Ecotoxicology (2010) 19:1551–1559
DOI 10.1007/s10646-010-0540-x

Oxidative stress and DNA damage in the earthworm *Eisenia fetida* induced by toluene, ethylbenzene and xylene

Yao Liu · Qixing Zhou · Xiujie Xie · Dasong Lin · Luxi Dong

Accepted: 23 August 2010 / Published online: 14 September 2010

Abstract Superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and the comet assay (SCGE) were used as biomarkers to evaluate the oxidative stress and genotoxicity of toluene, ethylbenzene and xylene in earthworms (*Eisenia fetida*). The results indicated that the exposure of the three pollutants caused a stress response of the three enzymes, an approximate bell-shaped change (a tendency of inducement firstly and then inhibition with increasing concentrations of the pollutants) was mostly found. The three enzymes tested differed in their sensitivity to different pollutants. While the activity of POD was not significantly changed within the concentration range, the concentration thresholds for significant ($P < 0.05$) responses to toluene based on SOD and CAT were 5 mg kg^{-1}, respectively. Similarly, the concentration thresholds for significant ($P < 0.05$) responses to ethylbenzene based on CAT and POD were 10 and 5 mg kg^{-1}, respectively, while the activity of SOD was not significantly changed within the concentration range. Significant responses to xylene based on CAT and POD were 5 mg kg^{-1}, respectively, while the activity of SOD was significantly ($P < 0.05$) induced at 10 mg kg^{-1}. The SCGE assay results showed that these three pollutants could significantly ($P < 0.01$) induce DNA damage in earthworms and the clear dose-dependent relationships were displayed, indicating potential genotoxic effects of toluene, ethylbenzene, and xylene on *E. fetida*. The inducement of DNA damage may be attributed to the oxidative attack of toluene, ethylbenzene, and xylene. Toluene seemed to be more genotoxic as it could induce the higher extent of DNA damage than ethylbenzene and xylene. The results suggest that the SCGE assay of earthworms is simple and efficient for diagnosing the genotoxicity of pollutants in terrestrial environment.

Keywords Toluene · Ethylbenzene · Xylene · Oxidative stress · Genotoxicity · Earthworm (*Eisenia fetida*) · Comet assay (SCGE)

Introduction

Soil pollution by petroleum has become a significant environmental concern due to the widespread occurrences of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution stations (Mater et al. 2006). Monocyclic aromatic hydrocarbons such as toluene, ethylbenzene and three isomers of xylene are commonly associated with crude petroleum and petroleum products such as gasoline and diesel fuels. For example, it has been reported that toluene, ethylbenzene and xylene make up about 18% by volume in gasoline (An 2004; Plaza et al. 2007). Because of the relatively high water solubility and low K_{ow} values, these compounds are highly mobile in the environment (Dou et al. 2008). The presence of these hydrocarbons in the environment is a hazard to public health and an ecological concern due to their toxicity and ability to bioaccumulate through food chain, and they are classified as

Y. Liu · Q. Zhou · X. Xie · D. Lin · L. Dong
Key Laboratory of Pollution Processes and Environmental Criteria, Ministry of Education/College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China

Q. Zhou (✉)
Key Laboratory of Terrestrial Ecological Process, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China
e-mail: Zhouqx@nankai.edu.cn

environmental priority pollutants in many countries (Plaza et al. 2007). Toluene, ethylbenzene, and xylene can cause nonspecific narcosis above certain level in tissue (particularly membrane) lipids. Toxicity to the central nervous system is the main human health concern of these pollutants (Mehlman 1992; McGregor 1994; Murata et al. 1999). Several standard toxicity studies concerning physiology and survival effects on different soil organisms have been performed in order to estimate the toxicity of these compounds and to prevent the potential risk of these compounds on terrestrial ecosystems (Salanitro et al. 1997; An 2004; An and Lee 2008). However, the biochemical responses (i.e., oxidative stress and genotoxicity) of these compounds to earthworms have not yet been investigated integratedly.

Biomarkers can be used as a complementary approach to standard toxicity tests (i.e., mortality and reproduction rates) to investigate the effects of contaminant toxicity on living organisms at earlier stages and lower concentrations (Lukkari et al. 2004; Gastaldi et al. 2007; Schreck et al. 2008). It has been reported that the contaminant stress in living organisms often results in the production of reactive oxygen species (ROS). The overproduction of ROS such as H_2O_2 and superoxide radical ($^{\cdot}O_2^-$) cause oxidative stress and produce useless molecular debris and sometimes cell death (Radic et al. 2009). The toxic effects of ROS can be counteracted by cellular antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POD) and catalase (CAT). Therefore, the changes of these enzymatic activities should indirectly indicate the toxic effects of contaminants on living organisms (Li et al. 2008; Schreck et al. 2008). The single-cell gel electrophoresis (SCGE) assay, also called the comet assay, has recently emerged as an important biomarker of DNA damage in individual cells (Fourie et al. 2007). Because of its sensitivity for detecting DNA single strain breaks (SSBs) and alkali-sensitive sites damage at very low levels and the relative ease of application, the SCGE assay has been used to evaluate various physical and chemical genotoxic agents in a wide variety of eukaryotic cells (Tice et al. 2000; Dhawan et al. 2009). Coelomocytes located around the intestinal tract of earthworms, are particularly exposed to soil pollutants and are involved in the process of cell immunity (Bonnard et al. 2009). The SCGE assay employing coelomocytes from earthworms (*Eisenia fetida*) has been proved to be a useful tool to detect genotoxic agents in contaminated soils (Verschaeve and Gilles 1995; Salagovic et al. 1996; Reinecke and Reinecke 2004; Xiao et al. 2006; Song et al. 2009). Therefore, the objective of the present study was to evaluate the oxidative stress and genotoxicity of toluene, ethylbenzene, and xylene in earthworms (*E. fetida*) using SOD, POD, CAT, and the SCGE assay as biomarkers.

Materials and methods

Chemical and reagents

Toluene, ethylbenzene, and xylene with greater than 95% purity were obtained from the Tianjin Jindong Tianzheng Chemical Co. Ltd. All other chemicals were of reagent grade and purchased from the Genview Co.

Toluene, ethylbenzene, and xylene were first dissolved in Tween-80 (mole fraction $< 2 \times 10^{-3}$) and diluted in distilled water to different concentrations. The solvent control was prepared as the same mole fraction of Tween-80 solution in soil toxicological tests.

Earthworms and test soil preparation

The earthworm *E. fetida* was selected as a test organism, and was purchased from an earthworm culturing farm in Tianjin, China. Healthy earthworms with adult clitellum and weighing about 350 mg were used for our experiments. There was no mortality observed while the experiments were in progress.

The soil was taken from the clean urban area of Tianjin, China. The main physical and chemical properties of soils are listed as: pH 8.06, organic matter 22.34 g kg^{-1}, cation exchange capacity (CEC) 25.48 cmol kg^{-1}, sand 1.34%, silt 56.74%, clay 39.18%. After having sieved (2 mm mesh) and homogenized, soil samples were freeze-dried and stored at −20°C until use.

Toxicological tests

According to the result of preliminary tests, four concentration of each compound were used (in mg kg^{-1}): 5, 10, 50, and 100 for toluene, 5, 10, 30, and 60 for ethylbenzene, and 5, 10, 20, and 40 for xylene, which were lower than the minimum lethal concentration (unpublished data from our lab) of each compound.

Soil toxicity tests were conducted in a closed soil microcosm (CSM) described by An (2005). The CSM was a closed 1-l, and wide-mouth glass jar test unit. Each test unit contained 500 g soil samples as dry weight and 125 ml of test solution with a specific concentration of toluene, ethylbenzene, and xylene. Test solutions were mixed with the soil samples as dropwise additions and aluminum foil lids were tightened to prevent volatilization of these pollutants. Three CSMs were used for each concentration and ten earthworms were added to each CSM. According to An (2005), because of the rapid movement from soil to air phase after volatile organic matters (VOCs) are released from soil, acute toxicity assessment of VOCs may be more important than chronic toxicity evaluation, so the test

duration was set as 48 h in our experiment. The test unit was placed in an incubator (20 ± 1°C) in darkness. After incubation periods of 48 h, six earthworms for enzymes and comet assays were collected from each replicate CSM and prepared for the next experiments (enzyme assays and alkaline comet assay).

Enzyme assays

Three earthworms (collected from the same CSM) were placed into a prechilled mortar and pestled under ice-cold conditions in 50 mM Tris-sucrose buffer (1:9, w/v, pH 7.5). The homogenate was centrifuged at 3,000 rpm at 4°C for 15 min. The supernatant was used for further analysis.

The protein concentration in the supernatant was determined by the dye-binding method according to Bradford (1976) using bovine serum albumin (BSA) as the standard. The activity of enzymes is expressed as U mg^{-1} protein.

The activity of SOD was determined by measuring its ability to inhibit photochemical reduction of pyrogallol as described by Marklund and Marklund (1974) with a few modifications. The rate of pyrogallol reduction was measured at 325 nm. One unit of enzyme activity was defined as the amount of the enzyme that results in 50% inhibition of the auto-oxidation rate of pyrogallol at 25°C.

The activity of POD was determined using guaiacol substrates according to the method of Song et al. (2009) with a slight modification. The reaction process was measured by recording absorbance at 470 nm as soon as 50 µl of the supernatant was added to reaction mixture containing 2.9 ml potassium phosphate buffer (0.2 M, pH 6.0), 1 ml 0.2% H_2O_2 and 1 ml guaiacol.

The activity of CAT was determined as described by Song et al. (2009). In this assay, a solution of H_2O_2 was used as substrate for the enzyme. The enzyme activity was calculated from the decrease in ultraviolet absorption with time at 250 nm, following degradation of H_2O_2 by CAT present in the sample. One unit of CAT activity was defined as the enzyme quantity required to consume half of H_2O_2 in 100 s at 25°C.

Alkaline comet assay

After exposure to the pollutants, earthworm coelomocytes were obtained using the non-invasive extrusion method described by Eyambe et al. (1991) with a slight modification. Three earthworms (collected from the same CSM) were rinsed in the chilled extrusion medium (5% ethanol, 95% saline, 2.5 mg ml^{-1} EDTA, 10 mg ml^{-1} guaiacol glyceryl ether, pH 7.3) for 3 min. Coelomocytes were spontaneously secreted in the medium, and then the extrusion medium was centrifuged (4°C, 9,000 rpm, 10 min) and the supernatant was removed. Coelomocytes were washed three times in PBS and the final cell density was adjusted to 1×10^5–1×10^6 cells ml^{-1} with PBS. The viability of the cells obtained by the trypan blue exclusion method was in the range of 90–98% for all groups.

The alkaline comet assay was performed as described originally by Singh et al. (1988) with a slight modification. All steps were conducted under dim yellow light and performed at 4°C to prevent additional DNA damage. The cell suspension (50 µl) was mixed with 50 µl of 1.0% (w/v in PBS) low melting agar (LMA) at 37°C and pipette onto normal microscope slides precoated with a layer of 100 µl of 0.6% (w/v in PBS) normal melting agar (NMA). After solidification at 4°C for 15 min, cover slips were removed and another layer of 85 µl LMA was added, slides were left at 4°C again for 15 min to solidify and the cover slips were removed. Then slides were immersed into a lysis solution for 2 h (4°C, 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, 1% Na-sarcosinate, supplemented with 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100 just before use, pH 10). Slides were then placed in an electrophoresis tank containing 300 mM NaOH with 1 mM Na_2EDTA (pH > 13) for 30 min at 4°C prior to electrophoresis in the same buffer for 20 min at 150 mA, 15 V (1 V cm^{-1}). The slides were then neutralized (0.4 M Tris, pH 7.5) thrice at 5 min intervals and fixed with absolute ethanol for 10 min, dried at room temperature and kept under refrigeration until analyses.

Slides were stained with 40 µl ethidium bromide (20 µg ml^{-1}) and viewed using a fluorescence microscope (Zeiss, Axio Imager Z1) equipped with a CCD camera. For each parallel set of slides, 50 randomly select and non-overlapping cells were captured at 400× magnification and a total of 100 cells were scored for each sample. The captured images were analyzed using CASP (Końca et al. 2003). The parameter used to quantify the extent of DNA damage was the olive tail moment (OTM). OTM is the product of the distance between the center of gravity of the head and the center of gravity of the tail and percent tail DNA.

Statistical analysis

Each treatment was performed in triplicate, and all the values are presented as means ± standard deviation (SD). In enzyme assays, one-way analysis of variance (ANOVA) followed by the Tukey test was employed to evaluate the statistical significance ($P < 0.05$) of the results between the exposed and control groups by using SPSS 13.0. Comet assay data were found to be abnormally distributed and therefore non-parametric statistical methods were employed. Significant differences ($P < 0.01$) between

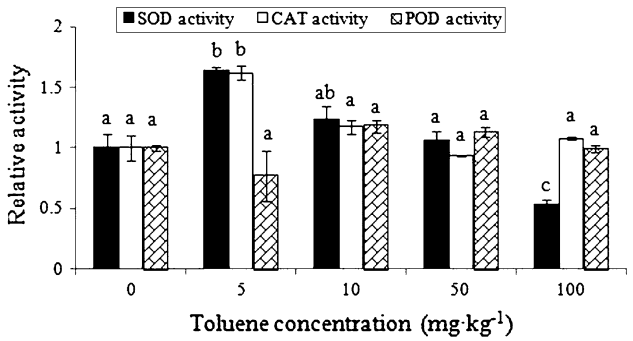

Fig. 1 The activity of SOD, CAT, and POD in earthworms exposed to toluene. Enzymatic activities in toluene treatment groups are represented as the relative activity compared with that in the control. Within the same type of enzymes, means sharing the same letter are not significantly different (Tukey test, $P < 0.05$)

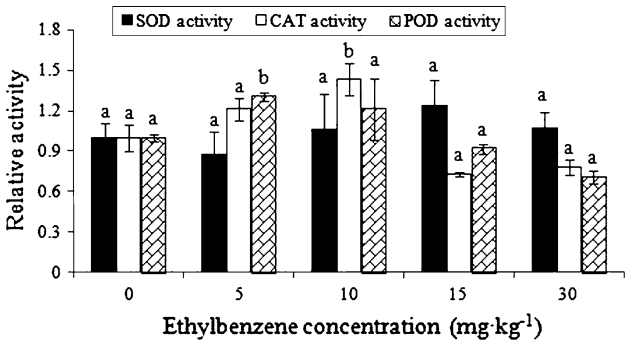

Fig. 2 The activity of SOD, CAT, and POD in earthworms exposed to ethylbenzene. Enzymatic activities in ethylbenzene treatment groups are represented as the relative activity compared with that in the control. Within the same type of enzymes, means sharing the same letter are not significantly different (Tukey test, $P < 0.05$)

treatment groups and the control were determined using the Mann–Whitney *U*-(Two-tailed) test.

Results

Effects of toluene on the activity of SOD, CAT, and POD in earthworms

As shown in Fig. 1, the activity of SOD was significantly higher (164% of the control, $P < 0.05$) at 5 mg kg^{-1} toluene, and decreased with the increasing concentration of toluene from 5 to 100 mg kg^{-1}, but still higher than that of the control at 10 and 50 mg kg^{-1} (123% and 106% of the control, respectively). Compared with the control and other treatment groups, the activity of SOD by 100 mg kg^{-1} toluene was significantly low (53% of the control, $P < 0.05$).

The activity of CAT increased approximately 162% by 5 mg kg^{-1} toluene, which was significantly higher ($P < 0.05$) than that in the control, and then significantly decreased ($P < 0.05$) to the level in the control while the concentration of toluene was 10, 50 and 100 mg kg^{-1}, respectively.

Within the range of test concentrations, POD activity did not exhibit any significant responses to toluene exposure, only some slight changes were found. The activity of POD was slightly changed (77% of the control) while treated with 5 mg kg^{-1} toluene and then changed approximately 118, 113, and 99% of the control with higher dose of the pollutant (10, 50 and 100 mg kg^{-1}, respectively).

In general, exposure of earthworms to 5 mg kg^{-1} toluene yielded significant increases ($P < 0.05$) in SOD and CAT activity, while the activity of POD was slightly inhibited. When exposed to 10 and 50 mg kg^{-1} toluene, the activities of three enzymes showed slight changes around control levels. The activities of CAT and POD were still remained at control levels at 100 mg kg^{-1} toluene, while the activity of SOD was significantly inhibited ($P < 0.05$).

Effects of ethylbenzene on the activity of SOD, CAT, and POD in earthworms

As shown in Fig. 2, the activity of SOD changed approximately 13% at 5 mg kg^{-1} ethylbenzene in comparison with that in the control. Although the activity of SOD was higher than that in the control at higher concentration groups (10, 15, and 30 mg kg^{-1}, respectively), there were no significant differences between the higher concentration groups and the control.

The activity of CAT in 5 and 10 mg kg^{-1} ethylbenzene treatments was higher than that in the control, and the difference in 10 mg kg^{-1} ethylbenzene treatment was significant (144% of the control, $P < 0.05$). The activity of CAT was lower than that in the control at 15 and 30 mg kg^{-1}, respectively.

The activity of POD in 5 mg kg^{-1} ethylbenzene treatment increased approximately 125%, which was significantly higher ($P < 0.05$) than that in the control, and then significantly decreased ($P < 0.05$) with the increasing concentration of ethylbenzene from 5 to 30 mg kg^{-1}.

In general, exposure of earthworms to 5 mg kg^{-1} ethylbenzene yielded increases in the activity of CAT and POD (the increase in POD activity was significant, $P < 0.05$), while the activity of SOD was slightly inhibited. The activity of the three enzymes was higher than that in the control (the increase in CAT activity was significant, $P < 0.05$) at 10 mg kg^{-1} ethylbenzene. While exposed to 15 and 30 mg kg^{-1} ethylbenzene, the activity of SOD was higher than that in the control, while the activity of CAT and POD was lower than that in the control.

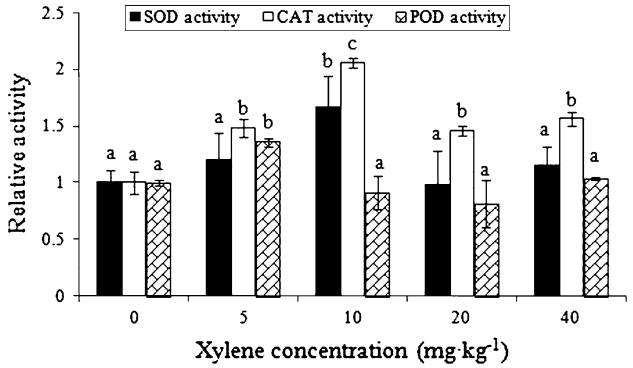

Fig. 3 The activity of SOD, CAT, and POD in earthworms exposed to xylene. Enzymatic activities in xylene treatment groups are represented as the relative activity compared with that in the control. Within the same type of enzymes, means sharing the same letter are not significantly different (Tukey test, $P < 0.05$)

Effects of xylene on the activity of SOD, CAT, and POD in earthworms

As shown in Fig. 3, the activity of SOD at 5 mg kg^{-1} xylene still remained at the control level. While exposed to 10 mg kg^{-1} xylene, the activity of SOD was significantly higher ($P < 0.05$) than that in the control and other treatment groups, and finally decreased to the control level at 20 and 40 mg kg^{-1}, respectively.

The activity of CAT at all concentrations of xylene increased significantly ($P < 0.05$) while compared with that in the control, and reached the peak at 206% of the control under the condition of 10 mg kg^{-1} xylene.

The activity of POD in 5 mg kg^{-1} xylene treatment increased approximately 126%, which was significantly higher ($P < 0.05$) than that in the control, and significantly decreased ($P < 0.05$) to the control level at 10, 20 and 40 mg kg^{-1}, respectively.

In general, exposure of earthworms to 5 mg kg^{-1} xylene yielded significant ($P < 0.05$) increases in the activity of CAT and POD, and the activity of SOD remained at the control level. The activity of SOD and CAT was significantly higher ($P < 0.05$) than that in the control at 10 mg kg^{-1} xylene. While exposed to 20 and 40 mg kg^{-1} xylene, the activity of SOD and CAT was higher than that in the control, in particular, the increase in the activity of CAT was significant ($P < 0.05$), while the activity of POD was lower than that in the control.

DNA damage induced by toluene, ethylbenzene and xylene

As shown in Table 1, all values for OTM in treatment groups had statistically significant ($P < 0.01$) increase versus the controls. The analyses of OTM showed a strong positive response as the concentration of toluene increased, while weak but also statistically significant ($P < 0.01$) responses were observed as the concentrations of ethylbenzene and xylene increased. Under the same concentrations (5 and 10 mg kg^{-1}, respectively), earthworms exposed to toluene had greater DNA damage than ethylbenzene and xylene groups.

There was a strong linear correlation between the mean OTM and concentrations of these three pollutants: toluene, $y = 10.15 + 0.468x$, $R^2 = 0.932$; ethylbenzene, $y = 7.43 + 0.233x$, $R^2 = 0.773$; xylene, $y = 6.83 + 0.265x$, $R^2 = 0.901$; and OTM data are also presented as the distribution of cells (%) (Fig. 4). Of the total cells observed in the solvent control group, 95.7% were in the category <10 OTM, 4.35% were in the category 10-20 OTM, indicating a very low level of DNA damage in solvent control group.

Of the total cells observed, 36.1% were in the category <10 OTM at 5 mg kg^{-1} toluene. However, only 11.6% were in the category <10 OTM at 100 mg kg^{-1} toluene, while 29.0% were in the category >80 OTM, reflecting the severity of DNA damage in this group. Similarly, the increases in the number of damaged cells were observed with increasing concentrations of ethylbenzene and xylene.

Discussion

SOD is a key enzyme in protecting cells against oxidative stress, which converts $\cdot O_2^-$ into O_2 and H_2O_2 (Singh et al. 2006). In the present study the activity of SOD in earthworms was significantly ($P < 0.05$) stimulated by toluene

Table 1 DNA damage in earthworm coelomocytes treated with toluene, ethylbenzene and xylene

Toluene (mg kg^{-1})	OTM	Ethylbenzene (mg kg^{-1})	OTM	Xylene, (mg kg^{-1})	OTM
0	3.21 ± 3.09^{a}	0	3.21 ± 3.09^{a}	0	3.21 ± 3.09^{a}
5	13.85 ± 10.04^{b}	5	8.87 ± 5.93^{b}	5	8.45 ± 4.40^{b}
10	22.50 ± 15.64^{c}	10	12.51 ± 7.74^{c}	10	11.35 ± 10.30^{bc}
50	30.99 ± 27.77^{c}	30	17.58 ± 12.26^{c}	20	12.51 ± 7.79^{c}
100	57.35 ± 47.61^{d}	60	19.38 ± 15.03^{c}	40	16.72 ± 11.53^{c}

Means followed by the same letter within the same column are not significantly different at the level of $P < 0.01$

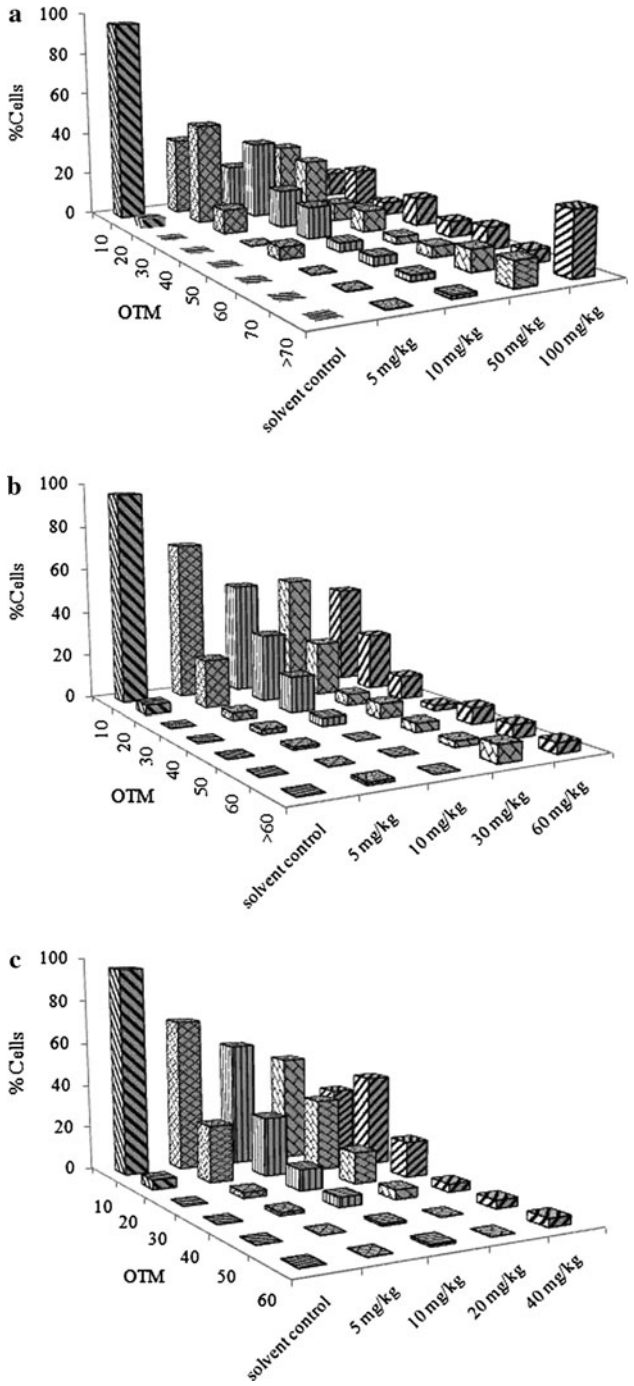

Fig. 4 Effects of toluene (**a**), ethylbenzene (**b**), and xylene (**c**) on the percentage distribution of cells with respect to the OTM in coelomocytes of *E. fetida*

with the low concentration (5 mg kg^{-1}) compared with that in the control. This increase in the activity of SOD had better protection against oxidant damage, and might be related to an increase in the expression level of mRNA or the post-transcriptional activation (Costa et al. 1997), indicated that exposure to low concentration of toluene induced the formation of $O_2^{\cdot-}$ leading to the oxidative stress. However, while exposed to the high concentration of toluene (10 and 50 mg kg^{-1}, respectively), the activity of SOD decreased, but the inducement still occurred. According to Sun et al. (2007), $O_2^{\cdot-}$ could be reduced by other antioxidant defense systems, such as GSH and GST, or metabolized to other radicals, for example, $^{\cdot}OH$ to decrease the SOD activity. The excess $O_2^{\cdot-}$ can insult the activity of SOD and make them inactivated (Sun et al. 2007), a significant ($P < 0.05$) inhibition was observed at 100 mg kg^{-1} compared with that in the control, this possibly as a result of a more severe degree of toluene toxicity, indicating oxidative stress occurred. In the present study, the SOD activity changes induced by toluene were similar to the previous study on arsenate and cadmium where SOD activity increased in response to exposure of low concentration pollutants, but high concentration of pollutants inhibited the activity of SOD (Hartley-Whitaker et al. 2001; Lin et al. 2007). H_2O_2 is also toxic to cells and has to be further detoxified. In the antioxidant enzymatic system, CAT scavenges H_2O_2 into H_2O and O_2, which is found in peroxisomes, cytosol and mitochondria. POD decomposes H_2O_2 by oxidation of co-substrates such as guaiacol or ascorbate as the electron donors (Zhang et al. 2007). While exposed to the low concentration of toluene (5 mg kg^{-1}), the activity of CAT was significantly stimulated ($P < 0.05$) compared with that in the control. However, the level of the POD activity at 5 mg kg^{-1} was similar to that in the control. Due to elevated activities of SOD and CAT, the mechanism of antioxidative defense was still active. While exposed to the high concentration of toluene (10, 50 and 100 mg kg^{-1}, respectively), the activity of both CAT and POD exhibited a slight induction, indicating the generation of H_2O_2 was still within the CAT and POD elimination capacity. It has been reported that the sensitivity of antioxidant enzymes (SOD, CAT and POD) to various oxidative stress varies greatly, indicating that they might respond to oxidative attack in different ways (Holocská et al. 1998). The results in our study indicate that the activity of the three antioxidant enzymes had different responses to the toxicities of toluene within the tested concentration of toluene. The concentration thresholds of the response for SOD and CAT were 5 mg kg^{-1}, the role of POD in earthworms seemed to be limited in the toxicological test of toluene as there was no significant increase in the activity of POD within the range of the tested toluene concentration. The activity of SOD coordinated with the activity of CAT play a central protective role in the $O_2^{\cdot-}$ and H_2O_2 scavenging process. Therefore, the sensibility of these enzymes to toluene could be ranked as follows: SOD (CAT) > POD.

In toxicological tests of ethylbenzene, the activity of SOD was stimulated at 10, 15 and 30 mg kg^{-1} ethylbenzene. This increase in the activity of SOD indicated that

exposure to ethylbenzene induced the formation of $^{\cdot}O_2^-$ leading to the oxidative stress. The activity of CAT was significantly stimulated ($P < 0.05$) at 10 mg kg^{-1} and the activity of POD was significantly stimulated ($P < 0.05$) at 5 mg kg^{-1} ethylbenzene, indicating the formation of H_2O_2. Then the activity of both CAT and POD was inhibited at the high concentration of ethylbenzene (15 and 30 mg kg^{-1}, respectively), this inhibition could be attributed to the H_2O_2 accumulation and indicated that the H_2O_2 scavenging function of CAT and POD was impaired. The activity of SOD, CAT and POD showed a tendency of induction firstly and then inhibition with increasing concentration of ethylbenzene, this bell-shaped response indicated that as far as the balance in the cell functions is maintained, these biomarkers in earthworms could respond to the development of pollutant-induced stress syndrome (Gastaldi et al. 2007). The concentration thresholds of responses for CAT and POD were 10 and 5 mg kg^{-1}, respectively, the activity of SOD was not significantly induced within the range of the tested ethylbenzene concentration. Therefore, the sensibility to ethylbenzene followed the sequence of POD > CAT > SOD.

In toxicological tests of xylene, the activity of SOD was significantly ($P < 0.05$) stimulated at 10 mg kg^{-1} xylene, suggesting that this increase in SOD had better protection against oxidant damage, and then decreased to the level of the control at 20 and 40 mg kg^{-1}, respectively, suggesting that there was a balance in cell compartment between the production of $^{\cdot}O_2^-$ and SOD. The activity of CAT was significantly ($P < 0.05$) stimulated at all concentrations of xylene, especially at 10 mg kg^{-1}, indicating the production of H_2O_2. The activity of POD was significantly ($P < 0.05$) stimulated at 5 mg kg^{-1}, and then decreased to the control level at 10, 20 and 40 mg kg^{-1}, respectively. This decrease was compensated by the induction of CAT activity, and showed that these two enzymes were functioning concurrently to remove H_2O_2, which was similar to the effect of heavy metals on plants (Rizhsky et al. 2002; Cao et al. 2004). The concentration thresholds of responses for SOD, CAT, and POD were 10, 5, and 5 mg kg^{-1}, respectively. Therefore, the sensibility to xylene followed the sequence of CAT > POD > SOD.

Since the comet assay was first described by Ostling and Johanson (1984) and developed by Singh et al. (1988), it has become accepted as a rapid, simple, and sensitive method for measuring DNA damage and repair mechanisms, biomonitoring, and determination of genotoxicity (Singh et al. 1988; Tice et al. 2000; Dhawan et al. 2009). Many parameters (i.e., tail length, % tail DNA, tail moment) have been used to evaluate the extent of DNA migration and DNA damage in the results of the comet assay. Among these parameters, olive tail moment (OTM) incorporates two most reliable primary measurements (amount of damaged DNA and the distance of migration of the genetic material in the tail), has been proved very reliable and can give a good correlation in genotoxicity studies (Olive et al. 1990; Kumaravel and Jha 2006). In the present study, we used OTM to express DNA damage in *E. fetida* exposed to different concentrations of toluene, ethylbenzene, and xylene.

According to US Environmental Protection Agency (US EPA 1999, 2002, 2005), the results from genotoxicity tests of toluene, ethylbenzene, and xylene on animals and bacteria were negative, and these pollutants have been classified as Group D, not classifiable as to human carcinogencity. However, some studies on aquatic organisms by SCGE had demonstrated that the crude and industrial petroleum effluents can induce high level of DNA damage (Hamoutene et al. 2002; Taban et al. 2004; Vanzella et al. 2007). Similarly, in the present study, the results in the comet assay revealed a significant ($P < 0.01$) dose-dependent increase in DNA damage in coelomocytes of *E. fetida* exposed to toluene, ethylbenzene, and xylene as compared with the controls, number of comets in higher OTM category increased with increasing concentrations of the three pollutants, indicating potential genotoxic effects of toluene, ethylbenzene, and xylene on *E. fetida*. This result also showed that the comet assay using coelomocytes of an earthworm is a rapid and sensitive way to assess DNA damage induced by pollutants in terrestrial ecosystems.

The cell distribution of DNA damage in the present study showed that some cells remained undamaged even in the tested highest concentration of the three pollutants. However, even in the controls, some cells showed slightly DNA damage, which was similar to the results of measuring the genotoxicity of some sulfur dyes on tadpoles and a polluted river on fish and earthworm tissues (Rajaguru et al. 2001, 2003). In the present study, Tween-80 (mole fraction $< 2 \times 10^{-3}$) was used as the solvent control and a very low level of DNA damage was observed in this group, the similar results had been described by Zang et al. (2000) using Tween-80 (1%) as negative control in a comet assay.

The results of the present study indicated that toluene seemed to be more genotoxic as it could induce the higher extent of DNA damage than ethylbenzene and xylene. This might be attributed to the different physical and chemical properties they have. The ability of these compounds to dissolve in water will affect their bioavailability in environment (Luo et al. 1999). Water solubility of toluene, ethylbenzene, and xylene are 515, 152 and 160-220 mg l^{-1}, respectively (Mackay et al. 1999), therefore, toluene can be absorbed by *E. fetida* easier than ethylbenzene and xylene, and then show a higher genotoxicity. According to our results, the concentration thresholds required to induce significant DNA damage in

E. fetida exposed to toluene, ethylbenzene, and xylene were 5 mg kg^{-1}, indicating that the exposure concentration was probably not low enough to get the minimum concentration thresholds.

A number of studies have shown that during oxidative processes, the overproduction of ROS can induce many kinds of negative effects including membrane peroxidation, loss of ions, protein cleavage, and even DNA strand breakages (Mittler 2002; Cooke et al. 2003; Ahmad et al. 2009). Similarly, the DNA damage observed in *E. fetida* exposed to toluene, ethylbenzene, and xylene might be attributed to the oxidative attack. According to Vanzella et al. (2007), the monoaromatic hydrocarbons present in diesel water soluble fraction (DWSF) may have generated electrophilic compounds, which in association with DNA molecule sites caused several lesions.

In conclusion, some biomarkers were used in the present study to evaluate oxidative stress and genotoxicity of toluene, ethylbenzene and xylene in earthworms (*E. fetida*). The activity of SOD, CAT and POD could be responded to the exposure of the three pollutants, but the sensitivity of the three enzymes differed. The comet assay was very sensitive and results showed significant induction ($P < 0.01$) in DNA damage, indicating potential genotoxic effects of toluene, ethylbenzene, and xylene on the earthworm *E. fetida*. Therefore, the four biomarkers can be used as a battery to assess effects of the pollutants in terrestrial ecosystems at early stages and with low concentrations.

Acknowledgments The authors acknowledge the financial support by the National Natural Science Foundation of China as a general project (Grant No. 20777040 and Grant No. 20977053) and as a key project (Grant No. 40930739), and by the Ministry of Education, People's Republic of China as a grand fostering project (Grant No. 707011).

References

Ahmad P, Jaleel CA, Azooz MM, Gowher Nabi (2009) Generation of ROS and non-enzymatic antioxidants during abiotic stress in plants. Bot Res Int 2:11–20

An YJ (2004) Toxicity of benzene, toluene, ethylbenzene, and xylene (BTEX) mixtures to *Sorghum bicolor* and *Cucumis sativus*. Bull Environ Contam Toxicol 72:1006–1011

An YJ (2005) Assessing soil ecotoxicity of methyl tert-butyl ether using earthworm bioassay; closed soil microcosm test for volatile organic compounds. Environ Pollut 134:181–186

An YJ, Lee WM (2008) Comparative and combined toxicities of toluene and methyl tert-butyl ether to an Asian earthworm *Perionyx excavates*. Chemosphere 71:407–411

Bonnard M, Eom IC, Morel JL, Vasseur P (2009) Genotoxic and reproductive effects of an industrially contaminated soil on the earthworm *Eisenia fetida*. Environ Mol Mutagen 50:60–67

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Cao X, Ma LQ, Tu C (2004) Antioxidative responses to arsenic in the arsenic-hyperaccumulator Chinese brake fern (*Pteris vittata* L.). Environ Pollut 128:317–325

Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003) Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 17:1195–1214

Costa V, Amorim MA, Reis E, Quintanilha A, Moradas-Ferreira P (1997) Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. Microbiology 143:1649–1656

Dhawan A, Bajpayee M, Parmar D (2009) Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biol Toxicol 25:5–32

Dou J, Liu X, Hu Z, Deng D (2008) Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction. J Hazard Mater 151: 720–729

Eyambe GS, Goven AJ, Fitzpatrick LC, Venables BJ, Cooper EL (1991) A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies. Lab Anim 25:61–67

Fourie F, Reinecke SA, Reinecke AJ (2007) The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. Ecotoxicol Environ Saf 67:361–368

Gastaldi L, Ranzato E, Caprì F, Hankard P, Pérés G, Canesi L, Viarengo A, Pons G (2007) Application of a biomarker battery for the evaluation of the sublethal effects of pollutants in the earthworm *Eisenia Andrei*. Comp Biochem Physiol C 146: 398–405

Hamoutene D, Payne JF, Rahimtula A, Lee K (2002) Use of the comet assay to assess DNA damage in hemocytes and digestive gland cells of mussels and clams exposed to water contaminated with petroleum hydrocarbons. Mar Environ Res 54:471–474

Hartley-Whitaker J, Ainsworth G, Meharg AA (2001) Copper- and arsenate-induced oxidative stress in *Holcus lanatus L.*clones with differential sensitivity. Plant Cell Environ 24:713–722

Holocská K, Lenártová V, Rosival I, Kičinková M, Majerčiaková A, Legáth J (1998) Antioxidant and detoxifying enzymes in the liver and kindney of pheasants after intoxication by herbicides MCPA and ANITEN I. J Biochem Mol Toxicol 12:235–244

Końca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wojcik A (2003) A cross-platform public domain PC image-analysis program for the comet assay. Mutat Res 543: 15–20

Kumaravel TS, Jha AN (2006) Reliable comet assay measurements for detecting DNA damage induced by ionizing radiation and chemicals. Mutat Res 605:7–16

Li Y, Zhou Q, Li F, Liu X, Luo Y (2008) Effects of tetrabromobisphenol A as an emerging pollutant on wheat (*Triticum aestivum*) at biochemical levels. Chemosphere 74:119–124

Lin AJ, Zhang XH, Chen MM, Cao Q (2007) Oxidative stress and DNA damages induced by cadmium accumulation. J Environ Sci (China) 19:596–602

Lukkari T, Taavitsainen M, Soimasuo M, Oikari A, Haimi J (2004) Biomarker responses of the earthworm *Aporrectodea tuberculate* to copper and zinc exposure: differences between populations with and without earlier metal exposure. Environ Pollut 129: 377–386

Luo Y, Zang Y, Zhong Y, Kong ZM (1999) Toxicological study of two novel pesticides on earthworm *Eisenia fetida*. Chemosphere 39:2347–2356

Mackay D, Shiu WY, Ma KC, Lee SC (1999) Physical-chemical properties and environmental fate handbook. CRC netBase, Chapman & Hall, Boca Raton

Marklund S, Marklund G (1974) Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Mater L, Sperb RM, Madureira LAS, Rosin AP, Correa AXR, Radetski CM (2006) Proposal of a sequential treatment methodology for the safe reuse of oil sludge-contaminated soil. J Hazard Mater 136:967–971

McGregor D (1994) The genetic toxicology of toluene. Mutat Res 317:213–228

Mehlman MA (1992) Dangerous and cancer-causing properties of products and chemicals in the oil refining and petrochemical industry. VIII. Health effects of motor fuels: carcinogenicity of gasoline— Scientific update. Environ Res 59:238–249

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Murata M, Tsujikawa M, Kawanishi S (1999) Oxidative DNA damage by minor metabolites of toluene may lead to carcinogenesis and reproductive dysfunction. Biochem Biophys Res Commun 261:478–483

Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the 'comet' assay. Radiat Res 122:86–94

Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298

Plaza GA, Wypych J, Berry C, Brigmon RL (2007) Utilization of monocyclic aromatic hydrocarbons individually and in mixture by bacteria isolated from petroleum-contaminated soil. World J Microbiol Biotechnol 23:533–542

Radic S, Cvjetko P, Glavas K, Roje V, Pevalek-Kozlina B, Pavlica M (2009) Oxidative stress and DNA damage in broad bean (*Vicia faba* L.) seedlings induce by thallium. Environ Toxicol Chem 28:189–196

Rajaguru P, Kalpana R, Hema A, Suba S, Baskarasethupathi B, Kumar PA, Kalaiselvi K (2001) Genotoxicity of some sulfur dyes on tadpoles (*Rana hexadactyla*) measured using the comet assay. Environ Mol Mutagen 38:316–322

Rajaguru P, Suba S, Palanivel M, Kalaiselvi K (2003) Genotoxicity of a polluted river system measured using the alkaline comet assay on fish and earthworm tissues. Environ Mol Mutagen 41:85–91

Reinecke SA, Reinecke AJ (2004) The comet assay as biomarker of heavy metal genotoxicity in earthworms. Arch Environ Contam Toxicol 46:208–215

Rizhsky L, Hallak-Herr E, Van Breusegem F, Rachmilevitch S, Barr JE, Rodermel S, Inzé D, Mittler R (2002) Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate preoxidase or catalase. Plant J 32:329–342

Salagovic J, Gilles J, Verschaeve L, Kalina I (1996) The comet assay for the detection of genotoxic damage in the earthworms: a promising tool for assessing the biological hazards of polluted sites. Folia Biol 42:17–21

Salanitro JP, Dorn PB, Huesemann MH, Moore KO, Rhodes IA, Rice Jackson LM, Vipod TE, Western MM, Wisniewski HL (1997) Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. Environ Sci Technol 31:1769–1776

Schreck E, Geret F, Gontier L, Treilhou M (2008) Neurotoxic effects and metabolic responses induced by a mixture of six pesticides on the earthworm *Aporrectodea caliginosa nocturna*. Chemosphere 71:1832–1839

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in an aquatic plant, *Bacopa monnieri* L. Chemosphere 62:233–246

Song Y, Zhu LS, Wang J, Wang JH, Liu W, Xie H (2009) DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia fetida*) induced by atrazine. Soil Biol Biochem 41: 905–909

Sun Y, Yin G, Zhang J, Yu H, Wang X (2007) Bioaccumulation and ROS generation in liver of freshwater fish, goldfish *Carassius auratus* under HC Orange No.1 exposure. Environ Toxicol 22:256–263

Taban IC, Bechmann RK, Torgrimsen S, Sanni S (2004) Detection of DNA damage in mussels and sea urchins exposed to oil using comet assay. Mar Environ Res 58:701–705

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–211

US EPA (1999) IRIS toxicological review of ethylbenzene. US Environmental Protection Agency, Washington, DC

US EPA (2002) IRIS toxicological review and summary documents of xylenes (external review draft). US Environmental Protection Agency, Washington, DC

US EPA (2005) Toxicological review of toluene (CAS NO. 108–88-3). US EPA, Washington, DC

Vanzella TP, Martinez CBR, Cólus IMS (2007) Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species. Mutat Res 631:36–43

Verschaeve L, Gilles J (1995) Single cell gel electrophoresis assay in the earthworm for the detection of genotoxic compounds in soils. Bull Environ Contam Toxicol 54:112–119

Xiao RY, Wang Z, Wang CX, Yu G, Zhu YG (2006) Genotoxic risk identification of soil contamination at a major industrialized city in northeast China by a combination of in vitro and in vivo bioassays. Environ Sci Technol 40:6170–6175

Zang Y, Zhong Y, Luo Y, Kong ZM (2000) Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. Environ Pollut 108:271–278

Zhang FQ, Wang YS, Lou ZP, Dong JD (2007) Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere 67:44–50