

DNA Damage and Effects of Antioxidant Enzymes in Earthworm (*Eudrilus Eugeniae*) Exposed to Contaminated Soil with Organic Environmental Pollutant: Triclosan



V. Swabna and M. Vasanthy

Abstract Triclosan is a nonionic, phenolic, antimicrobial compound that is used all over the world, as a key ingredient in pharmaceutical and personal care products (PPCPs) such as disinfectants, soap, detergent, toothpaste, mouthwash, fabric, deodorant, shampoo, and plastic additives. It causes a range of adverse effects in animals and has a great impact on environment also. This chapter investigates the effect of triclosan in varying concentrations and triclosan-contaminated soil by using earthworms as bioindicators. The effects were calculated by analyzing their antioxidative enzymes and DNA damages. The antioxidant enzymes were estimated by analyzing the antioxidant properties of earthworm which include glutathione peroxidase and catalase. Comet assay was done to monitor the DNA damage of the earthworms with regard to triclosan toxicity. The investigation proves that when the concentration of triclosan increases, the effects in the earthworms were also radical. This study thus denotes that triclosan induces adverse consequences on earthworms which sever the antioxidant enzyme mechanisms and DNA damage.

Keywords Triclosan · Earthworm · Antioxidant enzymes · DNA damage · PPCPs

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1 Introduction

Organic contamination is briefly visualized as the exploitation of water resources by organic compounds from various sources by anthropogenic sources. Contamination occurs in varied ways that are persistent in nature which may conclude in a major concern that may lead to a great impact on the environment. Organic pollutants that may enter the fresh water resources increase day by day due to manmade activities, and these pollutants degrade naturally to less toxic or nontoxic forms (Focazio et al., 2008). However, these pollutants get accumulated or biomagnified in organisms through food chain which might alter the diversity (Thornton et al., 2001). Among the organic pollutants, the presence of pharmaceutical and personal care products (PPCPs) causes inimical effects toward the aquatic and terrestrial organisms. The major anthropogenic sources of pharmaceutical and personal care products (PPCPs) include communal waste and byproducts of fertilizers from farmlands. PPCPs such as diclofenac, iopamidol, carbamazepine, ibuprofen, clofibrac acid, triclosan, phthalates, and bisphenol A were classified as the major entrants that can cause detrimental effects (Ellis, 2008).

5-Chloro-2-(2, 4-dichlorophenoxy) phenol, commonly known as triclosan, is a synthetic antimicrobial, antifungal agent that has some antibiotic properties (Singer et al., 2002). It is an aromatic halogenated compound containing phenolic, diphenyl ether, and polychlorinated biphenyls (PCBs) as supporting structures. The chief mode of triclosan discharge into the environment is through pharmaceutical and personal care products (PPCPs) that are used by humans, such as toothpastes, shampoos, detergents, and cosmetics (McBain et al., 2002). Presently, triclosan and its by-products have settled all over the environment and even found in human breast milk. The health issues concerned with triclosan and its by-products include antibiotic resistance, dermal irritations and allergies, endocrine disruption, altered metabolism, and development of tumors (Latch et al., 2003).

Earthworms are considered as bioindicators for testing the contaminants and to check the toxicity of the soil. The toxic tests which are used to identify the toxic contaminants are quantified by the earthworm's survival and reproductive rates, biomass index, and behavioral changes. This assessment gives us knowledge about the contaminant uptake, its metabolism, and the by-product elimination which in other words is called as toxicokinetics. Earthworms are present in various types of soils ranging from temperate to tropical regions. Hence, they are aforesought to be decisive in food chains as these earthworms are contemplated as a prime source of food for many organisms including birds and mammals which have turned out to be the dominant impetus for ecologists and ecotoxicologists to explore the whereabouts of the earthworms (Lokke & Van Gestel, 1998). This is the major reason for which earthworms are habitually used as experimental organisms to analyze the chemicals deposited in the soil and also to find out the nature and characteristics of the chemicals (Oste et al., 2001).

The effect of triclosan in varying concentrations and PPCP-contaminated soil was analyzed by using earthworms as knockoff organism. This was done by

estimating their changes in antioxidative enzymes and DNA damages. The estimation of antioxidative enzymes and protein was done to perceive the antioxidant properties of the earthworm after introducing into the triclosan-contaminated soil. The antioxidant enzymes were analyzed for estimating the antioxidant properties of earthworm which include glutathione peroxidase and catalase. Comet assay was also done to check the DNA damage of the earthworms with respect to triclosan toxicity.

2 Materials and Methods

2.1 Earthworm Exposures

Earthworms used in this assay (*Eudrilus eugeniae*) were purchased from an earthworm culturing farm at ICAR-Krishi Vigyan Kendra (KVK) in Karur District, which were introduced into the artificial soil, which is composed of 10% kaolin clay, 20% of coir pith, and 70% of industrial soil. Triclosan was added to the soil in the concentration of 0 (control), 0.25 mg, 0.50 mg, 0.75 mg, 1.00 mg, and one bowl with PCP-contaminated soil obtained from river beds of Kaveri. For each concentration bowl, 15 earthworms were inoculated.

2.2 Enzyme Extraction

Earthworms were killed by introducing into the formaldehyde solution for a minute and placed in a dissection tray. It was dissected vertically, its gut was cleared, then placed into a prechilled mortar and pestle, and crushed under ice-cold condition with 0.5 M Phosphate buffer (pH 7.0). The homogenate was centrifuged at 8000 rpm at 4 °C for 30 min (Song et al., 2009).

2.3 Protein Estimation by Bradford Assay

Measurement of protein content was necessary to calculate enzymatic activities. The protein content was determined using the Bradford method (Bradford, 1976). It is widely used and more reliable for detecting 20-400 µg. To the clean test tubes, 2.50 ml of Bradford reagent was added. To the reagent, 10-20 µl of earthworm extract is added which produces a blue color by reaction. Its absorbance is measured at 595 nm.

2.4 Enzyme Assays

Catalase

Catalase (CAT) activity was determined as described by Jingbo et al. (1997). 1.5 ml of phosphate buffer is added into the test tube. 0.5 ml of hydrogen peroxide is added to the buffer. To the mixture, 100 μ l of earthworm extract is added. At this stage to the reaction mixture, 250 μ l of potassium dichromate is added which when added arrests the reaction at different time intervals. Then the whole set is kept in boiling water bath for 10 minutes. After 10 minutes, the reaction mixture develops a green color to which the absorbance is measured at 570 nm.

Glutathione Peroxidase

Glutathione peroxidase activities were assayed according to the method of Saint Denis et al. (1998). 200 μ l of EDTA is added to the centrifuge tubes. To that 100 μ l of sodium azide is added along with that 100 μ l of hydrogen peroxide was added. To the reaction mixture, reduced glutathione was mixed. 400 μ l of phosphate buffer is added simultaneously. To that, earthworm extract is added followed by an incubation of 37 °C for 10 minutes. Then the reaction is arrested by adding 500 μ l of 10% TCA. Subsequently the whole mixture is centrifuged at 3000 rpm for 3 minutes. The supernatant is transferred to a test tube and 3 ml of disodium hydrogen phosphate is added. Then 1 ml of DTNB was added which develops a yellow color. Absorbance is measured for the reaction mixture at 412 nm.

2.5 Comet Assay

The comet assay was performed by the method described by Eyambe et al. (1991). The earthworm cells were collected by trypsinization from the six tissue samples. The microscopic slides were sequentially coated with 200 μ L of 0.75% normal melting agarose as the first layer and 100 μ L of 0.5% low melting agarose as the second layer. Then 20 μ L of each cell suspension was added to 60 μ L of 0.5% low melting agarose, which was distributed on the slides as a third layer. Then the slides were incubated in cell lysis buffer (2.5 M NaCl, 0.2 M NaOH, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% dimethyl sulfoxide, pH =10.0) for overnight at 4 °C. After that, the slides were immersed in double distilled water for three times followed by 20 min incubation of unwinding solution (3 M NaOH). Subsequently, the slides were placed in a horizontal gel electrophoresis tank containing electrophoresis solution (1 mM Na₂EDTA and 300 mM NaOH, pH =13). The electrophoresis was conducted at 25 V (1 V/cm, 300 mA) for 25 min. Then the slides were incubated in neutralization buffer (0.4 M Tris-HCl, pH =7.5) for 10 min

followed by immersion in ultrapure water for three times and air-dry. The cells were stained with 50 μL of ethidium bromide (5 mg/L) and observed under fluorescent microscope. All steps were carried out under dim light to minimize extra DNA damage.

3 Results and Discussion

3.1 Mortality Table

The mortality of the earthworms was observed throughout the study period (Table 1). The mortality of the earthworms significantly increased with increase in the concentration of the triclosan. This signifies that triclosan induces mortality in the earthworms.

3.2 Effect of Triclosan on Protein Content

The total protein content was subsequently increased in the second week and decreased throughout the week in the control. There was a decrease in the protein levels (0.25, 0.50, 0.75, 1.00 mg kg^{-1}) from the first week to the fourth week. Decreased levels of protein content have also been noted in the PCP-contaminated



Fig. 1 Experimental setup of earthworms with different concentrations of triclosan. A - Control, B - 0.25 mg, C - 0.50 mg, D - 0.75 mg, E - 1.00 mg, F - contaminated soil

Fig. 2 Different stages of earthworm in 4 weeks (control)



Fig. 3 Different stages of earthworm in 4 weeks (contaminated soil)

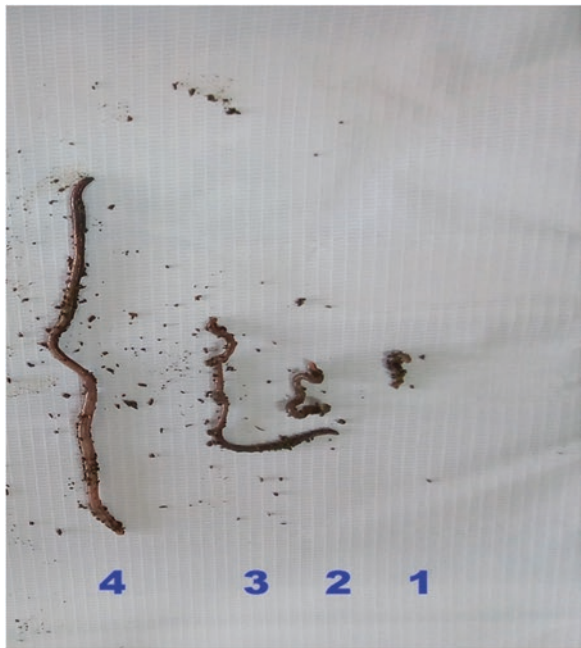
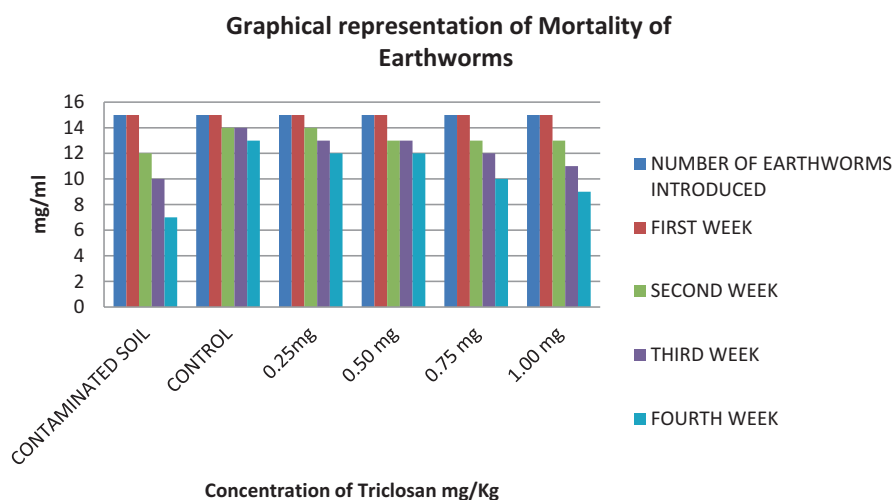


Table 1 Mortality of earthworms

Sl.. no	Concentrations	Number of earthworms introduced	First week	Second week	Third week	Fourth week
1	Contaminated soil	15	15	12	10	7
2	Control	15	15	14	14	13
3	0.25 mg	15	15	14	13	12
4	0.50 mg	15	15	13	13	12
5	0.75 mg	15	15	13	12	10
6	1.00 mg	15	15	13	11	9

**Fig. 4** Mortality of earthworms

soil. This could be due to the fact that triclosan may have induced and enhanced the gene transcription and expression of mRNA (Lescoat et al., 2000) (Table 2).

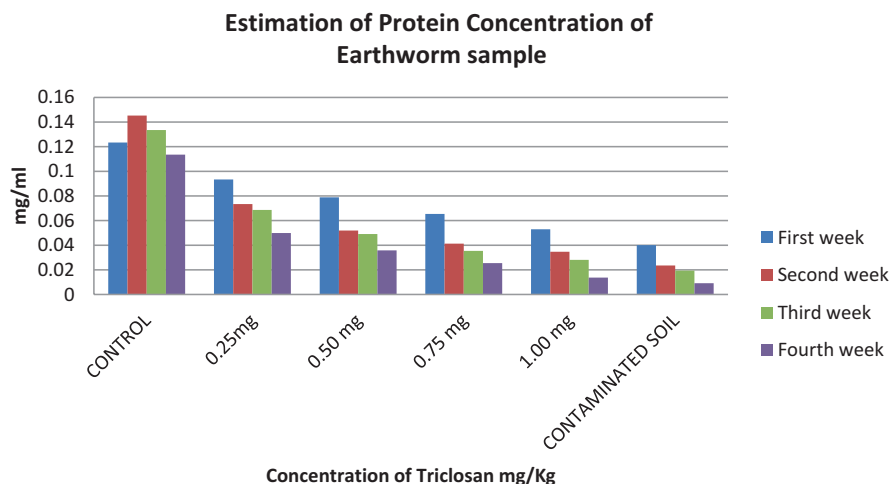
3.3 Enzyme Assays

Effect of Triclosan on Catalase Concentration

The catalase activity of the control has been increasing from the seventh day onward and increased weekly. But there is a marked increase in the other concentrations of soil with triclosan (0.25 mg, 0.50 mg, 0.75 mg, and 1 mg) concentration throughout the 4 weeks. The catalase activity tended to increase in the tissues of the earthworm at the start of the treatment and then they have been increased till the fourth week. Compared to the concentration, the catalase activity tends to be more effective in the

Table 2 Estimation of protein concentration of earthworm sample

Concentrations	First week	Second week	Third week	Fourth week
Control	0.1234	0.1453	0.1335	0.1136
0.25 mg	0.0934	0.0734	0.0686	0.0499
0.50 mg	0.0789	0.0519	0.0491	0.0358
0.75 mg	0.0654	0.0413	0.0354	0.0255
1.00 mg	0.0529	0.0347	0.0281	0.0137
Contaminated soil	0.0401	0.0235	0.0194	0.0091

**Fig. 5** Graphical representation of estimation of protein concentration of earthworm sample

contaminated soil. The highest catalase activity was observed in the triclosan-contaminated soil in the fourth week which is denoted with 1.996 IU/ml (Table 3 and Fig. 6).

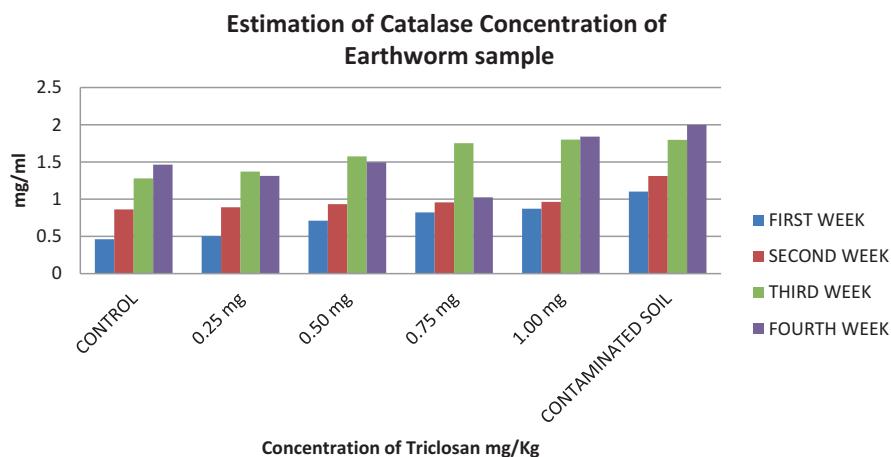
The catalase activity may be induced in the cells that were exposed to PCPs. The escalation and deterioration of catalase activity is regulated at the mRNA level of the earthworms (Mutoh & Hayashi, 1988).

Effect of Triclosan on Glutathione Peroxidase Concentration

There was significant increase in the GPx activity in all the four concentrations of triclosan (0.25, 0.50, 0.75, 1.00 $\mu\text{g kg}^{-1}$) and the contaminated soil when compared to the control (Table 4 and Fig. 7). The highest Gpx activity was signified in the earthworms present in the contaminated soil samples with the highest value of 2.108 IU/ml in the fourth week.

Table 3 Estimation of catalase concentration of earthworm sample

Concentrations	First week	Second week	Third week	Fourth week
Control	0.4618	0.8625	1.2797	1.4645
0.25 mg	0.503	0.8921	1.3705	1.3133
0.50 mg	0.7111	0.9333	1.5748	1.4954
0.75 mg	0.8218	0.9581	1.7524	1.0254
1.00 mg	0.8725	0.9639	1.8001	1.8408
Contaminated soil	1.1021	1.312	1.797	1.996

**Fig. 6** Graphical representation of estimation of catalase concentration of earthworm sample**Table 4** Estimation of glutathione peroxidase concentration of earthworm sample

Concentration	First week	Second week	Third week	Fourth week
Control	0.6646	0.6975	0.8426	0.9462
0.25 mg	0.6961	0.7234	0.9243	1.2129
0.50 mg	0.7321	0.8189	1.008	1.6498
0.75 mg	0.7871	0.9461	1.3515	1.8593
1.00 mg	0.8692	1.1234	1.6352	1.9961
Contaminated soil	0.9971	1.2254	1.7651	2.1098

The GPx activity increased from the first day and then throughout of the experiment period in all the groups prompting that antioxidant defense is overwhelmed by the reactive oxygen species. Glutathione has the ability to control the levels of peroxides in the body. Predominantly in a system, there should be a balance between the GPx production and peroxide level. In the earthworms, the system has lost its critical balance of redox states compared to control animals (Song et al., 2009).

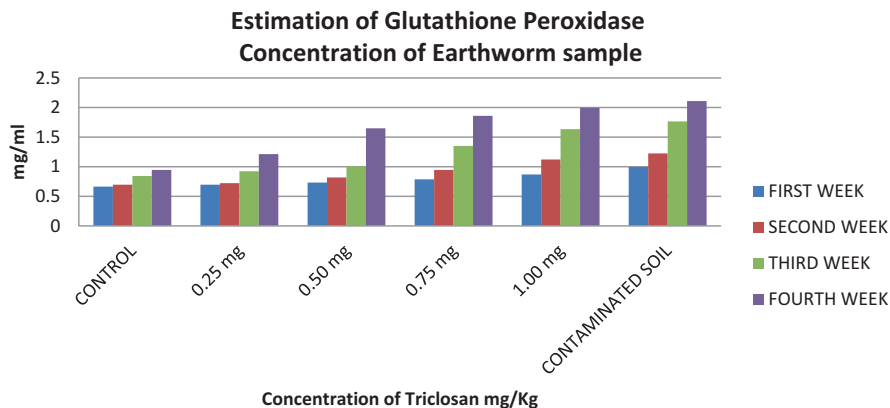


Fig. 7 Graphical representation of estimation of glutathione peroxidase concentration of earthworm sample

3.4 Comet Assay

Comet assay of the earthworm coelomocytes were tested on the seventh, 14th, 21st, and 28th day after treatment with different doses of triclosan. In the control group (Figs. 8, 9, 10, 11, 12, and 13), cells with intact DNA and few comets with very short tail length were seen. The extent of DNA damage along with number of comets was increased with increased doses of triclosan (control, contaminated soil, 0.25 mg, 0.50 mg, 0.75 mg, 1.00 mg) and triclosan-contaminated soil. These were significantly higher than those of the controls due to DNA fragmentation; therefore, DNA damage increased as triclosan concentration increased.

Oxidative stress induces many types of negative effects such as membrane peroxidation, loss of ions, protein cleavage, and DNA strand breakages, which could lead to cancer in humans or animals (Collins & Harrington, 2002). In this chapter, the induction of the DNA damage was majorly due to oxidative stress, which indicates that ROS accumulation in the earthworm tissues which may have caused subsequent DNA damage, or due to the activation of DNA repair mechanisms induced by triclosan in earthworm coelomocytes.

Numerous studies show that reactive oxygen species is the major reason for DNA damage which cause strand breaks, nucleotide removal, and modifications in the nucleotide bases. Although the cells had developed repair mechanisms to correct naturally occurring errors in DNA, enormous ROS can lead to a permanent error or damage of DNA (Mittler, 2002).

1. *Control*
2. *Contaminated soil*
3. *Earthworm (0.25 mg)*

Fig. 8 Comet assay – control

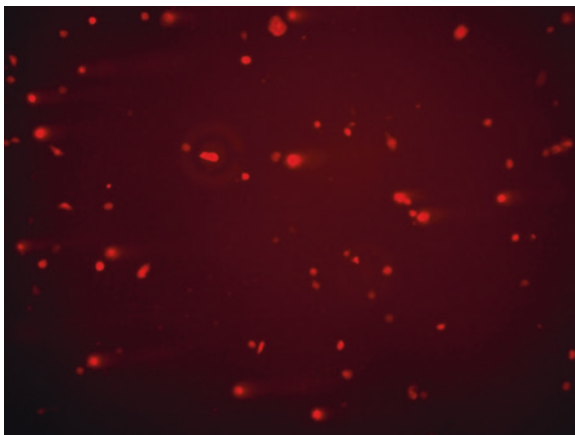


Fig. 9 Comet assay – contaminated soil

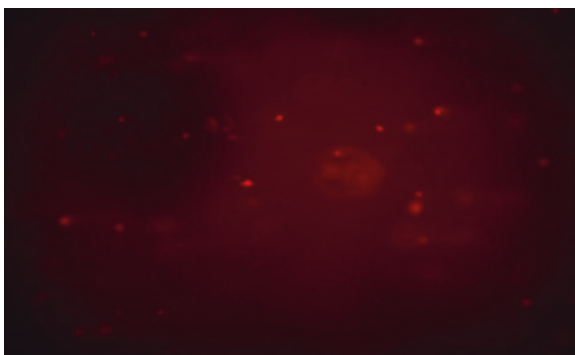


Fig. 10 Comet assay – 0.25 mg

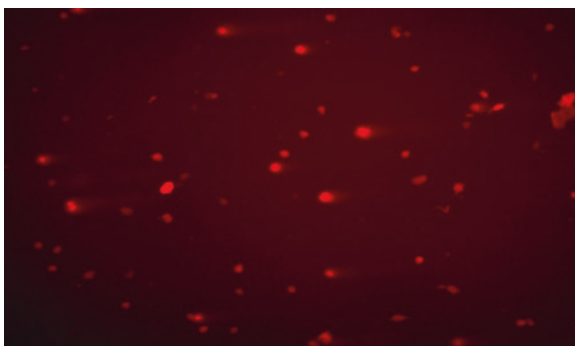


Fig. 11 Comet assay – 0.50 mg

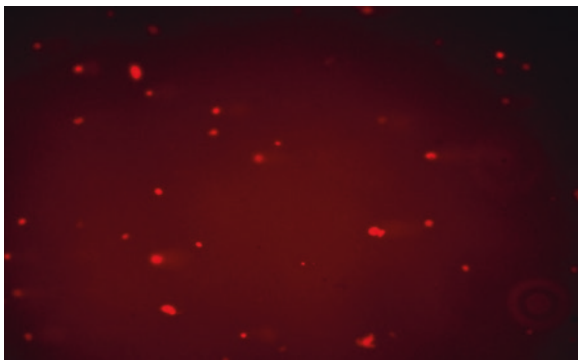


Fig. 12 Comet assay – 0.75 mg

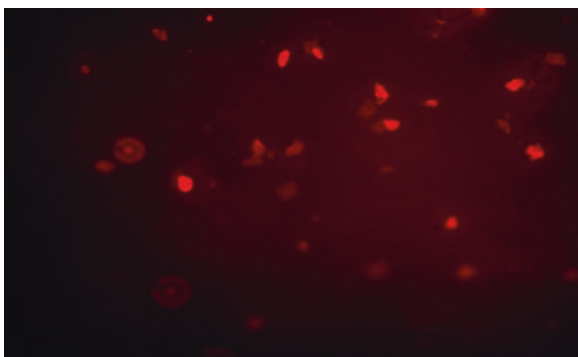


Fig. 13 Comet assay – 1.00 mg



4. *Earthworm (0.50 mg)*

5. *Earthworm (0.75 mg)*

6. *Earth worm (1.00 mg)*

4 Summary and Conclusion

Earthworms are the most suitable animal for testing the contaminants and to check the toxicity of the contaminated soil. Acute and chronic toxicity tests have been used traditionally to assess the toxicity of contaminants, with mortality and changes in biomass, reproduction rates, and behavioral responses representing endpoints. The uptake, accumulation, and elimination properties of metals by earthworm are the major part of toxicology, which is called toxicokinetics. Triclosan in the form of pharmaceutical and personal care products (PPCPs) is widely distributed in the environment, and it causes a range of adverse effects in animals. It has been shown to inhibit enzymes and alter biological membranes related to the formation of lipid peroxides.

The effect of triclosan in varying concentrations and triclosan-contaminated soil was analyzed by using earthworms as model organisms. This was done by estimating their antioxidative enzymes and DNA damages. The estimation of antioxidative enzymes and protein was done to perceive the antioxidant properties of the earthworm after introducing into the triclosan-contaminated soil. The antioxidant enzymes were analyzed for estimating the antioxidant properties of earthworm which include glutathione peroxidase and catalase. Comet assay was also done to check the DNA damage of the earthworms with regard to triclosan toxicity.

The investigation concludes that when concentration increases, the effects in the earthworms are also drastic. This shows that triclosan induces an adverse effect on earthworms which amputate the antioxidant enzyme mechanisms and DNA damage.

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