Applied Biological Research 26(3): 359-369; (2024)
DOI: 10.48165/abr.2024.26.01.41

PHYSIOLOGICAL AND GENOTOXIC RESPONSES OF EARTHWORM *Eudrilus eugeniae* (Kingberg) EXPOSED TO SUBLETHAL LEVELS OF INDUSTRIAL EFFLUENT FROM ERODE DISTRICT, TAMIL NADU (INDIA)

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(Received 23 May, 2024; accepted 26 August, 2024)

ABSTRACT

The ecological risk is mostly assessed by evaluating the standard toxicological endpoints in earthworm toxicity assays, such as mortality and reproductive effects. The processes of toxic pollutants cannot comprehensively be understood by solely considering their endpoints. In this study, the usual testing by OECD, analysis of the composition of coelomic fluid, evaluation of micro- and binucleated cells, and histological studies were employed to assess the effects of industrial effluent on earthworm tissues. An additional endpoint in earthworm (*Eudrilus eugeniae*) was used to quantify the impact of industrial effluent on stress levels. The Comet assay was performed to evaluate the genotoxicity of industrial effluent. The effluent-treated earthworms exhibited a concentration-dependent increase in the number of micro- and binucleated cells, as compared to the control. It postulates that industrial pollution has an effect on cellular metabolism. Upon comparing the earthworms exposed to industrial effluent with those unexposed ones, a notable rise in DNA damage was observed. It confirmed the effectiveness of existing methods in monitoring the industrial pollution through biological means. It showed that evaluating DNA damage and levels of micro- and binucleated cells is a reliable approach. Histological studies revealed that longitudinal muscles, peritoneal epithelium, epidermis, and chloragogen cells of earthworm experienced substantial damage. The study concluded that industrial effluents pose a serious threat to earthworms, and more studies are needed to assess the acute and long-term toxicity of industrial effluent in contaminated areas and its actual influence on the environment.

Keywords: Coelomocytes; DNA damage; Comet assay; histopathology, industrial effluent

INTRODUCTION

Earthworms are well-represented in soil ecosystem in terms of density and they react to a range of ecological and environmental factors, including the changes in soil chemistry and agricultural practices. Earthworms are regarded as promising soil health indicator as well as are used in biomonitoring the soil ecotoxicological risk assessments (Xiao *et al*., 2022). Certain categories of industrial wastes, like sugar mill waste, paper and pulp waste, tannery sludge, textile sludge, and food processing refuge, have effectively been stabilized using term technology (Bhat *et al*., 2018; Karmegam *et al.*, 2021). Earthworms are able to survive and reproduce effectively even in soils with heavy metal concentrations that exceed their critical thresholds (Stürzenbaum *et al.*, 2004). Nevertheless, Donner *et al*. (2010) in epigenetic studies asserted significant genetic and physiological harm to earthworms exposed to pollutants, which is fully understood from growth and reproduction

data. Consequently, a high survival and reproduction rate does not necessarily indicate that the species has successfully adapted and established itself in polluted substrates (Kille *et al.,* 2013). Principal reactions in genotoxicity studies are DNA methylation (DNA methyl transferase activity) and DNA damage (single cell gel electrophoresis or Comet assay) [Reinecke and Reinecke, 2004]. However, there is lack of documentation regarding the process of DNA methylation and DNA damage kinetics in earthworms during vermicomposting of industrial wastes. Furthermore, the studies on metal-induced oxidative stress, specifically the production of reactive oxygen species (ROS) and lipid peroxidation, and its control by cellular homeostasis in epigeic earthworms (*Eisenia fetida* and *Eudrilus eugeniae*) during vermicomposting has not sufficiently been performed so far. The functional roles of catalase, reduced glutathione, and superoxide dismutase are crucial in regulating the redox balance in cells by the removal of ROS (Ighodaro and Akinloye, 2018). Hence, the manipulation of these crucial components and the rate at which ROS are produced in epigeic earthworms residing in vermibeds derived from industrial waste should serve as a viable approach to target the antioxidant defence system of organism. Micronuclei (MN) frequency assays and DNA damage measurement have significantly increased the possibility of researching vulnerable populations. MN assay is a quick and sensitive test that locate genomic damage caused by the changes in mitotic spindle as well as chromosome-damaging effects known as clastogenicity (Gudi *et al*., 1992). The circulating leukocytes in coelomic cavity of earthworm's coelomocytes are crucial to immuno-logical protection. They are frequently employed to assess the impacts of genotoxic metals present in household industrial waste (Wang *et al*., 2009). The hemocytes in land snail *Eobania vermiculata* activate stress markers like DNA damage, ROS generation detection, and lipid peroxidation in response to the experimental and environmental challenges (Itziou and Dimitriadis, 2011).

Similar to surface water pollution, anthropogenic activities have led to increase in long term land contamination through faulty agricultural practices, garbage disposal, industrial operations, etc. It is impossible to infer toxicity toward terrestrial animals from aquatic ones. Therefore, it is necessary to use some specific methods and models for evaluating the soil contaminants that affect terrestrial biota. The earthworms have drawn greater attention due to their ecological significance as they make up 60-80% of total soil biomass (Ridaand Lawrence, 1994). Earthworms are essential, if not dominant, component of soil biomass and function as soil engineers, controlling the crucial soil processes including fertilization. Due to their robust connection and direct contact with soil, they are appropriate biomonitoring organisms. The earthworms are widely distributed, sensitive, and manageable in a variety of soil types, so are useful in monitoring the effluent interaction with living things as well as for determining the safety level in environment. Earthworms are excellent bioindicators for tracking metal-pollution in soil (Spurgeon and Hopkin, 1996). Not much is known about the toxicity and reproduction of soil contaminants, hence concerns regarding the possible impact of effluent use on reproductive health have grown during last ten years. The primary aim of this work was to get a deeper insight into the processes behind the impacts of industrial effluent on *Eudrilus eugeniae* and to supplement the existing knowledge on the toxicological effects of effluent in soil.

MATERIALS AND METHODS

Experimental animal and sample collection

Eudrilus eugeniae (Oligochaeta, Lumbricidae), used in present study, were procured from the vermi-composting unit at Tamil Nadu Agricultural University, Tamil Nadu (India). The earthworms were maintained in controlled environment using suitable soil and food to ensure their quick acclimatization and sound health. The effluent samples were collected within Erode district, Tamil Nadu (India) in clean sterile containers. The container was immediately labelled with essential information including location, date, and time. A detailed log was maintained, documenting conditions at the sampling site. The sample was carefully transported in an insulated container to maintain its temperature stability,

and preservation methods, as deemed necessary, were employed. The samples were immediately analysed as per the established protoc (Kannadasan *et al.,* 2021) to ensure the validity of research.

Preparation of test solutions and earthworm bioassay

The effluent was first dissolved in deionized water in 1:10 ratio to prepare a stock solution, and used to prepare test solutions of 5, 10 and 25 mL kg^{-1} concentrations. On the basis of sub-lethal dose, the test concentrations were selected as 5, 10, and 25 mL kg^{-1}.

Gut cells and coelomocytes collection

The gut sections of two randomly selected live treated and untreated earthworms were extracted using sharp scissors, and examined under a dissecting microscope (Leica EZ4 W). The finely chopped guts were put in a flask containing 10 mL dissociating solution (3:1; ethanol: acetic acid). After gentle stirring for 1 h the liquid was filtered through nylon filters having 250 - 60 mm diameter. The earthworms were exposed to 4.5 V electric current for 30 sec so as to discharge coelomic fluid along with coelomocytes through dorsal pores as per the protocol of Roch (1979). Each earthworm was weighed, cleaned, and dry blotted individually and placed in a Petri-dish with 1-4 mL extrusion fluid (forcing of a liquid through a die) depending on the weight of earthworm. To stop cell aggregation, phosphate buffer saline (PBS) mixed with 2.5 g L^{-1} EDTA was added to this fluid (Kurek and Plytycz, 2003). The gut cells and coelomocytes were counted using Neubauer hemocytometers, while being observed under a light microscope (BX53, Olympus, Japan). Cell concentrations were brought down to 10^5 cells mm^{-1}.

Nuclear abnormalities assay

The earthworm's coelomocyte and hemocyte nuclear abnormalities were assessed by using micronucleus test (Hooftman and De Raats,1982; Carrasco *et al*.,1990). For this, hemolymph was applied on spotless glass slides right away after sampling, allowed to dry for a night, fixed for 10 min with methanol, and then dyed with 5% Gimesa acetocarmine. Each slide had a total of 3000 hemocytes that were analyzed using an optical microscope (Leica A60 F, Germany) under10x magnification. Each experimental group mean frequencies of micronuclei (MN) and binucleated cells (BN) were computed and expressed per 100 cells.

Fluorescent analysis of cell death

A dye mixture comprising of 1 µL acridine orange (AO) in PBS was prepared in 1:1 v/v ratio. The solution was then applied to clean microscopic cover slips together with 900 µL cell suspension containing 10^3 cells mL^{-1}. The cells were collected and washed using phosphate-buffered saline (PBS). The procedure (Kavithaa *et al.,* 2016) involved the application of 10 µL AO and propidium iodide stain to the cells for 2 min, and then subsequently rinsed twice with phosphate-buffered saline (PBS). The fixed cells were subsequently examined using a fluorescence microscope (Lynx, LM-3501, Japan) at 20x magnification.

Assay for micronuclei

The aliquots of 20 µL coelomic and gut cell suspensions were applied to sterile slides, one slide for each replicate (three slides total per concentration). Following air-drying of slides, the cells underwent a 15-min fixation in 100% ethanol, 8-min staining with 5% Giemsa solution, carbol fuchsin and a tap-water washing. A binuclear microscope was used to observe the mitotic metaphase stage. The frequencies of micro- and binucleated cells were calculated as the number of anomalies 1000 $cell^{-1}$ as per Barsiene *et al*. (2010).

Histological analysis

Gut-cleaned worms were fixed in 4% paraformaldehyde solution for 24 h after being rinsed with distilled water. The tissue blocks were paraffin-embedded and sliced into 5-µm slices with a microtome after being dehydrated in a 30% sucrose solution for 3 days. All sections were stained with hematoxylin and eosin and examined under a light microscope.

Statistical analyses

The results were expressed as mean ± SD. The p-values < 0.05 were considered statistically significant. Significant differences of a treatment on nuclear abnormalities between treatments and controls were tested using a one-way analysis of variance. All statistics were performed using the SPSS-21 software.

RESULTS AND DISCUSSION

In present study, earthworms were exposed to sublethal concentrations of 5, 10, and 20 mL kg^{-1}, and morphological changes in coelomocytes, DNA damage, micronucleus damage and gut histological changes were observed after 28 days.

Damage and changes in morphology of coelomocytes

Damage and changes to the morphology of coelomocytes (a type of immune cell found in the coelomic cavity of earthworms) can be indicative of various environmental stressors or exposures to toxic substances. These changes, often studied in ecotoxicology, can provide valuable clue about the health of earthworms and the quality of their environment. In comparison to the corresponding control cells, coelomocytes of treated earthworms appear to have higher levels of all forms of cellular abnormalities, including membrane damage, vacuolation, membrane blebbing, binucleation, and micronucleation. The coelomocytes of earthworms treated with sub-lethal dose of textile effluent had highest proportion of coelomocyte membrane damage (27.6%) [Fig. 1b-d]. Highest binucleation frequency was observed in 20 mL kg^{-1} treated earthworms. The coelomocytes of treated earthworms showed changes in cellular and nuclear diameter as well as the nucleus-cytoplasmic ratio. Fig. 1 shows the hypertrophy of tiny agranulocytes, blast-like cells, plasmatocyte-like cells, and chloragocytes. The treatment exerted a subordinate influence on the morphology of coelomocytes. The rise in effluent levels has resulted in increase in toxicity. Moreover, this phenomenon resulted in cellular shrinkage, alterations in coelomocytes, and a decrease in the overall number of viable cells. The presented data illustrates the effluent induced apoptosis in cellular populations (Fig. 1b-d). In contrast, the coelomocytes in untreated control did not exhibit any significant impact (Fig. 1a).

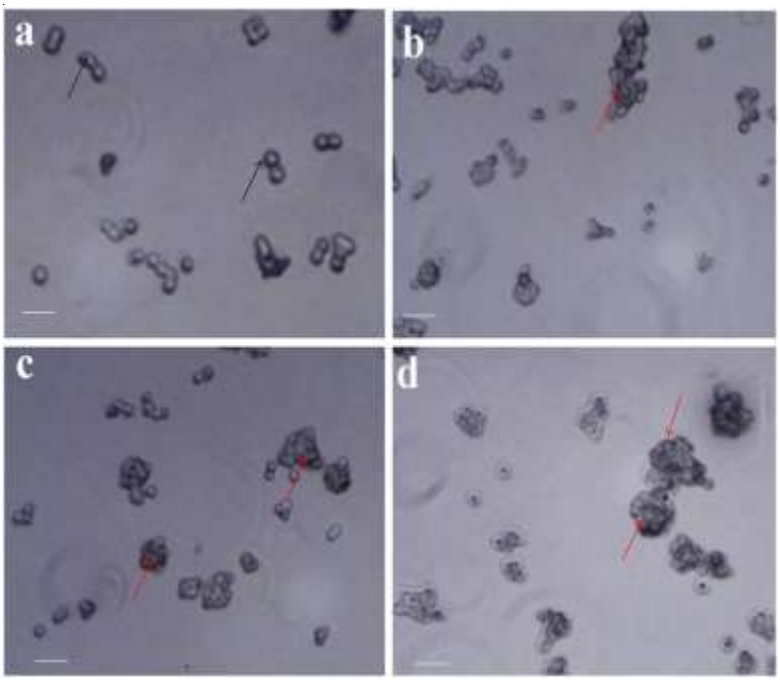

Fig. 1: Assessment of morphological changes in earthworm coelomic fluid cells (coelomocytes) treated with different concentrations of effluent. The cellular abnormalities in *E. eugeniae* after exposure to effluent for 28 days were recorded and representative micrographs were presented. a) Untreated; b) 5 mL kg^{-1} treated; c) 10 mL kg^{-1} treated; d) 20 mL kg^{-1} treated group; Black arrows indicate normal cellular morphology; Red arrows indicate altered cellular morphology indicating that due to the toxicity of effluent. The scale bar measure about 50 µm

Earlier works suggest that earthworms, as significant soil organisms, may be considered in the evaluations of soil health. Earthworm's cellular and molecular reactions have been proposed as potential indicators of toxicity of certain contaminants. Recent research has identified wastewater as emerging pollutants in soil (Simonin, 2016). The cellular and biochemical characteristics (coelomocytes) have not been given much attention as toxicity indicators, though traditional parameters such as survival, growth, and reproduction of earthworms are primarily considered (Wang *et al.*, 2022). Earthworm coelomocytes serve as their immune system and play a crucial role in maintaining physiological balance. Even extremely low levels of pollution have been demonstrated to have an impact on those species (Calisi *et al.*, 2016). Metaphireposthuma experienced a reduction in the overall number of coelomocytes when it was subjected to experimental dosages of a metal-contaminated fluid (Gautam *et al*., in 2018). Following a 14-day exposure to effluent, the coelomocyte density of *L. mauritii* decreased, suggesting a compromised immune system and corroborating the findings of earlier studies. Another crucial immunological response is phagocytosis, which involves the ingestion of foreign microbes and subsequent degradation. Earthworms exposed to pollutants can have an effect on phagocytosis (Singaram *et al.*, 2013). Bigorgne *et al.* (2011) have revealed that soils contaminated with chemicals from industries affected *Eisenia fetida* by inhibiting their phagocytic response of coelomocytes. In present study, it was observed that effluent decreased the phagocytic response of *L. mauritii* coelomocytes, indicating a state of poor health.

Micronuclei analysis

Fig. 2 and 3 show Giemsa and carbol fuchsin stained cells, displaying the quantities of micronuclei and binucleated cells observed in coelomocytes that were subjected to textile dye effluent treatment @ 5, 10, and 20 mL kg^{-1}. The results demonstrated that live coelomocytes exhibited an orange fluorescence, but deceased cells exhibited fluorescence with acridine orange, particularly There. was a notable rise in the quantity of binucleated cells observed in 5, 10, and 20 mL kg^{-1} treated earthworms. In contrast, the untreated control cells did not show multinucleation or binucleation. The impact of apoptogenic activity of effluent on coelomocytes was investigated by the utilization of fluorescence

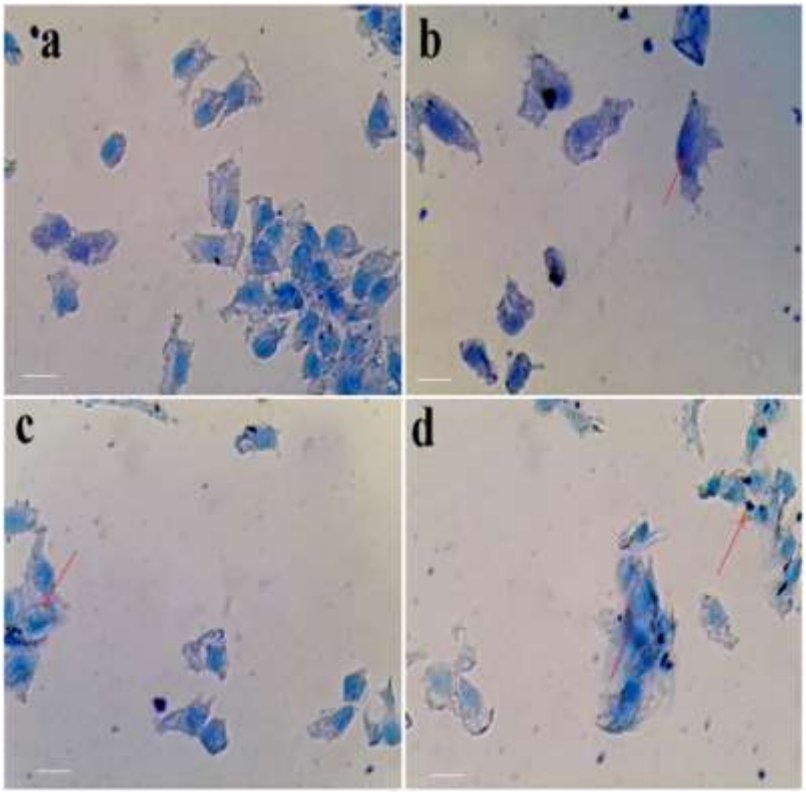

Fig. 2: Micronucleated cells of coelomocytes in *E. eugeniae* stained with 10% Giemsa stain after *in vivo* exposure to effluent for 28 days. Giemsa staining of a) healthy nuclei isolated from effluent treated earthworm cells. For the isolation of nuclei 20 volumes of fixative {methanol: glacial acid, 3:1} was slowly added. Nuclei were spread and fixed on a microscopic slide, immersed in freshly prepared 5% Giemsa stain for 20 min, flushed with tap water, left to dry and examined micro-scopically; b) 5 mL kg^{-1} treated; c) 10 mL kg^{-1} treated; d) 20 mL kg^{-1} treated group. Red arrows indicate disintegrated forms of nucleus and cytoplasm due to effluent toxicity. The scale bar measure 50 µm.

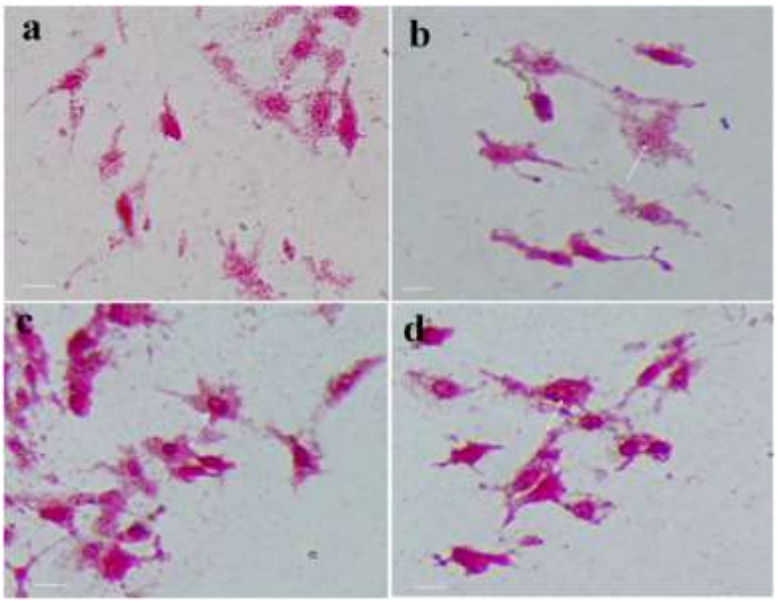

Fig. 3: Micronucleated cells of coelomocytes in *E. eugeniae* stained with Carbol fuchsin after *in vivo* exposure to effluent for 28 days. Carbol fuchsin staining of (a) cells of healthy earth worm isolated from effluent treated show normal healthy morphology. Apoptotic detection cells fixed on a microscope slide, immersed in freshly prepared 5% Carbol fuchsin stain for 20 min, flushed with tap water, left to dry and subjected to microscopy. b) 5 mL kg^{-1} treated; c) 10 mL kg^{-1} treated; d) 20 mL kg^{-1} treated group. (White arrows indicate disintegrated morphology of nucleus and cytoplasm due to the toxicity of effluent; whereas untreated cells seemed healthy and integrated). Scale bar used 50 µm

microscopy. No agglomeration of effluent was seen during study due to the dispersion of the majority of coelomocytes at all tested low concentrations. The assessment of apoptosis induction in coelomocytes was conducted by employing fluorescence microscopy and staining the cells with acridine orange/ethidium bromide (AO) The coelomocytes in untreated control exhibited a substantial quantity of viable cells (Fig. 4 and 5). In contrast, the observed specimens displayed a greater degree of cellular injury, characterized by the presence of nuclear shrinkage, membrane blabbing, and nucleus disintegration.

There are limited studies on genotoxicity of industrial effluent, especially with respect to their *in vivo* effects. Industrial effluents contain harmful substances that are

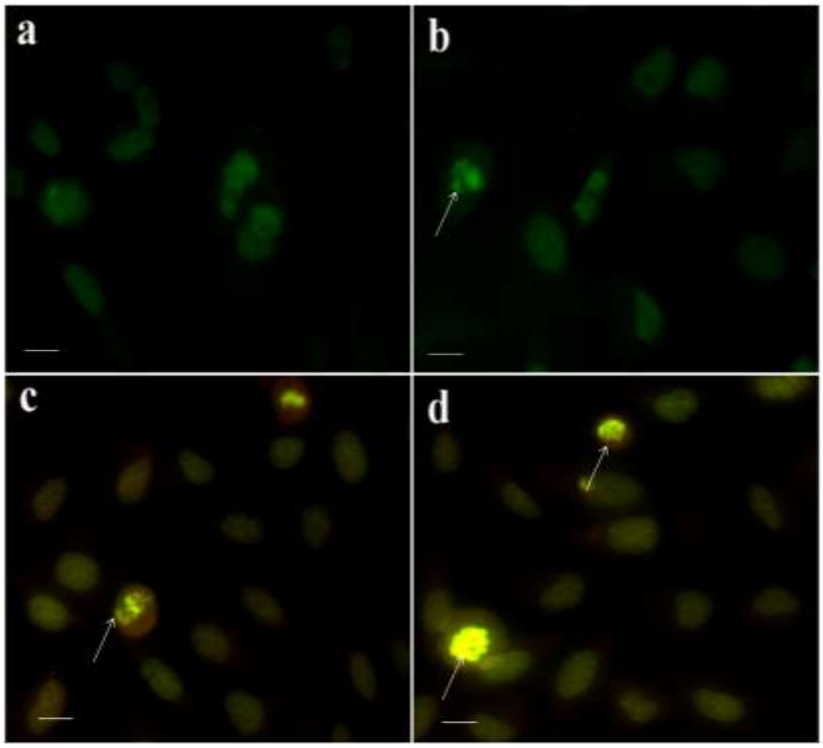

Fig. 4: Fluorescent microscopic AO images of multinucleated cells in *E. eugeniae* after *in vivo* exposure to effluent. *E. eugeniae* stained with acridine orange (AO) dye after *in vivo* exposure to effluent for 28 days. AO staining of healthy a) isolated from effluent treated earthworm cells exhibit normal healthy morphology. b) 5 mL kg^{-1} treated; c) 10 mL kg^{-1} treated; d) 20 mL kg^{-1} treated group; White arrows show disintegrated morphology of nucleus due to the toxicity of effluent; whereas the untreated cells seem healthy and integrated. Scale bar used 50 µm

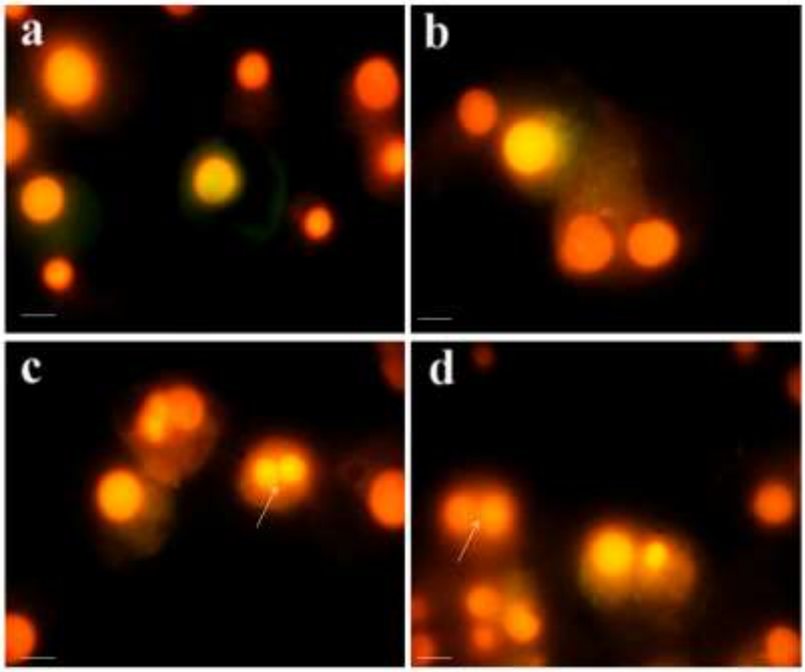

Fig. 5: Fluorescent microscopic propidium iodide (PI) images of multinucleated cells in *E. eugeniae* after *in vivo* exposure to the effluent. *E. eugeniae* stained with PI dye after *in vivo* exposure to effluent for 28 days. PI staining of healthy a) isolated from effluent treated earthworm cells exhibit normal healthy morphology; b) 5 mL kg^{-1} treated; c) 10 mL kg^{-1} treated; d) 20 mL kg^{-1} treated group. White arrows indicate binucleated morphology of nucleus due to the toxic effect of effluent; whereas the untreated cells display to be healthy and integrated. The scale bar measures 50 µm

smaller than 100 nm. These substances have the ability to enter cells and interact with large molecules like proteins and DNA (Asha Rani *et al.*, 2009). The nuclear abnormalities test revealed a significant increase in the presence of micronuclei (MN) and binuclei (BN) in coelomocytes of treated earthworms when they were subjected to various concentrations. The study showed that the coelomocytes of *E. euginea* were more sensitive and appropriate as a biomarker for evaluating the genotoxicity of industrial effluent in comparison to gut cells. The current study suggests that the industrial effluent stimulates the development of reactive oxygen species (ROS) in the coelomocytes of *E. euginea*, indicating oxidative damage. The Comet assay is a thoroughly studied method for evaluating genotoxicity, which has been utilized to assess the toxicities of environmental contaminants (Lourenço *et al.*, 2012). Wang *et al.* (2021) reported that an increase in cellular reactive oxygen species (ROS) leads to changes in nucleotides and breaks in DNA strands, ultimately causing chromosomal abnormalities. *Eisenia hortensis* coelomocytes exhibited elevated DNA damage when exposed to metals (Ciğerci *et al*., 2018).

DNA damage

After 28 days, the coelomocytes of earthworms were collected, and the amount of DNA damage measured (Fig. 6). As compared to the control, the treated earthworms showed maximum DNA damage during the experiment. After 28 day's exposure, the 20 mL kg^{-1} effluent showed a substantial increase in DNA damage. Fluorescent microscopic images revealed a comet tail in DNA samples, while control DNA displayed a thick, spherical comet head. The exposed specimens had fragmented DNA that travelled within a nucleus, exhibiting a more prominent comet tail. The genotoxicity in present experiment was revealed by the proportion of tDNA and OTM values obtained. Over time, tanneries produce toxic metals that gradually build up in soil and local populations of earthworms. In this scenario, it is possible that multiple species of earthworms may encounter a decrease in population and continue to face chemical stress. Accumulation of metals and the process of trophic magnification has the capacity to contaminate the food chain and give rise to the health issues in people. In addition, the process of mapping the soil quality in India's agricultural regions is experiencing delays, which sets it apart from many other advanced countries. Hence, the present study holds great significance in terms of earthworm preservation, assessment of metal toxicity and soil health.

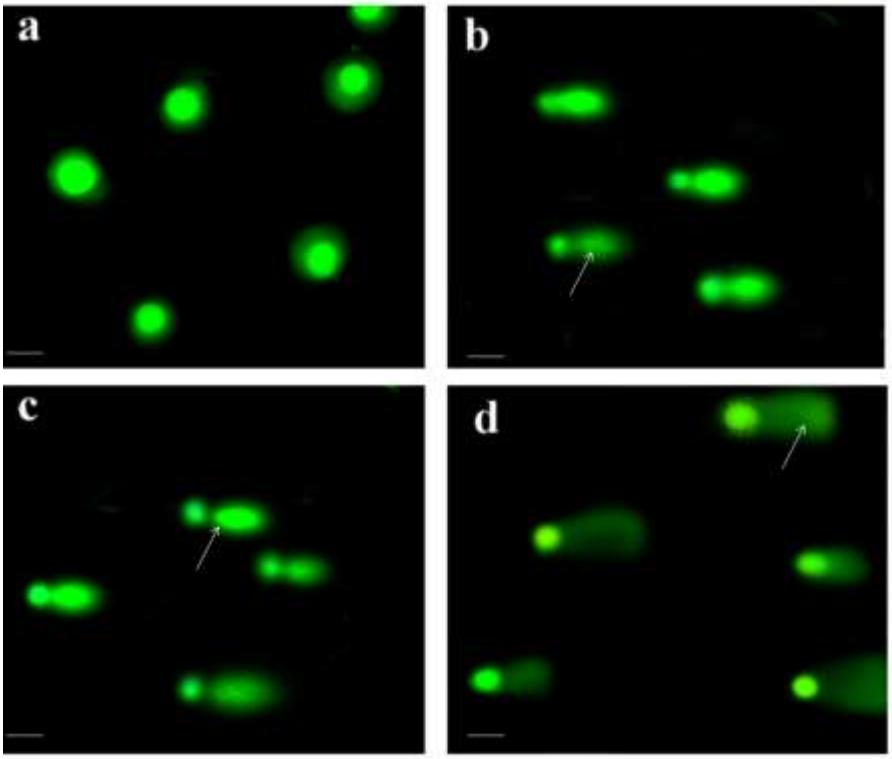

Fig. 6: DNA fragmentation analysis through Comet assay: The Comet assay in coelomocytes at various concentrations of effluents. The results are compared with untreated ones (control). The arrows indicate tail DNA length. When concentration was increased the tail DNA length also increased. The scale bar measure 50 µm.

Histological observation

After 28 days, the gut histology of the earthworms exposed to different concentrations of textile dye effluent (5, 10, and 20 mL kg^{-1}) as well as control earthworms were analysed (Fig. 6). The gut of textile dye effluent-treated earthworms showed complete erosion of epithelium in several locations, and in specific regions a noticeable diminishing of circular musculature was observed. Nevertheless, specific regions of body wall exhibited significant deterioration and cracks that affected the longitudinal muscles. The damage occurred to the peritoneal epithelium, which serves as the lining of colon. Hence, the present study holds significance in terms of earthworm preservation, assessment of metal toxicity, and monitoring soil health. Both ventral and dorsal blood arteries were observed. The ventral nerve cord suffered severe damage to the extent that its recognition was no longer possible. The detachment of Chloragogen cells from the peritoneal epithelium was observed in some regions.

The histological composition of *E. eugeniae* earthworm was remarkably influenced by the higher concentration of effluent in a short time. After comparing the control group with the experimental group, it was clear that the size of coelom cavity had decreased in the pre-typhlosolar region, while it expanded in post-typhlosolar zone. The muscle fibres connecting the longitudinal muscle of pre-typhlosolar area to the visceral peritoneum had undergone substantial degeneration and were undetectable. The surgical cut led to the augmentation in the length of longitudinal muscles. Fig. 7 illustrates that the cut made on the outermost layer of skin, which resulted in complete destruction of tissue that covered the colon. The earthworms were subjected to an elevated dosage for four hours, the minor fraction of total trial period. This was done to improve the detectability of tissue injury during observation. The purpose of minimizing the specimen preparation process was to optimize the accuracy of tissue observation. Further, the tissues sustained significant harm and became inappropriate for processing as a specimen after death, following the whole period of exposure. With an anticipated average duration of mortality of around 20 h, the material was exposed for 4 h. The heightened level of concentration led to notable detrimental outcomes.

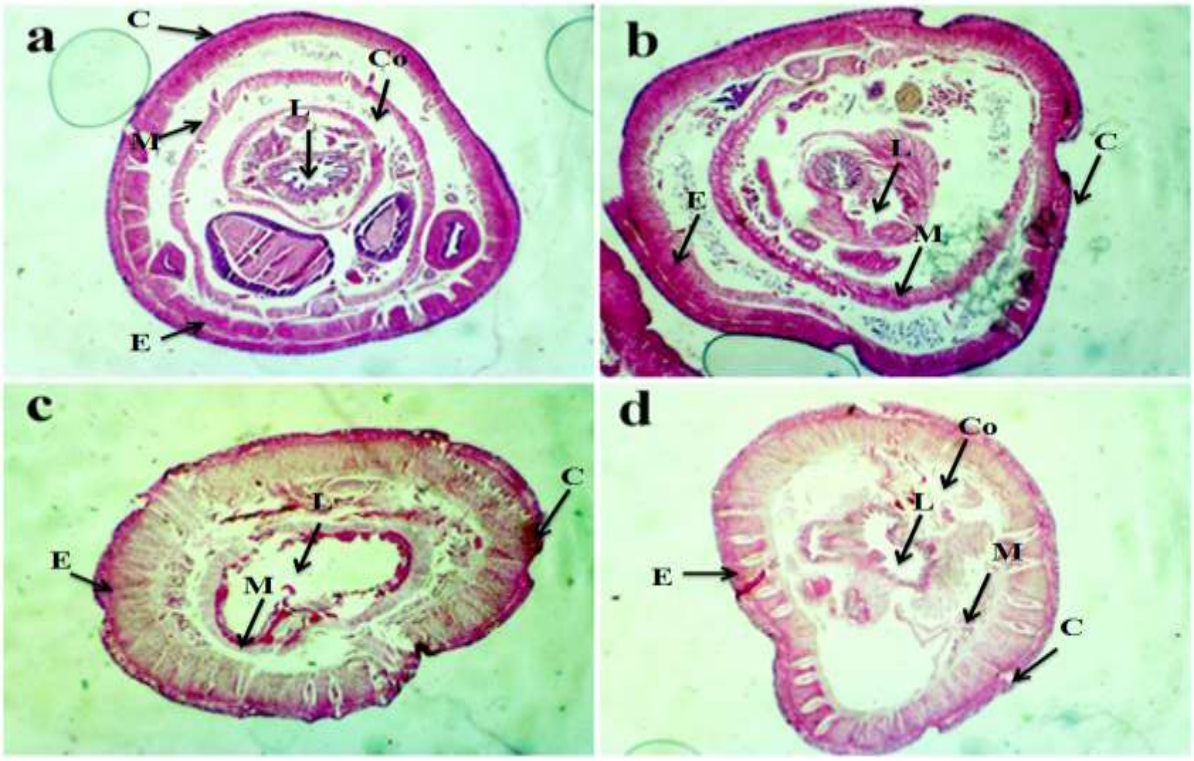

Fig. 7: Histology of Pre- and Post-typhlosolar regions of *Eudrilus eugeniae* exposed to effluent; a) Untreated; b) 5 mL kg treated; c) 10 mL kg treated; d) 20 mL kg treated group. L - Lumen, Co – Coelom, M - Muscles, E - Epithelium with secreting cells, C - Cuticle. The cuticle and epidermis of Pre-typhlosolar region are seen distinct and intact in the tissues of untreated control earthworm. A prominent layer of circular and longitudinal muscles can be seen with muscle strands. However, effluent treated earthworm longitudinal muscles were heavily damaged with fissures at some places in body wall. The peritoneal epithelium lining the intestine is damaged in several places. Greater degree of injury to the dorsal and ventral blood vessel is observed. Magnification at 10 X

Conclusion: To identify the presence of several metal toxins in soil, a range of specific biomarker indicators are used. The activities of coelomocytes were assessed by examining morphometry, cellular damages, and nuclear aberrations. New methods for evaluating toxicity included analysing the changes in density, studying the morphological characteristics, assessing the cell damage, and measuring the abnormal nuclear activity in coelomocytes. The probable soil and food chain contamination can have adverse effects on the well-being of consumers, including both human health and local biodiversity. The study suggests the use of a multi-parametric index-based risk method to regularly evaluate the soil quality in industrial effluent affected areas.

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