Short Communication

BISPHENOL S INDUCED CYTO-GENOTOXIC EVALUATION IN *Eisenia fetida* THROUGH MICRONUCLEUS AND COMET ASSAYS

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ABSTRACT

Bisphenol S (BPS), the most commonly used alternative to bisphenol A (BPA), is widely present in various consumer products. This study was designed to investigate the BPS-induced cyto-genotoxicity in *E. fetida*, an important soil indicator. Earthworms were categorized into four experimental groups and one control group (n=10/group), and exposed to different concentrations of BPS (50, 100, 150 and 200 mg/mL). The median lethal concentration (LC50) of BPS was calculated as 50 mg/mL using Probit analysis estimating the concentration corresponding to the 50% mortality. Both cytotoxic and genotoxic assays (micronucleus and comet) showed a concentration-dependent increase in micronuclei frequency and DNA damage in earthworms with significant differences ($p < 0.05$) between the control and BPS-treated groups. At 200 mg/mL, the greatest level of DNA damage (92.0 ± 1.41) and micronuclei frequency (113.5 ± 4.24) were observed. The findings of this study showcase the potential cyto-genotoxicity and hazardous risks due to BPS exposure and emphasize the need for its cautious use and stringent regulation regarding BPS in consumer products.

Keywords: Biomarkers, DNA Damage, Endocrine disrupter, Oxidative stress, Soil ecotoxicology

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INTRODUCTION

Bisphenols in daily-use plastic products are major contributors to health effects, which encourage the industries to produce safer products or alternatives such as "Bisphenol A (BPA)-free" (Cull *et al.,* 2025). Bisphenol S (BPS), as a substitute, also exhibits severe endocrine disrupting effects (Rochester *et al.,* 2015). BPS's decreased biodegradability has been majorly attributed to its heat and UV resistance. BPS and their interaction with microplastics and causing severe adverse health effects are growing concerns and need further investigation (Fang *et al.,* 2020).

BPA use has been restricted in Canada, the USA and many European Countries due to growing health concerns (Datta *et al.,* 2024). The structural similarities between BPA substitutes like BPS and BPF have demonstrated the potential toxic and endocrine-disruptive effects (Xiong *et al.,* 2024; Hwang *et al.,* 2025). In cells, BPS has estradiol-like potency which influences the cell growth and ultimately causing cell death.

Environmental microplastics (MPs) with the ability to interact with biota, chemicals and other pollutants badly affect the aquatic ecosystems (Tang *et al.,* 2025; Guven *et al.,* 2018). Nanoparticles such as iron oxide nanoparticles of several sizes and shapes have elucidated the similar genotoxic effects in *E. hortensis* (Ciğerci *et al.,* 2022). Furthermore, the cytogenotoxic effects of BPS have been examined in the meristematic cells of *A. cepa* (Ali *et al.,* 2022).

A significant increase in biodegradation of BPS (up to 98.6%), facilitated by earthworms, results its bioaccumulation in tissues and impairing their oxidative and anti-oxidative balance (Qian *et al.,* 2023). The potential risks associated with bisphenols extend beyond human health, as they can exert detrimental effects on various non-target species in the environment (Cull *et al.,* 2025; Datta *et al.,* 2024). Therefore, the present study aimed to investigate the micronucleus formation, DNA damage and the environmental risks caused by BPS on earthworms (*E. fetida*) using micronucleus and comet assays.

MATERIALS AND METHODS

Earthworm collections and chemicals: Earthworms (300-600 mg) were purchased from the National Agricultural Research Center (NARC), Islamabad. BPS (98% purity, CAS No. 80-09-01) was obtained from Sigma-Aldrich Co America. The experiments were conducted at the Department of Wildlife and Ecology and the Institute of Biochemistry and Biotechnology (IBBt), University of Veterinary and Animal Sciences (UVAS), Lahore.

BPS Exposure Bioassay: The earthworms were maintained under controlled laboratory conditions at 26±2 ºC in a dim light. An aqueous solution-based toxicity test was conducted on *E. fetida* for BPS exposure according to the protocol described by Ciğerci (2016), with minor modifications. Earthworms were divided into five groups: one control group and four experimental groups (10 earthworms per group). Distilled water was used as a control group (no chemical exposure). For experimental groups, BPS concentrations (50, 100, 150 and 200 mg/mL) were prepared by dissolving BPS in distilled water using the dilution equation $C_1V_1=C_2V_2$. Each group of earthworms was then placed in a separate petri dish containing 20 mL of the respective BPS concentrations under the same controlled laboratory conditions for 24 hours.

The median lethal concentration (LC_{50}) of mortality was computed using the Probit analysis method. The Probit regression analysis followed the Y=mx+c equation where y represents 50% mortality, m is the slope of log10 concentration, c is the intercept and x represents the $\log_{10}$ concentration (Kumari *et al.,* 2024).

Comet assay: According to Ciğerci (2016), coelomic fluid was extracted from the coelomic cavity with extrusion buffer (50 mL ethanol, 1.091g EDTA, 4.161g NaCl and 950 mL dH_2O to prepare 1 liter total solution, pH 7.5). Coelomic fluid was collected in a centrifuge tube of 1 mL**.** The coelomocytes were centrifuged for 5 min at 6000 rpm. The supernatant was discarded and washed with phosphate buffered saline (PBS 10X). The preheated slides were coated with normal melting agarose (NMA). Then, 10 µL of sample and 100 µL of low melting agarose (LMA) mixture was spread on slides. Slides were placed on ice slabs for 5 minutes then they were kept in ice-chilled lysis buffer (NaOH, EDTA; pH > 13) for 20 minutes. For gel electrophoresis, the slides were run in electrophoresis tank at 25V, 300 mA for 20 min. Staining was performed with 80 µL of 20 µg/mL ethidium bromide solution (Liman *et al.,* 2025). DNA damage was visualized using a fluorescent microscope (OLYMPUS CX41) at 40 × magnification and scored on an arbitrary scale ranging from 0 to 4, where 0 indicated no damage, 1 slight damage, 2 moderate damage, 3 extensive DNA damage and 4 complete DNA damage. The comet assay was performed in triplicate for each concentration and 100 comets were counted per slide. DNA damage induced by different BPS concentrations was assessed using the following formula:

$$\text{Arbitrary damage} = \Sigma^{4}_{i=0} (N_i \times i)$$

Here, N*i* denotes the number of cells at a specific degree of damage based on a 5-point scale while *i* indicates extent of degree of damage ranging from 0 to 4 (Liman *et al.,* 2025).

Micronucleus test: The micronucleus test was carried out according to the procedure described by Ciğerci (2022), with minor modifications. Coelomic fluid was treated with 1 mL of KCl and centrifuged (5-6 min, 1200 rpm). Then, 1 mL of fixative I (50 mL 0.09% NaCl + 50 mL fixative II) and 1 mL of fixative II (methanol 200 mL + glacial acetic acid 40 mL) were blended with coelomic fluid and centrifuged (5 min at 1200 rpm). The supernatant was discarded and cell suspension was smeared on a clean slide. Three slides were prepared for each concentration, air-dried and stained with Giemsa stain for 15 min. A total of 500 cells were counted per concentration using a compound microscope (LABOMED, USA, LX400).

Statistical Analysis: One-way ANOVA (SPSS version 20.0) and Tukey test were applied to analyze the outcomes and to compare different experimental groups with the control group. Results were evaluated as mean ± standard deviation for test and control groups at a significance level of $p < 0.05$.

RESULTS

In this study, the LC_{50} value for BPS was determined to be 50 mg/mL using Probit analysis of mortality. The concentration-dependent genotoxic effects on earthworms (*E. fetida*) were observed (**Fig. 1; Fig. 3**). Highest DNA damage (92.0±1.41) was found at 200 mg/mL, whereas least DNA damage (16.5±4.94) was observed in negative control. A statistically significant difference ($p < 0.05$) was observed between the control group and all BPS concentrations.

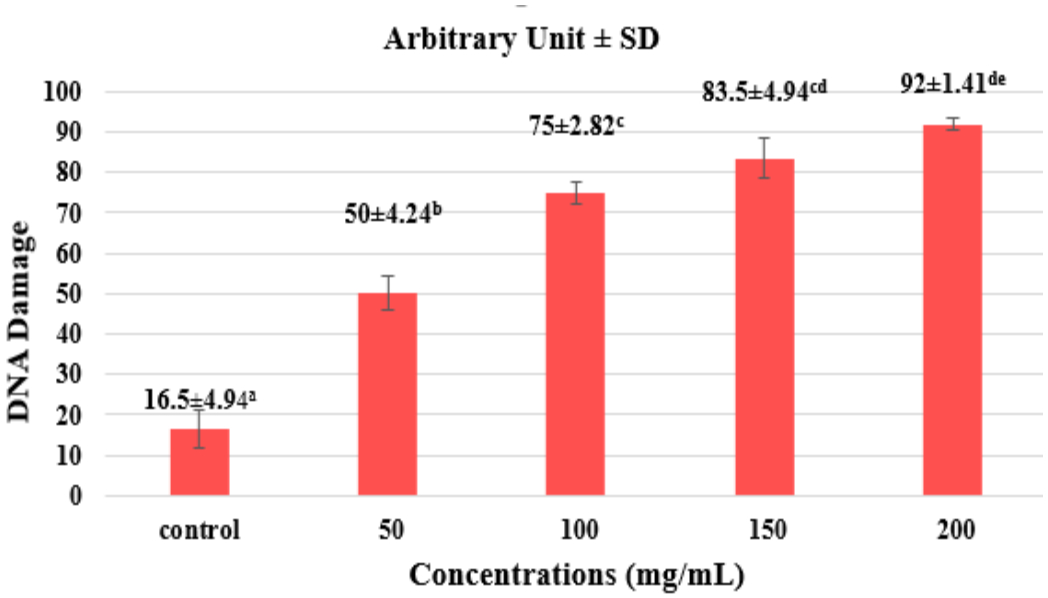

Fig. 1. Mean DNA damage score in earthworms exposed to various concentrations of BPS, significantly different from control group ($p < 0.05$). Error bars represent the standard deviation.

Micronuclei formation at different concentrations of BPS (**Fig. 4**). In the current study, BPS showed the concentration-dependent micronuclei formation. The highest frequency of micronuclei formation (113.5±4.24) was observed at 200 mg/mL while the lowest frequency was noted in the control group. (**Fig. 2**). A statistically significant difference ($p < 0.05$) was observed between the control and BPS-treated groups in micronucleus test.

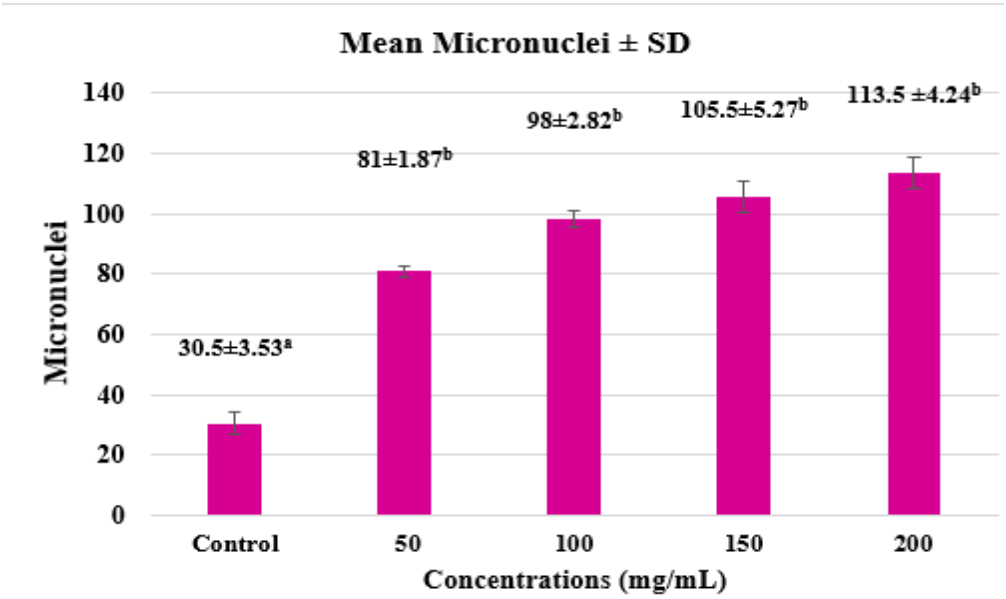

Fig. 2. Mean micronuclei in earthworms exposed to various concentrations of BPS, significantly different from control group ($p < 0.05$). Error bars represent the standard deviation.

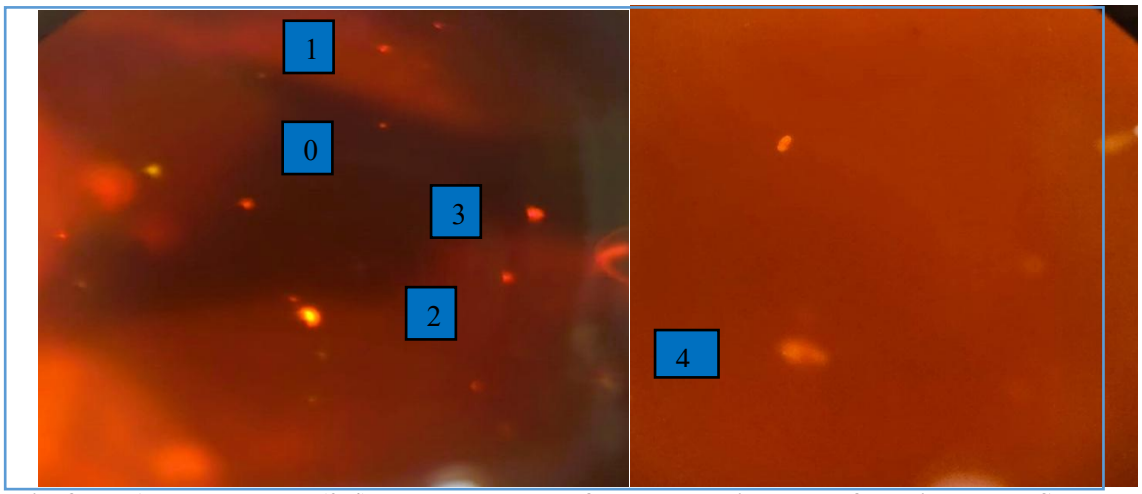

Fig. 3. DNA damage score (0-4) observed under a fluorescent microscope following the BPS exposure

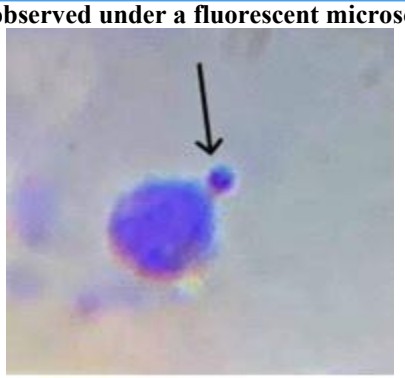

Fig. 4. Micronuclei Formation in earthworms following the BPS exposure

DISCUSSION

BPS, a BPA alternative, is commonly found in personal care products, thermal paper and food items (Li *et al.,* 2018). BPS decomposed quickly in soil, with a half-life of <1 day in clay loam soil (Choi and Lee, 2017). The composition and activity of soil microbiota significantly affect the degradation of organic pollutants (Ren *et al.,* 2018). BPS-contaminated products may affect soil organisms like earthworms by impairing their antioxidant defense enzymes including Superoxide Dismutase (SOD) and Peroxidase (POD), which contribute to protecting against oxidative stress and maintaining normal physiological functions (Olujimi *et al.,* 2020). A concentration of 50 mg/mL resulted 50% mortality and determined as LC_{50}.

Moreover, BPS may cause cyto-genotoxicity in earthworms as evidenced by micronucleus and comet assays. Oxadizon and Pendimethalin have shown the similar effects of chromosomal aberrations and DNA damage in earthworms (Ciğerci *et al.,* 2022). BPF exposure lowers liver fat in high-fat diet-fed mice by reducing lipid levels and disrupting lipid metabolism, leading to liver injury (Sun *et al.,* 2023), disturbs sperm functions and spermatogenesis (Gao *et al*., 2022). Nanoparticles (CeO_2 and MgO) and their ionic forms cause genotoxic effects on *E. hortensis* and similar results were observed in this study on *E. fetida* (Güneş *et al.*, 2022).

BPA exposure may change earthworm's genetic makeup, impairing their ability to detoxify, repair DNA damage and epigenetic modifications (Novo *et al.,* 2018). Harmful effects of BPA are well-studied; its analogues with estrogenic activity remain understudied, need further investigation (Chen *et al.,* 2016). The genotoxic results of this study also demonstrated the necessity of further investigation of BPS. BPS, BPF and BPA at 0.001 and 0.0001 mg/L did not affect the life span of worms but the environmental effects were considerable (Ficociello *et al.,* 2021). Therefore, studies using high concentrations such as the present study are important to reveal the potential chronic, sub-lethal effects beyond mortality. BPA exposure has adverse effects on *E. fetida* causing immune suppression, increased mortality, reproductive toxicity and decreased growth rate, declining at higher concentration (Verdú *et al.,* 2018). BPS may induce genotoxicity by producing oxidative stress, DNA strand breaks and chromosomal damage through disruption of DNA repair pathways.

Excessive BPS contamination in environmental media (sediments, indoor dust, sewage sludge, water) poses a risk of DNA interactions and potential harm to human health (Qiu *et al.,* 2019). To understand the ecological importance of soil species, particularly *E. fetida* as a key indicator of pollutant biodiversity and soil quality, the current study was designed to assess the genotoxic effects of BPS using *E. fetida* as a representative model. BPS exhibited genotoxic effects and potential genetic hazards at 50-200 mg/mL concentrations; therefore, further studies are needed at environmentally relevant exposure levels.

Conclusion: BPS exposure induced significant genotoxic effects in earthworms, as demonstrated by increased DNA damage and micronucleus formation. Both comet and micronucleus assays confirmed a statistically significant concentration-dependent increase in DNA damage and micronucleus frequency in *E. fetida* after BPS exposure. These findings highlight the potential genetic hazards of BPS, reinforcing the importance of monitoring BPS environmental exposure.

Conflict of Interest: The authors have declared that there is no conflict of interests.

Authors Contribution: AI and MMA designed the study, UN performed the research experiment, KA and AH devised ideas and supervised the whole experiment, HGF and SA helped in data analysis. SA and BA provided the critical revision and final approval of the article.

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