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Mutagenic and genotoxic effects of carbosulfan in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis

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ABSTRACT

Carbosulfan insecticide is widely used in agriculture and was recently proposed for treatment against pyrethroid-resistant mosquitoes. The mutagenic and genotoxic effect of carbosulfan was carried out in fish *Channa punctatus* using micronucleus (MN) test and comet assay. The 96 h LC_{50}, estimated by probit analysis in a semi-static bioassay experiment, was 0.268 mg l^{-1}. Based on the LC_{50} value, three sub-lethal concentrations of carbosulfan (1/4th LC_{50} = ~67 μg l^{-1}, 1/2nd LC_{50} = ~134 μg l^{-1} and 3/4th LC_{50} = ~201 μg l^{-1}) were selected and fishes were exposed to the said concentrations for 96 h and the samplings were done at regular intervals of 24 h for assessment of the MN frequencies and DNA damage. In general, significant effects ($P < 0.01$) from both concentrations and time of exposure were observed in exposed fishes. The MN induction was highest on 96 h at all the concentrations in the peripheral blood. Similar trend was observed for the DNA damage measured in terms of the percentage of tail DNA in the erythrocyte and gill cells. This study confirmed that the comet and micronucleus assays are useful tools in determining potential genotoxicity of water pollutants and might be appropriate as a part of monitoring program.

1. Introduction

Carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl[(dibutylamino)thio] methylcarbamate] belongs to the benzofuranyl methyl carbamate group of pesticide and has been widely used in agriculture for broad spectrum control of insect pests of crops such as caterpillars, green leafhoppers, white-backed plant hoppers, brown plant hoppers, gall midges, stem borers, leaf folder of paddy, white aphids of chilies (Giri et al., 2002). It has been reported to be effective against certain insect pests not controlled by organo-chlorine or organo-phosphorus insecticides (Sahoo et al., 1990) and has also been proposed for the control of pyrethroid-resistant mosquitoes (Guillet et al., 2001). It acts as neurotoxicant by affecting synaptic transmission in cholinergic parts of the nervous system of aquatic organisms (Tuker and Thompson, 1987). Widely used in the rural communities, carbosulfan reaches aquatic environment due to the proximities of the agricultural country sides to water places or directly due to careless application in such environments. Although carbosulfan is not stable under aquatic conditions and does not persist in the environment, bioaccumulation occurs to some extent in fish mainly due to their slow metabolism (IPCS, 1986). Its high water solubility, extensive usage in the environment and exposure to non-target organisms might pose potential hazards to aquatic organisms in the long run.

The use of fish as bio-indicators of pollutant effects is being more and more used since fishes are very sensitive to changes in their environment and play significant roles in assessing potential risk associated with contamination in aquatic environment of new chemicals (Lakra and Nagpure, 2009). Several ecotoxicological characteristics of *Channa punctatus* such as wide distribution in the freshwater environment, availability through out the seasons, easy acclimatization to laboratory conditions and commercial importance make this species an excellent test specimen for toxicity studies (Pandey et al., 2005).

Several studies have shown that the micronucleus (MN) test and comet assay (CA) are the two sensitive, rapid and extensively used methods in the detection of mutagenic and genotoxicity of chemicals and xenobiotics under field and laboratory conditions (Ateeq et al., 2002, 2005; Abdul-Farah et al., 2003; Jha, 2004; Cavas and Ergene-Gözükara, 2005a; Pandey et al., 2006; Talapatra et al., 2006; Sharma et al., 2007; Ergene-Gözükara et al., 2007; Nagpure et al., 2007; Ali et al., 2008, 2009; Xiao et al., 2008; Ventura Campo de et al., 2008).

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Although carbosulfan has been reported to induce micronucleus formation, sister chromatid exchange and chromosomal aberrations in human peripheral blood lymphocytes and bone marrow cells of rats (Sterhrer-Schmid and Wolf, 1995; Topaktas et al., 1996; Rencüzogullari and Topaktas, 2000; Giri et al., 2003), the information regarding the mutagenic and genotoxic nature of carbosulfan in aquatic organism is rare, especially the data pertaining to its effects on fishes. The present study investigates the mutagenic and genotoxic effects of carbosulfan using MN assay in erythrocytes and CA in erythrocytes and gill cells of *C. punctatus* exposed *in vivo*.

2. Materials and methods

2.1. Experimental fish specimen and chemicals

Freshwater air-breathing fish *C. punctatus* (Bloch, Family: Channidae, order: Channiformes) was procured from local sources. The specimens had an average weight and length of 16.50 ± 2.14 g and 11.40 ± 2.01 cm, respectively. Fish specimens were subjected to a prophylactic treatment by bathing twice in 0.05% Potassium permanganate ($KMnO_4$) for 2 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. The faecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. For the present study, commercial formulations of carbosulfan (25% EC) with trade name 'Aaatank', manufactured by Northern Minerals Ltd, India was purchased from the local market.

2.2. Determination of sub-lethal concentrations

To determine the 96 h LC_{50} value of carbosulfan, acute toxicity bioassay was conducted in semi-static system in the laboratory with the change of test solution on every alternate day to maintain the similar concentration of the chemical. Since carbosulfan was emulsifiable concentrate, it was directly added to the system. This study was conducted under the OECD guideline No. 203 in the semi-static test conditions (OECD, 1992). A set of 10 specimens were randomly exposed to each of the five carbosulfan concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mg l^{-1}), obtained after range finding test, and the experiment was set in triplicate to obtain the 96 h LC_{50} value of the test chemical for the species. The 96 h LC_{50} value of carbosulfan was determined as 0.0268 mg l^{-1} for *C. punctatus*, following the probit analysis method as described by Finney (1971). Based on the 96 h LC_{50} value, the three test concentrations of carbosulfan viz; sub-lethal concentration I (SL-I; 1/4th of LC_{50} = ~67 mg l^{-1}), concentration II (SL-II; 1/2nd of LC_{50} = ~134 mg l^{-1}) and concentration III (SL-III; 3/4th of LC_{50} = ~201 mg l^{-1}) were estimated and used for the *in vivo* experiment.

2.3. In vivo exposure experiment

The fish specimens were exposed to the three aforementioned test concentrations of carbosulfan in a semi-static system with the change of test water on every alternate day to maintain the concentration of the pesticide. The exposure was continued up to 96 h (4 days) and tissue sampling was done at the intervals of 24, 48, 72 and 96 h at the rate of five fish per interval. The specimens maintained in tap water and those exposed to cyclophosphamide (20 mg kg^{-1}) simultaneously were considered as the negative and positive controls, respectively. On each sampling day, the whole blood and gill cells were collected and immediately processed for MN and CA. Blood was collected by incising the lateral vein using heparinized syringe and was diluted 20-fold. About 0.5 ml of 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.

The physico-chemical properties of test water, namely temperature, pH, dissolved oxygen, conductivity and total hardness were analysed by standard methods (APHA, AWWA and WPCF, 1998).

2.4. Micronucleus (MN) assay

Peripheral blood samples obtained from the caudal vein were smeared on clean, grease free frosted glass slides. Slides were fixed in methanol for 10 min and left to air-dry at room temperature and finally stained with 6% Giemsa in Sorenson buffer (pH 6.9) for 20 min. After dehydration through graded alcohol and clearing in xylen, slides were mounted in a mixture of distyrene (Polystyrene), Plasticizer (Tricresyl phosphate) and xylene (DPX). Five fish were used for each concentration and from each fish three slides were prepared for micronucleus test. From each slide, 1500 cells were scored under light microscope (Leitz Wetzlar Germany, Type 307-083.103, Oil immersion lens, 100/1.25). Micronucleus frequency was calculated as follows:

$$\%\,\mathrm{MN} = \frac{\text{Number of cells containing micronuclei}}{\text{Total number of cells counted}} \times 100$$

2.5. Alkaline single-cell gel electrophoresis (SCGE)

The alkaline single-cell gel electrophoresis (SCGE)/comet assay was performed as a three layer procedure (Singh et al., 1988) with slight modifications (Klaude et al., 1996). The gill tissue was 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 (PBS). Viability of both the erythrocytes and gill cells was evaluated by the trypan blue exclusion test method (Anderson et al., 1994) and tissue samples showing cell viability exceeding 84% were further processed for comet assay. In brief, about 15 µl of cell suspension (approx 20,000 cells) was mixed with 85 µl of 0.5% low melting point agarose (LMPA) and layered on one end of a frosted glass slide, coated with a layer of 200 µl of 1% normal agarose. It was covered with a third layer of 100 µl LMPA. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% triton X-100, added fresh) overnight at 4 °C. After lysis, the slides were placed side by side in a horizontal electrophoresis unit containing fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2–EDTA and 0.2% DMSO, pH 13.5) and left in the solution for 20 min at 4 °C for DNA unwinding and conversion of alkali-labile sites to single-strand breaks.

Alkaline electrophoresis was performed using the same alkaline electrophoresis buffer for 20 min at 15 V (0.8 V/cm) and 300 mA at 4 °C. The slides were then washed three times for 5 min with neutralization buffer (0.4 M Tris, pH 7.5) to remove the excess alkali. The slides were stained with 75 µl ethidium bromide (20 µg/ml). For positive control, the erythrocyte and gill cells were treated *ex vivo* with 100 µM H_2O_2 for 10 min at 4 °C. Two slides per specimen were prepared and 25 cells per slide (250 cells per concentration) were scored randomly and analysed using an image analysis system (Komet – 5.5 Kinetic Imaging, UK) attached to florescent 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.

2.6. Statistical analysis

Statistical analysis was performed with the SPSS 10.1 computer program (SPPS Inc. Chicago, IL, USA). The results were expressed as mean ± SE. One-way analysis of variance was employed to compare the mean differences in % tail DNA between tissues within concentration, between concentrations within tissue and between durations within concentration and tissue. The percentage MN was compared between concentrations within durations using Mann–Whitney test. The *P* values less than 0.01 were considered statistically significant.

3. Results

3.1. Physico-chemical parameters of the test water

The physico-chemical characteristics of the test water are presented in Table 1. The water temperature varied from 23.60 to 25.20 °C and the pH ranged from 7.2 to 7.8. The dissolved oxygen concentration ranged from 6.3 to 6.9 mg l^{-1}, conductivity values ranged from 254 to 310 µM cm^{-1} while total hardness varied from 176–184 mg l^{-1} during the experimental period.

3.2. LC_{50} and application factor

In acute toxicity bioassay, the LC_{50} values (with 95% confidence limits) of different concentration of carbosulfan in *C. punctatus* (Table 2) were found to be 0.629 (0.50–1.017), 0.295 (0.264–0.325),

Table 1
Physico-chemical properties of the test water.

Characteristics	Unit	Mean	Range
Air temperature	°C	26.50	25.80–27.40
Water temperature	°C	24.80	23.60–25.20
pH	–	7.40	7.20–7.80
Dissolved oxygen	mg l^{-1}	6.50	6.30–6.90
Conductivity	µM cm^{-1}	256	254–310
Total hardness	mg l^{-1}	178	176–184

Table 2
Lethal concentrations of carbosulfan (mg l^{-1}) (95% confidence intervals) depending on exposure time for *C. punctatus* (n = 10 in three replicates).

Lethal concentration	Exposure time (h)			
	24	48	72	96
LC_{10}	0.215[a] (0.136–0.266)	0.178[b] (0.122–0.187)	0.162[b] (0.128–0.189)	0.160[b] (0.134–0.191)
LC_{50}	0.629[a] (0.503–1.017)	0.295[b] (0.264–0.325)	0.280[b] (0.252–0.307)	0.268[b] (0.243–0.293)
LC_{90}	1.846[a] (1.105–6.540)	0.548[b] (0.477–0.677)	0.482[b] (0.429–0.573)	0.435[b] (0.392–0.505)

Values with different alphabet superscript differ significantly ($P < 0.01$) between exposure time within lethal concentration.

0.280 (0.252–0.307) and 0.268 mg l^{-1} (0.243–0.293) for 24, 48, 72 and 96 h LC_{50}, respectively. A dose dependent increase and time dependent decrease were observed in mortality rate such that as the exposure time increases from 24 to 96 h, the median concentration required to kill the fish was reduced. The estimated safe levels of carbosulfan, as calculated by multiplying the 96 h LC_{50} with different application factors (AF), are given in Table 3. The values of safe level of carbosulfan in the fish species under study varied from 2.68×10^{-2} to 2.68×10^{-6} mg l^{-1}.

3.3. DNA damage and induction of micronucleus

The DNA damage measured as % tail DNA in the erythrocyte and gill tissues of the control and treatment groups (Table 4, Figs. 1 and 2a and b, and 3a–c) indicated that the fish specimens exposed to different concentrations of carbosulfan exhibited significantly higher DNA damage ($P < 0.01$) in their tissues than the control sample. The DNA damage in both tissues was found to be dose and time dependent with the highest DNA damage occurring in SL-III followed by SL-II and SL-I. A significant effect of duration of exposure ($P < 0.01$) was observed in fish specimen exposed to carbosulfan. The lowest DNA damage in both tissues was observed at 24 h and there was gradual non-linear increase in the DNA damage with progression of the experiment and the highest DNA damage was observed on 96 h treatment.

Comparing the DNA damage in both tissues, the gill cells exhibited comparatively higher DNA damage than the blood erythrocyte at all concentrations and durations (Fig. 3a–c). The highest DNA damage was observed on 96 h in gill cells (22.17%) followed by the erythrocytes (19.22%) at the highest concentration (Table 4).

The result of MN analysis in erythrocytes of *C. punctatus* at different concentrations and duration of carbosulfan are summarized in Table 5. There was significant induction of MN in the fish specimens due to exposure of different concentrations of carbosulfan than the control group. Increase in the concentration of the pesticide resulted in higher induction of MN with the highest MN frequency recorded at SL-III at 96 h. The lowest concentration of carbosulfan treatment in fish specimen induced MN frequency of 0.907% in blood erythrocyte in 24 h which was significantly increased to 1.864% with 96 h exposure. Similar trend was observed for SL-II in which the MN frequency of 1.875% in 24 h increased to 2.697% after 96 h. At the highest concentration, the MN frequency significantly increased from 2.960% at 24 h exposure to 4.977% after 96 h duration.

The observed MN (Fig. 4a and b) varied from cell to cell. In some cells MN were found attached to the cell wall or boundary while others were unattached and located near the main nucleus. In all, the incidence of micronucleated erythrocytes of the pesticide indicated positive dose response effects over the entire dose range tested (Fig. 5).

4. Discussion

Fish are often used as sentinel organism for ecotoxicological studies because they play a number of roles in the trophic web, accumulate toxic substances and respond to low concentration of mutagens (Cavas and Ergene-Gözükara, 2005a). Therefore, the use of fish biomarkers as indices of the effects of pollution, are of increasing importance and can permit early detection of aquatic environmental problems (Lopez-Barea, 1996; Van Der Oost et al., 2003).

The test result of the LC_{50} of the present study at 96 h was 0.268 mg l^{-1} which indicated that carbosulfan is very toxic to fish. Our estimate is slightly higher than the 96 h LC_{50} value of 0.231

Table 3
Estimate of safe levels of carbosulfan at 96 h exposure time.

Chemical	96 h LC_{50} (mg l^{-1})	Method	AF	Safe level (mg l^{-1})
Carbosulfan	0.268	Sprague (1971)	0.1	02.68×10^{-2}
		CWQC (1972)	0.01	02.68×10^{-3}
		NAS/NAE (1973)	0.1–0.00001	02.68×10^{-2} to 02.68×10^{-6}
		IJC (1977)	5% of 96 h LC_{50}	01.34×10^{-2}

Table 4
Mean ± SE percentage tail DNA in cells of *C. punctatus* exposed to different concentrations of carbosulfan.

Exposure time (h)	Erythrocytes			Gills		
	201 μg l^{-1}	134 μg l^{-1}	67 μg l^{-1}	201 μg l^{-1}	134 μg l^{-1}	67 μg l^{-1}
Control	3.632 ± 0.138^{A}	3.632 ± 0.138^{A}	3.632 ± 0.138^{A}	4.099 ± 0.154^{A}	4.099 ± 0.154^{A}	4.099 ± 0.154^{A}
24	9.308 ± 0.207^{aA1}	8.737 ± 0.209^{abA1}	8.381 ± 0.193^{bA1}	10.688 ± 0.101^{aA2}	10.433 ± 0.278^{abA2}	9.751 ± 0.165^{bA2}
48	10.435 ± 0.137^{aB1}	9.324 ± 0.123^{bA1}	8.492 ± 0.140^{cA1}	12.771 ± 0.176^{aB2}	10.972 ± 0.079^{bA2}	10.178 ± 0.093^{cB2}
72	13.342 ± 0.147^{aC2}	10.812 ± 0.066^{bB2}	8.650 ± 1.55^{cA1}	16.01 ± 0.206^{aC1}	14.073 ± 0.171^{bcB1}	11.198 ± 0.165^{cC2}
96	19.219 ± 0.195^{aD2}	12.880 ± 0.216^{bC2}	10.860 ± 0.256^{cB1}	22.174 ± 0.543^{aD1}	16.340 ± 0.257^{bC1}	11.365 ± 0.156^{cC1}

Values with different alphabet (lowercase) superscripts differ significantly ($P < 0.01$) between concentration within tissue and duration.
Values with different alphabets (uppercase) differ significantly ($P < 0.01$) between durations within concentration and tissue.
Values with different numeric superscripts differ significantly ($P < 0.01$) between tissues within concentration and duration.

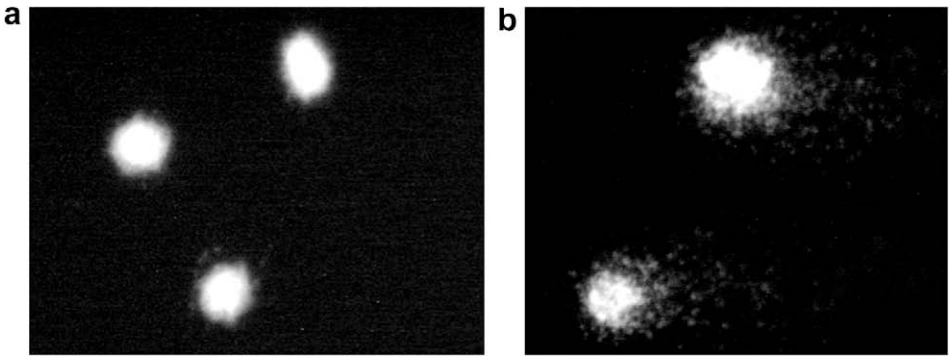

Fig. 1. (a) Control gill cells and (b) Gill cells after exposure to carbosulfan.

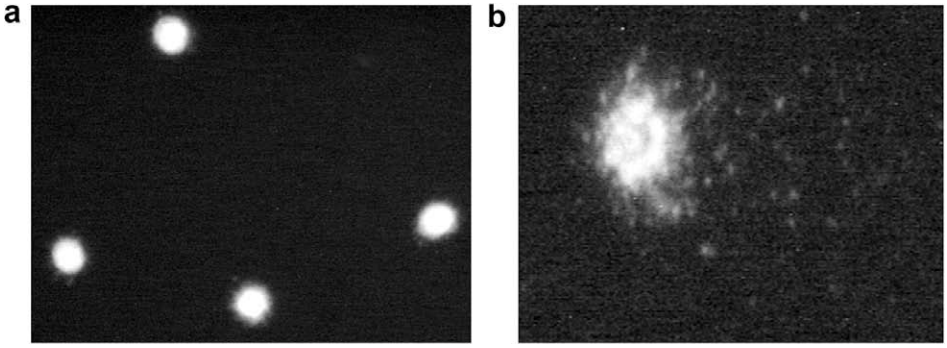

Fig. 2. (a) Control erythrocyte cells and (b) erythrocyte cells after exposure to carbosulfan.

and 0.122 mg l^{-1} estimated by Boran et al. (2007) for rainbow trout (*Oncorhrincus mykiss*) and guppy (*Poeicillia reticulata*) for the same pesticide, respectively. The variation may be due to the difference and hardiness of the test species and water quality parameters. The estimated safe levels of carbosulfan in *C. punctatus*, as calculated by multiplying the LC_{50} with application factor (AF) as recommended by different methods, varied from 2.68×10^{-2} to 2.68×10^{-6} mg l^{-1}. However, the large variation in safe levels determined by different methods has resulted in controversy over its acceptability (Buikema et al., 1982; Pandey et al., 2005). Mount and Stephan (1967) underscored the fact that extrapolation of laboratory data to field is not always meaningful and hence, it is difficult to decide an acceptable concentration based on the laboratory experiments that may be considered 'safe' in the field.

The CA has been considered as sensitive, rapid and reliable method of quantitatively measuring DNA damage in eukaryotic and prokaryotic cells (Cotelle and Ferard, 1999; Bajpayee et al., 2005). It is increasingly being used in testing of substances such as industrial chemicals, biocides, agrochemicals, food additives and pharmaceuticals for genotoxicity testing (Brendler-Schwaab et al., 2005). The assay is favoured among other cytogenetic methods (chromosome aberrations, sister chromatid exchange and micronucleus test) used for the detection of DNA damage (Buschini et al., 2003) as it is capable of detecting wide variety of DNA damage such as DNA single-strand breaks (Sharbel, 2004). We applied the alkaline comet assay to evaluate total DNA strand breaks in the erythrocyte and gill cells of *C. punctatus* exposed *in vivo* to different concentrations of carbosulfan. The data obtained showed that the frequencies of % tail DNA damage for all concentrations of carbosulfan, tested in both tissues, were significantly higher ($P < 0.01$) than the control. The % tail DNA damage was both dose and time dependent. For the tissues, the higher the concentration and duration of carbosulfan exposure, the higher the % tail DNA damage. The DNA damage or DNA stand breaks as detected in this study could be considered a kind of lesion potentially pre-mutagenic (Kamman et al., 2001), the production of breaks in the DNA stands being related to mutagenic and genotoxic properties of the pesticide (Frenzilli et al., 2000). The DNA damage detected in this study could have originated from DNA single-strand breaks, DNA double strand breaks, DNA adducts formations and DNA–DNA and DNA–protein cross-links resulting from the interaction of the pesticide or its metabolites with DNA (Fairbrairn et al., 1995). Furthermore, environmental contaminants such as carbosulfan are known to modulate antioxidant defensive systems and to cause oxidative damage in aquatic organisms by the production of reactive oxygen species (ROS) (Risso-de Facerney et al., 2001; Liu et al., 2006). ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) at supranormal levels have been shown to produce extensive damage such as DNA strand breaks, enzyme inactivation and even apoptosis (Peña-Llopis et al., 2003; Banudevi et al., 2006). Thus, it is possible that carbosulfan could cause alterations in DNA of *C. punctatus* resulting in formation of comets. ROS and oxidative stress have been demonstrated to be triggers of apoptosis (Shen and Liu, 2006). However organisms are equipped with independent cascade of enzymes to counteract the toxic action of xenobiotics like carbosulfan. In this cascade, glutathione (GSH) and glutathione dependent enzymes are the major enzymes in eliminating toxic substances formed during bioactivation of xenobiotics. Depletion of cellular GSH content below the critical level however prevents the conjugation of xenobiotics to GSH and enables them to freely combine covalently with DNA, RNA or cell proteins and thus lead to cell damage (Meister and Andersen, 1983). DNA damage could also be due to formation of GSH conjugates, which might deplete the cellular GSH content and induce oxidative stress (Yamano and Morita, 1995). Again, the genotoxic properties of pesticides also depend on chemical structures. Since there is no direct evidence, it is difficult to determine the actual cause for the observed DNA strand breaks in the present study

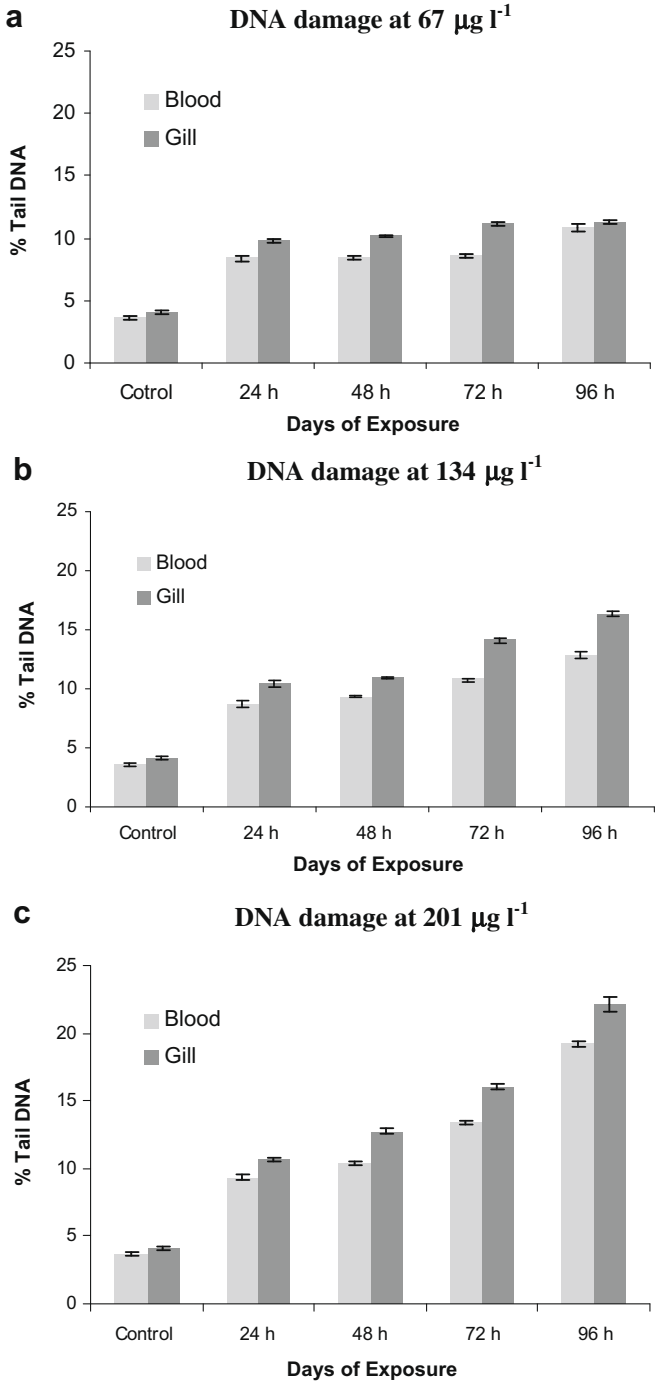

Fig. 3. DNA damage in blood and gill cells by (a) 67 µg l^{-1}, (b) 134 µg l^{-1}, and (c) 201 µg l^{-1} of carbosulfan.

and this will be addressed in further studies. There was variation in DNA damage in the two tissues of specimens exposed to pesticide. Tissue specific variation in DNA lesions occur due to variation in alkali-labile sites and cell types with different background levels of DNA single stand breaks due to variation in excision repair activity, metabolic activity, antioxidant concentrations or other factors (Lee and Steinert, 2003; Xiao et al., 2008). Compared with the blood erythrocytes, gill cells are more prone to injury caused by chemicals and xenobiotics as the gills are the most appropriate target organ that is directly and constantly exposed to the DNA-damaging chemicals dissolved in the water (Dzwonkowska and Hubner, 1986) whereas the blood receives the chemical in the course of circulation. This may be the explanation of higher % tail DNA damage in the gills than in the blood tissues in all tested concentrations. The observed tissue specific response may also be due to physiological activities distinctive to these organs, with respect to either the activation or detoxification of particular pollutants or the repair of the different types of strand breaks. Previous investigations on different fish species indicated the higher sensitivity of the gill cells to DNA damage than the blood erythrocytes, lymphocytes, liver or kidney cells (Pandey et al., 2006; Ateeq et al., 2005; Sharma et al., 2007; Ali et al., 2008, 2009).

The MN test has potential for detecting clastogenic substances in aqueous media and has been used in different types of fishes. It is favoured due to the presence of nucleated erythrocytes, large number of which is ensured by more blood volume (about 1–3 × 10^6) per ml (Ateeq et al., 2002). Small, non-refractive, circular or ovoid intracytoplasmic masses of chromatin resulting from chromosome breaks after clastogenic action, whole chromosome do not migrate during anaphase as a result of aneugenic effect are regarded as micronuclei (Ferraro et al., 2004; Cavas et al., 2005). Carbamate and dithiocarbamate pesticides have been reported to induce MN formation in animals (Cid et al., 1990; Chauhan et al., 2000; Giri et al., 2002). In the present study, all concentrations of carbosulfan induced significantly ($P < 0.01$) higher number of MN than the control and its frequency increased with concentrations and durations. A dose dependent increase in the induction of MN in peripheral blood of *Heteropneustes fossilis* has been reported by Das and Nanda (1986) in response to mitomycin C and paper mill effluents. Abdul-Farah et al. (2003) reported time dependent increase in MN induction in the peripheral blood of *C. punctatus* exposed to PCP and 2,4-D, an effect corroborated by the present work. Cavas and Ergene-Gözükara (2005b) reported tissues specific variation in MN frequencies with gill cells exhibiting higher MN than erythrocytes. Oliveria et al. (2005) investigated the genotoxic effect of arsenic in the gill cells of Zebra fish (*Danio rerio*) and reported that MN frequency increased more than 56 times than in the control. The results of this study emphasized the importance of the peripheral blood MN assay and suggest its broader application as an early biological maker of exposure of fish to clastogenic pollutants in the aquatic environments. The current study, thus, indicated that the CA and MN assays are sensitive tools for evaluating the mutagenic and genotoxic effects of commercial

Table 5
MN frequencies in erythrocytes of *C. punctatus* exposed to carbosulfan at different concentrations and exposure times.

Experiment groups	% MN frequencies (mean ± SE)				
	Concentration	24 h	48 h	72 h	96 h
Negative control (NC)	–	0.406 ± 0.027^{a1}	0.412 ± 0.026^{a1}	0.416 ± 0.026^{a1}	0.417 ± 0.017^{a1}
Positive control (PC)	–	0.705 ± 0.338^{a2}	0.846 ± 0.071^{a2}	1.038 ± 0.028^{b2}	1.157 ± 0.136^{b2}
Carbosulfan	67 (µg/l)	0.907 ± 0.130^{a2}	1.337 ± 0.252^{ab2}	1.743 ± 0.096^{b3}	1.864 ± 0.132^{b3}
	134 (µg/l)	1.875 ± 0.175^{a3}	2.348 ± 0.103^{b3}	2.506 ± 0.152^{b4}	2.697 ± 0.17^{b4}
	201 (µg/l)	2.960 ± 0.305^{a4}	3.846 ± 0.292^{a4}	4.661 ± 0.180^{b5}	4.977 ± 0.054^{b5}

Values with numeric superscript differ significantly ($P < 0.01$) between durations within concentrations.
Values with alphabet superscript differ significantly ($P < 0.01$) between concentrations within durations.

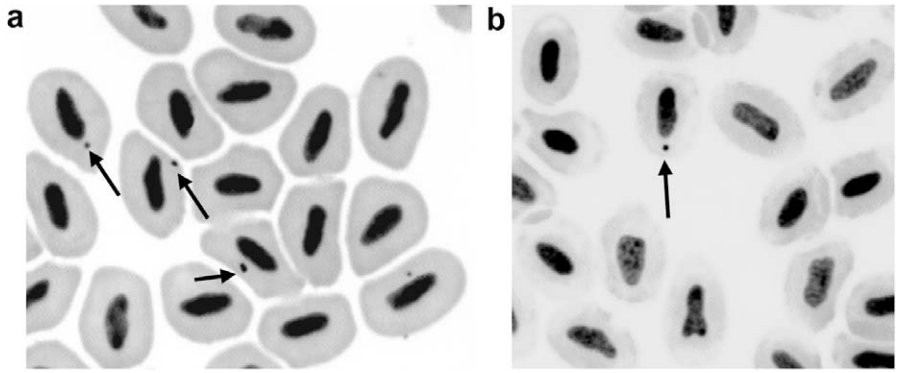

Fig. 4. Micronuclei formations in the erythrocytes after exposure to: (a) cyclophosphamide and (b) carbosulfan.

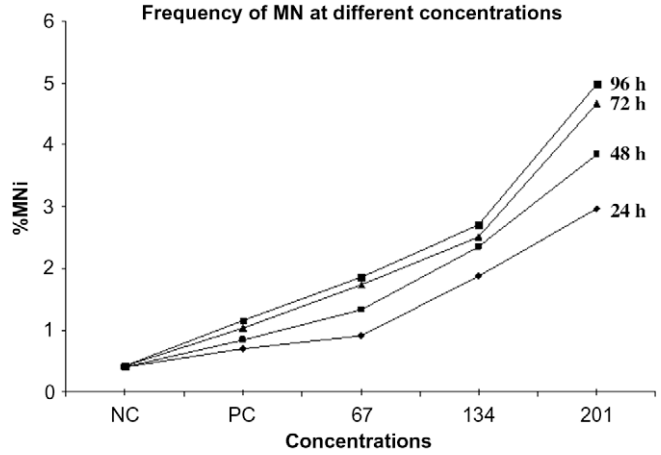

Fig. 5. Dose response relationship of MN frequency by carbosulfan in the erythrocyte of *C. punctatus* for multiple sampling times. NC, negative control; PC, positive control.

formulation of carbosulfan in different fish tissues. Our results give further credence to other reports where carbosulfan induce mitotic aneuploidy in *S. cerevisae* (Wiedenmann et al., 1990) and has mutagenic and genotoxic effects on animals hence exposure to and use of carbosulfan should be restricted.

5. Conclusion

Considering the mutagenic and genotoxic effects of commercial formulation of carbosulfan (Aaatank) on *C. punctatus* as obtained in this study by MN and CA assays, there is serious apprehension about the potential danger of this pesticide to aquatic organisms especially with the reports about its use for the treatment against pyrethroid-resistant mosquitoes. However, detailed studies using other assays having different end points may be needed to confirm the mutagenic and genotoxic status of the pesticide and further explore the mechanism and interactions with the DNA metabolism in different aquatic organisms, especially fish.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Abdul-Farah, M., Ateeq, B., Ali, M.N., Ahmad, W., 2003. Evaluation of genotoxicity of PCP and 2,4-dichlorophenoxyacetic acid by micronucleus test in fresh water fish *Channa punctatus* (Bloch). Ecotoxicol. Environ. Saf. 54, 25–29.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., 2008. Genotoxicity assessment of acute exposure of chlorpyriphos to fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71, 1823–1831.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., Lakra, W.S., 2009. Assessment of genotoxic and mutagenic effects of chlorpyriphos in fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 47, 650–656.

Anderson, D., Yu, T.W., Philips, B.J., Schmerzer, P., 1994. The effects of various antioxidants and other modifying agents on Oxygen radical-generated DNA damage in human lymphocytes in COMET assay. Mutat. Res. 307, 261–271.

APHA, AWWA, WPCF, 1998. Standard Methods for Examination of Water and Wastewater, 20th ed. America Public Health Association, New York.

Ateeq, B., Abul-Farah, M., Ali, M.N., Ahmad, W., 2002. Induction of micronuclei and erythrocyte alterations in the catfish *Clarias batrachus* by 2,4-dichlorophenoxyacetic acid and butachlor. Mutat. Res. 518, 135–144.

Ateeq, B., Farah, M.A., Ahmad, W., 2005. Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlorophenoxyacetic acid and butachlor exposed erythrocytes of *Clarias batruchus*. Ecotoxicol. Environ. Saf. 62, 348–354.

Bajpayee, M., Pandey, A.K., Parmar, D., Marthur, N., Seth, P.K., Dhawan, A., 2005. Comet assay responds in human lymphocytes are not influenced by the menstrual cycle: a case study in healthy Indian females. Mutat. Res. 565, 163–172.

Banudevi, S., Krishnamoorthy, G., Venkatataman, P., Vignesh, C., Aruldhas, M.M., Arunakaran, J., 2006. Role of α-tocopherol on antioxidant status in liver, lung and kidney of PCP exposed male albino rats. Food Chem. Toxicol. 44, 2040–2046.

Boran, M., Altinok, I., Capkin, E., Karacam, H., BIcer, V., 2007. Acute toxicity of carbaryl, methiocarb and carbosulfan to the rainbow trout (*Oncorhinchus myskiss*) and guppy (*Poecilia reticulata*). Turk. J. Vet. Anim. Sci. 31, 39–45.

Brendler-Schwaab, S., Hartman, A., Pfuhler, S., Speit, G., 2005. The *in vivo* comet assay: use and status in genotoxicity testing. Mutagenesis 20, 245–254.

Buikema, J.R., Naider-Lehner, A.L., Cairns, J.R., 1982. Biological monitoring: part IV. Toxicity testing. Environ. Mol. Mutagen. 33, 287–292.

Buschini, A., Carboni, P., Martino, A., Poli, P., Rossi, C., 2003. Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of *Dreissena polymorpha*. Mutat. Res. 537, 81–92.

Cavas, T., Ergene-Gözükara, S., 2005a. Micronucleus test in fish cells: a bioassay for in situ monitoring of genotoxic pollution in the marine environment. Environ. Mol. Mutagen. 46, 64–70.

Cavas, T., Ergene-Gözükara, S., 2005b. Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. Aquat. Toxicol. 74, 264–271.

Cavas, T., Garanko, N.N., Arkhipehuk, V.V., 2005. Induction of micronucleus and binuclei in blood, gill and liver cells sub-chronically exposed to Cadmium chloride and Copper sulphate. Food Chem. Toxicol. 43, 569–574.

Chauhan, L.K.S., Pant, N., Gupta, S.K., Srivastava, S.P., 2000. Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbosulfan exposure. Mutat. Res. 465, 123–129.

Cid, M.G., Loria, D., Matos, E., 1990. Genotoxicity of the pesticide propoxur and its nitroso derivative, NO-propoxur, on human lymphocytes in vitro. Mutat. Res. 232, 45–48.
Cotelle, S., Ferard, J.F., 1999. Comet assay in genetic ecotoxicology: a review. Environ. Mol. Mutagen. 34, 246–255.
Committee on Water Quality Criteria (CWQC), 1972. A Report of the Committee on Water Quality Criteria. Ecol. Res. Series. EPA-R3-003.
Das, R.K., Nanda, N.K., 1986. Induction of micronuclei in peripheral erythrocytes of fish *Heteropneustes fossilis* by mitomycin-C and paper mill effluent. Mutat. Res. 175, 67–71.
Dzwonkowska, A., Hubner, H., 1986. Induction of chromosomal aberrations in the Syrian hamster by insecticides tested *in vivo*. Arch. Toxicol. 58, 152–156.
Ergene-Gözükara, S., Cavas, T., Celik, A., Koleli, N., Kaya, F., Karahan, A., 2007. Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. Ecotoxicology 16, 385–391.
Fairbrairn, D.W., Olive, P.L., O'Neill, K.L., 1995. The comet assay: a comprehensive review. Mutat. Res. 399, 37–59.
Ferraro, M.V.M., Fenocchio, A.S., Mantovani, M.S., deO Ribeiro, C., Cestari, M.M., 2004. Mutagenic effects of tributyltin and inorganic lead (Pb 11) on the fish *H. malabaricus* as evaluated using the comet assay and piscine micronucleus and chromosome aberration tests. Gen. Mol. Biol. 27, 103–107.
Finney, D.J., 1971. Probit Analysis. Cambridge University Press, Cambridge. p. 333.
Frenzilli, G., Bosco, E., Barale, R., 2000. Validation of single cell gel assay in human leukocytes with 18 reference compounds. Mutat. Res. 35, 206–221.
Giri, S., Giri, A., Sharma, G.D., Prasad, S.B., 2002. Mutagenic effects of carbosulfan, a carbamate pesticide. Mutat. Res. 519, 75–82.
Giri, S., Giri, A., Sharma, G.D., Prasad, S.B., 2003. Induction of sister chromatid exchanges by cypermethrin and carbosulfan in bone marrow cells of mice *in vivo*. Mutat. Res. 18, 53–58.
Guillet, P., N'Guessan, R., Darriet, F., Traore-Lamizana, M., Chanve, F., Carnevale, P., 2001. Combined pyrethroid and carbamate two-in-one treated mosquito nets: field efficacy against pyrethroid resistant *Anopheles gambie* and *Culex quiquefasciatus*. Med. Vet. Entemol. 15, 102–112.
IJC, 1977. New and Revised Great Lakes Water Quality Objectives. Windsor, Ontario, P.I.
International programme on chemical safety (IPCS), 1986. Carbamate Pesticide: A General Introduction, Environmental Health Criteria 64. Office of Publications, World Health Organization, Geneva, Switzerland.
Jha, A.N., 2004. Genotoxicological studies in aquatic organisms: an overview. Mutat. Res. 552, 1–17.
Kamman, M., Bunke, H., Steinhart, N., Theobald, A., 2001. A permanent fish cell line (EPC) for genotoxicity testing of marine sediments with comet assay. Mutat. Res. 498, 67–77.
Klaude, M., Erikson, S., Nygren, J., Ahnstrong, G., 1996. The comet assay: mechanisms and technical considerations. Mutat. Res. 363, 89–96.
Lakra, W.S., Nagpure, N.S., 2009. Genotoxicological studies in fishes: a review. Indian J. Anim. Sci. 79, 93–98.
Lee, R.F., Steinert, S., 2003. Use of single-cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and fresh water) animals. Mutat. Res. 544, 43–64.
Liu, Y., Zhang, Y., Liu, J., Huang, D., 2006. The role of reactive oxygen species in the herbicide acetachlor-induced DNA damage on *Bufo-raddei* tadpole liver. Aquat. Toxicol. 78, 21–26.
Lopez-Barea, J., 1996. Biomarkers to detect environmental pollution. Toxicol. Lett. 88, 79.
Meister, A., Andersen, M.E., 1983. Glutathione. Ann. Rev. Biochem. 52, 711–760.
Mount, D.I., Stephan, C.E., 1967. A method for stabilizing acceptable toxicant limits for fish: malathion and butoxyethanol ester of 2,4-D. Trans. Am. Fish. Soc. 96, 185.
Nagpure, N.S., Kumar, R., Kushwaha, B., Singh, P.J., Sirivastava, S.K., Lakra, W.S., 2007. Genotoxicity Assessment in fishes; A Practical Approach. NBFGR, Lucknow, India. p. 126.
NAS/NAE, 1973. Water Quality Criteria. US Government Printing Office, Washington DC. WQC 1972, EPA-R3-033.
Organization for Economic Cooperation and Development (OECD), 1992. Guideline for the Testing of Chemicals: Fish, Acute Toxicity Test, Document 203.
Oliveria, A., Ramirez, B., G arcia, F.P., 2005. Genotoxic damage in Zebra fish (*Danio rerio*) by arsenic in waters from Zimapan, Hidalgo, Mexico. Mutagenesis 20, 291–295.
Pandey, S., Kumar, R., Sharma, S., Nagpure, N.S., Srivastava, S.K., Verma, M.S., 2005. Acute toxicity bioassays of mercuric chloride and malathion on air breathing fish *Channa punctatus* (Bloch). Ecotoxicol. Environ. Saf. 61, 114–120.
Pandey, S., Nagpure, N.S., Kumar, R., Sharma, S., Srivastava, S.K., Verma, M.S., 2006. Genotoxicity evaluation of acute doses of endosulfan to freshwater teleost *Channa puctatus* (Bloch) by alkaline single-cell gel electrophoresis. Ecotoxicol. Environ. Saf. 65, 56–61.
Peña-Llopis, S., Ferrando, M.D., Peña, J.B., 2003. Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by N-acetylcysteine. Aquat. Toxicol. 65, 337–360.
Rencüzogullari, E., Topaktas, M., 2000. Chromosomal aberrations in cultured human lymphocytes treated with the mixtures of carbosulfan, ethyl carbamate and ethyl methanosulfonate. Cytologia 65, 83–92.
Risso-de Facerney, C., Devaux, A., Lafaurie, M., Girard, J.P., Bailly, B., Rahmani, R., 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygen species. Aquat. Toxicol. 53, 65–76.
Sahoo, A., Sahu, S.K., Sharmila, M., Sethunathan, N., 1990. Persistence of carbamate insecticides, carbosulfan and carbofuran in soils as influenced by temperature and microbial activity. Bull. Environ. Contam. Toxicol. 44, 948–954.
Sharbel, W.M., 2004. Monitoring DNA damage following radiation exposure using cytokinesis-block micronucleus method and alkaline single-cell gel electrophoresis. Clin. Chem. Acta 347, 15–24.
Sharma, S., Nagpure, N.S., Kumar, R., Pandey, S., Srivastava, S.K., Singh, P.J., Mathur, P.K., 2007. Studies on the genotoxicity of endosulfan in different tissues of fresh water fish *Mystus vittatus* using the comet assay. Arch. Environ. Contam. Toxicol. 53, 617–623.
Shen, H.M., Liu, Z.G., 2006. JNK signaling pathway is a key modification in cell death mediated by reactive oxygen and nitrogen species. Free Radic. Biol. Med. 40, 928–939.
Singh, N.P., McCay, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cell. Exp. Cell. Res. 175, 184–191.
Sprague, J.B., 1971. Measurement of pollutant toxicity to fish: 1. Bioassay methods for acute toxicity. Water Res. 3, 793–821.
Sterhrer-Schmid, P., Wolf, H.U., 1995. Genotoxic evaluation of three heterocyclic N-methylcarbamate pesticides using the mouse bone marrow micronucleus assay and *Saccharomyces cerevisiae* strain D7 and D61. Mutat. Res. 345, 111–125.
Talapatra, S.N., Ganguly, P., Mukhopadhyyay, A., Banerjee, S., 2006. Assessment of genetic biomarkers with special reference to micronucleated and binucleated erythrocytes in two fish species grown at industrial vicinity of thermal power plants, Kolkata, India. Asian J. Water Environ. Poll. 4, 139–144.
Tiano, L., Fedeli, D., Santori, A.M., Villarini, M., Engman, L., Falcioni, G., 2000. Effect of diary telluride and an organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro. Mutat. Res. 464, 269–277.
Topaktas, M., Rencüzogullari, E., Ila, H.B., 1996. In vivo chromosomal aberrations in bone marrow cells of rats with marshal. Mutat. Res. 371, 259–264.
Tuker, J.W., Thompson, C.Q., 1987. Dangers of using organophosphorous pesticides and diesel oil in fish ponds. Aquacult. Mag. 13, 62–63.
Van Der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharm. 13, 57–149.
Ventura Campo de, B., Angelis de Fransceschi de, D., Marin-Morales, M.A., 2008. Mutagenic and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic. Biochem. Physiol. 90, 42–51.
Wiedenmann, D., Stehrerer-Schmide, P., Wolf, H.U., 1990. Mutagenic effects of carbosulfan and furathiocarb in the Ames test and yeast assay. Naunyn Schmiedeberg's Arch. Pharmakol. 341, R29.
Xiao, H.Y., Shao, N.L., Zhang, L., Guo, N.Z., Hui, S.Z., 2008. Evaluation of DNA damage in Chinese toad (*Bufo bufo gargarizans*) after in vivo exposure to sublethal concentrations of four herbicides using the comet assay. Ecotoxicology 17, 280–286.
Yamano, T., Morita, S., 1995. Effects of pesticides on isolated hepatocytes, mitochondria and microsomes 11. Arch. Environ. Contam. Toxicol. 28, 1–7.