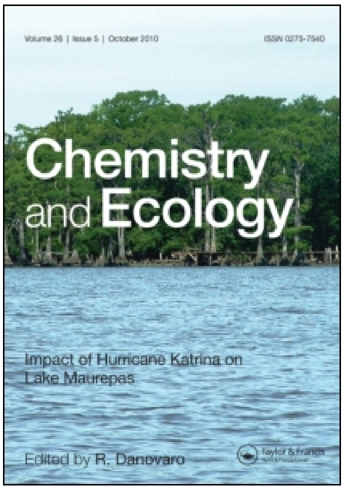

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Influences of environmental factors and antidote addition on glyphosate toxicity to freshwater fish, *Labeo rohita* (Hamilton)

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Influences of environmental factors and antidote addition on glyphosate toxicity to freshwater fish, *Labeo rohita* (Hamilton)

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The effect of glyphosate (a potential herbicide: trade name glycel™, isopropyl amine salt of glyphosate, 41% w/w and inert materials 59% w/w) has been investigated on the survival rate of a freshwater fish *Labeo rohita*. In the present study, we also analysed the influence of some environmental factors (e.g. pH, calcium, and salinity levels of water) and of antidote application (fresh cow dung) on the glyphosate toxicity. A concentration of glyphosate at 12.3 l a.i. ha^{-1} m^{-1} was very effective in inhibiting the growth of some aquatic submerged macrophytes, and so this dose was chosen as a toxicant to the fish. Survival rate of the fish was considered as an index of glyphosate toxicity. The results showed that the toxicity of glyphosate persisted for *c*. 18 d after which the herbicide degraded, and the survival rate of *Labeo rohita* (mean weight 24.5 ± 0.3 g and mean length 14.5 ± 0.25 cm) was 100%. We observed the total mortality of the fish up to the 12 d of the experiment. The toxic effect of the glyphosate was significantly reduced ($P < 0.05$) at pH 6.8–7.6, at salinity 1.0 ppt, and at water calcium level *c*. 200 mg l^{-1}. Our results showed that a dose of 30 ml l^{-1} (9990 kg $ha^{-1}m^{-1}$) of fresh cow dung (3.33% w/v in water) reduced the toxicity of glyphosate and the survival rate of the fish reached 100% up to 96 h from the application.

Keywords: Glyphosate; *Labeo rohita*; Environmental factors; Antidote

1. Introduction

Fish forms a part of daily diet in most Indian families. It is one of the most important and low-cost sources of protein available. Therefore, the quality of fish must be safe for human consumption. Because of the indiscriminate use of pesticides, there is a high possibility of contamination of aquatic bodies and consequently of fish, thus being unsuitable for human consumption. Glyphosate is a widely used herbicide to control the growth of a number of submerged macrophytes in freshwater bodies [1] and has a profuse chance of polluting water bodies and fish. Changes in some environmental factors in the water such as temperature, pH,

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hardness, alkalinity, calcium level, and salinity may alter or (with a few exceptions) reduce the toxic effects of pesticides. Some studies [2–6] have reported that the toxicological effects of endosulfan decrease at a high pH (10.0 units), whereas different results have been observed in the case of triazophos. The toxicity of endosulfan decreases with increasing exposure period and hardness of water. These results indicate that the degradation of endosulfan takes place at a high pH and with an increase in the hardness of water, but in the case of triazophos its toxicity increased under similar environmental conditions [7]. Antidotes may play a key role in the studies of management and monitoring of toxicity. The efficacy of ascorbic acid (vitamin C) for removal of toxicity caused by different pollutants including pesticides has been reported in various studies [8, 9]. Some authors [10] suggest an approach to reduce organochlorine pesticide content based on the culinary treatments of fish. A new trend to reduce the herbicidal/pesticidal toxicity is based on the use of different types of organic matter. Thus, it is evident that environmental parameters and antidote addition can ameliorate the toxic effect of pesticides. Therefore, an attempt has been made in the present investigation to study the effect of glyphosate on the survival rate of a freshwater fish, *Labeo rohita*, and also to evaluate the influence of some environmental factors and antidote addition on glyphosate toxicity to the fish.

2. Materials and methods

2.1 *Effect of glyphosate toxicity on survival rate of* Labeo rohita

The experiment was carried out in 25-l glass aquaria using 20 l of dechlorinated tap water in each aquarium. Glyphosate at 12.3 l a.i. ha^{-1} m^{-1} was very effective in inhibiting the growth of some aquatic submerged macrophytes such as *Nechamandra alternifolia*, *Hydrilla verticillata*, and *Ottelia alismoides* (to an extent of 98, 89, and 100%, respectively within 15 d [1]). We also observed that a few more days were required to clear the entire mass with the same dose in the aquaria where 100% decay did not occur in 15 d. As this dose of glyphosate is effective in controlling the distribution of the submerged aquatic weeds in fish ponds, this dose of glyphosate has been chosen to evaluate the effect of this herbicide on fish in the present study. The concentration of glyphosate used for this experiment was 0.3 ml l^{-1} water, which is equivalent to 12.3 l a.i. ha^{-1} m^{-1}. Glyphosate (Glycel™), 41% S.L., manufactured by Excel Industries, Mumbai, was used. The chemical composition of glyphosate was isopropyl amine salt of glyphosate 41% w/w (weight by weight) and inert materials 59% w/w. Both the treatments and controls were maintained in triplicates. Fish specimens (*Labeo rohita*) were collected from the fish farm of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Orissa, India. The average size and weight of the fish were 14.5 ± 0.25 cm and 24.5 ± 0.3 g, respectively. The fish were disinfected by dipping them in 1% $KMNO_4$ solution for 1 min, after which they were acclimatized in laboratory under natural light and temperature conditions for 7 d, before the start of our experiment. Fish were added to the experimental jars on day 0, 6, 12, and 18. Each time, six fish were added in each jar, and the survival rate of each fish was reported.

2.2 *Regulation of environmental parameters on glyphosate toxicity to* Labeo rohita

The effects of the three different environmental parameters pH, calcium, and salinity of water were tested on glyphosate toxicity to *Labeo rohita*. Fish specimens were collected from CIFA fish ponds and disinfected by giving them a 1-min dip treatment in 1% $KMNO_4$ solution. After the initial acclimatization in a polyfibre tank, only healthy and active specimens were used for the different investigations.

The study was carried out in 40-l plastic tubs filled with 30 l of dechlorinated tap water. *Labeo rohita* of average weight 24.5 ± 0.3 g and length 14.5 ± 0.25 cm were used. Six fish were kept in each tub, and the tubs were aerated using aerators. A period of 1 week was allowed for the fish to acclimatize. Feeding was withheld 1 day prior to the experiment.

2.2.1 Water pH. Four different values of water pH, i.e. 6, 7, 8, and 9, were maintained in different tubs. Three replications were maintained for each pH value and also for the control. Different pH solutions were prepared and buffered following the standard methods. For pH 6, 19.0 ml l^{-1} 1.0 N NaOH + 80.0 ml l^{-1} of 1.0 M KH_2PO_4, for pH 7, 19.0 ml l^{-1} 1.0 N NaOH + 30.0 ml l^{-1} 1.0 M KH_2PO_4, for pH 8, 19.0 ml l^{-1} 1.0 N NaOH, and for pH 9.0, 8.8 ml l^{-1} 1 N NaOH + 30 ml l^{-1} 0.5 M H_3BO_3 reagent-grade chemicals were added to aerated freshwater to buffer the required pH (ml l^{-1} indicates 1 millilitre per litre of water used). A lethal dose of glyphosate, i.e. 12.31 a.i. ha^{-1} m^{-1}, was used for the study. The solubility of glyphosate in water was 12 g l^{-1} at 25 °C. Based on this, the nominal concentration of glyphosate in the water could be 14 μg l^{-1}. The experiment was conducted for 96 h, and the percentage mortality was reported.

2.2.2 Water calcium. Five sets, viz. (1) control (no glyphosate+no calcium), (2) control (with glyphosate + no calcium) (A), (3) A + 100 mg l^{-1} Ca^{2+}, (4) A + 200 mg l^{-1} Ca^{2+}, and (5) A + 300 mg l^{-1} Ca^{2+}, each with two replications, were maintained. A lethal dose of glyphosate, i.e. 12.31 a.i. ha^{-1} m^{-1}, was used. One hundred (100) mg l^{-1} Ca^{2+} was obtained from 12.41 g of $CaSO_4 \cdot 2H_2O$, 200 mg l^{-1} from 25.82 g, and 300 mg l^{-1} from 38 g of $CaSO_4 \cdot 2H_2O$, respectively. The experiment continued for 96 h, after which the percentage mortality was counted. Hardness and alkalinity were measured following standard procedures [11].

2.2.3 Water salinity. Six different sets, viz. (1) Control (no salt + no glyphosate), (2) control (with glyphosate lethal dose) (A), (3) A + 15 g NaCl + 15 g KCl, (4) A + 30 g KCl, (5) A + 45 g NaCl + 45 g KCl, and (6) A + 60 g NaCl + 60 g KCl, and each set with three replications were maintained. The experiment was carried out for up to 96 h, during which time observations of fish mortality were made, and data were reported at the end of the experiment. The conductivity and salinity of the water were measured using an Autoranging Conductivity Meter (Chemito 130) and Salinometer, after 96 h, according to standard procedures. The survival rate was considered as an index of the effect of environmental parameters. The observations were made up to 96 h according to standard protocols. The rearing tanks were not supplied with a filtration system. During the exposure of glyphosate for 96 h to the tank, no food was added. The 96-h study was a static bioassay test. Thus, chemical parameters (NO_2, NO_3) have not been tested. However, the NO_3 content of the initial water was 0.01 mg l^{-1}, while NO_2 was not detected.

2.3 *Methods for assessment of efficacy of an antidote to glyphosate toxicity to* Labeo rohita

This investigation was carried out in a 40-l plastic tub filled up with 30 l of dechlorinated tap water. The *Labeo rohita* juveniles of average length 14.5 ± 0.25 cm and average weight 24.5 ± 0.3 g collected from the CIFA fish ponds were used for the study. The fish were disinfected by giving them a 1-min dip treatment in 1% $KMNO_4$ solution, after which they were acclimatized to laboratory conditions in natural light and temperature conditions for a period of 7 d. In each tub, six fish were kept. The feeding and aeration were stopped during the experimental period.

Cow dung is organic manure used in fish ponds for plankton production and is an age-old practice in Indian aquaculture. Thus, cow dung was used to study its ameliorative action against glyphosate toxicity. Fresh cow dung was collected from the CIFA dairy, and a stock solution of 3.33% w/v in water was prepared using the raw cow dung. The chemical composition of cow dung was 80 ± 5% moisture, 12 ± 2% organic materials, 0.40 ± 0.05% N, 0.20 ± 0.05% P_2O_5, and 0.10 ± 0.05% K_2O. Three different doses, viz. 10 ml l^{-1} of stock solution (3330 kg ha^{-1} m^{-1} cow dung), 20 ml l^{-1} of stock solution (6660 kg ha^{-1} m^{-1} cow dung), and 30 ml l^{-1} of stock solution (9990 kg ha^{1} m^{-1} cow dung), were added to each tub. Three replications for each dose as well as for the control were maintained. Glyphosate at 12.3 l a.i. ha^{-1} m^{-1} water was used as toxicant. Fish survival rates (%) were observed 24, 48, 72, and 96 h, respectively, after the beginning of the experiment.

2.4 *Statistical analysis*

Student's *t* test was used to determine whether the environmental parameters (pH, calcium, and salinity) and the antidote cow dung had any significant effect in ameliorating the toxicity of glyphosate to fish [12].

3. Results

The effect of glyphosate toxicity on the survival rate of Labeo *rohita* is reported in table 1. Fish mortality was observed on days 0, 6, and 12 when they were exposed to glyphosate. However, on the 18th day, 100% survival of the fish was observed when they were exposed to the herbicide treatment. No mortality of fish was reported in any of the control jars (without herbicide) during the experiment.

The effect of pH on glyphosate toxicity to *Labeo rohita* after 96 h of exposure is reported in table 2. A lower fish mortality rate at the lethal level of glyphosate was observed at pH 6.8 and 7.6, whereas the total fish mortality was observed at pH above 7.6. At pH 6.8, 50% of fish mortality was recorded, while at pH 7.6, 30% fish mortality was observed at the lethal dose of

Table 1. Effect of glyphosate toxicity on survivability of *Labeo rohita.*

Treatment	Day of observations	R_1	R_2	R_3	Percentage survivability
Control (no glyphosate)	0	6	6	6	100
	6	6	6	6	100
	12	6	6	6	100
	18	6	6	6	100
12.3 l a.i. ha^{-1} m^{-1} glyphosate	0	0	0	0	0
	6	0	0	0	0
	12	0	0	0	0
	18	6	6	6	100

Note: R: replication.

Table 2. Effect of pH on glyphosate toxicity to *Labeo rohita* after 96 h of exposure (lethal dose: 12.3 l a.i. ha^{-1} m^{-1} of glyphosate).

pH of the medium before addition of glyphosate	pH of the medium after addition of glyphosate (lethal dose)	Percentage mortality R_1	R_2	R_3	t-test value (0.05)
Control (no glyphosate)	6.8	0	0	0	
6.0	6.8	40 ± 10	60 ± 10	50 ± 10	27.71 S
7.0	7.6	30 ± 10	20 ± 10	40 ± 10	19.08 S
8.0	8.8	100	100	100	
9.0	9.6	100	100	100	

Note: S: significant.

glyphosate. These results showed that the toxic effect of glyphosate decreased at near neutral to slightly alkaline pH (pH 7.6). No fish mortality occurred in the control.

The effect of calcium level of water on glyphosate toxicity to *Labeo rohita* after 96 h of exposure is shown in table 3. Concentrations of *c*. 200–300 mg l^{-1} calcium completely reduced the toxicity of glyphosate as in this range of calcium 100% fish survived at up to 96 h of exposure to the lethal level of glyphosate. At lower calcium levels (*c*. 50 mg l^{-1}), only 50% fish survived. These results demonstrate that glyphosate toxicity could be limited and completely disappears at higher calcium concentrations (above 200 mg l^{-1}). In the control (without glyphosate and without calcium), there was no mortality, whereas in the control (only with glyphosate and no calcium), there was total mortality of the fish *Labeo rohita*.

The effect of salinity on glyphosate toxicity to *Labeo rohita* after 96 h of exposure period is summarized in table 4. At a salinity level of 1.0 ppt, 100% survival rate of the fish was reported at the lethal dose of glyphosate after 96 h of exposure. Above a salinity level of 1.0 ppt, the fish mortality rate increased, and at a salinity level of 3 ppt, 100% fish mortality was recorded at the lethal dose of glyphosate after 96 h of exposure. This shows that 1.0 ppt salinity could reduce the glyphosate toxicity. No fish mortality occurred in the control (without salt and glyphosate), whereas there was 100% mortality in the control (with glyphosate and without salt).

The efficacy of cow dung on glyphosate toxicity using the survival rate of *Labeo rohita* is reported in table 5. It is evident from the results that fresh cow dung (3.33% w/v in water) at 30 ml l^{-1} was most effective in reducing the toxicity of the lethal dose of glyphosate to *Labeo rohita* in terms of survival rate, and at this dose of cow dung, 100% survival of the fish was recorded at the lethal dose of glyphosate up to 96 h and beyond. The fresh cow dung at 10 ml l^{-1} could alleviate the toxic action of glyphosate up to 48 h, beyond which the fish died.

Table 3. Effect of calcium on glyphosate toxicity to rohu (*Labeo rohita*) after 96 h of exposure (lethal dose: 12.3 l a.i. ha^{-1} m^{-1} of glyphosate).

Nature of solution	Hardness (ppm)	Alkalinity (ppm)	Percentage mortality R_1	R_2	R_3	t-test value (0.01)
Control (no glyphosate + no calcium)	148	124	0	0	0	
Control (with glyphosate-lethal dose)-A	160	116	90 ± 10	100	100	
3. A + 100 ppm Ca^{2+}	360	164	40 ± 10	50 ± 10	50 ± 10	36.12 S
4. A + 200 ppm Ca^{2+}	580	140	10 ± 10	0	0	53.02 S
5. A + 300 ppm Ca^{2+}	760	100	0	0	0	

Note: S: significant; R: replication.

Table 4. Effect of salinity on glyphosate toxicity to rohu (*Labeo rohita)* after 96 h of exposure (lethal dose: 12.3 l a.i. ha^{-1} m^{-1} of glyphosate).

Nature of solution	Conductivity (dSm^{-1})	Salinity (ppt)	Percentage mortality			t-test value (0.05)
			R_1	R_2	R_3	
1. Control (no salt + no glyphosate)	0.271	BDL	0	0	0	
2. Control (glyphosate with lethal dose) A	0.285	BDL	100	90 ± 10	100	
3. A + 15 g NaCl + 15 g KCl	1.430	1.0	0	10 ± 10	0	17.21 S
4. A + 30 g NaCl + 30 g KCl	2.560	1.5	40 ± 10	50 ± 10	40 ± 10	33.64 S
5. A + 45 g NaCl + 45 g KCl	3.500	2.0	50 ± 10	60 ± 10	50 ± 10	32.28 S
6. A + 60 g NaCl + 60 g KCl	5.050	3.0	100	100	90 ± 10	9.18 n.s.

Note: BDL: below detection limit; S: significant; R: replication.

Table 5. Efficacy of cow dung to glyphosate toxicity on survival of rohu (*Labeo rohita*) (lethal dose: 12.3 l a.i. ha^{-1} m^{-1} of glyphosate).

Observation at different time intervals	Survival (%) in control (without glyphosate)	Survival (%) in presence of lethal dose of glyphosate	Survival (%) in lethal dose of glyphosate + cow dung (3.33% (w/v) in water)		
			10 ml l^{-1}	20 ml l^{-1}	30 ml l^{-1}
24 h	100	0	100	100	100
48 h	100	0	40 ± 10	100	100
72 h	100	0	0	60 ± 10	100
96 h	100	0	0	20 ± 10	100
t-test value (0.05)				8.16 S	37.17 S

Note: S: significant.

The cow dung at 20 ml l^{-1} reduced the toxic actions of glyphosate up to 96 h in terms of a 20% survivability of the fish. The effectiveness of fresh cow dung at various concentrations in ameliorating the toxicity of glyphosate to the fish was statistically significant ($P < 0.05$).

4. Discussion

The results of the effect of glyphosate on the survival rate of *Labeo rohita* indicated that the toxicity of this herbicide persisted in the water for at least 12 d. As no fish mortality was recorded on the 18th day, it could be assumed that the degradation of the herbicide occurred between the 13th and 18th days. There are several studies indicating that the different herbicides persist in water for different periods. The accumulation of radioactivity (concentration 2,4-D) and glyphosate in carp and tilapia were studied using labelled and unlabelled chemicals at a concentration of approximately one-hundredth and one-thousandth of the 48-h LC_{50} values[13]. The authors reported that approximately 83 (at a concentration of 0.5 ppm) and 91% (of 0.05 ppm) of the radioactivity was in the water until 14 d from the beginning of the experiment, but only 17.2% of glyphosate (0.05 ppm concentration of glyphosate) was observed in the water. No significant effects of glyphosate on fish mortality were reported from 2 to 7 d. They also observed that glyphosate disappeared within 3 d in water under sunlight; the radio chemicals in the water hyacinth remained constant up to the 14th day. Pendimethalin and anilofos herbicides were moderately persistent, with an average half-life of 56–60 d [14] and 11–15 d in water, respectively [15]. The organochlorine herbicide, butachlor, persisted in water for 140 d, as indicated by the survival of the fish [1]. The rate of degradation of herbicides in different aquatic systems varied with edaphic and climatic conditions as well as with the properties of the chemical itself. Persistence of pendimethalin was inversely related to soil-water content [16]. Glyphosate isopropylamine was practically non-toxic, producing no mortality among any of the four species of south-western Australian frogs: *Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis*, and *Litoria moorei* over 48 h, at concentrations between 503 and 684 $mg\,l^{-1}$ (343 and 466 $mg\,l^{-1}$ glyphosate acid equivalent, AE) [17]. They also reported that the toxicity of technical-grade glyphosate acid (48 h LC_{50}, 81.2–121 $mg\,l^{-1}$) was likely due to acid intolerance. The slight differences in species sensitivity were evident, with *L. moorei* tadpoles showing greater sensitivity than tadpoles of the other four species. Adult and newly emergent metamorphose were less sensitive than tadpoles.

In the present study, the toxicological effects of glyphosate decreased at near neural to slightly alkaline pH (pH 6.8–7.6) of the test water. When the calcium level of water was examined, the toxicity showed a decreasing pattern with increasing calcium concentration in water. Regarding salinity, the toxicity of glyphosate decreased at 1 ppt salinity for the test water. These results indicated that the degradation of glyphosate took place at neutral to slightly alkaline pH; at 1 ppt salinity and at high (100–200 $mg\,l^{-1}$) calcium level in the water. The isopropyl amine salt of glyphosate herbicide was more toxic to rainbow trout and bluegills at pH 7.5 than at pH 6.5. Moreover, the toxicity did not increase at pH 8.5 or 9.5 [18]. High values of pH (6–9) and concentration of suspended sediment (0–200 $mg\,l^{-1}$) significantly increased the toxicity of glyphosate-based herbicides (e.g. Roundup) to *Ceriodaphnia dubia* [19]. The toxicological effects of endosulfan to *Heteropneustes fossilis* decreased at high pH (pH 10) and at a high water-hardness level (200 $mg\,l^{-1}$) [20]. The decrease in toxicity of endosulfan was also reported in relation to *Cirrihinus mrigala* in systems characterized by a high pH and water hardness [4]. Conversely, Rawat *et al.* [20] reported that the toxicity of triazophos to *Heteropneutes fosslis* increased at a higher pH and water hardness. The increase in toxicity at low pH and at high pH might be attributed to the hydrolysis of the toxicants resulting in production of isomers which are comparatively more toxic [21].

In the present study, the fresh cow dung (3.33% w/v in water), at 30 $ml\ l^{-1}$ (9990 kg $ha^{-1}\ m^{-1}$), was the most effective antidote in ameliorating the toxic actions of lethal concentration of glyphosate to *Labeo rohita* (100% survivability after a 96-h exposure period). The protective action of cow dung against glyphosate toxicity could be either due to its preferential reaction (flocculation and precipitation) with toxic ingredient of glyphosate, or due to its action as a precursor of enzymatic activities. The cow dung may be enabling to restore tissue supply of such enzymes which may otherwise be depleted in situations involving inhibition of such enzymes which leaves the cells susceptible to oxidative stress. In the present study, the dissolved organic carbon (DOC) of the water was 2.0 $mg\,l^{-1}$. Both dissolved (DOC) and particulate organic carbon (POC) in the test water could also affect the bioavailability of insecticide to fish. This effect has been demonstrated for the accumulation of pyrethroids, viz. deltamethrin, fenvalerate, and cyhalomethrin, by *Daphnia magna* [22]. However, it has been reported that in natural waters, POC was likely to influence the amount of pyrethroids available to rainbow trout, but low concentrations of DOC (1–10 $mg\,l^{-1}$ carbon concentration used in that study) could be expected to have minimal effects on pyrethroid bioavailability [23]. An increased temperature and optimum level of dissolved oxygen (by aerator) could decrease the effect of pesticides [24]. Also, Das [25] has reported on the application of lime to increase the pH and reduce the toxicity of pesticides. Vitamins may have antitoxic effects against insecticide poisoning. In fact, a mixture of vitamins such as Macraberin Forte may be used as an antitoxicant against malathion poisoning to *Channa punctatus* [26].

5. Conclusion

Water pH in the range of 6.8–7.6 resulted in a higher fish survival rate at lethal concentrations of glyphosate, whereas near 1.0 ppt salinity, this could ameliorate glyphosate toxicity. Glyphosate toxicity could also be reduced significantly at higher (above $200\,\mathrm{mg\,l^{-1}}$) calcium levels in water. Fresh cow dung (3.33% w/v in water) at $30\,\mathrm{ml\,l^{-1}}$ ($9990\,\mathrm{kg\,ha^{-1}\,m^{-1}}$) could be one of the most effective antidotes in ameliorating the toxic actions of lethal concentration of glyphosate to the fish in terms of survival rate. In freshwater pond aquaculture, growth of the fish is optimal at a slightly alkaline pH. The application of cow dung is an age-old practice in Indian aquaculture. Thus, cow-dung application to fish ponds could protect fish from glyphosate toxicity. However, cellular and molecular studies are required to assess the ameliorative role of cow dung on fish health and to study the detoxification pathway or any stress that could have been activated in fish following cow-dung treatments.

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