+/K^+-ATPase activation throughout acute and sublethal exposure. Herbicides, including glyphosate, can disrupt cellular and ionic homeostasis, as well as salt uptake, when absorbed in the gill region. The observed changes in plasma ion concentration may be credited to alterations in gill accessibility of membranes caused by herbicide toxicity.

CONCLUSIONS

The findings of the study indicate the Na^+/K^+-ATPase enzyme acts as critical involvement in the regulation of branchial ion transportation and the preservation of osmotic and ionic cellular metabolism in freshwater fish. The toxicant-induced repression of this enzyme action can have consequences for the preservation of the balance of ions and osmolality and the level of this metabolite can be utilized to detect impact on fish ionic and osmoregulation. Hence, employing less persistent glyphosate, developing herbicides with specific targets, and implementing molecular regulation measures it may reduce in hazard to humans and other living things.

FUTURE SCOPE

Glyphosate affected in grass carp gill and ion regulation in the present research may be further to affects for fish enzymes and DNA damages.

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Conflict of Interest. None.

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