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DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*

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ABSTRACT

The present study was undertaken to evaluate the genotoxic and oxidative stress modulatory effects of commercial formulation of glyphosate-based herbicide (Roundup®) in freshwater fish *Channa punctatus*. Three sublethal test concentrations of the herbicide *viz.*, SL-I (1/10th of LC_{50} = ~3.25 mg L^{-1}), SL-II (1/8th of LC_{50} = ~4.07 mg L^{-1}) and SL-III (1/5th of LC_{50} = ~6.51 mg L^{-1}) were calculated using 96-LC_{50} value and the test specimens were exposed to these concentrations. Blood and gill cells of the exposed specimens were sampled on day 1, 7, 14, 21, 28 and 35 to examine the DNA damage using comet assay and to assess the alteration in lipid peroxidation and antioxidant enzymes activities. The highest DNA damage was observed on day 14 at all test concentrations followed by gradual non-linear decline. Induction of oxidative stress in the blood and gill cells were evidenced by increased lipid peroxidation level, while antioxidants namely superoxide dismutase, catalase and glutathione reductase responded in a concentration-dependent manner. The results supported the integrated use of comet and antioxidant assays in determining the toxicity of water pollutants which could be used as part of monitoring programs.

1. Introduction

Glyphosate, [N-(phosphoromethyl)glycine], is a broad spectrum herbicide primarily used in agricultural applications for the control of a great variety of annual, biennial and perennial grasses, sedges, broad leaved weeds and woody shrubs (Ayoola, 2008). It is also used for the control of aquatic weed in fish ponds, lakes, canals and slow running water (Tsui and Chu, 2008). It is probably the most important herbicide ever developed (WHO, 1994). Glyphosate is very slightly to moderately toxic to the aquatic animals, but the commercial formulation, Roundup®, is considered more toxic due to the addition of the surfactant polyoxyethylene amine (POEA) (Brausch and Smith, 2007). It reaches aquatic environment due to the proximities of the agricultural fields along the water bodies.

Fish have served as bio-indicators of environmental pollution and played significant roles in assessing potential risk associated with contamination in aquatic environment (Lakra and Nagpure, 2009). The use of fish as bioindicators for assessing the effects of pollution are of increasing importance and

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permit early detection of aquatic environmental problems (Lopez-Barea, 1996; Van Der Oost et al., 2003). The fish species *Channa punctatus* was selected for the test because of some ecotoxicological characteristics such as wide distribution in the freshwater environment, non-invasive, availability throughout the season and easy acclimation to laboratory conditions (Kumar et al., 2010). Further, the fish is a bottom dweller which makes it to be in contact with xenobiotics in water as well as in sediment and is also sensitive to variations in water quality.

The comet assay (CA) is a standard, quick, simple, sensitive, rapid and reliable method for quantitatively measuring DNA damage in the exposed organism (Bajpayee et al., 2005). It is increasingly being used for genotoxicity testing of substances, like industrial chemicals, biocides, agrochemical, food additives and pharmaceuticals (Brendler-Schwaab et al., 2005; Guilherme et al., 2009). Further, many enzyme activities can be used as a biomarker for assessment of pesticide contamination in water (Chandrasekara and Pathirantne, 2005). Biochemical markers, like lipid peroxidation (LPO) and antioxidant enzymes, are widely used to assess the toxic stress, integrity of the immune system and tissue damage in different organisms (Ansari et al., 2011; Dabas et al., 2012). They have the advantage of being sensitive, highly conserved between species and often easier to measure as stress indices (Agrahari et al., 2007). The antioxidant enzymes have been shown to work in synergistic manner to protect against oxidative stress and tissue specific damage. Oxidative stress develops when there is imbalance between pro-oxidants and anti-oxidants ratio, leading to the generation of reactive oxygen species (ROS). Environmental contaminants, such as herbicides, heavy metals and insecticides, are known to modulate antioxidant defensive systems and to cause oxidative damage in aquatic organisms by ROS production (Liu et al., 2006; Monteiro et al., 2006). Cavalcante et al. (2008) reported that ROS generated by the metabolism of herbicides could interact with DNA of exposed fish resulting in the lesions detected by comet assay. Saleha et al. (2001) reported that besides ROS-dependent processes, organophosphate pesticides can cause DNA strand breaks by inhibiting enzymes involved in DNA repair or interacting with DNA.

Recently, there are few literatures available on the integrated genotoxic as well as oxidative stress modulatory effects of glyphosate-based herbicide (Roundup®) in fish (Guilherme et al., 2010, 2012a, 2012b), but most of which are concerned with short term exposure. Further, application of a more sensitive approach, especially on chronic exposure, using battery of biomarkers is essential in evaluating the real environmental risk of herbicides. Hence, the present study is aimed to investigate the genotoxic and oxidative stress modulatory effects of glyphosate-based herbicide (Roundup®) in freshwater fish *C. punctatus* after *in vivo* chronic exposure.

2. Materials and methods

2.1. Experimental fish and chemical

The juveniles of freshwater air-breathing fish *C. punctatus* (Bloch, Family: Channidae, Order: Perciformes) were caught from nearby ponds and lakes with the help of local fishermen. The fish specimens had an average (±S.D) wet weight and length of 11.81 (±0.11) g and 9.98 (±0.11) cm, respectively. Specimens were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate ($KMnO_4$) for two min to avoid any dermal infections. The specimens were then acclimatized for 2 weeks under laboratory conditions in semi-static systems. They were fed boiled eggs, minced goat liver and poultry waste materials during acclimatization. The fecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. Commercial formulation of glyphosate (41% EC), manufactured by Monsanto India Ltd with CAS NO 1071-83-6 and trade name Roundup®, was purchased from the local market and used for the present study.

2.2. Determination of sub-lethal concentrations

The 96 h LC_{50} of the glyphosate based test chemical Roundup, i.e. 32.54 mg L^{-1}, for the present study on *C. punctatus* was taken from Nwani et al. (2010). Based on this value, three sublethal test concentrations of glyphosate, *viz.* SL-I (1/10th LC_{50} = ~3.25 mg L^{-1}), SL-II (1/8th LC_{50} = ~4.07 mg L^{-1}) and SL-III (1/5th LC_{50} = ~6.51 mg L^{-1}) were estimated and used for *in vivo* experiment.

2.3. In vivo exposure for genotoxicity and biochemical assay

The specimens were exposed to each of the three aforementioned test concentrations in a semi-static system with the change of test solution on every alternate day to maintain the test concentration of the chemical. The specimens maintained simultaneously in tap water (without test chemical) were considered as 'control'. The fish were not fed 24 h prior to commencement of exposure to the test chemical to prevent the interference of feces in the process of the assays. To avoid mortality due to starvation during 35 days of exposure, the fish were fed daily small quantity of food of approximately 1% of total body weight about an hour before the test solution was renewed. The exposure was continued for 35-days and whole blood and gill tissues were collected on day 1, 7, 14, 21, 28 and 35 of exposure from five test specimens each time. On each sampling day, the collected tissue were used for comet and biochemical assays. For CA, the collected blood was diluted 20-fold and about 0.5 ml of the diluted blood was added to an isotonic solution (10 ml) for further dilution (Tiano et al., 2000) in dark or dim light to prevent the occurrence of additional DNA damage. For biochemical analysis, tissue samples were homogenized in chilled sodium phosphate buffer (0.1 M, pH 7.4) using Potter-Elvehjem homogenizer for TBARS quantification. Part of the homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C using Sigma refrigerated centrifuge and the resulting supernatant was centrifuged again at 12,500 × *g* for 10 min at 4 °C to prepare the post-mitochondrial supernatant (PMS), which was further used for various enzymatic determinations. The physico-chemical properties of test water viz., temperature, dissolved oxygen, chloride, pH, total hardness, conductivity and total alkalinity were analyzed using standard methods (APHA, AWWA, WPCE, 2005).

Table 1 – Mean ± SE% tail DNA in blood and gill cells of *C. punctatus* exposed to different concentrations (mg L^{-1}) of glyphosate (n = 250 cells/concentration were scored).

Tissues	Test concentration (mg L^{-1})	Exposure durations (in days)					
		1	7	14	21	28	35
Blood	Control	3.45 ± 0.49^{a1A}	3.54 ± 0.49^{a1A}	3.54 ± 0.49^{a1A}	3.54 ± 0.49^{a1A}	3.54 ± 0.44^{a1A}	3.54 ± 0.49^{a1A}
	SL-I	4.41 ± 0.36^{ad1A}	10.15 ± 2.38^{b1A}	11.22 ± 0.98^{b1A}	7.39 ± 0.43^{cd1A}	5.69 ± 0.89^{bd1A}	3.81 ± 0.45^{a1A}
	SL-II	5.53 ± 0.89^{ac1A}	11.48 ± 0.93^{b1A}	13.19 ± 1.05^{b1A}	7.97 ± 0.82^{c1A}	6.63 ± 0.92^{c1A}	4.19 ± 0.61^{a1A}
	SL-III	6.52 ± 0.73^{b1A}	12.30 ± 1.10^{c1A}	13.25 ± 1.23^{c1A}	8.68 ± 0.68^{b1A}	6.98 ± 0.84^{b1A}	4.39 ± 0.66^{a1A}
Gill	Control	3.74 ± 0.51^{a1A}	3.74 ± 0.51^{a1A}	3.74 ± 0.51^{a1A}	3.74 ± 0.51^{a1A}	3.74 ± 0.51^{a1A}	3.74 ± 0.51^{a1A}
	SL-I	5.33 ± 0.73^{ac1A}	10.71 ± 0.81^{b2A}	11.54 ± 1.51^{b1A}	7.69 ± 0.79^{c1A}	6.23 ± 0.81^{cd1A}	4.86 ± 0.73^{ad1A}
	SL-II	7.41 ± 0.91^{b1A}	12.10 ± 1.02^{c1A}	15.69 ± 1.10^{d2B}	8.53 ± 1.05^{b1A}	7.58 ± 0.99^{b1A}	5.42 ± 0.74^{ab1A}
	SL-III	10.34 ± 0.83^{b2B}	14.00 ± 1.20^{c1A}	17.29 ± 1.52^{d2B}	9.29 ± 1.12^{b1A}	7.98 ± 0.89^{ae1A}	5.84 ± 0.88^{a1A}

Values with different alphabetic (lower case) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with different numeric superscripts differ significantly ($p < 0.05$) between concentrations within exposure duration and tissue. Values with different alphabetic (uppercase) superscripts differ significantly ($p < 0.01$) between tissues within exposure duration and concentration.

2.4. *Comet assay*

The CA or alkaline single-cell gel electrophoresis (SCGE) was performed as a three layer procedure (Singh et al., 1988) with slight modifications (Klaude et al., 1996). The gill tissue was removed with the help of scissor and homogenized in ice-cold homogenization buffer [1X Hanks' balanced salt solution (HBSS), 20 mM EDTA, 10% dimethyl sulfoxide (DMSO), pH 7.0–7.5], followed by centrifugation at 3000 rpm at 4 °C for 5 min. The cell pellet was then suspended in chilled phosphate buffered saline. Viability of both the blood and gill cells was evaluated by the trypan blue exclusion test method (Anderson et al., 1994) and the cells showing >84% viability were further processed for CA according to the method described by Ali et al. (2008).

Two slides per specimen were prepared and 25 cells per slide (250 cells per concentration) were scored randomly and analyzed using an image analysis system (Komet-5.5 Kinetic Imaging, UK) attached to a fluorescent microscope (Leica) equipped with appropriate filters. The parameter selected for quantification of DNA damage was percent tail DNA (% tail DNA = 100 − % Head DNA), as determined by the software.

2.5. *Oxidative stress and antioxidant enzymes assay*

LPO was measured by estimation of thiobarbituric acid reactive substances (TBARS), as described by Sharma and Krishna-Murti (1968). Catalase (CAT) activity was estimated as per Aebi (1984). Total superoxide dismutase (SOD) activity was estimated by measuring inhibition of auto-oxidation of epinephrine, according to the method of Misra and Fridovich (1972). Glutathione reductase (GR) activity was determined spectrophotometrically by measuring NADPH oxidation at 340 nm, as described by Tayarani et al. (1989). The protein contents in the samples were estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA, Sigma) as standard.

2.6. *Statistical analysis*

Statistical analysis was performed using SPSS (version 16.0) computer software (SPSS Inc. Chicago, IL, USA). The results were expressed as means ± SE. Three-way analysis of variance was employed to determine significant difference between groups and to compare the mean differences in % tail DNA among different tissues, concentrations and sampling durations. A p-value less than 0.01 were considered statistically significant.

3. Results

3.1. *Physico-chemical properties of the test water*

The physico-chemical parameters of the test water used during the experiment were: temperature 26.70–27.65 °C, pH 7.14–7.76, nitrate 23.0–26 mg L^{-1}, nitrite 0.04–0.07 mg L^{-1}, ammonia 0.08–0.11 mg L^{-1} and conductivity 254–286 μM cm^{-1}. The dissolved oxygen concentration ranged from 6.14 to

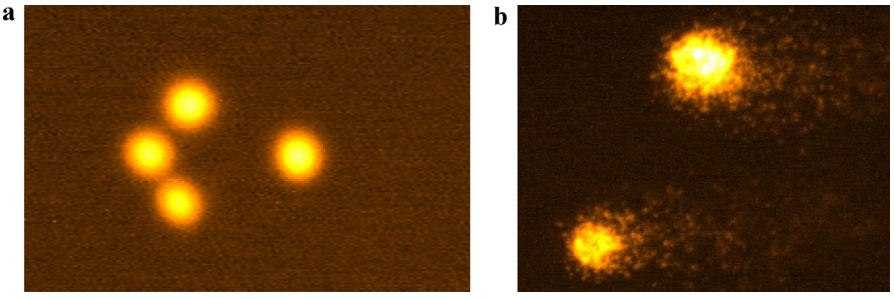

Fig. 1 – Blood cells after comet assay from control (a) and glyphosate exposed (b) specimens of *C. punctatus*.

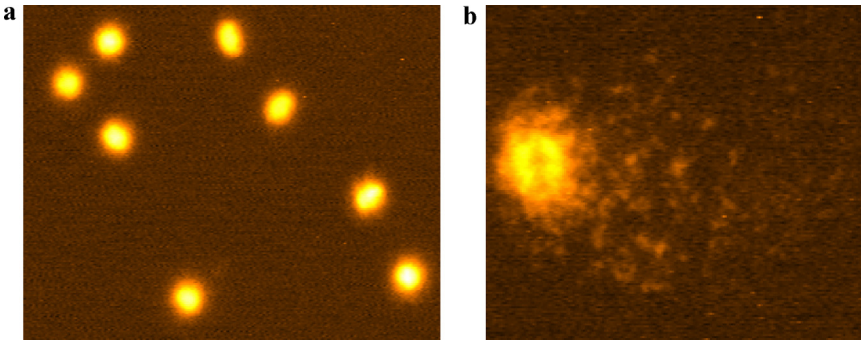

Fig. 2 – Gill cells after comet assay from control (a) and glyphosate exposed (b) specimens of *C. punctatus*.

6.86 mg L^{-1}, while total hardness and total conductivity ranged from 200 to 240 mg L^{-1} and 270–300 as $CaCO_3$, respectively.

3.2. *DNA damage in exposed tissues*

The result of the DNA damage measured as % tail DNA in the blood and gill cells of the control and treated groups of *C. punctatus* (Table 1 and Figs. 1 and 2) indicated that the fish specimens exposed to different test concentrations exhibited significantly higher DNA damage ($p < 0.01$) in their tissues as compared to the control group. Further, the DNA damage was found to be time and concentration dependent with the highest DNA damage recorded on day 14 at all test concentrations and in both the tissues. After reaching maximum DNA damage on day 14, there was a gradual non-linear decline with the progression of the experiment. Between the tissues, the gill cells exhibited comparatively higher damage than the blood cells at all test concentrations and exposure durations. Among the test concentrations, the highest damages were observed at SL-III in blood (13.25%) and gill cells (17.29%) on day 14 of exposure.

3.3. *Effect on lipid peroxidation*

The effects of different test concentrations of glyphosate on LPO in form of TBARS formation in the blood and gill cells of *C. punctatus* are presented in Table 2. LPO induction was both concentration and exposure duration dependent. The lowest TBARS formation was observed on day 1 of exposure at SL-I and there was gradual non-linear increase with highest formation on day 35 in both the tissues. At SL-III, TBARS formation increased by a factor of 2.03 and 1.29 on day 1 and 7.02 and 5.59 on day 35 in gill and blood cells, respectively.

3.4. *Effect on antioxidant enzymes*

The activities of CAT enzyme in the blood and gill cells of *C. punctatus* exposed to test chemical are presented in Table 3. The activity was significantly reduced in a concentration and time dependent manner in both tissues at all test concentrations as compared to the control. At SL-III concentration, the CAT activity reduced by 7.59 and 29.43% in the blood and gill cells, respectively, on day 1, and was further reduced by 25.86 and 51.24% in the same tissues on day 35 compared to the control. Similarly, exposure of *C. punctatus* to test chemical for 35 days reduced SOD activity in both the tissues, but the effects

Table 2 – Effects of glyphosate on lipid peroxidation in blood and gill cells of *C. punctatus* (values are expressed as means ± SE nmol TBARS/mg protein).

Tissues	Test concentration (mg L^{-1})	Exposure durations (in days)					
		1	7	14	21	28	35
Blood	Control	1.01 ± 0.06^{a1A}	1.04 ± 0.07^{a1A}	1.03 ± 0.05^{a1A}	1.01 ± 0.07^{a1A}	1.00 ± 0.07^{a1A}	1.02 ± 0.07^{a1A}
	SL-I	1.16 ± 0.04^{a1A}	1.18 ± 0.05^{a1A}	1.42 ± 0.16^{a1A}	2.43 ± 0.12^{a1A}	2.50 ± 0.12^{a1A}	2.52 ± 0.40^{a1A}
	SL-II	1.19 ± 0.07^{a1A}	1.28 ± 0.19^{a1A}	1.85 ± 0.08^{a1A}	3.40 ± 0.11^{b2A}	3.68 ± 0.17^{b2A}	3.68 ± 0.93^{b2A}
	SL-III	1.30 ± 0.05^{a1A}	1.77 ± 0.07^{a1A}	2.65 ± 0.10^{a2A}	3.68 ± 0.13^{b2B}	5.68 ± 0.17^{b2A}	5.70 ± 0.86^{b2A}
Gill	Control	1.08 ± 0.02^{a1A}	1.06 ± 0.02^{a1A}	1.06 ± 0.01^{a1A}	1.03 ± 0.02^{a1A}	1.01 ± 0.01^{a1A}	1.02 ± 0.03^{a1A}
	SL-I	2.04 ± 0.03^{a1A}	2.09 ± 0.02^{a1A}	3.11 ± 0.09^{a2A}	3.24 ± 0.19^{a2A}	3.80 ± 0.60^{a2A}	4.98 ± 0.78^{b2B}
	SL-II	2.09 ± 0.06^{a1A}	2.11 ± 0.60^{a1A}	3.24 ± 0.06^{a3A}	4.94 ± 0.81^{b3A}	5.03 ± 0.84^{b3A}	5.80 ± 0.64^{b3B}
	SL-III	2.19 ± 0.04^{a1A}	2.23 ± 0.19^{a1A}	3.53 ± 0.64^{a3A}	5.06 ± 0.99^{b4A}	6.58 ± 0.30^{b4A}	7.16 ± 0.76^{b4B}

Values with different alphabetic (lowercase) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within exposure duration and tissue. Values with different alphabetic (uppercase) superscripts differ significantly ($p < 0.01$) between tissues within exposure duration and concentration.

Table 3 – Effect of glyphosate on catalase enzyme in blood and gill cells of *C. punctatus* (values are expressed as means ± SE U/mg protein).

Tissues	Test concentration ($mg\,L^{-1}$)	Exposure durations (in days)					
		1	7	14	21	28	35
Blood	Control	130.76 ± 0.66^{a1A}	131.53 ± 0.34^{a1A}	130.41 ± 0.83^{a1A}	131.09 ± 0.58^{a1A}	130.90 ± 0.15^{a1A}	130.88 ± 0.47^{a1A}
	SL-I	128.94 ± 1.92^{a1A}	128.01 ± 0.52^{a1A}	127.23 ± 0.42^{a1A}	125.29 ± 1.61^{b1A}	118.49 ± 1.41^{c1A}	112.99 ± 0.46^{d1A}
	SL-II	128.64 ± 6.44^{a1A}	123.71 ± 3.22^{a2A}	120.41 ± 1.72^{b2A}	115.58 ± 1.52^{b2A}	108.31 ± 1.10^{c2A}	102.90 ± 0.97^{c2A}
	SL-III	120.83 ± 6.82^{a2A}	114.65 ± 3.51^{b3A}	101.77 ± 2.41^{c3A}	100.51 ± 2.63^{c3A}	100.03 ± 4.10^{c3A}	97.04 ± 1.20^{c2A}
Gill	Control	127.76 ± 2.60^{a1B}	127.09 ± 3.54^{a1B}	126.83 ± 1.99^{a1B}	126.14 ± 1.11^{a1B}	126.03 ± 2.06^{a1B}	126.01 ± 0.97^{a1B}
	SL-I	112.41 ± 1.11^{a2B}	109.02 ± 1.31^{a2B}	104.31 ± 1.08^{a2B}	100.22 ± 0.91^{b2B}	100.03 ± 0.84^{c2B}	99.54 ± 1.32^{c2B}
	SL-II	101.23 ± 0.71^{a3B}	100.40 ± 1.02^{b3B}	99.53 ± 3.04^{c3B}	96.31 ± 2.31^{d3B}	91.52 ± 1.61^{d3B}	83.54 ± 1.51^{e3B}
	SL-III	90.15 ± 0.15^{a4B}	86.41 ± 1.21^{b4B}	85.91 ± 1.31^{c4B}	83.08 ± 1.33^{d4B}	70.53 ± 1.06^{e4B}	66.44 ± 1.11^{f4A}

Values with different alphabetic (lowercase) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within exposure duration and tissue. Values with different alphabetic (uppercase) superscripts differ significantly ($p < 0.01$) between tissues within exposure duration and concentration.

Table 4 – Effect of glyphosate on superoxide dismutase in blood and gill cells of *C. punctatus* (values are expressed as means ± SE U/mg protein).

Tissues	Test concentration ($mg\,L^{-1}$)	Exposure durations (in days)					
		1	7	14	21	28	35
Blood	Control	17.95 ± 0.23^{a1A}	17.90 ± 0.11^{a1A}	17.90 ± 0.63^{a1A}	17.88 ± 0.21^{a1A}	17.86 ± 0.10^{a1A}	17.85 ± 0.09^{a1A}
	SL-I	16.78 ± 1.91^{a1A}	15.82 ± 0.71^{a1A}	14.79 ± 0.68^{a2A}	12.42 ± 0.34^{b2A}	11.27 ± 0.37^{b2A}	9.96 ± 0.16^{b2A}
	SL-II	15.57 ± 0.22^{a1A}	13.78 ± 0.17^{a2A}	12.79 ± 1.16^{b2A}	10.53 ± 0.44^{bc2A}	10.11 ± 0.58^{c2A}	8.57 ± 0.46^{cd2A}
	SL-III	12.67 ± 1.70^{a2A}	10.59 ± 0.56^{a3A}	10.08 ± 2.41^{a3A}	9.85 ± 1.11^{b3A}	8.58 ± 0.57^{bc2A}	8.01 ± 0.20^{c2A}
Gill	Control	17.59 ± 0.83^{a1A}	17.38 ± 0.91^{a1A}	17.22 ± 1.10^{a1A}	17.17 ± 1.01^{a1A}	17.15 ± 0.93^{a1A}	17.11 ± 1.03^{a1A}
	SL-I	16.55 ± 1.22^{a1A}	14.15 ± 0.64^{a2A}	14.03 ± 0.73^{a2A}	12.03 ± 1.01^{b2A}	10.30 ± 0.66^{bc2A}	9.54 ± 0.83^{c2A}
	SL-II	15.06 ± 0.15^{a1A}	12.37 ± 1.51^{b3A}	12.01 ± 0.93^{b3A}	9.63 ± 0.77^{c3A}	8.51 ± 0.71^{cd3A}	8.04 ± 0.29^{d3A}
	SL-III	11.83 ± 1.14^{a2A}	10.08 ± 0.83^{a4A}	9.53 ± 0.96^{ab4A}	8.86 ± 1.14^{b4A}	8.01 ± 0.62^{b4A}	7.29 ± 0.98^{bc4A}

Values with different alphabetic (lowercase) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within exposure duration and tissue. Values with different alphabetic (uppercase) superscripts differ significantly ($p < 0.01$) between tissues within exposure duration and concentration.

were somewhat different in each tissue. The decrease in SOD activity was 30–55% in the blood cells and 33–60% in the gills at different exposure levels (Table 4). The activity of GR was reduced in both the tissues in treated specimens as compared to the control (Table 5). At SL-III concentration, the GR activity was reduced by 29.42 and 32.74% on day 1, which further reduced by 55.13 and 57.39% on day 35 in blood and gill cells, respectively, as compared to control.

Table 5 – Effect of glyphosate on glutathione reductase in blood and gill cells of *C. punctatus* (values are expressed as means ± SE nmol/mg protein).

Tissues	Test concentration ($mg\,L^{-1}$)	Exposure durations (in days)					
		1	7	14	21	28	35
Blood	Control	10.05 ± 0.38^{a1A}	10.06 ± 0.15^{a1A}	10.24 ± 0.23^{a1A}	10.21 ± 0.33^{a1A}	10.05 ± 0.04^{a1A}	10.04 ± 0.57^{a1A}
	SL-I	10.00 ± 1.32^{a1A}	9.45 ± 0.48^{a1A}	9.16 ± 0.85^{a1A}	8.33 ± 0.53^{a1A}	8.04 ± 0.40^{a1A}	7.45 ± 0.29^{b1A}
	SL-II	9.42 ± 1.22^{a1A}	9.36 ± 0.85^{a1A}	8.21 ± 0.77^{a1A}	8.07 ± 0.92^{a1A}	7.99 ± 0.28^{a1A}	7.21 ± 0.46^{b2A}
	SL-III	9.05 ± 1.15^{a1A}	8.48 ± 0.67^{a1A}	8.10 ± 1.42^{a1A}	7.06 ± 1.24^{a2A}	6.90 ± 0.35^{b2A}	6.64 ± 0.50^{b2A}
Gill	Control	10.02 ± 0.16^{a1A}	10.02 ± 0.19^{a1A}	10.01 ± 0.41^{a1A}	10.01 ± 0.66^{a1A}	9.89 ± 0.83^{a1A}	9.97 ± 0.77^{a1A}
	SL-I	9.96 ± 0.71^{a1A}	9.63 ± 0.34^{a1A}	9.03 ± 0.31^{a1A}	7.42 ± 0.33^{a1A}	7.20 ± 0.38^{a1A}	7.06 ± 0.22^{b1A}
	SL-II	9.51 ± 0.26^{a1A}	8.93 ± 0.71^{a1A}	8.42 ± 0.11^{a1A}	7.13 ± 0.14^{a2A}	7.01 ± 0.81^{a1A}	7.01 ± 0.99^{a2A}
	SL-III	8.71 ± 0.63^{a1A}	8.03 ± 0.74^{a1A}	7.55 ± 0.71^{a2A}	7.02 ± 0.91^{a3A}	6.72 ± 0.84^{a2A}	6.04 ± 0.36^{b3A}

Values with different alphabetic (lowercase) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within exposure duration and tissue. Values with different alphabetic (uppercase) superscripts differ significantly ($p < 0.01$) between tissues within exposure duration and concentration.

4. Discussion

The present study elucidates the genotoxicity and oxidative stress modulatory effects of commercial formulation of glyphosate (Roundup®) in freshwater fish *C. punctatus*. Toxicity of pollutants to the aquatic organisms has been reported to be affected by temperature, pH, dissolved oxygen, size and age, type of fish species, water quality, concentration and formulations of chemicals (Gupta et al., 1981; Young, 2000). Our tested sublethal concentrations (3.25, 4.07 and 6.51 mg L^{-1}) in the present study could be environmental relevant considering that glyphosate has been detected at sublethal level up to 328 μg L^{-1} in some water bodies in USA (Battaglin et al., 2009) and between 0.36 and 2.16 mg L^{-1} in Brazil (Rodrigues and Almeida, 2005). Our tested concentration though exceeded the freshwater aquatic life standard of 65 μg L^{-1} and the 0.7 mg L^{-1} maximum contaminated level (MCL) (EPA, 2010), but in view of the repeated applications of the pesticide in most developing countries, the concentration in the aquatic ecosystem may be higher, thus, suggesting the relevance of test concentrations.

Fish and aquatic invertebrates have been considered to be efficient and cost effective model systems for studying the toxic, mutagenic and carcinogenic potential of pollutants due to their ability to metabolize, concentrate and store waterborne pollutants (Osman et al., 2008). We applied the alkaline SCGE to evaluate total DNA strand breaks in the blood and gill cells of *C. punctatus* exposed *in vivo* to different sublethal concentrations of glyphosate for 35 days. The long term genotoxicity studies can be important approach for achieving greater insight into the organisms DNA repair ability and other protective mechanisms for excreting toxic chemicals. The results of our study showed that exposure of *C. punctatus* to sublethal test concentrations induced significant ($p < 0.01$) higher DNA damage in both the blood and gill cells than the control and, thus, indicated the genotoxic potential of the pesticide to aquatic organisms. A concentration and time dependent increase in DNA damage in the form of comet induction followed by a time dependent decrease in DNA damage was observed. The DNA damage as observed in the present study is consistent with the findings of Cavaş and Könen (2007) on the blood cells of gold fish (*Carassuis auratus*) after exposure to 5, 10 and 15 mg L^{-1} of glyphosate and those of Cavalcante et al. (2008) for the blood and gill cells of *Prochilodus lineatus* exposed to 10 mg L^{-1} of the same herbicide.

The time dependent decrease in DNA damage after 14 days of exposure might indicate repair of damaged DNA, loss of heavily damaged cells or both (Miyamae et al., 1998; Saleha et al., 2001). Similar repair mechanism was observed in our previous work when we exposed the same fish species to atrazine (Nwani et al., 2011). The DNA damage detected in the present study could have originated from DNA single-strand breaks, DNA double-strand breaks, DNA–DNA/DNA-protein cross-linking or inhibition of the enzymes involved in DNA repair resulting from the interaction of the pesticide or its metabolites with DNA (Guilherme et al., 2012b). It is also possible that the DNA damage observed in the fish following exposure to Roudup® could be due to ROS generated by the metabolism of the herbicide which could have interacted with DNA of exposed fish resulting in the lesions detected by comet assay.

Many xenobiotics, including glyphosate, induce redox cyling and, thus, causing oxidative cellular damage (Parvez and Raisuddin, 2005; Goetz and Luch, 2008). Oxidative stress is caused by an imbalance between the production of ROS and an organism's ability to detoxify them or repair the resulting damage. Free radicals can be formed as a result, thus, causing DNA damage and other impairment in fish. A number of studies have demonstrated that glyphosate causes the generation of ROS and oxidative stress in bullfrog tadpoles (Costa et al., 2008), rat liver cells (El-Shenawy, 2009), mouse kidney and liver cells (Bolognesi et al., 1997). Furthermore, the test chemical has been reported to cause oxidative stress leading to LPO in different tissues of other fish species (Glusczak et al., 2007; Langiano and Martinez, 2008; Modesto and Martinez, 2010). In the present study, the dose and time dependent increase in LPO was also accompanied by increase incidence of DNA damage in the blood and gill cells of the fish. Thus, it is possible that glyphosate could have caused alterations in DNA of *C. punctatus* resulting in formation of comets. The decline in DNA damage after day 14 of exposure did not affect LPO concentration probably due to the influx of superfluous ROS produced during the exposure. Organisms are, however, equipped with independent cascades of enzymes to alleviate oxidative stress and repair damaged macro-molecules, produced during normal metabolism or due to exposure to xenobiotics. In this cascade, SOD and CAT are major enzymes in eliminating ROS and the induction of SOD/CAT provides a first line of defense against ROS. SOD converts the superoxide anion radical to hydrogen, while CAT converts hydrogen peroxide to water and molecular oxygen (Shao et al., 2012). Another antioxidant GR is also activated to counteract the negative effects of ROS (Parvez and Raisuddin, 2005). Under treatment with glyphosate in the present study, LPO increased, while CAT, SOD and GR decreased at all test concentrations. This phenomenon points out clearly an increased pro-oxidant status in both tissues that can be explained as the basis of the observed DNA damage in the fish. Our results suggest enzyme inhibition as a potential mechanism through which this herbicide can induce oxidative stress. Organophosphate pesticides have been reported to reduce antioxidant enzyme activity, enhance the production of lipid peroxides and reduce the level of cellular antioxidants (Julka et al., 1992). Modesto and Martinez (2010) reported similar decrease in antioxidant enzymes (SOD, CAT and GPX) in fish *P. lineatus* exposed to different concentrations of glyphosate. Lushchak et al. (2009) also reported the suppression of SOD, GR and GST in *C. auratus* exposed to 2.5–20 mg L^{-1} of Roundup®. Contrary to our results Guilherme et al. (2010) reported that the DNA damaging effects induced by Roundup® in the European eel *Anguilla anguilla* were not directly related with an increased pro-oxidant state. This however, may be attributed to the low concentration of the herbicide used.

There was variation both in DNA damage and oxidative stress response in the blood and gill cells of specimens exposed to different concentrations of glyphosate. Compare to the blood, the gill cells were more prone to injury caused by chemicals and xenobiotics as the gills are the most appropriate target organ which is directly and constantly exposed to the DNA damaging chemicals dissolved in

water (Dzowonkowska and Hubner, 1986), whereas the blood receives the chemical during the course of circulation. Gills are also reported to be weak in terms of their antioxidant potential (Sayeed et al., 2003). This may be the explanation for the higher LPO and % tail DNA damage and the corresponding higher inhibition of the antioxidants in the gills than the blood in all tested concentrations of glyphosate in *C. punctatus*. Fish gills have also been reported to be more vulnerable toward oxidative damage than other organs and may respond earlier to a pollutant-induced proxidant challenge (Ahmad et al., 2004; Santos et al., 2006). Thus, this makes the fish gills more adequate for genotoxic risk assessment in aquatic system in the presence of moderate concentrations of herbicides (Guilherme et al., 2012a).

The present finding establishes the genotoxic and oxidative-stress modulatory effects of glyphosate in *C. punctatus*. Glyphosate and its formulated products, including Roundup®, are currently used tremendously in many countries. Further, studies should be carried out to understand the toxico-kinetic profile of glyphosate and its formulated products including on both short- and long-term exposures to aquatic organisms.

5. Conclusion

The results of the present study, which involved an integrated examination of the genotoxic effects and possible alterations in lipid peroxidation and antioxidant defenses, demonstrated that sublethal exposure of glyphosate to *C. punctatus* stimulated oxidative stress leading to elevated LPO, suppressed antioxidant enzymes and induced genotoxic damage in the subject fish species. Further, work is, however, needed to establish the component of the formulated product, glyphosate or POEA, responsible for ROS generation and DNA damage in organisms. In all, while we focused on organophosphate exposures in this investigation, the capacity of other classes of pesticides to induce similar patterns of oxidative stress and DNA damage should be investigated.

Ethical statement

All experiments were carried out in accordance with the guidelines for the care of experimental animals as approved by the National Bureau of Fish Genetic Resources (Indian Council of Agricultural Research), Canal Ring Road, P.O Dilkusha, Lucknow (UP) 226 002, UP, India.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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