

DNA DAMAGE CAUSED BY A TEXTILE DYE (ACID RED) IN COELOMOCYTES OF EARTHWORM, *EISENIA FETIDA*

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ABSTRACT

The genotoxic effect of a commercially-available textile dye on earthworms was assessed using the alkaline comet assay. Earthworms were exposed to sub-lethal concentrations of the dye for 24 hours and coelomocytes were processed for comet assay. Nucleoids were visually scored and categorised into various degrees of damage. Significant increases ($p < 0.05$) in the percentage of damaged nucleoids in treated groups were recorded. The distributions in the damage grades in treated groups were significantly different ($p < 0.05$) from the control. The degrees of DNA damage between different treatments were not significantly different. However, the damage distribution was consistent over all the concentrations, showing that commercial acid red dye tested has the potential to induce genotoxicity in the coelomocytes of earthworms. Continued usage of such commercial dyes with such cryptic molecular toxicity can be detrimental to the ecosystem. Further studies on the genotoxic effects of more commercially available dyes on various test systems are warranted.

Key words: acid red dye; earthworm; comet assay; genotoxicity.

The disruption of ecological balance by diverse environmental pollutants is increasing. Out of the various environmental pollutants encountered, the dye and dye intermediates comprise the most important and critical group of compounds, since most of them are not characterised for purity, composition, structure, toxicity and impact on health and environment (Mathur and Bhatnagar 2007). Commercial use of these dyes in textile, leather, paint and food industries makes it a subject of concern. The Central Pollution Control Board in India has classified this industry as one of the most heavily polluting industries (CPCB 1990). Many dyes are persistent substances; most of them have been in the market for years and come under the existing chemicals directive and are not fully tested for their potential health effects (Walters et al. 2005). Toxicological information is scarce as most were released in the market before 1983 (Schneider 2004). In various parts of India, especially the rural sectors, commercial dyes are produced and used in unknown quantities. Unfortunately, there is no information with respect to the chemical structure, purity or composition of these commercial dyes; continuous use of such dyes can lead to grave consequences to exposed organisms (Mathur et al. 2005). Pollution caused by these dyes may lead to detrimental effects in the terrestrial soil biota.

Risk assessment of these pollutants is generally difficult due to the complex environmental exposures confronted by the organisms of the ecosystem; in addition, sensitive molecular assays detecting the genetic integrity of these organisms, that can be practically applied to different cell systems for environmental biomonitoring, are still not available (Tarazona and Vega 2002; Borrás and Nadal 2004). The emergence of the comet assay in the past two decades has facilitated the measurement of DNA-damage induction in many types of cells (Walsh et al. 1995; Rajaguru et al. 2003, Fairbairn et al. 1995) over a broad spectrum of species and exposures.

We chose earthworms for the testing of textile dyes since they are in intimate contact with a diverse range of environmental compartments and are prone to mixed contaminant exposure (Tarazona and Vega 2002; Langdon et al. 2003). Rajaguru et al. (2003) stated that for ecotoxicological investigations it is relevant to use native fauna as indicators of environmental contamination. Earthworms are efficient prospectors in soil within which they account for a significant proportion of the biotic biomass (Saint Denis 1999) and therefore they are suitable indicators of soil pollution. Earthworms constitute an important component of the terrestrial ecosystem and enhance productivity by improving soil structure (Edwards and Bohlen 1996). The easy availability and culturability of earthworms make them ideal test species for scientific and regulatory investigations (Reinecke and Reinecke 2004; OECD 1984). They are ideal sentinel species being ubiquitous and abundant, they bioaccumulate toxicants and act as indicators of the soil-borne genotoxic contaminants, and hence earthworms were selected for the present study. *Eisenia fetida*, being the standard earthworm species used for *in vivo* and *in vitro* bioassays, was employed for the study (OECD 1984).

The comet assay in earthworms is employed for monitoring and detection of DNA damage by chemicals in terrestrial ecosystems (Salagovic et al. 1996; Zang et al. 2000). It has emerged as a sensitive (one break per 10^{10} Da of DNA, Gedik et al. 1992) and consistent biomarker detecting DNA damage, finding its application in earthworm ecotoxicology (Tice et al. 2000). The alkaline comet assay detects DNA single strand breaks (Martin et al. 1999; Yared et al. 2002).

So far, textile dyes of known and unknown purity have been tested *in vitro* (Mathur et al. 2005, Bakshi and Sharma 2003; Tsuboy et al. 2007; Mansour et al. 2009) and *in vivo* test systems (Rajaguru et al. 1999) for genotoxicity; however, there are no data on the effects of these dyes on

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Table 1. The distribution of nucleoids in different damage categories.

Group	Dose (mg/kg soil)	DNA damage categories					% DNA damage	DNA damage score (AU)
		0	1	2	3	4		
Control	0	78.1 ± 4.067	21.1 ± 4.09	0.8 ± 0.63	0 ± 0.00	0 ± 0.00	21.9 ± 4.07	22.7 ^a
ARD	0.02	32 ± 2.45	41.8 ± 2.44	19.1 ± 2.77	6.1 ± 2.51	1 ± 1.15	68 ± 2.45*	102.3 ^b
ARD	0.2	33.3 ± 2.91	39.2 ± 3.26	18.5 ± 4.74	7.1 ± 2.77	1.9 ± 1.97	66.7 ± 2.91*	105.1 ^{b,c}
ARD	2.0	25.3 ± 3.47	36.7 ± 1.95	34.2 ± 4.26	3.5 ± 1.90	0.3 ± 0.67	74.7 ± 3.47*	116.8 ^{c,d}
ARD	20	27.5 ± 2.42	26.4 ± 2.01	28.2 ± 2.78	13.1 ± 2.60	4.8 ± 1.48	72.5 ± 2.42*	141.3 ^d
CP	100	0.3 ± 0.48	1.8 ± 1.87	2.6 ± 1.96	28 ± 6.68	67.3 ± 6.98	99.7 ± 17.49*	360.2 ^c

ARD - Acid Red Dye. CP - Cyclophosphamide – Positive mutagen

* - Statistically significant values as evaluated by t test.

DNA Damage Categories: 0 – Undamaged, 1 - Mild damage, 2 - Moderate damage, 3 - Severe damage, 4 - Complete damage

AU – Arbitrary Unit - Unit used for the expression of DNA damage. It is an arbitrary value which is calculated using the formula,

$$4$$

$$\text{Arbitrary unit} = \sum_{i=0}^4 Ni \times i$$

Where N is the number of cells in the damage category *i* (*i* = 0, 1, 2, 3 or 4).

AU values between groups were compared (Kruskal–Wallis test, *p*<0.05). Values with the same letter are not statistically different from each other.

earthworms, which comprise an important component of the terrestrial ecosystem. The lack of data on the genotoxicity of commercial dyes on earthworms prompted the present investigation. We initiated studies on one such commercial dye, Acid Red. Acid red belongs to the acid dyes, which are widely used in dyeing of textiles, especially protein fibres. However, we have no data on the composition or purity of the dye used in the present investigation. Such commercial dyes are being sold in local markets without any data on their safety, and used for dyeing of textiles in various parts of India. In general, acid dyes carry a sulfo or carboxy group in their structure, which makes them water-soluble. Acid dyes share a similar structure with azo dyes. Azo dyes in purified form are not directly mutagenic or carcinogenic, except for some azo dyes with free amino groups (Brown and De Vito 1993). However, reduction of azo dyes, i.e., cleavage of the dye’s azo linkages, leads to formation of aromatic amines and several aromatic amines are known mutagens and carcinogens (Walters et al. 2005). With this background, the present study was designed to analyse the genotoxicity of acid red on earthworms using the alkaline comet assay.

Acid red dye was obtained commercially from a local market in Chennai, India. No data on purity, composition or chemical structure are available. Proper precautionary methods were taken while handling the chemical. Laboratory-bred earthworms (*Eisenia fetida*) weighing approximately 350 mg were used for the experiments. Ten earthworms were released in artificial soil (Industrial sand: Kaolinite clay: Sphagnum peat at 70:20:10) containing various concentrations of the dye (0.02, 0.2, 2, 20 and 200 mg/kg soil). Positive (Cyclophosphamide (Sigma, USA) 100 mg/kg soil) and untreated control groups were maintained. Standard lighting (400–800 lux) and temperature (18 - 22°C) was maintained throughout the course of the experiment.

The doses were selected based on a preliminary range-finding study (data not shown). After 24 h of exposure, the coelomocytes were obtained by non-invasive extrusion (Eyambe et al. 1991). Individual earthworms were rinsed in extrusion medium. Coelomocytes were spontaneously secreted in the medium and washed in phosphate- buffered saline. The cells were collected by centrifugation (800 g, 3 min) and kept in ice till further processing. The comet assay was performed as described by Singh et al. (1988). Briefly, 1 h lysis, 20 min alkaline unwinding and 20 min electrophoresis (300 mA, 25 V) was performed. Ethidium bromide-stained nucleoids were examined with a fluorescence microscope (Axiostar plus, Carl Zeiss, Germany) and classified according to Collins et al. (1995). One hundred comets on each slide were scored visually as belonging to one of five classes of 0, 1, 2, 3 or 4 (from undamaged 0, to maximally damaged, 4) (Figure 1). Thus, the total score for 100 comets could range from 0 (all undamaged) to 400 (all maximally damaged). The percentage of damaged cells was calculated and the results analysed using Student’s ‘t’ test. An “arbitrary unit” (AU) was used to express the extent of DNA damage and was calculated as follows:

$$4$$

$$\text{Arbitrary unit (AU)} = \sum_{i=0}^4 Ni \times i$$

Where N_i = Number of cells in *i* degree; *i* = degree of damage (0, 1, 2, 3, 4).

The number of nucleoids in each grade, the percentage of damaged nucleoids and DNA damage score of each group are presented in Table 1. Significant increases (*p*<0.05) in the percentage of damaged nucleoids in all dye-treated groups over control were recorded. AU values between groups were compared (Kruskal –Wallis test, *p*<0.05). AU values

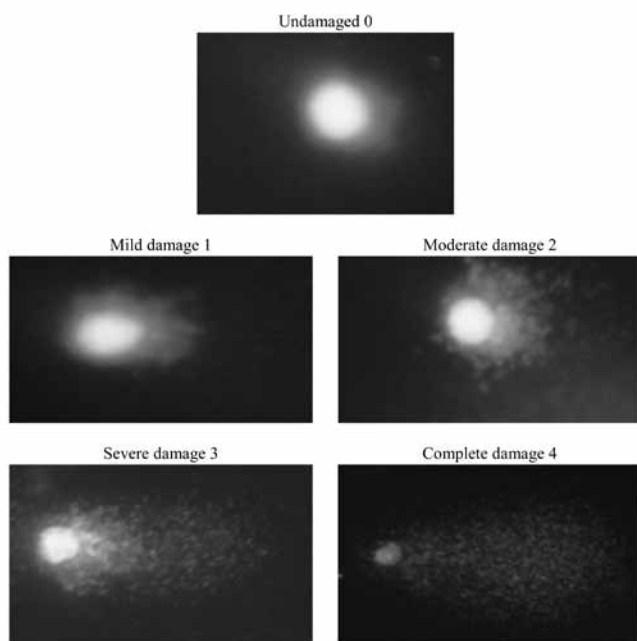


Figure 1. Photomicrographs of undamaged through damaged nucleoids of different degrees of damage.

0 – Undamaged; 1 –Mild damage; 2- Moderate damage; 3- Severe damage; 4- Complete damage.

of treated groups were significantly different from controls. Values marked with the same letter of the alphabet are not statistically different from each other. Box plots (Figure 2) were used to depict the damage distribution in the various treatment concentrations. The box represents 50% values i.e., the inter-quartile range of the data. The bottom and top of the box represent the 25th percentile and 75th percentile values of the data. The bars extending from the box represent the whiskers. Values were statistically analysed using the Kruskal-Wallis multiple comparison test. In all the damage categories, the treated groups were statistically significant ($p < 0.05$) over control. Figure 3 represents the dose-effect relationship. All the treatment values were statistically significant over control (Kruskal Wallis test, $p < 0.05$). The dose effect response was not linear but a plateau. However, considering the damage distribution profile and the statistical significance observed, it could be concluded that acid red dye has the potential to induce single strand breaks in the coelomocytes of earthworms.

The comet assay in coelomocytes has been utilised for the detection of genotoxicity of various chemicals (Verschaeve and Gilles 1995) including pesticides (Bustos–Obregon and Gicochea 2002; Zang et al. 2000), polluted sites (Salagovic et al. 1996; Xiao et al. 2006), river sediments (Rajaguru et al. 2003), heavy metals (Reineicke and Reineicke 2004) and species sensitivity to genotoxicants (Fourie et al. 2007). Martin et al. (2005) suggested that earthworm tissues may be incorporated into genotoxicity assays to facilitate hazard identification within terrestrial ecosystems.

Damage to DNA results in a variety of lesions ranging from strand breaks to mutations, progressing to cancer and other

syndromes (Shugart 2000; Kurelec 1993). Such incidences in biotic factors of the ecosystem can cause imbalances in the ecological niche (Klobucar et al. 2003). DNA damage in the form of single strand breaks is a direct indication of genotoxicity (Sardas et al. 1998). From the results, it is clear that acid red dye is genotoxic as it induces DNA damage (single strand breaks) in the coelomocytes of earthworms. Strand breaks may result from incomplete excision repair of DNA adducts, cross-links and alkali labile sites (Pfuhrer and Wolf 1996; De Boeck et al. 2000). The alkaline version of the comet assay employed can detect such diverse types of DNA damage. The comet assay is capable of examining DNA strand breaks in individual eukaryotic cells after *in vivo* or *in vitro* exposure and is considered to be a sensitive biomarker for the quantification and identification of genotoxicity (Faust et al. 2004). Assessment of the genotoxicity of compounds in terrestrial ecosystems presents a number of challenges, due to the diverse and complex nature of these environments (Qiao et al. 2007); however, the utility of artificial soils and *in vivo* bioassays like the comet assay make such investigations possible.

The effect of a chemical on the DNA of earthworms is worth studying as they facilitate major interactions in soil and channel contaminants to predators at higher trophic levels within the ecosystems (Langdon et al. 2003), and toxicity to earthworms at either lethal or sublethal levels of exposure may lead to detrimental effects in the consumers. The assessment of sub-lethal effects of contaminants using biomarkers is an important component of ecotoxicology (Van Gestel and Van Brummelen 1996).

From the present study, it is concluded that the commercially-available dye, acid red, is genotoxic to earthworm even at sublethal concentrations. Continued usage of such commercial dyes with such cryptic molecular toxicity can be detrimental to the ecosystem. Further studies on genotoxic effects of more commercially-available dyes of the same and different classes on various test systems (fish, Daphnia, animal models) are warranted, an investigation which is underway in our laboratory.

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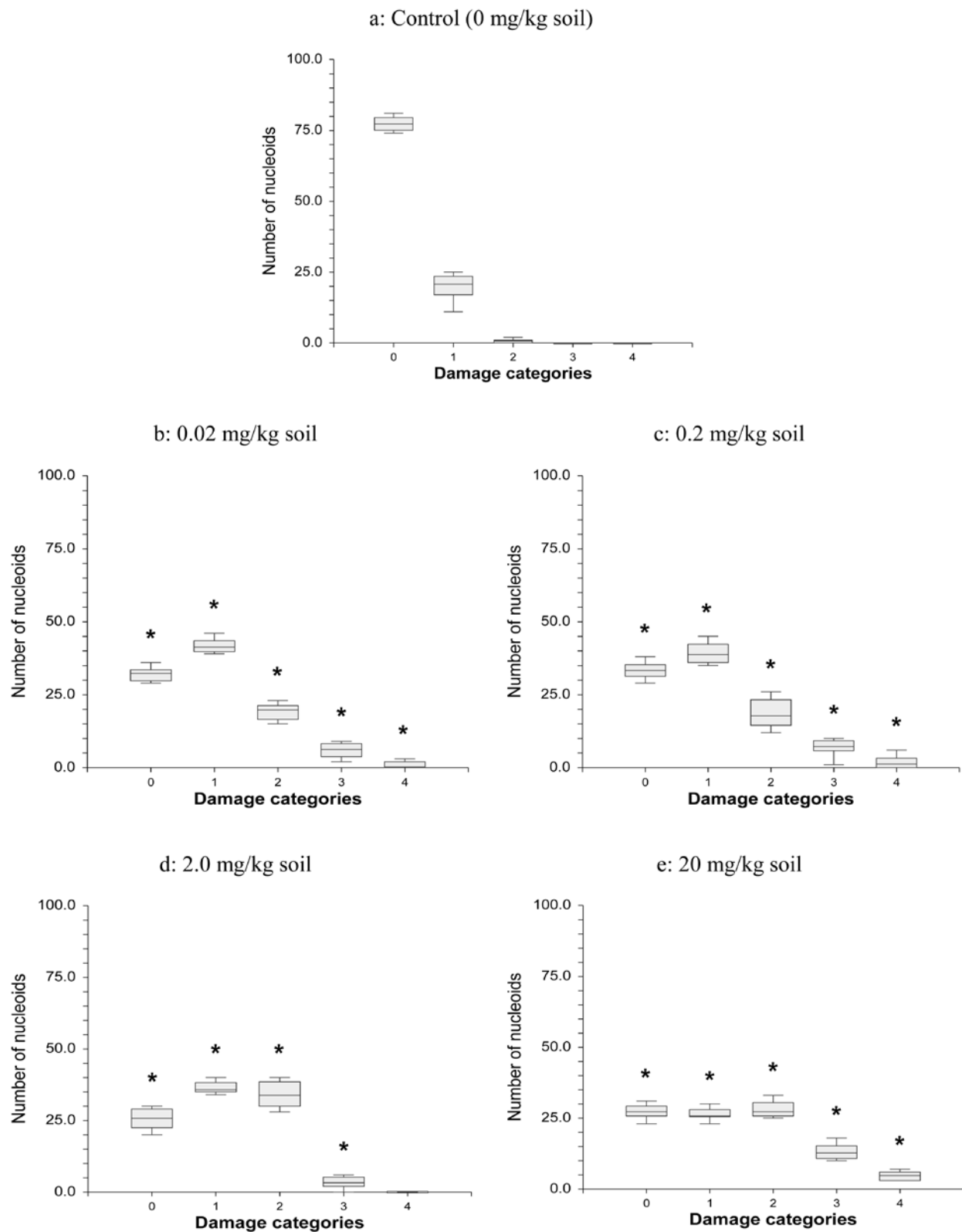


Figure 2. Distribution of undamaged and damaged nucleoids in control and acid dye treated groups.

a: Control (0 mg/kg soil). **b:** 0.02 mg/kg soil. **c:** 0.2 mg/kg soil. **d:** 2.0 mg/kg soil. **e:** 20 mg/kg soil.

The box represents 50% values i.e., the inter-quartile range of the data. The bottom and top of the box represent the 25th percentile and 75th percentile values of the data. The bars extending from the box represent the whiskers.

* represents statistically significant values at $p < 0.05$ (Kruskal Wallis multiple comparison test) against the respective damage category distribution in the control group.

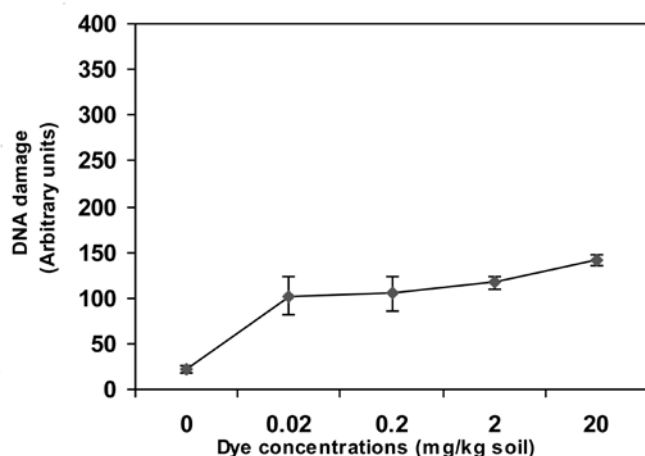


Figure 3. Dose-effect relationship between concentration of Acid Red dye and damage to earthworm coelomocytes.

The scale on the Y axis represents arbitrary units ranging from 0 - 400. The points represent mean arbitrary units and the error bars represent the standard deviations of values from 10 earthworms. All values were statistically different from control (refer Table 1).

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