

# Evaluation of the Genotoxicity of Chlorpyrifos in Common Indus Valley Toad, *Bufo stomaticus* Using Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

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## Abstract

Chlorpyrifos is a commonly used pesticide of organophosphate group, which causes toxicological effects in non-target organisms especially fish and frogs. In the present study, the genotoxicity of sublethal concentrations of chlorpyrifos was observed in the erythrocytes of common Indus valley toad, *Bufo stomaticus*, using the Alkaline Single-Cell Gel Electrophoresis (Comet) assay. In the first step, acute toxicity of chlorpyrifos was evaluated by exposing the tadpoles to high concentrations of the pesticide. The acute LC<sub>50</sub> value of chlorpyrifos, calculated by Trimmed Spearman-Kärber (TSK) in static bioassay, was found to be 930.0 µg/L. On the basis of acute LC<sub>50</sub> value, the tadpoles were exposed to three sublethal concentrations (155, 233 and 465 µg/L) of chlorpyrifos for 96 h. Blood cells were collected at every 24 h interval and were subjected to the Alkaline Single-Cell Gel Electrophoresis assay. The observed DNA damage was concentration and time-dependent, and those levels of DNA damage in between the tested concentrations and times were significantly different ( $p < 0.05$ ). The tadpoles exposed to different concentrations of chlorpyrifos also showed different morphological abnormalities. It was concluded that chlorpyrifos is a genotoxic pesticide causing DNA damage in *Bufo stomaticus*.

## Keywords

Pesticide; Chlorpyrifos; Tadpoles; DNA Damage; Comet Assay

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## 1. Introduction

Pesticides are widely used in agricultural areas to improve the crop yield, but the indiscriminate use of these chemicals in the environment causes toxicity to the non-target organisms. These pesticides tend to reach into the adjacent small water bodies (e.g., ponds, creeks, and drainage ditches) and may exhibit toxicity and genotoxicity to aquatic organisms.

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a wide-ranging organophosphate insecticide used to control the agricultural and household pests [1]. It inhibits the acetylcholine esterase (AChE) enzyme and affects the nervous system of the aquatic organisms [2]. The insecticides are transported to the freshwater water bodies through run-off and storm water [3] [4] and severely affect the aquatic biodiversity of that area. These chemicals not only have negative impacts on the physiology of organisms but also interact with their DNA [5].

Amphibians are found in the aquatic, wetlands and agricultural ecosystems and are very important natural enemies of many agricultural insect pests. As the amphibians in their larval stages live in aquatic environment, they are regarded as bio-indicators [6] [7] and are widely used as test specimens in the assessment of toxic effects of pesticides on aquatic ecosystem [8]. A number of studies have demonstrated that the amphibians are sensitive and suitable organisms for the assessment of genotoxicity of pollutants [5] [9] [10].

Chlorpyrifos has already been found genotoxic in the root meristematic cells of *Crepis capillaries* [11]. It has been reported genotoxic in gill cells and lymphocytes of freshwater fish *Channa punctatus* (Bloch) [12] and in leucocytes of Swiss albino mice [13]. Genotoxicity of chlorpyrifos has also been evaluated in liver cells and erythrocytes of Chinese toad (*Bufo bufo gargarizans*) tadpoles [14].

Among different techniques used for the evaluation of DNA strand breakage, we selected to employ alkaline single-cell gel electrophoresis/comet assay to quantify DNA damage in the erythrocytes of *Bufo stomaticus* exposed to sublethal concentrations of chlorpyrifos. Comet assay has many advantages over other cytogenetic assays like micronucleus test, chromosomal aberration test and sister chromatid exchanges to detect DNA damage. Comet assay permits the visualization of DNA damage at an individual cell level [15]; relatively few cells are required and any cell having a nucleus can be used in this assay. The comet assay is very sensitive (detecting 1 strand break in  $1 \times 10^{10}$  Da of DNA) [16] and shows a clear dose dependent response. It is less expensive and less time-consuming than other biomonitoring assays [5].

There was no study on the toxicity and genotoxicity of chlorpyrifos in *Bufo stomaticus*. The present study was conducted to investigate the acute toxicity and genotoxicity of chlorpyrifos in *Bufo stomaticus* in a static bioassay. This study may also direct to reinforce the fact of the effectiveness of the Comet assay as a sensitive biomarker of DNA damage in the erythrocytes of *Bufo stomaticus*.

## 2. Materials and Methods

### 2.1. Chemicals

Normal melting point (NMP) agarose was purchased from Invitrogen Life Technologies Ltd. (Paisley, UK). Low melting point (LMP) agarose was obtained from Promega Corporation (Madison, USA). Methyl methane-sulfonate (MMS) and Ethidium bromide (EtBr) were supplied by Sigma-Aldrich, (St. Louis, MO, USA). Triton X-100 was obtained from AppliChem GmbH (Darmstadt, Germany). Phosphate buffered saline (PBS) was purchased from Invitrogen (Carlsbad, USA). Ethylenediamine tetraacetic acid (EDTA) disodium salt was obtained from GIBCO Life technologies Inc., (New York, USA). Dimethyl sulphoxide (DMSO) was supplied by Labscan Asia Co Ltd., (Bangkok, Thailand). Trizma base, NaOH, HCl, NaCl,  $MgSO_4 \cdot 7H_2O$ ,  $K_2HPO_4$ , and all other chemicals used were of analytical grade. Commercial formulation of chlorpyrifos product (40% EC), named as "Chlorpyrifos" (manufactured by M/s. K & N Efthymiadis, Greece), was purchased from the local market. It was observed that the chlorpyrifos of this grade is mostly employed in the fields.

### 2.2. Experimental Specimen

Tadpoles of *Bufo stomaticus* were obtained from NIBGE Faisalabad fields and shifted to the glass aquaria. These specimens were acclimatized for 15 days prior to the pesticide exposure with a 16:8 h (light:dark) photoperiod. The specimens were fed with boiled lettuce ad libitum, and feeding was stopped 24 h prior to the pesticide exposure till the end of the experiment.

### 2.3. Determination of Acute Toxic and Sublethal Concentrations

Stock solution of the insecticide was prepared by dissolving chlorpyrifos in acetone. Five working chlorpyrifos concentrations (0.4, 0.8, 1.2, 1.6 and 2.0 mg/L) were prepared in the solvent (acetone) from the stock solution. To determine the acute LC<sub>50</sub> value of chlorpyrifos, static bioassay was employed. Six groups of tadpoles, each containing 10 individuals, were selected at random and placed in the aquaria. Glass aquaria (with dimensions of 30 cm depth, 30 cm width and 45 cm length), with the capacity of about 40 L was filled with 15 L of water. The temperature of the water was regulated at 26°C ± 1°C. The electric conductivity and the pH of water were 2.62 - 2.76 mS and 8.77 - 9.29, respectively. The above mentioned concentrations were added to different aquaria containing specimens, keeping one as solvent control receiving no pesticide but maximum acetone that any dosing solution contain. Acute toxicity assay was performed following the guidelines of Organization for Economic Co-operation and Development (OECD) for testing of chemicals [17].

Acute LC<sub>50</sub> (96h) value of chlorpyrifos for tadpoles was calculated as 930.0 µg/L (95% Confident limits: 710.0 - 1220 µg/L) using a computer program, TSK (Trimmed Spearman-Kärber) [18]. Using the LC<sub>50</sub> value, three nominal sub-lethal concentrations viz., sub-lethal I (SL-I, 1/2<sup>nd</sup> of LC<sub>50</sub> = 465 µg/L), sub-lethal II (SL-II, 1/4<sup>th</sup> of LC<sub>50</sub> = 233 µg/L) and sub-lethal III (SL-III, 1/6<sup>th</sup> of LC<sub>50</sub> = 155 µg/L) were prepared.

### 2.4. In Vivo Sublethal Exposure Experiment

The tadpole were exposed to three above mentioned sublethal concentrations of chlorpyrifos in a static bioassay system for 96 h with a 16:8 h (light:dark) photoperiod. Blood samples were obtained at the intervals of 24, 48, 72 and 96 h from the two tadpoles per concentration per interval. Dechlorinated tap water and methyl methanesulfonate (MMS) (5 mg/L) were used as negative and positive controls, respectively.

Physico-chemical properties of water (pH, temperature, dissolved oxygen, electrical conductivity and total hardness) were analyzed at the start and at the completion of experiment.

### 2.5. Alkaline Single Cell Gel Electrophoresis (SCGE)

The alkaline single cell gel electrophoresis (SCGE)/Comet assay was performed using the procedure of Singh *et al.* [19] with slight modifications of Ralph *et al.* [10]. The tadpoles were decapitated and placed in 1 ml chilled phosphate buffered saline (PBS, Calcium and Magnesium-free) for 5 min. All the specimens were treated separately. The erythrocytes of the tadpoles were selected for DNA damage as these cells are much more numerous in the circulatory system and are nucleated in amphibians. The viability of the cells was assessed using the Trypan blue exclusion method [20]. Cell samples exhibiting cell viability more than 84% were further processed for comet assay.

About 20 µl of the cell suspension was mixed with 80 µl of 0.5% LMP agarose and pipetted on a slide already coated with 200 µl of 0.9% NMP agarose. After solidification, slides were coated with a third layer of 100 µl of 0.5% LMP agarose and covered with cover slips. The slides were placed at 4°C for 20 min to allow for complete polymerization of agarose. After the polymerization of the agarose, the cover slips were removed and the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100, pH 10.0) for 2 h at 4°C. Then the slides were placed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na<sub>2</sub> EDTA, pH > 13) in a horizontal gel electrophoresis unit for 25 min at 4°C, allowing for DNA unwinding and salt equilibrium. Electrophoresis was carried out in the same solution at 25V (~0.73 V/cm), 300 mA for 25 min. The slides were then neutralized with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min to remove excess alkali. This step was repeated three times. Then the slides were dried and stained with 20 µl of ethidium bromide (20 µg/ml). Slides were observed under an epifluorescent microscope (Labomed Lx400, Labo America, Inc., USA) equipped with an excitation filter 515 - 560 nm and emission filter 590 nm. Total 100 individual cells were observed per tadpole per concentration (50 cells per slide). The cells with no DNA damage have nucleoids, whereas the cells with DNA damage have comet-like appearance. The cells having DNA damage with no head or a dispersed head were regarded as apoptotic cells and were not considered for the analysis. The length of the migrated DNA in the Comet tail was measured using an ocular micrometer [21]. DNA damage was measured at individual cell level with the help of the following formula:

$$\text{Comet tail length } (\mu\text{m}) = \text{total comet length} - \text{head diameter}$$

## 2.6. Statistical Analysis

Statistical analysis of the data was carried using Statistix 8.1 computer software. Analysis of variance (ANOVA) was employed to compare the mean differences in the tail length between different concentrations within time durations and between time durations within concentrations. A  $p$  value less than 0.05 was considered as statistically significant.

## 3. Results

Physico-chemical characteristics of the water used in the experiments recorded, as pH:  $8.2 \pm 0.1$ ; temperature:  $26^\circ\text{C} \pm 1^\circ\text{C}$ ; dissolved oxygen:  $8.6 \pm 0.2$ ; electrical conductivity: 2.65 - 2.75 mS/m; total hardness: 86 - 96 as mg  $\text{CaCO}_3/\text{L}$ , were according to USEPA specifications [22]. Acute LC50 values (with 95% confidence limits) of chlorpyrifos to *Bufo stomaticus* at 24 h, 48 h, 72 h and 96 h were 1070  $\mu\text{g}/\text{L}$  (820 - 1410), 1040  $\mu\text{g}/\text{L}$  (800 - 1360), 970  $\mu\text{g}/\text{L}$  (740 - 1280) and 930  $\mu\text{g}/\text{L}$  (710 - 1220) respectively. In addition to the lethal effects of chlorpyrifos, the pesticide also has negative effects on the morphology of the tadpoles during the experimental period. These morphological abnormalities include tail deformities, fins shrunken and head edema.

Erythrocytes of the *Bufo stomaticus* tadpoles were used to assess the genotoxicity of chlorpyrifos using the comet assay. The tail length ( $\mu\text{m}$ ) of the comet was measured as the degree of DNA damage. Significantly higher ( $p < 0.05$ ) levels of DNA damage were observed in the erythrocytes of tadpoles exposed to all the three sublethal concentrations of chlorpyrifos relative to the negative control (Table 1). It was found that the increase in DNA damage was significantly concentration dependent. DNA damage observed at the lowest concentration of chlorpyrifos (155  $\mu\text{g}/\text{L}$ ) was  $53.61 \pm 5.71 \mu\text{m}$ , significantly increased to  $71.22 \pm 5.21 \mu\text{m}$  at the highest concentration (465  $\mu\text{g}/\text{L}$ ) at 24 h exposure (Table 1). Same trend was observed at other three exposures time durations. It was also found that the increase in DNA damage was exposure time dependent. At the lowest sublethal concentration of chlorpyrifos (155  $\mu\text{g}/\text{L}$ ), there was no significant increase in DNA damage was observed at all exposure time intervals. Whereas, at the sublethal concentration 233  $\mu\text{g}/\text{L}$ , DNA damage was significantly ( $p < 0.05$ ) increased from  $57.88 \pm 6.38 \mu\text{m}$  at 24 h to  $74.13 \pm 8.67 \mu\text{m}$  at 96 h. The same trend in significant increase in DNA damage was observed at the highest concentration of chlorpyrifos (465  $\mu\text{g}/\text{L}$ ) from 24 h to 96 h time intervals.

## 4. Discussion

Tadpoles are useful experimental organisms for environmental bio-monitoring studies due to their availability in sufficient numbers, ease to collect, confinement to water bodies and direct exposure to contaminants in water and vegetation [23]. In this study, the acute toxicity and genotoxicity of chlorpyrifos were evaluated in *Bufo stomaticus* tadpoles. The acute LC<sub>50</sub> (96 h) value of chlorpyrifos in *Bufo stomaticus* was calculated as 930.0  $\mu\text{g}/\text{L}$  (0.93 mg/L) (95% confident limits: 710.0 - 1220  $\mu\text{g}/\text{L}$ ). The results showed that chlorpyrifos is moderately toxic to *Bufo stomaticus*. The LC<sub>50</sub> value of chlorpyrifos in Chinese toad (*Xenopus laevis*) tadpoles was 0.56 mg/L and it was found that the tadpoles exposed to the insecticide during metamorphs were more sensitive than those exposed at pre-metamorphs [24]. In another study, the acute LC<sub>50</sub> (48 h) value of chlorpyrifos to *Rana limnocharis* was found to be 2.40 mg/L [25].

**Table 1.** Mean comet tail length ( $\mu\text{m}$ ),  $\pm\text{SD}$ , of erythrocytes of *Bufo stomaticus* exposed to different concentrations of chlorpyrifos at different time intervals.

Concentrations ( $\mu\text{g}/\text{L}$ )	24 h	48 h	72 h	96 h
Control	$40.37 \pm 1.27$ <sup>a1</sup>	$40.72 \pm 1.20$ <sup>a1</sup>	$40.25 \pm 1.42$ <sup>a1</sup>	$40.61 \pm 1.52$ <sup>a1</sup>
155	$53.61 \pm 5.71$ <sup>b1</sup>	$56.38 \pm 4.60$ <sup>b1</sup>	$59.22 \pm 4.92$ <sup>b1</sup>	$62.59 \pm 6.48$ <sup>b1</sup>
233	$57.88 \pm 6.38$ <sup>b1</sup>	$62.64 \pm 5.12$ <sup>b12</sup>	$67.75 \pm 7.47$ <sup>b12</sup>	$74.13 \pm 8.67$ <sup>b2</sup>
465	$71.22 \pm 5.21$ <sup>c1</sup>	$76.31 \pm 5.18$ <sup>c12</sup>	$85.75 \pm 5.42$ <sup>c23</sup>	$93.13 \pm 6.22$ <sup>c3</sup>
MMS	$71.81 \pm 3.19$ <sup>c1</sup>	$76.46 \pm 4.72$ <sup>c1</sup>	$80.55 \pm 6.97$ <sup>c12</sup>	$88.52 \pm 7.43$ <sup>c2</sup>

Note: values with different alphabet superscripts differ significantly ( $p < 0.05$ ) between concentrations within duration. The values with different numeric superscripts differ significantly ( $p < 0.05$ ) between durations within concentration.

The alkaline single-cell gel electrophoresis/comet assay is a commonly known biomarker of genotoxicity in organisms exposed to environmental genotoxic pollutants and the greater the number of comets is associated with the serious health consequences in the organisms [26]-[28]. Chlorpyrifos was not found to be genotoxic in any of these assays (Ames test, rat lymphocyte chromosomal aberration test, CHO/HGPRT assay, mouse bone marrow Micronucleus assay, cytogenetic abnormalities) [29]. But later on, it was reported that chlorpyrifos induce *in-vivo* genotoxicity in leucocytes of Swiss albino mice using Comet assay [13]. Chlorpyrifos has also been observed genotoxic in root meristematic cells of *Crepis capillaries* [11], and in lymphocytes and gill cells of freshwater fish *Channa punctatus* (Bloch) [12].

A number of studies have reported the genotoxicity of pesticides in amphibians [14] [30]-[33]. Chlorpyrifos induced the genotoxicity in erythrocytes and liver cells of Chinese toad (*Bufo gargarizans*) when the tadpoles were exposed to the sublethal concentrations of chlorpyrifos (80.0 µg/L, 160.0 µg/L, 320.0 µg/L, 640.0 µg/L) [33]. A concentration-dependent increase in DNA damage was observed in the erythrocytes and liver cells of Chinese toad. Our results also showed an increase in DNA damage (tail length) in erythrocytes of *Bufo stomaticus* exposed to sublethal concentrations of chlorpyrifos and this increase in DNA damage was concentration and time-dependent (Table 1).

In this study, chlorpyrifos induced a significantly ( $p < 0.05$ ) higher DNA damage in the erythrocytes of *Bufo stomaticus* tadpoles as compared to negative control, depicting the genotoxic nature of chlorpyrifos to amphibians. A significantly concentration-dependent increase in DNA damage was observed at all the exposure concentrations of chlorpyrifos. The lower two sublethal concentrations of chlorpyrifos (155 and 233 µg/L) showed significant DNA damages as compared to negative control, but did not show any significantly ( $p < 0.05$ ) different results with respect to each other. The highest concentration (465 µg/L) induced significantly ( $p < 0.05$ ) higher DNA damages as compared to negative control and other two chlorpyrifos concentrations at all the exposure time durations (Table 1). Our observations are in accordance with the previous studies in which different pesticides induced concentration-dependent increase in DNA damages in different amphibian species [14] [30] [33].

Induction in DNA damages in the erythrocytes of *Bufo stomaticus* tadpoles was mostly time-dependent. Observed DNA damages were significantly ( $p < 0.05$ ) increased at most of the time durations in the higher two chlorpyrifos concentrations (Table 1). Same-time dependent increase in DNA damage was found in *Xenopus laevis* and *Pleurodeles waltil* tadpoles when exposed to Captan (a fungicide) [32].

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