Biomarker Responses in Earthworms *Lumbricus Terrestris* L. (Annelida: Lumbricidae) to Artificial Soils Contaminated with Dimethyl Phthalate

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Abstract

In ecotoxicological studies, oxidative damages and antioxidant defense responses in earthworms have been used as hallmarks for assessing the environmental pollution. Phthalates, which are esters of phthalic acid (PAEs) are primarily used to enhance plasticity of industrial polymers, and are an unavoidable part of modern life. In the present study, ecotoxicological effects of dimethyl phthalate (DMP) were investigated in the earthworms *Lumbricus terrestris* L., at different concentrations (0, 0.1, 1, 10 and 50 mg kg^{-1}) on the 7^{th} and 14^{th} days of exposure in artificial soils. We evaluated the effects of DMP on oxidative stress (OS) biomarkers including malondialdehyde (MDA/LPO) level and the activities of antioxidant enzymes catalase (CAT) and glutathione S-transferase (GST), and carbonylated proteins (CP) content in earthworm's whole body extract. The neutral red retention (NRR)-time of coelomocytes and total antioxidant capacity (TAC) were also measured in the earthworm's coelomic fluid (ECF). Upon exposure to DMP, OS status was induced in a time and concentration-dependent manner. The MDA level, CAT and GST activities, and CP content were found to be increased, but the TAC value and NRR-times were decreased in all DMP-treated groups on 7^{th} and 14^{th} days of exposure. The toxicity of DMP was generally greater in the groups with 14 day toxicant exposure. Briefly, our resultes defined that DMP is a source of oxidative damages in the earthworms. However, for detailed elucidation of the mechanisms associated with DMP ecotoxicity and its adverse impacts in the soli organisms, further unraveling is needed.

Introduction

Transport of the harmful chemicals through disposal of industrial wastes is the main cause in soil contamination (Mirsal 2008). Over the past decade, an increasing number of studies have reported the continuous exposure to different types of chemicals liberated from the plastic sources in the terrestrial environment (USEPA 1980; Liang et al. 2008; Kim et al. 2019). Phthalates are the dialkyl or alkyl aryl esters of 1, 2-benzendicarboxylic acid (phthalic acid) and also known as PAEs. These chemicals have been released and detected in various environments including soils, sediments, landfill leachates (Schwarzbauer et al. 2002; Zheng et al. 2007). Studies showed that ~ 6 million tons of PAEs are consumed globally each year (Niu et al. 2014). The PAEs are widely use in a number of consumer end products such as toys, paints, adhesives, lubricants, packaging and building materials, electronics, medical devices, and are an unavoidable part of modern life. PAEs are used mainly as plasticizers to improve the flexibility, durability, and transparency of plastics (Gao et al. 2014), not chemically bonded to the plastics polymer and can therefore eventually migrate from the plastics into the environment. Two major manufacturers of PAEs, paper and plastic industries, discharge them into the environment causing environmental pollution (Shea 2003; Liang et al. 2008; Gao and Wen 2016).

PAEs and their metabolites have been found to be potentially harmful for human and environment due to their hepatotoxic, teratogenic and carcinogenic characteristics (Matsumoto et al. 2008). The United States Environmental Protection Agency (USEPA) has categorized the following six PAEs as priority pollutants: diethylhexyl phthalate (DEHP), di-*n*-butyl phthalate (DBP), dimethyl phthalate (DMP), diethyl

phthalate (DEP), di-*n*-octylphthalate (DOP) and butyl benzyl phthalate (BBP) (US EPA 1980; Song et al. 2019). PAEs with lesser molecular weights, such as DEP have greater bioaccumulation factors (BAFs), while larger PAEs such as DEHP tend to have lesser BAFs in the environment (Staples, 1997). The reported data show that the total concentration of PAEs including DMP, DEP, and DEHP in the agricultural soils is in the range of 0.51–7.16 mg kg^{-1} (Ma et al., 2015). Among them, DMP is a colorless liquid that is majorly used in polyvinyl chloride (PVC) production as a plasticizer, the coating of cellulose films, and insect repellents (Zhao et al. 2004). The content of DMP in the different insect repellents preparations varies from 30 to 80%. DMP-containing lotions are widely used as synthetic insect repellent which are effective against biting insects such as mosquitoes (Khoobdel et al. 2007). The allowable value of soil cleanup objective of DMP in USA is 2.0 mg kg^{-1} (NYSDEC 2003). In human, the major pathway of exposure to PAEs is the oral route (Adibi et al. 2003).

Soil is the environmental compartment where most of the pollutants released into the biosphere are accumulated (Köhne et al., 2009). Among soil organisms, earthworms as bioindicators are one of the best organisms for use in soil toxicity evaluation. Their activity is strongly dependent on the quality of soil and occurrence of pollutants in it (Sohrabi et al. 2021). Thus, they are included in a group of key indicators for ecotoxicological testing of industrial chemicals determined by the Organization for Economic Co-operation and Development and the European Economic Community (EEC) (Capowiez et al. 2003). Meanwhile, manipulation of earthworms is relatively simple; this facilitates the measurement of different life-cycle parameters e.g. growth and reproduction, as well as accumulation and excretion of pollutants, and biochemical responses. These organisms ingest large amounts of soil; therefore they are continuously exposed to contaminants adsorbed to solid particles through their alimentary tract (Morgan et al. 2004).

In environmental toxicology, biomarkers are defined as molecular, biochemical, physiological or histological indicators of contaminant exposure or effects (Van Gestel and Van Brummelen 1996). Biomarkers are useful for evaluating exposure and toxic effects of chemicals on invertebrates. They have also been useful to distinguish acute toxicity from long-term effects (Hagger et al. 2009). The use of biomarkers can be a complementary approach to standard toxicity tests because it provides more information about the organism's stress response to individual toxicants and mixtures. Biochemical responses of organisms to environmental stress are regarded as early warning signs of pollution in the environment. The oxidative damages and the activities of certain enzymes as biomarkers in earthworms have been considered as fast and prognostic indices of individual reaction to the environmental stress (Laszczyca et al. 2004; OECD, 1984; Song et al. 2008; Sohrabi et al. 2021). In cells, both enzymatic and non-enzymatic antioxidant defense systems are functioning against cellular oxidative damages caused by reactive oxygen species (ROS). The enzymatic antioxidants include catalase (CAT), glutathione S-transferase (GST), superoxide dismutase (SOD), and etc. Ascorbate, glutathione (GSH), carotenoids, and tocopherols serve as potent non-enzymatic antioxidants within the cells. All unstable metabolites of molecular oxygen like superoxide radical ($^{\bullet}O_2^-$) and hydroxyl radical ($^{\bullet}OH$) are defined as ROS (Halliwell and Gutteridge 1984). The accumulated ROS react with cellular components, causing oxidative damage

to the cellular biomolecules such as lipids, proteins and DNA (Lopez et al. 2006). Any decrease in the cellular antioxidant content leads to an increase in the ROS production, resulting in oxidative stress (OS) status (Golovanova et al. 1999). Once the OS is occurred, lipid peroxidation (LPO) process is the initial step of cellular membrane damage, and considered to be a valuable indicator of oxidative damage of cellular components via the extraction of hydrogen atom from polyunsaturated fatty acids (PUFA) of membrane phospholipids. Elevation in the level of malondialdehyde (MDA) as a by-product of LPO process reflects membrane degradation in a variety of pathological conditions and proposed to appraise the health status of exposed species. Excessive accumulation of MDA in organisms changed the structure and permeability of cell membranes, caused pollutants to enter cells and damage to DNA, thereby resulting in apoptosis or even necrosis (Halliwell and Gutteridge 1984; Farber 1994; Ferreira et al. 2005; Song et al. 2019).

Lysosomes are subcellular organelles containing hydrolytic enzymes capable of processing damaged or redundant cellular components. They are also able to accumulate and detoxify a wide range of toxic metals and organic pollutants, capable of damaging cells (Moore 2006). The neutral red retention (NRR) time is a commonly used non-specific biomarker technique that measures changes in lysosomal membrane stability, especially the increased membrane permeability in response to environmental stresses. The NRR-time assay is a histochemical technique based on the principle that only healthy lysosomes can retain the acidotrophic, red dye after an initial uptake (Weeks and Svendsen 1996; Gupta 2000; Ali and Naaz 2013; Trivedi et al. 2020). Feng et al. 2016 suggested that NRR-time assay can be used as a biomarker for monitoring levels of PAE contamination in soils. They demonstrated that PAEs cause a significant reduction in lysosomal membrane stability of earthworm's coelomocytes.

Previous investigations have documented the ecotoxicological effects of PAEs on earthworm's viability in the soils (Wang et al. 2013; Du et al. 2015; Feng et al. 2016; Ma et al. 2017; Kim et al., 2019). In 2016, it was revealed that soils contaminated with PAEs can harm populations of earthworms (Feng et al. 2016). The potential transfer of PAEs from soils to animals is also significant, highlighting the importance of evaluating their ecological toxicity in soils (He et al. 2015; Ma et al. 2016). According to a study by Wang et al. (2018), under PAEs stress, earthworms developed genotoxic and oxidative damages. They showed that PAEs elevated ROS generation which had a dose-effect relationship. In 2019, Song et al. revealed that MDA content and DNA breaks were markedly increased in DMP-exposed earthworms, indicating occurrence of oxidative damages. They also demonstrated that during initial exposure to DMP, earthworms produced large amounts of ROS. Meanwhile, under DMP-induced OS, a significant elevation was observed in the activities of antioxidant enzymes CAT and GST at dose 50 mg kg^{-1} (Song et al. 2019).

To date, less is understood about DMP toxicity in the terrestrial environment. The present study aimed to investigate the ecotoxicological effects of DMP at different concentrations 0, 0.1, 1, 10 and 50 mg kg^{-1} on the 7th and 14th days of exposure in earthworms *Lumbricus terrestris* L. in a artificial soil model. OS biomarkers including MDA/LPO level, activities of CAT and GST, and also CP content will determine in the earthworm's whole body extract. Besides, lysosomal membrane stability of coelomocytes (NRR-time) and

TAC value will determine in the earthworm's coelomic fluid (ECF). Hence, this study is of significance for information on the ecotoxicological effects of DMP on the soil organisms.

Materials And Methods

Chemicals

DMP (CAS No. 131-11-3, 99% purity) and other chemicals in reagent grade were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Animal model

Sexually mature earthworms *Lumbricus terrestris* L. (Annelida: Lumbricidae), with a well-developed clitellum and the mean weight about 3-4.2 gr (fresh weight), were obtained from Soroush Rouyesh Salamat (SRS) limited Co., Shahrekord, Iran. Earthworms were acclimated to the laboratory conditions with a photoperiod of 12/12- h at 20±2 ºC for 14 days.

Exposure method

The toxic effects of DMP on earthworms were investigated by artificial soil tests according to the OECD normal method (OECD 1984). The artificial soil was prepared with 70% fine sand, 20 % kaolin clay, and 10% sphagnum peat at pH 7.0±0.2. Experiments were performed in triplicate, and operated in 1L glass jar with 500 g artificial soil and 20 earthworms. Based on the previous similar studies (Du et al., 2015; Wang et al., 2018; Song et al. 2019), the selected DMP concentrations were set as 0, 0.1, 1, 10, and 50 mg kg^{-1}, dissolved in acetone, and sprayed on the surface of the test soil and thoroughly mixed. The DMP-treated artificial soils were placed in a well-ventilated fume hood and turned daily for 7 days in order to evaporate acetone. Then, all soil samples were rehydrated to 35 % moisture and left 1 day to equilibrate. The earthworms were cultivated for 24 h in untreated artificial soil and then they were put in the DMP treated soils.

Preparation of earthworms' homogenates and mitochondria suspension

After 7 and 14 days, earthworms were thoroughly washed with distilled water before being placed on damp filter paper for 12h to avoid their gut contents (OECD, 1984). The earthworms were put on ice to immobile, and then were cut into pieces and mixed with chilled 50 mM potassium phosphate buffer (1:8, w/v; pH 7.0) containing 0.5 mM EDTA. Homogenization was carried out with Ultrasonic processor (JY-250, Zhejiang, China). The resulting homogenate was divided into two portions, i) for measuring malondialdehyde (MDA) in the LPO assay, carbonylated proteins (CP) content, and total protein content, and ii) centrifuged at 10,000 rpm for 10 min at 4°C to obtain the post-mitochondrial supernatant for antioxidant enzymes (CAT and GST) analyses (Velisek et al. 2011; Hu et al. 2012).

Collection of earthworm's coelomic fluid (ECF)

In order to collect the ECF, electric shock method was conducted (Mohamed Jaabir et al. 2011; Chellathurai Vasantha et al. 2019). Earthworms suspended in the media containing Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) (1:8, w/v, pH 7.0), and then were subjected to electric shock (3V, 30 sec, 1 min interval for recovery and repeated 5 times). Under shocks, the bio-fluid CF was collected and then filtered through Whatman number 1 filter paper. The filtrate was then centrifuged at 8000 rpm for 10 min, resulting supernatant stored at -20 °C for determination of TAC value. The sediment containing coelomocytes was gently diluted in HBSS/PBS medium and used for NRR-time assay.

Assay of oxidative stress biomarkers

Determination of malondialdehyde (MDA/LPO) level

The TBARS assay was performed to determination the MDA/LPO level in the homogenate aliquots (Buege and Aust 1978). In brief, 500 µl of aliquots was mixed with 1 ml of chilled 30% (w/v) trichlroacetic acid (TCA) and centrifuged at 5000 ×g, 10 min (Lushchak et al. 2005). Control samples contained distilled water instead of homogenate aliquot. The resulting aliquot used for measuring the amounts of MDA, by reaction with thiobarbituric acid (TBA) at 532 nm, using UV-1700 spectrophotometer (Shimadzu, Japan). The MDA level was calculated by using molar extinction coefficient of chromophore TBA ($156\times10^3\ M^{-1}cm^{-1}$) and expressed as n moles MDA per mg protein.

Determination of catalase (CAT) activity

The activity of antioxidant enzyme catalase (CAT; EC 1.11.1.6) was measured on the basis of the breakdown of hydrogen peroxide (H_2O_2) at 240 nm (Luck 1965). Briefly, assay mixture consisting of 3 ml H_2O_2 phosphate buffer and 0.50 ml of 10% supernatant of earthworm's homogenate, and change in absorbance was recorded at 240 nm by UV-1700 Spectrophotometer (Shimadzu, Japan). The results were expressed as IU H_2O_2 decomposed per mg protein, following degradation of H_2O_2 by CAT activity present in the samples.

Determination of glutathione S-transferase (GST) activity

The determination of GST (GST; EC 2.5.1.18) activity refers to the method of Habig et al. (1974), using S-2, 4-dinitrophenyl glutathione (CDNB) as a substrate. The principle of the method is based on measurement of the conjugation of CDNB with GSH. The formation of adduct of DNB, S-2,4-dinitrophenyl glutathione (DNPG) was monitored by measuring the rate of increase in absorbance at 340 nm for 4 min. The enzyme activity was calculated from the extinction coefficient of GSH-CDNB and expressed as n moles DNPG produced per mg protein.

Determination of carbonylated proteins (CP) content

According to the mthod of Levine et al (1994), the CP content was measured spectrophotometrically in the earthworm's homogenate supernatants. Briefly, 0.5 ml of supernatants were treated with 0.5 ml of 10

mM 2,4-dinitrophenylhydrazine (DNPH) in 2M HCl, or with 0.5 ml of 2M HCl alone for the blank. Samples were then incubated for 1 h at room temperature in the dark, and then 10% trichloroacetic acid (TCA) was added and centrifuged at 3,000 ×g for 20 min. The pellet was washed three times in ethanol/ethylacetate and solubilized in 1 ml 6M guanidine in 20 mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid; the resulting solution was incubated at 37 °C for 15 min. Carbonyl concentrations were determined from the difference in absorbance at 370 nm between DNPH-treated and HCl-treated samples, with ε_{370} = 22,000 $M^{-1}cm^{-1}$. The content of CP were expressed as n moles CP per mg protein.

Determination of total antioxidant capacity (TAC)

To measure the changes in the TAC value, the method of ferric reducing antioxidant power (FRAP) was applied in the ECF (Benzie and Strain 1996). During the assay, yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) is reduced to blue ferrous complex (Fe (II)-TPTZ) by the action of electron-donating antioxidants in ECF samples. The resulting blue color is linearly related to the total reducing capacity of antioxidants in solutions: i) Reagents: 1) Acetate buffer 300 mM pH 3.6: Weigh 3.1 g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1L with distilled water; 2) TPTZ (2, 4, 6-tripyridyl-s- triazine), 10 mM in 40 mM HCl; 3) $FeCl_3$. $6H_2O$, 20 mM. The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1 just before testing. Standard was $FeSO_4.7H_2O$: 0.1-1.5 mM in methanol. ii) Procedure: FRAP solution (1.5 ml) was incubated at 37° C for 5 min. Then this solution mixed with 0.1 ml of diluted ECF (1:10) and incubated at 37° C for 10 min. The end product (Fe^{2+}-TPTZ) had blue color. The absorbance of the reaction mixture was measured at 593 nm for 4 min. For construction of the calibration curve, five concentrations of $FeSO_4$, $7H_2O$ (0.1, 0.4, 0.8, 1, 1.12, 1.5 mM) were used and the absorbance values were measured as for sample solutions. The values obtained were expressed as mM Fe^{2+} produced per L.

Determination of neutral red retention time (NRR-time)

The NRR-time assay was performed to evaluate the lysosomal membrane stability in earthworm's coelomocytes (Weeks and Svendsen 1996; Xiao et al. 2006). As healthy cell lysosomes retain the neutral red dye for some length of time, dye retention times represent a marker of membrane permeability. The filtrate of CF was centrifuged at 8000 rpm for 10 min and resulting sediment containing coelomocytes diluted in HBSS/PBS medium and gently used for this assay. The stock solution of red dye was made by dissolving 28.8 mg of dye, $C_{15}H_{16}N_4$.HCl, in 1 ml of dimethyl sulfoxide (DMSO). The working solution was prepared by diluting 5 µl of stock solution in 5 ml of physiological saline. A droplet of the sediment was transferred to a glass slide before 20 µl of working dye solution was added. The slides were scanned for non-leaking and leaking coelomocytes at 2 min intervals under a light microscope with magnification of 400, ideally 1 min per slide maximum. Tests were terminated when the dye loss was evident in at least 50% of the coelomocytes after 90 min. In the majority of the control earthworms, the number of leaking cells never reached this level within the maximum observation time of 90 min.

Determination of total protein content

The total protein content was quantificated by the method of Bradford using bovine serum albumin (BSA) as standard at absorbance of 595 nm, and presented as mg protein per ml of whole body extract of earthworms (Bradford, 1976).

Statistical Analysis

The experimental results were analyzed with the SPSS software (2019 v26). The standard ANOVA techniques, followed by Tukey's HSD post hoc test, were used to examine the differences among treatment groups and control groups. A probability level of $p<0.05$ was considered as significant. Data were presented as Mean±Standard Deviation (SD) (n=20).

Results

Malondialdehyde (MDA/LPO) level

In the Fig. 1, the MDA/LPO level was found to be increased significantly under DMP stress over a time exposure of 7 and 14 days (excluding the 0.1 and 1 mg kg^{-1} groups), compared with the control groups. The increases in the level of MDA/LPO followed a time and concentration-dependent trend. The highest MDA level was obtained in 50 mg kg^{-1} DMP-treated groups after 14 days by 76.56 %, that which was dramatically different from the control ($p<0.05$). The elevated MDA level was greater in the groups with 14 day DMP treatment (Fig. 1). We observed a positive correlation between DMP increasing concentrations and elevated MDA level ($r = 0.89$, $p<0.05$).

CAT activity

Our results regarding changes in the CAT activity indicate that in DMP-treated earthworm, the enzyme activity was elevated compared to controls in a time and concentration-dependent manner (Fig. 2). This increment was found to be meaningful only in 10 and 50 mg kg^{-1} groups after 7 and 14 days. The highest CAT activity was obtained in 50 mg kg^{-1} DMP-treated groups after 14 days (38.14 %, $p<0.05$). The recorded increase in the CAT activity was greater in 14 than 7 day exposed groups. The correlation between MDA increasing level and elevated CAT activity was $r = 0.76$ ($p<0.05$).

GST activity

Significant alterations in the GST activity in the DMP-treated earthworms have been found at higher concentrations (10 and 50 mg kg^{-1}) of the toxicant ($p<0.05$) after 7 and 14 days of exposure (Fig. 3). Our data indicate that maximum elevation in the GST activity was seen in 50 mg kg^{-1}-treated group at the end of 14 day exposure to DMP by 81.59 % ($p<0.05$). Here, a time and concentration-dependent trend was detected in the increasing GST activity which was greater in 14 day exposure to DMP (Fig. 3). The

positive value of correlation between increased MDA level and elevation in the GST activity was r = 0.82 ($p<0.05$).

CP content

As depicted in Fig. 4, the production of CPs in earthworms was induced with increases in the DMP concentration in a time-dependent manner. A positive strong correlation was observed between increase in the CP content and elevated MDA level (r = 0.91, $p<0.001$). The CP content was significantly increased in earthworms exposed to 10 and 50 mg kg^{-1} DMP concentrations after 7 and 14 day time points, compared to controls. The highest CP value was obtained in 50 mg kg^{-1}-treated group after 14 days of exposure to toxicant by 54.04 % ($p<0.05$). These increases were greater in group treated with 14 day than 7 day DMP exposure.

TAC value

The observed changes depicted in Fig. 4 indicate that the TAC value in ECF was lowered significantly following treatment with DMP concentrations over a time exposure of 7 and 14 days (excluding the 0.1 mg kg^{-1} groups), compared with the controls. A time and concentration-dependent trend was observed in the lowered TAC value. This lowering in the TAC value was greater in 14 day treated groups with toxicant. The maximum elevation in the TAC value was seen in 50 mg kg^{-1}-treated group at the end of 14 day exposure to DMP by -40% ($p<0.05$) (Fig. 5). A negative value of correlation between elevated MDA level and lowering in TAC value was observed (r = - 0.93, $p<0.001$).

NRR-time

Following 7 and 14 days, the changes in the NRR-time for coelomocytes of earthworm exposed to increasing concentrations of DBP is depicted in Fig. 6. The long NRR-times found in earthworms exposed to control soils. The values of NRR-times were significantly lower than the control at all different DMP concentrations and time points ($p<0.05$). The results show a clear time and concentration response relationship between DMP treatments and NRR-times. The NRR-times of control group were at initial level of 90±14 min, which declined steadily to 44±13 min (- 51.11 %, $p<0.05$) at the highest DMP concentration 50 mg kg^{-1} after 14 days. Here, the NRR-time assay results suggested that the toxicity of DMP was generally greater in the groups with 14 than 7 day DMP exposure (Fig. 6). A negative correlation was observed between decreased NRR-times and the elevated MDA level (r = 0.93, $p<0.001$).

Discussion

PAEs, a group of man-made chemicals, have no covalent bond exists between PAEs and the surrounding matrix, these compounds easily leach into the environment and affect habitant organisms (Sicinska et al. 2020). In ecosystems, the biomarker responses to chemicals depends on animal species including age, life stage of individuals, and their ability to adapt, and on the bioavailability and mode of action of the

chemicals (Wang et al. 2018). The popularity of earthworms as excellent bioindicators makes them a robust model for assessing soil pollution (OECD 1984; Ali and Naaz 2013).

Malondialdehyde (MDA/LPO) level

The MDA/LPO level can be used as a biomarker of OS which reflect degree of damages from ROS insult on the earthworm's body indirectly (Halliwell and Gutteridge 1984; Xue et al. 2009; Song et al. 2019). Our results concerning changes in the LPO content indicate an elevation in MDA level, in a time and concentration-dependent manner. Higher MDA is definitely due to induction of peroxidation chain reactions followed by oxidative damages in the earthworm's body exposed to DMP. As earlier noted, accumulation of MDA in organism's tissues results in changes in the structure and permeability of cell membrane leading to cell dysfunction (Halliwell and Gutteridge 1984; Ferreira et al. 2005). The elevation in MDA level can most likely be ascribed to an excessive production of ROS, which could be related to altered function of antioxidant enzymes following addition of DMP. Results from this study are similar to the findings of Song et al. (2019), who reported elevated level of MDA and oxidative damages in earthworms *Eisenia fetida* after 7 and 14 day exposure to DMP suggesting production of large amounts of ROS. Besides, they reported that due to the defensive effects of antioxidant enzymes, ROS content decreased later on and returned to the control level. This is also consistent with the findings of Wang et al. (2018) who reported an increase in MDA level and LPO propagation in the tissues of earthworms *Eisenia fetida* exposed to DBP. Our results are also supported by the findings of Ma et al. (2017) that reported increased MDA content in earthworms under the DEP exposure.

CAT and GST activities

It has been hypothesized that the accumulation of MDA level followed by increase in the oxidized lipids can be a cellular signal of OS that, in turn, regulates antioxidant potential and trigger enhanced defenses (Halliwell and Gutteridge 1984). In the present study, the activities of antioxidant defense systems CAT and GST were found to be elevated in the earthworms exposed to the DMP at concentrations of 10 and 50 mg kg^{-1} after 7 and 14 days. This result is consistent with the findings of the study in which under DMP-induced OS, a significant elevation was observed in the activities of CAT and GST at high dose 50 mg kg^{-1} (Song et al. 2019). The increase in MDA level and antioxidant enzyme activities clearly indicated that DMP was able to cause OS and related damages. CAT is an enzymatic antioxidant extensively distributed in all animal tissues which help decomposes H_2O_2 and protects the tissue from highly reactive hydroxyl radicals and reduction of the cell membrane fluidity (Winston and Giulio 1991). Inhibition of CAT may enhance sensitivity to free radical-induced cellular damages. GSTs are a family of multifunctional antioxidant enzymes involved in the detoxification of a broad variety of xenobiotics and reactive endogenous compounds by adding reduced glutathione (GSH), making them more water-soluble and, thus, excreted more easily (Moorhouse and Casida 1992; Reineme et al. 1996). However, we may assume that such an increase in the GST activity may indicate sufficient detoxification of DMP in the treated earthworms. Wang et al. (2018) demonstrated that in earthworms upon stimulation with phthalates to eliminate oxidative damage due to their toxic substances, the CAT and GST enzyme activities were

increased. According to the theory of ROS (Mittler 2002), we can postulate that at higher concentrations, DMP exposure produced large amounts of H_2O_2 and lipid radicals in earthworms and adapted to the environment by increasing the activities of CAT and GST, respectively.

CP content

The CP content was evaluated to detect the level of protein oxidation in the DMP-treated earthworms. We showed that DMP could increase significantly the CP content at concentrations 10 and 50 mg kg^{-1} after 7 and 14 day exposure. It can be assumed that elevated MDA level following DMP-induced OS may result in accumulation of CP in the treated earthworms. We also revealed that elevated MDA level was significantly associated with the augmented content of CP. This finding indicates that DMP has potency to modify the protein structures via oxidative damages in earthworms. The presence of carbonyl groups in proteins has been considered as the biomarker of ROS mediated protein oxidation in earthworms exposed to certain pollutants (Bourdineaud et al. 2021). The by-products of LPO, such as MDA and 4-hydroxy-2-nonenal, can react with the amino groups of certain amino acids forming reactive carbonyl groups (aldehydes and ketones) that cause protein oxidation via secondary reactions leading to cell inactivation. Therefore, if the MDA level increases, it is possible that protein damage occurs due to exposure to carbonyl groups. Many studies have employed CP content to evaluate the detrimental effects of OS-induced protein oxidation. The CPs are more susceptible to proteolytic degradation than their non-oxidized counterparts. Besides, the CP content has been identified as a main factor in protein function and removal in aging and diabetic animals (Levine et al. 1994; Nystrom 2005; Cecarini et al. 2007; Purdel et al. 2014). Amara et al. (2019) revealed that increased free radical production by DEHP would result in elevated CP content leading to an enhanced dose-dependent cardiotoxicity in mice. Furthermore, it has been demonstrated that the depletion of GSH was associated with the elevation of OS and increased CP content in mouse kidney treated with DEHP (Amara et al. 2020).

TAC value

Newly, ECF is considered as a bio-fluid which is accounted as a novel sensitive biomarker to monitor contaminations in environment (Griffith et al. 2019). The ECF possesses different active molecules and cells, coelomocytes, which involve in the phagocytosis of pathogens. Besides, ECF synthesizes and secretes the antibacterial molecules, cytotoxic proteins and some enzymes (Calisi et al. 2014; Ramian et al. 2018). Due to, ECF is responsible for pollutant disposition and tissue distribution to the whole organism, it is particularly interesting from a toxicological perspective. ECF also contains different ions, vitamins, and defense components including cytolytic and bactericidal substances (Hatti et al. 2010; Packialakshmi 2012). According to our results it was cleared that TAC value in DMP-treated earthworms was found to be lowered with increasing exposure time and concentration. The potency of DMP intoxication to induce OS caused the lowering the power of antioxidant defense systems in the ECF. The organism's body has several basic antioxidative mechanisms to counteract the oxidative damages. Antioxidants are stable enough to neutralize free radicals by donating electrons (Gutteridge 1995). It appears that water and fat-soluble vitamins vitamin C and E, respectively, are present in the ECF. These

vitamins scavenge and quench free radicals, but are oxidized and inactivated in the process. Each of these antioxidant nutrients has specific activities, and they often work synergistically to enhance the overall antioxidant capacity of the body (Sies and Stahl 1995). Moreover, it is well documented that pollutants cause reduction in the level of glutamine as a precursor of GSH in the ECF (Griffith et al., 2019). Therefore, it can be postulated that DMP played a toxic role to earthworms resulting in lowering the level of precursors of antioxidant defense system such as glutamine in the ECF.

NRR-time

Lysosomal membrane stability through the NRR-time assay is a general subcellular biomarker for the action of toxic pollutants that has been found to be reliable and dose-related in different earthworm species (Ma et al. 2016). The importance of this assay as a predictor of adverse effect of contaminants on the coelomocyte lysosomes were pointed out in previous studies as an early response to PAEs exposure in earthworms (Lee and Kim 2009; Klobučar et al. 2011; Muangphra et al. 2016; Ma et al. 2016; Wang et al. 2016). In the present study, exposure to DMP caused a significant and concentration-dependent reduction in the coelomocyte NRR-time of earthworms *Lumbricus terrestris* L. at different time points. Observation of the loss of red dye from these lysosomes into the surrounding cytosol has enabled the quantification of the degree of lysosomal damage caused to earthworms with exposure to an increasing range of soil DMP concentrations.

Ma et al. (2016) found that cell death in the form of apoptosis was increased in coelomic coelomocytes with increase in the PAE concentration. It is known that PAE induced Ca^{2+} influx in human granulocytes (Palleschi et al. 2009). The increase in intracellular Ca^{2+} concentration might be associated with the NRR-time results because the loss of lysosomal membrane stability may make facilitate the entry of the coelomocytes by Ca^{2+} and leads to further disruption of signal transduction (Ma et al. 2017). Other researchers believed that the NRR-time assay reflects the efflux of the neutral red into the cytosol following damage to the membrane and, possibly, impairment of the H^{+} ion pump following environmental contaminations (Lowe et al., 1994). Feng et al. 2016 demonstrated that NRR-time was reduced at concentration 150 mg kg^{-1} after 14 day exposure to PAE in earthworms *Eisenia fetida* resulting in harming earthworm populations. Similarly, Wang et al. (2018) reported that under the PAE stimulation ECF coelomocyte produced lots of ROS and peroxides resulting in damages to the cellular function and structure. In this regards, we may assume that molecular processes governing lysosomal function are a common target for PAEs, which can perturb normal function of these organelles. However, the dysfunction of the lysosomal membrane, recorded by NRR-time assay, may be recognized as a potential mechanism of DMP toxicity in the terrestrial organisms.

Conclusions

Our laboratory findings regarding biomarker responses in the earthworms *Lumbricus terrestris* L. exposed to DMP can provide sufficient data to elucidate the mechanisms of the impact of this PAE on these beneficial soil organisms. Overall, our results revealed that DMP has potency to induce OS. OS biomarkers, namley, MDA/LPO level, CAT and GST activities, and CP content were evaluated with increase in the concentrations of DMP. In addition, the TAC value and NRR-time of coelomocytes in the ECF were found to be lowered with a dose-effect relationship. In this study, the toxicity of DMP was generally greater in the groups with 14 than 7 day toxicant exposure. These findings suggest that under DMP stress, earthworms developed oxidative damages. However, for detailed elucidation of DMP-related impairments in the soli organisms, further unraveling is needed.

Declarations

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals (*Lumbricus terrestris*) were followed.

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Figures

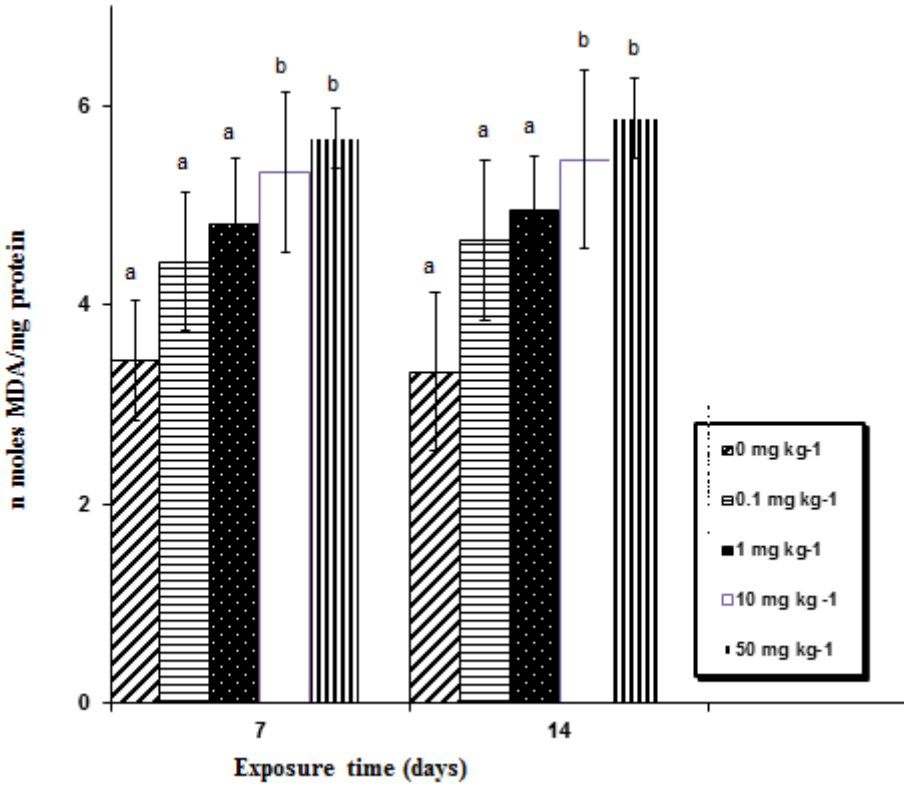

Figure 1

The level of MDA/LPO in earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

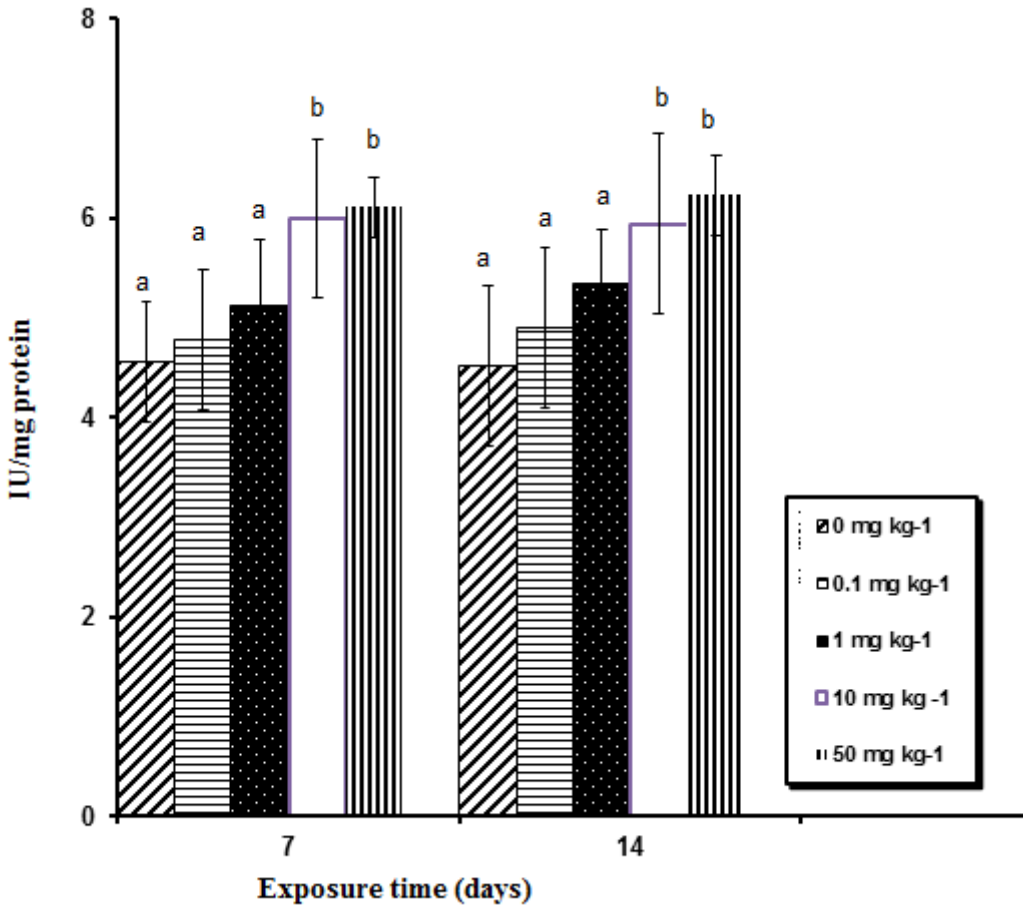

Figure 2

The activity of catalase (CAT) earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

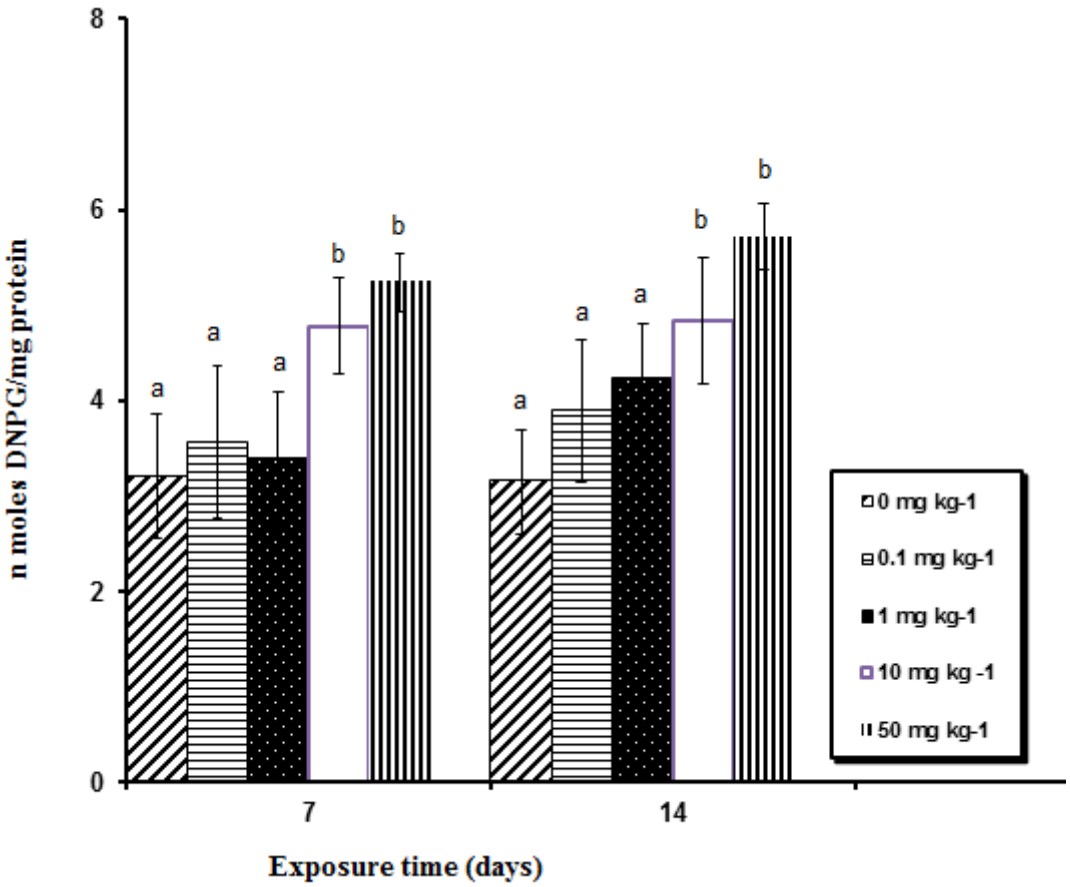

Figure 3

The activity of glutathione S-transferase (GST) in earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

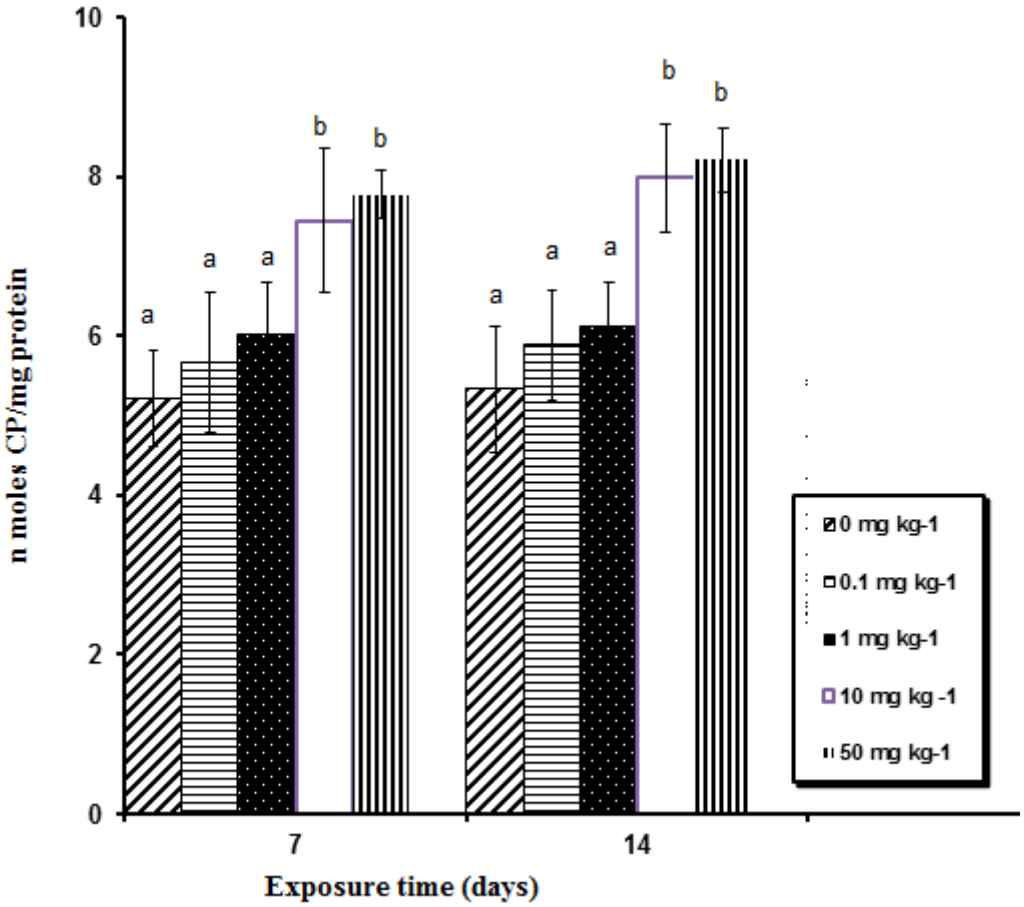

Figure 4

The content of carbonylated proteins (CP) in earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

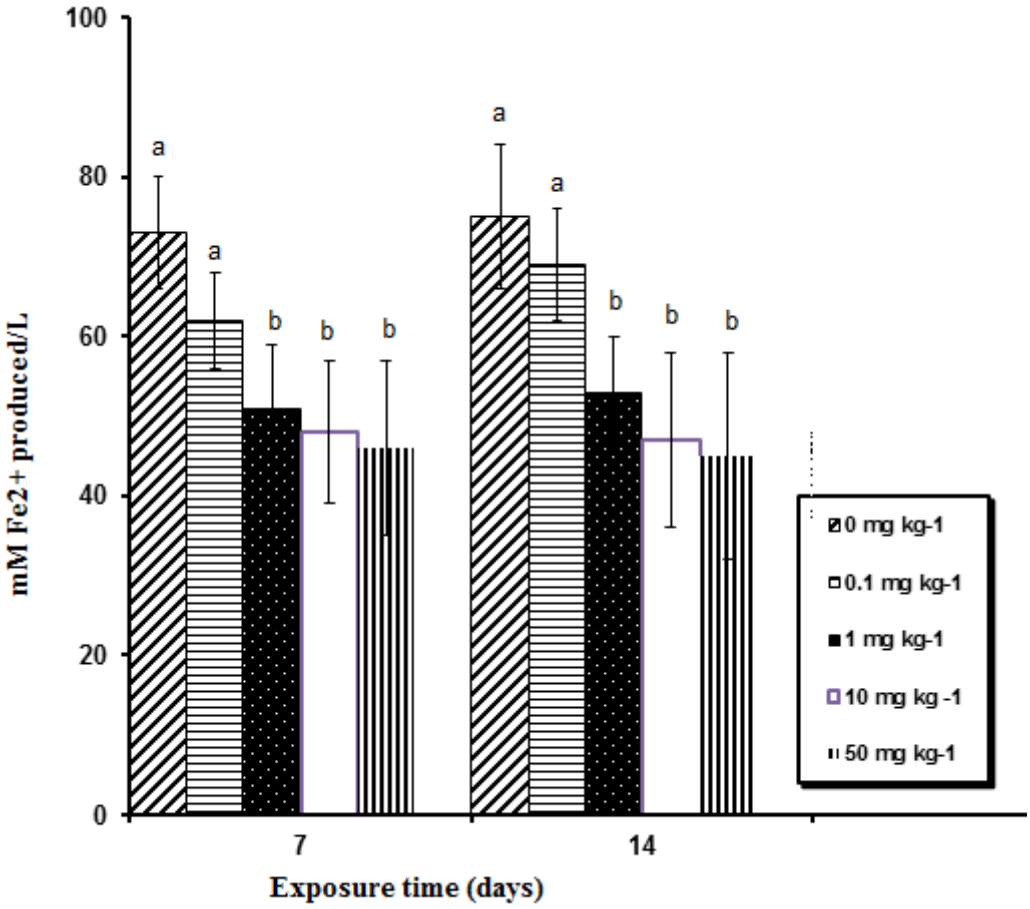

Figure 5

The total antioxidant capacity (TAC) value of coelomic fluid in earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

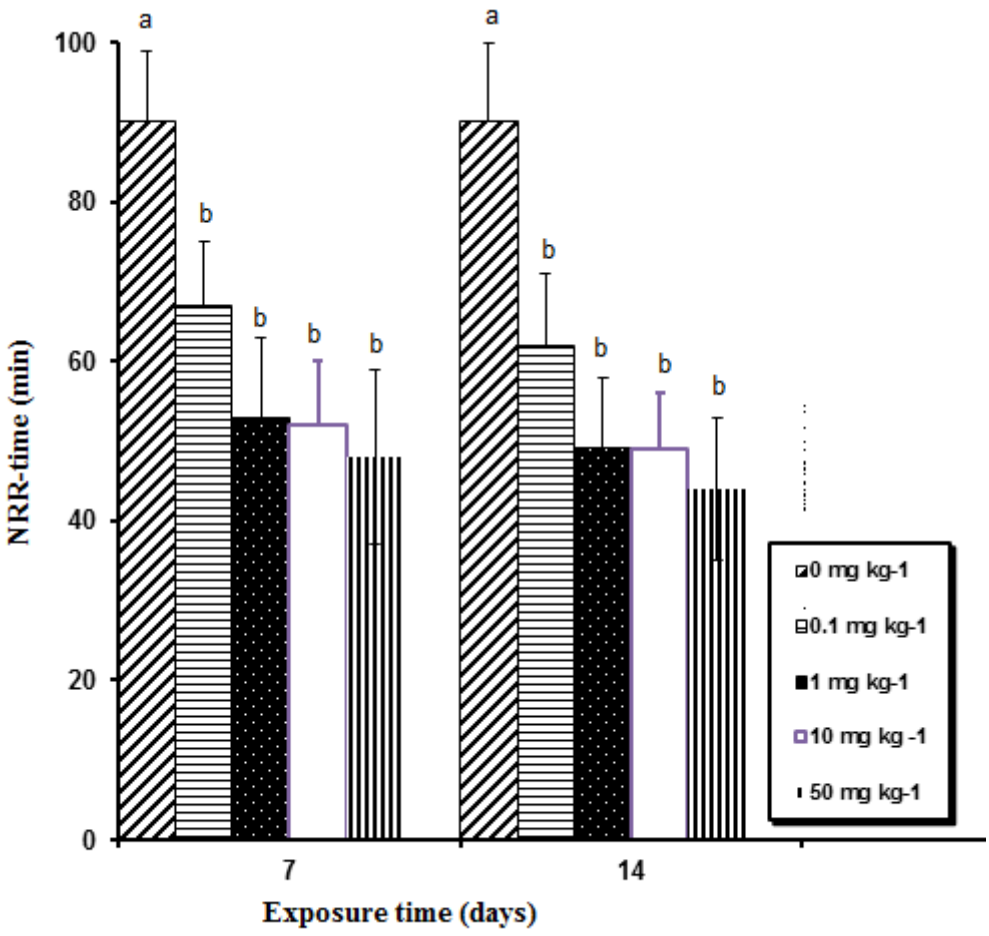

Figure 6

The netural red retention (NRR) time of coelomocytes in earthworm Lumbricus terrestris L. exposed to DMP after 7 and 14 days. a,b: data not sharing a common letter are significantly different ($p<0.05$).

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