The Lethal Impacts of Roundup® (glyphosate) on the Fingerlings of Guppy, *Poecilia reticulata* Peters, 1859

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Abstract

Weedicides bring about adverse effects to non-targeted animal species in the agricultural fields and associated habitats. Among these, Roundup® (glyphosate) is one of the commonest that is used all over the world. The present laboratory investigation examined the effects of Roundup® on fingerlings of guppies (*Poecilia reticulata*). In this investigation, the 24-hr and 96-hr LC_{50} of Roundup® for the guppy fingerlings were determined by exposing them to a series of Roundup® test preparations ranging from 3.6 $mg^.L^{-1}$ to 36 $mg^.L^{-1}$. Further, the behavioural changes of the fingerlings at these test preparations were studied and the histopathological changes of their gills were examined. The results showed that the 24-hr and 96-hr LC_{50} for Roundup® were 15.1 $mg^.L^{-1}$ and 9.76 $mg^.L^{-1}$ respectively. These values are well below the recommended field concentration of Roundup®, which is 2,592 $mg^.L^{-1}$. Fingerlings preferred to rest more at the surface of test preparations than resting elsewhere in the tanks. In contrast, such a behavioural change was not observed in the control. The histopathological study showed that the fingerlings exposed to all the Roundup® concentrations developed gill hyperplasia where its severity was concentration dependent. It is evident that the toxic concentration of Roundup® on guppy fingerlings is much lower than its recommended field concentration. At these toxic concentrations, Roundup® brings about stress and severe gill damage in guppies.

Introduction

Weedicides are extensively used to combat weed invasions (Carlisle and Trevors, 1986). One of the commonest and most effective weedicides used all over the world is glyphosate ($C_3H_8NO_5P$-N-(phosphometyl) glycine) (Gasnier et al. 2009). Glyphosate effectively kills the weed plant by interfering with the production of its aromatic amino acids resulting in a protein and carbon shortage (Hedberg and Wallin, 2009). In spite of its effectiveness and short life span in the environment (Giesy et al. 2000; Jiraungkoorskul et al. 2002), glyphosate brings about adverse effects to non-targeted animal species in the agricultural fields and the associated habitats.

Because of the leakages of weedicides occurring due to runoff from the crop fields, the surrounding aquatic environment can be contaminated so that fish and other aquatic organisms are affected (Jiraungkoorskul et al. 2001). For example, sub-lethal concentrations of glyphosate, corresponding to less than 2% of the LC_{50}, have caused ultra structural damage in the liver tissue of

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Cyprinus carpio (Szarek et al. 2000). Histological alterations were also observed in liver, gills and kidneys of *Oreochromis niloticus* after acute and chronic exposure to sub-lethal concentrations (Jiraungkoorskul et al. 2001, 2002). Further, effect of glyphosate on physiological activities of many fishes has also been tested. For example, Glusczak et al. (2006) showed that when glyphosate was tested against *Leporinus obtusidens*, the brain acetyl choline esterase activity and muscle glycogen and glucose level significantly decreased together with drastic alterations in hematological parameters. In addition, glyphosate also caused changes in swimming, breathing, migration and reproductive behaviours of fishes (Camargo and Martinez, 2006). Toxicological studies have also been carried out against amphibians (Relyea, 2004 and 2005) where the results obtained were more or less similar to those of fishes. Glyphosate has resulted in significant population losses of a number of terrestrial species through habitat and food supply destruction and thus caused a threat to endangered species and biodiversity (Kreutz et al. 2010).

Although acute toxicity of glyphosate itself is considered to be low, commercial glyphosate formulations such as Roundup[®] are more toxic (Langiano and Martinez, 2007) as they contain a surfactant called poly-oxy ethylene amine. This surfactant is used to promote penetration of glyphosate through plant cuticle (Glusczak et al. 2006) thereby enhancing its efficiency (Tsui and Chu, 2003; Relyea, 2005).

The present laboratory investigation examined the effect of Roundup[®] on the fingerlings of guppies. Guppies are commonly found in aquatic habitats associated with agricultural fields, and are considered as a hardy fish species and a very good biological controlling agent of mosquito larvae and a pollution indicator species (Costa, 1985).

Materials and Methods

Guppy fingerlings ("variety - sunset"; length 1-1.5 cm and weight 0.25-0.4 g) were purchased from a commercial aquarium and acclimatised to laboratory conditions in aged tap water for 14 days under natural photoperiod. Continuous aeration was provided and the fingerlings were fed with a common commercial fish feed daily at 4% of their body weight. During the acclimatising period, unconsumed food pellets and faeces were siphoned out daily to maintain the water quality at optimum levels.

A commercial Roundup[®] formulation was purchased from a local distributor of agro-chemicals. The glyphosate concentration in this product was 360 $g^. L^{-1}$. Instructions were printed in the product label and the recommended concentration to be used in a paddy field was 2,592 $mg^. L^{-1}$ (dilution factor; 1:140) and 4 $L^. ha^{-1}$ of this preparation should be sprayed.

Range Finding Test

Initially a range finding test was carried out to determine the range of Roundup[®] concentrations at which fingerling mortality occurs. For this, four Roundup[®] solutions i.e., 360 $mg^.L^{-1}$, 36 $mg^.L^{-1}$, 3.6 $mg^.L^{-1}$ and 0.36 $mg^.L^{-1}$, together with a control, were prepared by diluting the commercial formulation with aged tap water as required. The concentration of the strongest solution of this series i.e., 360 $mg^.L^{-1}$, is nearly one-seventh of the recommended field concentration. A batch of 10 acclimatised healthy guppy fingerlings were introduced into each solution, and the percentage mortality was determined after exposing them for 96 hours with continuous aeration. Results of the range finding tests revealed that 100% mortality occurred at 360 $mg^.L^{-1}$ and 36 $mg^.L^{-1}$ solutions while it was 60% at the 3.6 $mg^.L^{-1}$ and 0% for 0.36 $mg^.L^{-1}$ and for the control (Table 1). Based on the range finding test results, the concentration range of Roundup[®] that should be used in the acute toxicity test was between 3.6 $mg^.L^{-1}$ and 36 $mg^.L^{-1}$.

Table 1. The percentage mortality of guppy fingerlings after 96 hour exposure period at the five Roundup[®] concentrations in the range finding test.

Roundup[®] concentration $mg^.L^{-1}$	Percentage mortality of guppy fingerlings
0 (control)	0
0.36	0
3.6	60
36	100
360	100

Acute toxicity test

The acute toxicity test determines the LC_{50} of Roundup[®]. In this test, ten Roundup[®] preparations with concentrations of 3.6 $mg^.L^{-1}$, 7.2 $mg^.L^{-1}$, 10.8 $mg^.L^{-1}$, 14.4 $mg^.L^{-1}$, 18 $mg^.L^{-1}$, 21.6 $mg^.L^{-1}$, 25.2 $mg^.L^{-1}$, 28.8 $mg^.L^{-1}$, 32.4 $mg^.L^{-1}$ and 36 $mg^.L^{-1}$, including a control set up, were prepared in rectangular glass tanks by mixing the Roundup[®] stock solution as required with aged tap water to a volume of 30 L each. As three test tanks from each concentration were prepared, there were three replicate experimental set ups in this acute toxicity test. Immediately after the test tanks were prepared, a group of 20 acclimatised healthy fingerlings were placed in each tank and exposed for 96 hours. During the entire experimentation period the fingerlings were not fed, but the tanks were aerated continually and the dissolved oxygen concentration in each tank was maintained around 6.5 $mg^.L^{-1}$.

The temperature (mercury glass bulb thermometer), pH (WTW; pH 315i), dissolved oxygen concentration (WTW; Oxi 315i), and ammonia concentration (UV spectrophotometer; model CECIL/CE 1021 at 630 nm) of the test water in each tank were measured daily around 10.00 am respectively. The number of dead fish at each tank was also recorded daily and the dead fishes were removed.

Of the fingerlings that were introduced into the 18 $mg^.L^{-1}$ solution, some survived for 24 hours, but the remainder succumbed within the first few hours. Further, fingerlings that were introduced into Roundup® solutions with the concentrations above 21.6 $mg^.L^{-1}$ succumbed within an hour so that those solutions were disregarded for further experimentation or analysis.

Fingerlings that were resting near the surface gulping for air in the 0 $mg^.L^{-1}$, 3.6 $mg^.L^{-1}$, 7.2 $mg^.L^{-1}$, 10.8 $mg^.L^{-1}$, 14.4 $mg^.L^{-1}$, 18 $mg^.L^{-1}$ solutions were carefully counted during the first 24 hour period of exposure. Further, three surviving fingerlings from each solution after the 96 hour exposure period was caught randomly, sacrificed and the gills were removed. These gills were then preserved, sectioned and stained following standard histological techniques for microscopic observations for abnormalities of the gill tissues including hyperplasia to assess the tissue damage caused by Roundup®.

Guppy mortality data in the corresponding Roundup® concentrations were analysed using the Probit analysis to determine the LC_{50} (Finney, 1971). Roundup® concentrations with zero or 100% mortality were excluded in the analysis. Therefore, four Roundup® concentrations i.e. 7.2 $mg^.L^{-1}$, 10.8 $mg^.L^{-1}$, 14.4 $mg^.L^{-1}$and 18 $mg^.L^{-1}$, and the corresponding mean percentage mortality data (n = 3) were considered to determine the overall 24-hr LC_{50} value and the 95% confidence limits while Roundup® concentrations of 3.2 $mg^.L^{-1}$, 7.2 $mg^.L^{-1}$, 10.8 $mg^.L^{-1}$ and 14.4 $mg^.L^{-1}$ and the corresponding mean percentage mortality data (n = 3) were considered to determine the overall 96-hr LC_{50} value and the 95% confidence limits.

The guppy fingerlings observed to be resting at the water surface between the treatments in the acute toxicity test were analysed using one-way ANOVA followed by Tukey's pairwise comparison tests. The temperature, DO, pH and NH_3 between the test tanks for the 96-hr exposure period in the static acute toxicity test were analysed separately using two-way ANOVA. All the data were analysed using Minitab for Windows (version 14) statistical software package at $\alpha = 0.05$.

Results

The percentage mortality of the guppy fingerlings for the 24-hr and 96-hr exposure period in the Roundup® concentrations tested are shown in Fig. 1. The data clearly showed that the percentage mortality increased with the increasing Roundup® concentration and exposure period. Guppy mortality rate increased to 100% in the test solutions where the concentration was 18 $mg^.L^{-1}$ and above after the 96-hr exposure period.

The 24-hr LC_{50} for the three replicates were 15.14 $mg^.L^{-1}$, 15.81 $mg^.L^{-1}$ and 14.37 $mg^.L^{-1}$ respectively. As the 95% confidence limits of these LC_{50} values were narrow and overlapping, the means of these three replicates could be used to determine the overall LC_{50} in the Probit analysis. Thus, the overall LC_{50} for the 24-hr exposure period was determined as 15 $mg^.L^{-1}$ while its 95% confidence limits were 14.2 - 16.12 $mg^.L^{-1}$ (Fig.2).
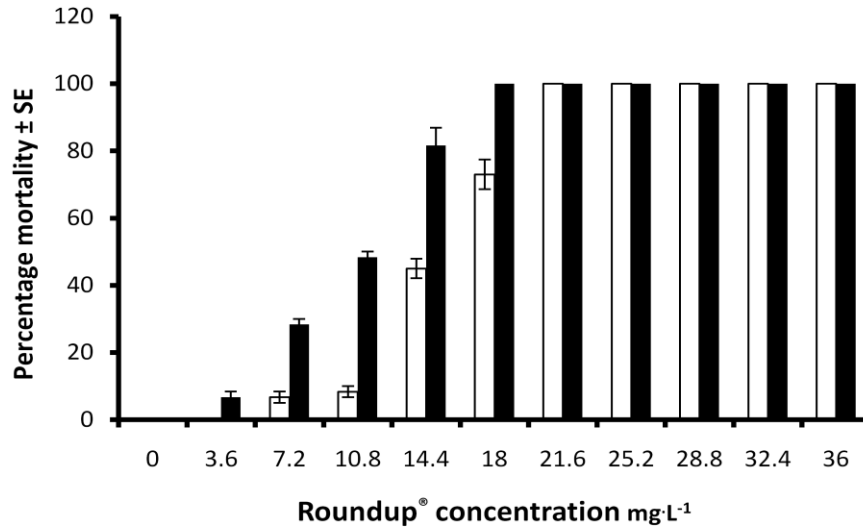
Fig. 1. Variation of the percentage guppy mortality with the increasing Roundup®
concentration in the static acute toxicity test after the 24-hr (white bars) and 96-hr
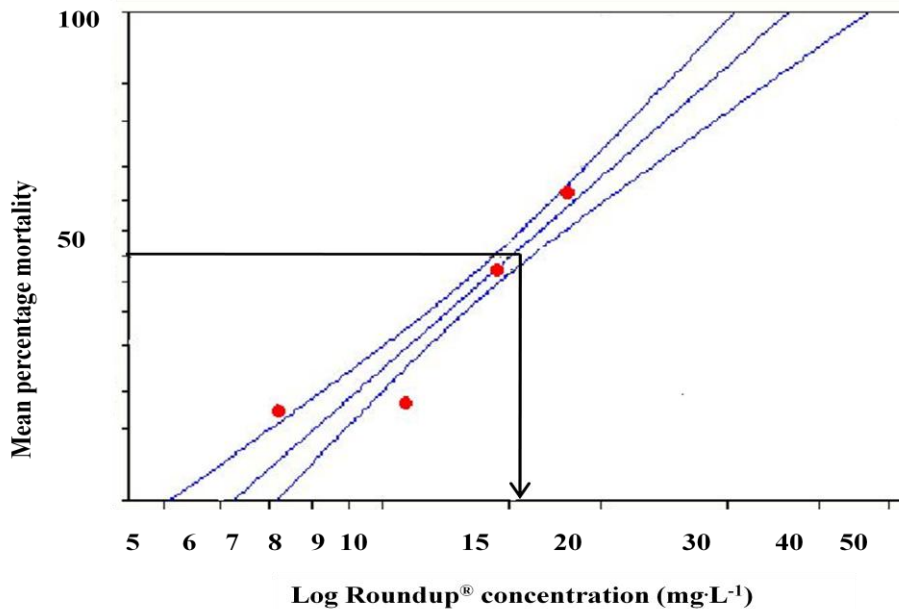(black bars) exposure periods. Mean percentage mortality ± SE are presented.

Fig. 2. The relationship between the mean percentage mortality of guppy fingerlings
and the log Roundup® concentration for 24-hr exposure period. The overall 24-hr LC_{50}
is 15.1 mg·L^{-1}.

The LC_{50} for the three replicates for the 96-hr exposure period were 9.0 mg·L^{-1}, 10.25 mg·L^{-1}
and 10.08 mg·L^{-1} respectively. As in the 24-hr exposure period, the 95% confidence limits of these
three LC_{50} values are narrow and overlapping, their means could be used to determine the overall

96-hr LC_{50} in the Probit analysis. Thus, the overall 96-hr LC_{50} was determined as 9.76 $mg^.L^{-1}$ while its 95% confidence limits were 8.98 - 10.67 $mg^.L^{-1}$ (Fig.3).
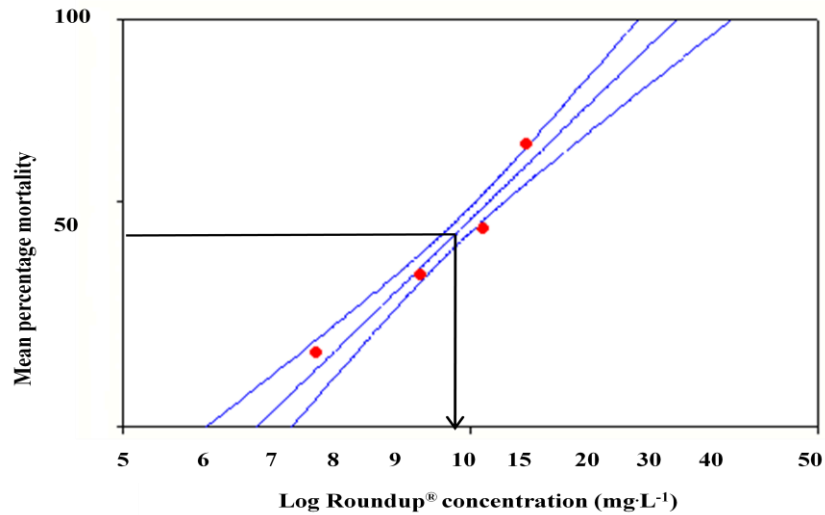

Fig. 3. The relationship between the mean percentage mortality of guppy fingerlings and the log Roundup[®] concentration for the 96-hr exposure period. The overall 96-hr LC_{50} is 9.76 $mg^.L^{-1}$.

Immediately after the fingerlings were introduced into the test tanks, it was observed that those in the control tanks gradually calmed down within a few minutes while the others in the test tanks began rapid erratic swimming movements but eventually rested by the surface in a head up vertical manner, probably gulping for air as they seemed to have breathing difficulties. Fingerlings in all the test solutions preferred to rest more at the surface of water than in the control ($P < 0.05$, Tukey's pairwise tests after one-way ANOVA) (Table 2). The opercular movement rates of these fishes were also observed to be higher than those in the control tanks.

Further, it was observed that the body colouration of the fingerlings in the Roundup[®] test tanks gradually changed from the initial yellowish to brownish body colour into a pale colouration. The intensity of colour change increased towards the latter part of the experiment.

Table 2. The number of fingerlings expected to be dispersed throughout the tank and observed resting near the surface of test tanks during the first 24-hr exposure period. Mean ± SE are presented (n = 3). Subscripts a, b and c indicate the significant differences detected by the Tukey's multiple comparison test after ANOVA. The rates of oppercular movements are also presented.

Roundup[®] Concentration $mg^.L^{-1}$	Number of fingerlings expected to be dispersed throughout the tank	Mean number of fingerlings remained at the surface of tank water ± SE	Rate of oppercular movements
0	20	$(1.2 \pm .02)^a$	Normal
3.6	20	$(12.5 \pm 2.8)^b$	High
7.2	20	$(14.2 \pm 2.4)^b$	High
10.8	20	$(16.4 \pm 2.5)^b$	High
14.4	20	$(19.5 \pm 0.1)^c$	Higher
18.0	20	$(19.8 \pm 0.2)^c$	Higher

In the static acute toxicity test the physico-chemical parameters i.e., DO (range: 6.4 - 6.6 $mg^{.}L^{-1}$), NH_3, (range: 0.68 - 0.70 $mg^{.}L^{-1}$) pH (range: 6.7 - 6.9) and temperature (range: 29 - 30°C) varied only slightly among the treatments during the 96 hr exposure period. However, none of these variations were significant between the treatments or between the four days experimentation period (p>0.05, two-way ANOVA).

The guppy fingerlings developed gill hyperplasia upon exposure to all the Roundup$^{®}$ concentrations tested in the study. Further, the severity of this condition is concentration dependent. For example, the severity of gill hyperplasia was mild in the lowest concentration (3.6 $mg^{.}L^{-1)}$) while it was severe at the concentration 18 $mg^{.}L^{-1}$ (Plate 1). At higher concentrations above 18 $mg^{.}L^{-1}$, the severity of the gill damage was extremely high where the gills appeared to be glued together so that they were even unable to process for histological sectioning.
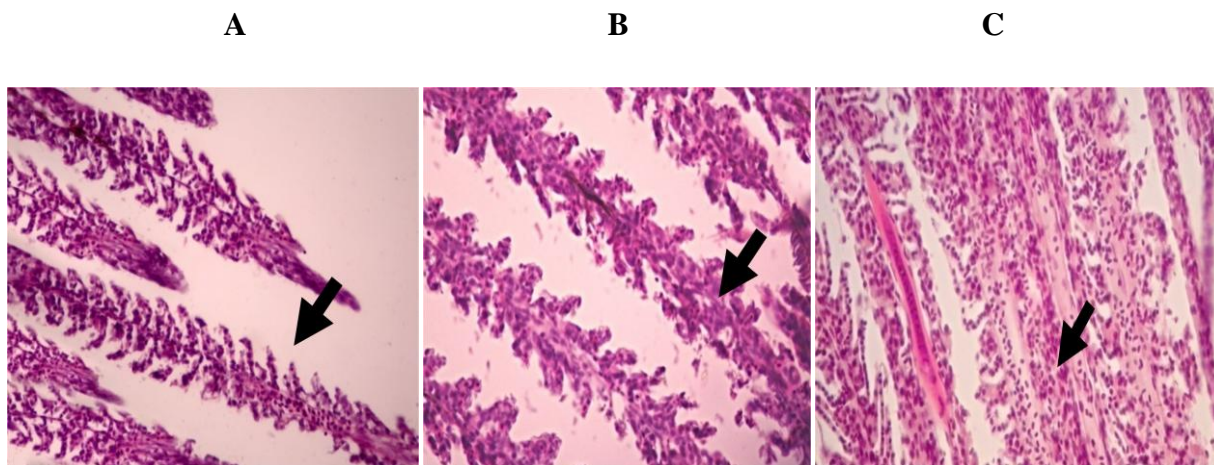

Plate 1. Photos showing the histopathological changes of gills at different concentrations of Roundup$^{®}$. The arrow in 'A' shows the healthy gills of guppies in the control (×400). The arrow in 'B' shows the mild gill hyperplasia at 3.6 $mg^{.}L^{-1}$ (x 400) while the arrow in 'C' shows the severe gill hyperplasia at 18 $mg^{.}L^{-1}$ (x 400).

Discussion

Weedicides are widely used to effectively control weeds (Carlisle and Trevors, 1986). Among these weedicides Roundup$^{®}$ is one of the most popular one and used all over the world today. In spite of its effectiveness, Roundup$^{®}$ brings about adverse effects to non-targeted animals in the agricultural fields and associated habitats.

Studies addressing the effect of Roundup$^{®}$ on non-targeted species are quite common. For example, Tsui and Chu (2003), Relyea (2004, 2005) and Langiano and Martinez (2007) studied the toxicity effects of Roundup$^{®}$ on the neotropical fish species *Prochilodus lineatus*, crustaceans and frogs and determined the LC_{50} values using static toxicity tests. However, the effects of Roundup$^{®}$ on the guppy have never been studied. Therefore, the present study was carried out with a view to determine the 24-hr and 96-hr LC_{50} values, study their behavioural changes and the

histopathological changes that would take place in their gills upon exposure to Roundup[®].

The recommended field concentration of Roundup[®] is a very high value. For example, a Roundup[®] concentration as high as 2,592 mg$\cdot$L^{-1} should be used to control weeds in a paddy field. Further, Carlisle and Trevors (1986) found that its half life is considerably high where it remains from 3 to 141 days in soil and 12 to 70 days in pond water respectively. This is further aggravated by the impropers pray of Roundup[®] by farmers (Peterson and Hulting, 2004) where they spray this weedicide many times above recommended levels and also at higher doses owing to the fact that they somehow need to rapidly eradicate the weed problem from their crop fields. They sometimes spray weedicide just before the rains so that most of it is carried away by the rain water runoff into freshwater habitats. Sometimes farmers spray the weedicide to the bunds adjacent to streams, while others spray concentrated weedicide directly into the water. For example, Tsui and Chu (2003) reported that weedicides are directly sprayed into sea weed culture ponds. Therefore, it appears that there is a very high chance of Roundup[®] being accumulated at extremely high levels and resides for a considerably longer period of time in the agricultural fields and associated streams and ponds causing unwanted effects to many life forms inhabiting there.

The present study showed that the 24-hr and 96-hr LC$_{50}$ of Roundup[®] against guppy fingerlings were very low values, i.e. 15.1mg$\cdot$L^{-1} and 9.76 mg$\cdot$L^{-1} respectively. Therefore it is evident that fishes living in the waters that are contaminated with high level of Roundup[®] become easy victims of its toxicity effects at concentrations as low as 9.76 mg$\cdot$L^{-1} or 15.1mg$\cdot$L^{-1}. The present study also showed that 100% mortality occurs within 24 hours at a Roundup[®] concentration as low as 21.6 mg$\cdot$L^{-1}. More or less similar results have also been achieved elsewhere. For example, the LC$_{50}$ values obtained for 6 hr, 24 hr and 96 hr for the neotropical fish *Leporinus obtusidens*, were 7.5 mg$\cdot$L^{-1}, 10 mg$\cdot$L^{-1} and 13.69 mg$\cdot$L^{-1} respectively (Glusczak et al. 2006) and for five species of frogs the LC$_{50}$ values for 16 days ranged from 1.3 to 2.5 mg$\cdot$L^{-1} (Relyea, 2005).

In the acute toxicity test, 100% fingerling mortality occurred in all the Roundup[®] preparations above the 21.6 mg$\cdot$L^{-1} concentration before completing the 24 hours exposure period and, above 18.0 mg$\cdot$L^{-1} concentrations before completing the 96-hr exposure respectively. Therefore, the Roundup[®] preparations of 18.0 mg$\cdot$L^{-1}, 21.6 mg$\cdot$L^{-1}, 25.2 mg$\cdot$L^{-1}, 28.8 mg$\cdot$L^{-1}, 32.4 mg$\cdot$L^{-1} and 36 mg$\cdot$L^{-1} had to be excluded from the Probit analysis. This had occurred, perhaps due to the over-estimation of the upper range of Roundup[®] concentration, i.e. 36 mg$\cdot$L^{-1}, in the range finding test. Had the acute toxicity test been carried out within a narrower range between 3.6 mg$\cdot$L^{-1} and 21.6 mg$\cdot$L^{-1} than the above, this error could have been avoided and the ten preparations could have been prepared effectively within the above range. Therefore, the mortality values at those concentrations could be included in the Probit analysis to obtain more accurate LC$_{50}$ values.

The mortality of guppies is higher upon prolonged exposure to low concentrations of Roundup[®] than exposure to higher concentrations for a shorter period. This was the case in the present experiment where the 24-hr LC$_{50}$ value i.e. 15.1 mg$\cdot$L^{-1}, is higher than that of the 96-hr LC$_{50}$

value which is 9.76 mg$^.$L^{-1}. Therefore the toxicity of Roundup$^®$ to guppy fingerlings appears to depend upon the exposure period.

The toxicity of Roundup$^®$ increases with the elevation of pH and temperature in the water (Tsui and Chu, 2003). In the present experiment however, pH and temperature as well as the DO and NH$_3$, remained unchanged between the control and the test preparations. This suggests that the physico-chemical parameters of water have no synergy effect on the toxicity of Roundup$^®$. Therefore, it appears that the fish mortality was brought about by the sole toxicity effect of Roundup$^®$ but not by changes of any physico-chemical parameters measured.

In the present study, it was observed that a sudden change of the swimming movements of the fingerlings occurred upon introducing to Roundup$^®$ preparations. Further, fingerlings that were introduced into the test preparations preferred to rest more at the surface in a head up vertical manner than in the control. Also their opercular movements became faster than in the control tanks. Generally, the fishes show the above behavioural changes when the water contains low levels of oxygen. Arunachalam and Palanichamy (1982) have also observed similar changes of behaviour in breathing in *Macropodus cupanus* upon introducing to the agrochemical carbaryl. In the present experiment, however, the test tanks were continuously aerated during the exposure period and found to be at optimal levels as high as 6.22 mg$^.$L^{-1}. Further, the dissolved oxygen level remained unchanged throughout the experimental period. It is a known fact that fish behaviour is innate and any change in their behaviour is directly related to a change in the environment. The environmental changes bring about physiological changes in the fish so that alterations of their behaviour are evident (Kramer, 1987). Therefore, changes in the fish behaviour as was seen in the present study may be due to the influence of Roundup$^®$ that caused histopathological changes in the gills or physiological changes in the body.

In the histopathological study, mild, moderate or severe hyperplasia were observed in all the fishes examined. However, the severity of the hypoplasia was dependent upon the Roundup$^®$ concentration. For example, the most severe hyperplasia in the gills were observed in fishes that were exposed to high concentrations of Roundup$^®$, as high as 18.0 mg$^.$L^{-1} and above. When the fishes were exposed to low concentrations of Roundup$^®$ (3.6 mg$^.$L^{-1}) hyperplasia was still evident, but the severity of this condition was mild. In hyperplasia, the secondary gill lamellae fuse together making it harder for the fishes to exchange respiratory gasses so that they directly gulp for air resulting in a change in the behaviour. Similar gill hypoplasia have also been observed in the fishes exposed to insecticides such as carbomates and organophosphates (Cengiz and Ünlü, 2001; Benli and Özkul, 2009).

In addition to the histopathological changes that took place in the gills, hypertrophy of the skin mucus glands could also be suspected due to the toxic effects of Roundup$^®$. For example, Ayoola (2008) observed that excessive mucus secretion took place in juvenile tilapia upon exposure to Roundup$^®$ for 96 hours. Thus, the tremendous discharge of mucus forms a protective coating over

the surface of the scales in order to minimise the contact of the pollutants to the skin. This mechanism is employed as one of the defense mechanisms of the fish against toxicants. However, this aspect was not studied in the present study.

Further, the body colouration of the fingerlings was also changed where the initial yellowish to brownish body colour gradually turned pale towards the latter part of the experiment. Hedberg and Wallin (2010) showed that Roundup[®] inhibits intracellular transport through disassembly of the cytoskeleton of the melanopores of the fish, *Xenopus laevis*. Therefore, the retrograde transport of melanosomes in the melanopores are affected so that the body colouration of the fish is changed. Similarly, the colour change in integument pigmentation of the guppies in the present experiment may be due to the toxicity effect of Roundup[®].

It has been found that Roundup[®] could bring about changes not only on the gills but also in other parts of the body. For example, Jiraungkoorskul et al. (2001) and Benli and Ozkul (2009) showed that Roundup[®] caused physiological changes of liver cells and kidney cells of Nile tilapia. Langiano and Martinez (2007) showed that plasma glucose levels of neotropical fishes increase due to exposure to Roundup[®]. Also Glusczak et al. (2006) have found that the acetyl choline esterase activity of brain cells of silver cat-fishes exposed to Roundup[®] was reduced by about 25-27 % but its activity on muscle cells did not change. Soso et al. (2006) showed that chronic exposure to sub-lethal concentration of Roundup[®] alters hormone profiles and negatively affects reproduction of female *Rhamdia quelen*. Therefore, there is much scope to study the physiological changes of guppies too, upon exposure to Roundup[®]. Guppies are abundant in paddy fields and streams near the crop fields. They are considered as a hardy fish and a very good biological controlling agent of mosquito larvae (Costa, 1985). Most importantly, since guppies are known as hardy fishes, any effects caused by Roundup[®] on them will be a clear indication where more sensitive endemic and naturally occurring fish species in the environment are affected.

Conclusion

The present study revealed that Roundup[®] brings about adverse effect on the guppy fingerlings at concentrations well below its recommended field concentrations. Fingerlings preferred to rest more at or near the surface of water than resting elsewhere in the Roundup[®] containing tanks due to gill hyperplasia and probably due to stress caused by Roundup[®]. It can therefore be concluded that gill damage and stress caused by Roundup[®] could contribute to the mortality of guppy fingerlings.

Acknowledgements

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