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A Battery of Bioassays for the Evaluation of Phenanthrene Biotoxicity in Soil

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Abstract A battery of bioassays was used to assess the ecotoxicological risk of soil spiked with a range of phenanthrene levels (0.95, 6.29, 38.5, 58.7, 122, and 303 μg g^{-1} dry soil) and aged for 69 days. Multiple species (viz. *Brassica rapa*, *Eisenia feotida*, *Vibrio fischeri*), representing different trophic levels, were used as bioindicator organisms. Among acute toxicity assays tested, the *V. fischeri* luminescence inhibition assay was the most sensitive indicator of phenanthrene biotoxicity. More than 15 % light inhibition was found at the lowest phenanthrene level (0.95 μg g^{-1}). Furthermore, comet assay using *E. fetida* was applied to assess genotoxicity of phenanthrene. The strong correlation ($r^2 \geq 0.94$) between phenanthrene concentration and DNA damage indicated that comet assay is appropriate for testing the genotoxic effects of phenanthrene-contaminated soil. In the light of these results, we conclude that the Microtox test and comet assay are robust and sensitive bioassays to be employed for the risk evaluation of polycyclic aromatic hydrocarbon-contaminated soil.

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Intensive use of biocides, urban wastes, and industrial activities lead to the discharge of a wide range of toxic chemicals, including heavy metals and organic pollutants, in soils (Lors et al.. 2010; Khan et al. 2012b), which disturbs the balance between soil flora and fauna, contaminates foods and groundwater, and constitutes a threat for living organisms. Phenanthrene, a polycyclic aromatic hydrocarbon (PAH), is usually introduced into the environment through natural (e.g., thermal geologic production) and anthropogenic sources (e.g., forest fires, combustion of fossil fuels, and other organic material combustion) (Reid et al. 2000; Semple et al. 2003; Manzo et al. 2008). There are concerns over the presence of phenanthrene in the environment due to its low aqueous solubility, ability to adsorb or to be absorbed by solid materials or soil organic matter, and persistent nature (Macleod and Semple 2000; Doick et al. 2005). Hence, phenanthrene poses toxic, genotoxic, mutagenic, and/or carcinogenic effects to living organisms (Armstrong et al. 2004; Khan et al. 2011).

The traditional approach to risk assessment of polluted soil, based on analysis of the concentrations of pollutants in the soil, is not enough for biological assessment and does not provide indication of toxic effects of pollutants on the biota (Calisi et al. 2011). Hence, new biological approaches to soil monitoring, such as the measurement of biochemical and cellular responses to pollutants (i.e., biomarkers) on soil organisms (i.e., bioindicators), have become increasingly important in hazard assessment and remediation for determining clean-up end points (Calisi et al. 2011). Many researchers around the world have developed a range of biological assays to examine the ecotoxicological effects of hydrocarbon in soils (Juhasz et al. 2010; Gandolfi et al. 2010; Lors et al. 2010, 2011; Khan et al. 2012b; Tang et al. 2011, 2012).

Plant bioassays, such as seed germination and seedling growth, have been used to assess the biotoxicity of soils polluted with PAHs and other hydrocarbons (Chouychai et al. 2007; Lors et al. 2010; Tang et al. 2011, 2012; Kummerova et al. 2013). Many indicator plants, such as *Lolium perenne* (Cheema et al. 2009), *Brassica rapa* (Song et al. 2005; Khan et al. 2012b), and *Lactuca sativa* (Lors et al. 2010, 2011), have been used to evaluate the health of PAH-polluted soils.

Due to their key role in organic matter breakdown, nutrient recycling, and soil formation (Calisi et al. 2011), earthworms have long been used as a key bioindicator organism in ecotoxicological studies (Gandolfi et al. 2010; Amorim et al. 2011; Wu et al. 2012; Sforzini et al. 2012). *Eisenia fetida* is the standard test species recommended by the Organization for Economic Cooperation and Development (2003) and has been used to evaluate PAHs and other hydrocarbon pollution (Eom et al. 2007; Lors et al. 2011; Tang et al. 2011; Manier et al. 2012; Khan et al. 2012a, b). The single-cell gel electrophoresis assay, also known as the "comet assay," which employs coelomcytes from earthworms, is known to be one of the most sensitive methods available to evaluate various levels of genotoxic agents in polluted soils by detecting DNA double-strand breaks (DSBs) and damaged alkali-sensitive sites (Cui et al. 2009; Gandolfi et al. 2010; Khan et al. 2012b).

Because of their rapid response to the bioavailable fraction of contaminants, bacteria are commonly used to assess the ecological health of polluted environments (Hayat et al. 2002). Luminescence-based bacterial biosensors are sensitive, simple, and reproducible and are widely used indicators of microbial response to polluted soil porewaters (Manzo et al. 2008; Tang et al. 2011, 2012; Khan et al. 2012b; Steliga et al. 2012).

Most of the previous studies focused only on a single indicator organism (Ma et al. 1999; Reinecke and Reinecke 2004; Zheng et al. 2008; Amorim et al. 2011) or a single concentration of pollution (Eom et al. 2007) to compare toxicity end points of PAH-contaminated soils. Thus, there are research gaps in the selection of appropriate toxicity indicators according to the level of soil contamination. Furthermore, the comparisons between different tests to determine the soil ecological integrity by employing the biological indicators are also scarcely found in the literature (Khan et al. 2012b). Hence, to fill the knowledge gaps and to address these research issues, the main purpose of this study was to evaluate the acute toxicity, subchronic toxicity, and genotoxicity of soil contaminated with PAHs and to assess the comparative sensitivity of bioassays for the evaluation of biotoxicity of PAH-polluted soil. For this purpose, two separate sets of experiments were performed using a series of concentrations of pyrene and phenanthrene in soil. The results of pyrene toxicity studies have been previously described elsewhere (Khan et al. 2012b). Here, we only reported the results of bioassays evaluating phenanthrene biotoxicity.

Materials and Methods

Chemicals

Phenanthrene of 99.9 % purity was obtained from Sigma Aldrich, UK. All of the other chemicals used in the study were of analytical grade.

Soil Collection and Preparation

An uncontaminated soil was collected from the upper 15-cm layer of an experimental rice field at Hua Jia Chi campus of Zhejiang University, Hangzhou, China (31°16/N, 120°12/E). The soil was collected and prepared according to the procedure described in Khan et al. (2012b). The soil properties, such as particle size distribution (50.5 % sand, 37 % silt, and 12.5 % clay), identified the soil as a sandy loam soil. The organic matter was 2.1 %, and the pH was 5.95. The cation exchange capacity was 7.76 cmol kg^{-1}, and electrical conductivity was 254.5 μS cm^{-1}. The nutrient levels were 17.8 μg g^{-1} total nitrogen, 9.39 μg g^{-1} total phosphorous, and 9.81 μg g^{-1} total potassium.

Soil Spiking and Storage

Six different levels of phenanthrene, viz. 1.12, 8.52, 44.5, 73, 136, and 335 μg g^{-1}, were used in triplicate for soil spiking. Soil spiking procedure and storage conditions were the same as described in Khan et al. (2012b). After 69 days of aging, Soxhlet-extracted concentrations of phenanthrene in soil were 0.95, 6.29, 38.5, 58.7, 122, and 303 μg g^{-1}.

Plant Assay

Plant assay using Chinese cabbage (*B. rapa*) was performed as described in Khan et al. (2012b). The light-to-dark temperatures were maintained at 26/24 °C, and the relative humidity was set at 85 %. The experiment was performed in triplicate.

*E. fet*ida Growth and Survival Test

Earthworms (*E. fetida*) were exposed to spiked and unspiked soil samples according to the procedure described in Khan et al. (2012b). During exposure tests, water was added every day to replenish water lost. Controls were also prepared in triplicate. Earthworm biomass and survival tests were also

performed in triplicate according to Khan et al. (2012b). In lethality tests (end point survival), results can be expressed as lower–observed adverse effect concentrations (LOAEC values [μg g^{-1}]) compared with controls.

Comet Assay

After exposure to phenanthrene in soil, noninvasive extrusion method was used to obtain the earthworm coelomocytes (Rajaguru et al. 2003). Comet assay was performed according to the method described in Khan et al. (2012b). All of the experimental steps were performed under dim red light at 4 °C to avoid additional DNA damage.

Comet images were obtained using a Nikon digital camera (Nikon corporation, Tokyo, Japan). The five comet parameters—tail length (TL) (distance from nuclear center to the end of the comet tail), tail DNA percentage (TD% [expressed by the percent of fluorescent intensity in tail]), head DNA percentage (HD% [expressed by the percent of fluorescent intensity in head]), olive tail moment (OTM) (product of the distance between the center of gravity of the head and the center of the gravity of the tail and percent tail DNA), and tail moment (TM) (product of TL and TD)—were recorded (Khan et al. 2012b) and calculated by image-analysis system comet assay software project (CASP), developed by Konca et al. (2003). TM was quantified using the computerized image-analysis system, covered both the length of DNA migration in the comet tail and the tail intensity, and is considered to be one of the best indices of DNA damage (De Boeck et al. 2000).

Microtox Test

V. fischeri (strain NRRLB-11177), the marine Gram-negative luminescent bacteria, were used throughout this experiment. The experimental conditions were similar to those of Ma et al. (1999) and Khan et al. (2012b). The assay was performed using the following concentration of 0.1 ml bacterial suspension, 0.9 ml test medium, and 1.9 salt media (28.1 g l^{-1} NaCl, 0.77 g l^{-1} KCl, 1.6 g l^{-1} $CaCl_2{\cdot}2H_2O$, 4.8 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.11 g l^{-1} $NaHCO_3$, and 3.5 g l^{-1} $MgSO_4{\cdot}7H_2O$ supplemented with 10 g l^{-1} meat extract and 10 g l^{-1} peptone for bacteriology) to a glass tube. The whole suspension was mixed thoroughly, and Toxicity Analyzer DXY-2 (Nanjing Institute of Soil Science, Chinese Academy of Sciences) at 22 ± 1 °C was used to measure the initial light unit. The decrease in bioluminescence was measured at a constant temperature of 22 ± 1 °C after 5 and 15 min. The percent inhibition of light emission from a treated aliquot corrected for loss of light in the control was used to quantify toxicity using the following formula (Eq. 1):

$$\%\ \text{Inhibition} = 100 \times \left[\left(I^0 - I^t\right)/I^0\right], \quad (1)$$

where I^0 is the initial bacterial luminescence, and I^t is the luminescence after addition of the toxic compound in the bacterial suspension.

Statistical Analysis

All of the results were analysed using Statistical Package for the Social Sciences 16.0 (SPSS, Chicago, IL) for Windows (George and Mallery 2009). Significant differences ($P < 0.05$) between controls and treated samples were calculated by one-way analysis of variance. Correlation analysis was performed using Pearson correlation.

Results and Discussion

Acute and Subchronic Toxicity Assays

Seed germination and root elongation of B. rapa

B. rapa was tested for its ability to germinate and grow in soil artificially contaminated with different concentrations of phenanthrene (viz. 0.95, 6.29, 58.7, and 303 μg g^{-1}). As listed in Table 1, the inhibitory effect of phenanthrene on seed germination percentage of *B. rapa* was not significant compared with the control. Similarly, no significant effect of phenanthrene was observed on the germination index of *B. rapa* compared with that of the control (Table 1). The results suggest that the seed-germination test is a poor indicator of phenanthrene pollution. Similar to our results, other studies have reported that seed germination is not a suitable test to be used for hydrocarbon pollution (Gong et al. 2001; Smith et al. 2006; Juhasz et al. 2010). However, recently, Lors et al. (2011), using lettuce as experimental plant, found that the seed germination test was good indicator and more sensitive compared with lettuce growth–inhibition bioassay or earthworm mortality.

The root elongation test is a convenient and good indicator of phytotoxicity (Kummerova et al. 2013). In the present study, a significant ($P < 0.05$) inhibitory effect of phenanthrene on root elongation of *B. rapa* was found compared with the control (Table 1). In addition, a concentration-dependent response of root elongation in *B. rapa* was observed under our experimental conditions. Increasing phenanthrene concentrations in soil significantly decreased the root elongation in *B. rapa.* These results are in agreement with those of Song et al. (2005) and Eom et al. (2007), who observed similar responses of *B. rapa* to pyrene or other PAHs and concluded that root elongation assay of *B. rapa* was sensitive and suitable biotest for the evaluation of PAH-contamination

Table 1 Seed germination, germination index, and root length of *B. rapa* (mean ± SE) grown in soil aged 69 days spiked with varying levels of phenanthrene

Phenanthrene concentration in soil (μg g^{-1})	Percent seed germination		Germination index	Root length (cm)
	24 h	48 h	48 h	48 h
0	95.0 ± 5.0[a]	99.3 ± 1.9[a]	19.4 ± 0.60[a]	2.71 ± 0.14[a]
0.95	95.0 ± 5.0[a]	96.7 ± 2.9[a]	19.2 ± 0.76[a]	2.57 ± 0.08[a]
6.29	91.7 ± 2.9[a]	96.7 ± 2.9[a]	18.8 ± 0.58[a]	2.40 ± 0.05[ab]
58.7	88.3 ± 7.6[a]	95.0 ± 0.0[a]	18.3 ± 0.76[a]	2.20 ± 0.07[bc]
303	90.0 ± 5.5[a]	96.7 ± 2.9[a]	18.7 ± 0.29[a]	2.15 ± 0.03[c]

Variants with the same lower-case superscript letter in a column are not significantly different at $P < 0.05$

PAHs are known to cause genetic mutation and slow growth as well as enhance the sensitivity of the plant to other stresses (Chouychai et al. 2007). Ogboghodo et al. (2004) investigated that phenanthrene induces perturbations in soil and plants that have a negative impact on root elongation. Phenanthrene is a group of chemicals with hydrophobic nature. Hydrophobic properties in any compound have the ability to prohibit or decrease water and gas exchange and nutrient absorption. The toxicity of hydrocarbons in barley and field pea can easily damage cell membranes. This damage in membranes ultimately decreases metabolic transport and respiration rate (Xu and Johnson 1995). The results of the phenanthrene toxicity in present study are in concordant with the previous studies performed by Eom et al. (2007) on seed germination or Kummerova et al. (2013) on root elongation using either the same or different plants species.

Growth of E. fetida

E. fetida was selected as a representative terrestrial invertebrate to assess the adverse effects of phenanthrene on survival and growth of animal. Earthworm weight alteration can depict pollutant stress and link pollutant effects to energy dynamics and finally growth inhibition (Khan et al. 2012b). After 28 days incubation in phenanthrene-polluted soil, no significant ($P < 0.05$) difference of frozen dry-weight loss of earthworms was observed for different levels of phenanthrene (Table 2). Nevertheless, worm fresh weight was significantly decreased at 6.29 μg g^{-1} (i.e., 0.206 ± 0.012 g) compared with the control (i.e., 0.235 ± 0.019 g), and the percent decrease in fresh weight after 28 days of incubation in 6.29 μg g^{-1} soil was 28.4 %, which is significantly greater than that of control (i.e., 8.04 %) level (Table 2). These results are in contrary to our previous findings (Khan et al. (2012b), where the effect of pyrene on fresh weight of *E. fetida* was not significant. In addition, a high value of fresh weight loss (i.e., 8.04 % compared with initial fresh-weight value) in the control group was found, showing that the decrease in worm weight was not only due to contaminant toxicity but might also be due to some other unknown factors (i.e., nutrition) (Khan et al. 2012b). Furthermore, this decrease in earthworm fresh weight maybe related to earthworms' decreased food intake to avoid contaminants (Wu et al. 2012).

In the present study, after 28 days of exposure, the phenanthrene LOAEC value (μg g^{-1}) of earthworm weight loss was 6.29 (Table 3). The effect of greater concentrations of phenanthrene (ranging from 6.29 to 303 μg g^{-1}) on the change in fresh weight of *E. fetida* was significant. Similar to our findings, Zheng et al. (2008) found that the mean weight loss of earthworm was significantly greater in soil amended with 10 μg g^{-1} phenanthrene compared with the control. Similar result were shown by Tang et al. (2011) and by Wu et al. (2012), who both reported that with the increasing concentrations of hydrocarbons, the inhibition of growth enhanced greatly. In this study, however, the earthworm weight-loss biotest did not show high sensitivity to phenanthrene in soil compared with other bioassays tested.

Survival of E. fetida

As a biomarker, earthworm survival is an important tool for soil biomonitoring and ecotoxicological studies (Juhasz et al. 2010; Calisi et al. 2011). In this study, no significant negative influence on *E. fetida* survival was found at the lower concentrations (viz. 0.095, 6.29 and 58.7 μg g^{-1}) of phenanthrene throughout the experiment (Fig. 1). Nevertheless, at greater levels of phenanthrene a time-dependent response of mortality in *E. fetida* was found. After 1 week of incubation, 0 and 13 % mortality were found in 122 and 303 μg g^{-1} phenanthrene-amended soil, respectively (Fig. 1). A significant increase in mortality (13 %) was observed after 2 weeks of exposure at 122 μg g^{-1}; later, after 28 days of exposure, mortality increased to 60 % at the same concentration of phenanthrene (i.e., 122 μg g^{-1}). One hundred percent mortality was found at a level of 303 μg g^{-1} phenanthrene after 14- and/or 28-day

Table 2 Fresh weight, change in fresh weight, and freeze-dried weight of *E. fetida* after 28-day incubation in phenanthrene-polluted soil

Phenanthrene concentration in soil (μg g^{-1})	FW ew^{-1} before exposure (g)	FW ew^{-1} after exposure (g)	FW loss after exposure (%)	DW ew^{-1} after exposure (g)
0	0.342 ± 0.031[a]	0.235 ± 0.019[a]	8.04 ± 1.7[b]	0.043 ± 0.002[a]
0.95	0.345 ± 0.020[a]	0.233 ± 0.031[a]	9.85 ± 9.6[b]	0.038 ± 0.006[a]
6.29	0.347 ± 0.037[a]	0.206 ± 0.012[a]	28.4 ± 2.05[a]	0.037 ± 0.002[a]
58.7	0.366 ± 0.012[a]	0.219 ± 0.017[a]	30.5 ± 6.56[a]	0.037 ± 0.006[a]
122	0.349 ± 0.020[a]	0.210 ± 0.010[a]	27.3 ± 4.58[a]	0.036 ± 0.001[a]
303	0.349 ± 0.035[a]	ND	ND	ND

FW fresh weight, *DW* dry weight, *ew* earthworm, *ND* not detected (earthworm did not survive at this concentration)

Values are the means of three replications ± SE. Variants with the same lower-case *superscript letter* in a column are not significantly different from each other at $P < 0.05$

Table 3 LOAEC values (μg g^{-1}) and relative sensitivity of bioassays performed on phenanthrene-polluted soil

Values and sensitivity	Luminescence inhibition (min)		Earthworm survival (d)			Earthworm weight loss		Chinese cabbage seed germination (h)		Chinese cabbage root elongation (h)
	5	15	7	14	28	Fresh	Dry	24	48	48
LOAEC (μg g^{-1})	0.95	0.95	303	122	122	6.29	303	303	303	6.29
Sensitivity (TU)	1.70	0.82	–	0.33	0.82	0.33	0.33	–	–	0.33

$TU = 100/EC_{50}$

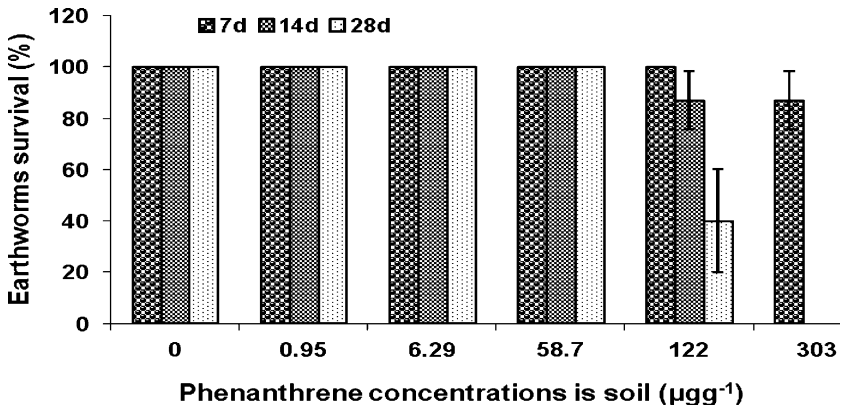

Fig. 1 Percent survival of *E. fetida* at different incubation times (i.e., 7, 14, and 28 days) under varying levels of phenanthrene (i.e. 0 [control], 0.95, 6.29, 58.7, 122, and 303 μg g^{-1}). *Columns* and *error bars* represent means and SDs ($n = 3$), respectively

incubation periods. The LOAEC value (μg g^{-1}) of phenanthrene was 122 at longer incubation times (14 and 28 days) as listed in Table 3. The mean calculated LC_{50} value for phenanthrene after 28 days of exposure was 122 μg g^{-1}.

The results of the present study indicate that earthworm survival assay is a more sensitive biotest than fresh- or dry-weight loss of earthworm. Nevertheless, in the present study, we found that the earthworm survival test was less sensitive compared with other bioassays (Microtox). Our these results agree with those of Eom et al. (2007) and Lors et al. (2011), who reported that bioassays of other terrestrial organisms was more sensitive than the earthworm survival test. In addition, these findings are consistent with results of our previous studies on pyrene biotoxicity (Khan et al. 2012b). In addition, due to its lower sensitivity, the earthworm survival assay was not recommended for soil-risk evaluation (Van Gestel and Weeks 2004).

Bioluminescence Inhibition of *V. fischeri*

In this study, a range of phenanthrene concentrations (from 0.095 to 303 μg g^{-1}) in soil was used to evaluate the suitability and sensitivity of Microtox acute test. A concentration- and time-dependent change in light levels and resulting change in the toxicity was found in phenanthrene-contaminated soil (Fig. 2). Percent luminescence inhibition of *V. fischeri* increased with increase in phenanthrene

levels. Percent luminescence inhibition was maximal (56.6 %) at the highest level (i.e., 303 μg g^{-1}) of phenanthrene. The LOAEC value (μg g^{-1}) of phenanthrene on the luminescence inhibition of *V. fischeri* was 0.95 at both 5- and 15-min assay as listed in Table 3. The mean calculated EC_{50} value for phenanthrene was 58.7 μg g^{-1}.

In several recent studies, Microtox test of *V. fischeri* proved to be the most sensitive assay employed as part of a battery of tests (Gandolfi et al. 2010; Tang et al. 2011; Steliga et al. 2012). The results of present study also indicate that Microtox test was the most sensitive test system among the tested biological assays. The findings of current study do not support the results of studies performed by Macken et al. (2008) and Matejczyk et al. (2011), who found Microtox test to be a poor indicator of PAH toxicity. However, in these research studies, tests were performed on different PAH concentrations under different experimental conditions.

Furthermore, results of Microtox test showed that the toxicity of phenanthrene decreased with increasing incubation time (Fig. 2). These findings support the results of Macken et al. (2008) and Khan et al. (2012b), who both reported that toxicity of PAHs to *V. fischeri* decreased with lengthening incubation time. However, in a review article by Salizzato et al. (1997), it was shown that a 5-min EC_{50} value is sufficient to report for organic pollutants. Our present results do not support the findings of Salizzato et al. (1997) because the toxicity of phenanthrene to *V. fischeri* changed with incubation time (Fig. 2).

In brief, the results of different biological assays showed their varying sensitivities to soils polluted with phenanthrene. Although end points are different in ecotoxicological significance, comparisons are made between the responses of the different indicator organisms used in this study (Table 3). The results of the acute-toxicity values, expressed in toxic units (TU = 100/EC_{50}), showed greater sensitivity of Microtox test than the sensitivities of either the earthworm survival or growth tests. The sensitivity ranking for phenanthrene toxicity in the current study was in the following increasing order: luminescence inhibition (5 min) > luminescence inhibition (15 min) > earthworm survival (28 days) > earthworm survival (14 days) > root elongation of Chinese cabbage > earthworm fresh weight loss > earthworm dry-weight loss (Table 3). These findings are consistent with previously published reports that underline the sensitivity of acute and subchronic end points (Mendonca and Picado 2002; Tang et al. 2011; Khan et al. 2012b). In the light of comparative results of sensitivity, it is concluded that Microtox (5-min exposure) was the most sensitive test among all of the biological assays used in the current study.

Genotoxicity Assays

DNA Damage in E. fetida

The analysis of comet TLs and damage classes was traditionally used for determination of DNA damage levels in earthworm coelomocytes (Reinecke and Reinecke 2004). In this study, TL, TM, OTM, HD%, and TD% were selected to show the final comet assay results. Significantly greater DNA damage was detected in earthworms exposed to phenanthrene-polluted soil compared with the control soil (Table 4). The values of TL, TM, OTM, and TD% of DNA damage in the control soil were low and consistent. A concentration-dependent response of DSBs in earthworms was found with DNA damage enhancing with increasing levels of phenanthrene in soil (Table 4). Greater DSBs, with mean TM ranging from 9.51 to 15.7 μm, were observed at the 38.5 and 58.7 μg g^{-1} levels of phenanthrene, respectively. The mean TM value at 0.95 μg g^{-1} of phenanthrene was still greater than the mean TM value of the control. Likewise, the mean OTM values were 15, 29, 59, and 82 times greater at 0.95, 6.29, 38.5, and 58.7 (μg g^{-1}) phenanthrene, respectively, than the value of OTM at control. Similarly, TL values of DNA damage

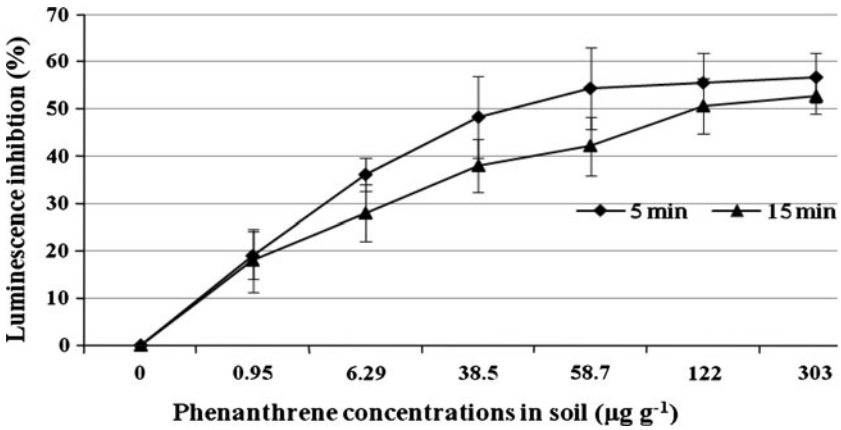

Fig. 2 Percent luminescence inhibition of *V. fischeri* under varying levels of phenanthrene (0 [control], 0.95, 6.29, 58.7, 122, and 303 μg g^{-1}) after 5 (*black diamond*) and 15 (*black triangle*) minutes of incubation. *Error bars* represent SDs ($n = 3$)

Table 4 Distribution and mean values of DSBs in coelomocytes of *E. fetida* exposed to soil (aged 69 days spiked with varying concentrations of phenanthrene) for 2-day in vivo bioassay

PHE[a] (μg g^{-1})	TL (μm)	TM (μm)	OTM (μm)	HD (%)	TD (%)
0	15.1 ± 0.59[e]	0.13 ± 0.23[d]	0.21 ± 0.23[e]	99.6 ± 0.59[a]	0.23 ± 0.22[e]
0.95	29.5 ± 3.32[d]	1.63 ± 1.58[d]	3.24 ± 2.74[d]	96.1 ± 3.32[b]	4.25 ± 2.82[d]
6.29	41.5 ± 4.71[c]	4.37 ± 3.44[c]	6.17 ± 4.13[c]	92.4 ± 4.71[c]	7.03 ± 4.89[c]
38.5	61.4 ± 5.33[b]	9.51 ± 4.76[b]	12.4 ± 5.69[b]	87.8 ± 5.33[d]	12.1 ± 5.48[b]
58.7	73.6 ± 11.3[a]	15.7 ± 12.1[a]	17.3 ± 11.7[a]	81.7 ± 11.3[e]	17.1 ± 11.8[a]

Values are the means of 50 comet readings ± SE. Variants with the same lower-case superscript letter in each column are not significantly different from each other at $P < 0.05$

PHE phenanthrene levels

Table 5 Correlation between phenanthrene concentrations in soil and *E. fetida* DNA damage parameters

Parameters[a]	Pearson correlation coefficients (r^2)				
	TL	TM	OTM	HD	TD
PHE	0.94*	0.98**	0.97**	–0.95*	0.95*
TL		0.97**	0.99**	–0.99**	0.99**
TM			0.99**	–0.99**	0.98**
OTM				–0.99**	0.99**
HD					–0.99**

PHE phenanthrene levels

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

were also 4- to 5-fold greater at the greater phenanthrene levels (viz. 38.5 and 58.7 μg g^{-1}) compared with the control. In brief, the different parameters exhibited the greater values for all of the phenanthrene levels (ranging from 0.95 to 58.7 μg g^{-1}) compared with the control. The results suggest that genotoxicity bioassay should be considered as part of the battery of tests for the risk assessment of PAH-polluted environments.

Although evaluation of genotoxic effects of contaminants is challenging due to the diverse and complex nature of soil (Donnelly et al. 2004), the comet assay is capable of detecting DSBs in individual cells after in vivo or in vitro exposure (Khan et al. 2012b; Sforzini et al. 2012). In addition, comet assay is considered to be a rapid and sensitive biomarker for the quantification of genotoxic effects (Gandolfi et al. 2010; Khan et al. 2012b; Sforzini et al. 2012). Several studies reported heterogeneity in DNA-damage parameters and showed that the peaks of the distributions shifted upward with increasing concentrations of the pollutant (Qiao et al. 2007; Khan et al. 2012b). Likewise, in the present study, both nondamaged DNA and seriously fragmented DNA were obtained with heterogeneous distribution (Table 4). The invariable low DSBs values in the controls are assumed to be background values derived from endogenous and unavoidable exogenous sources (Qiao et al. 2007; Khan et al. 2012b). Nevertheless, DSBs, compared with the controls after phenanthrene exposure, yielded significantly greater levels, which points to an exogenous genotoxic-pollutant as a major cause of the genetic damage (Khan et al. 2012b).

As listed in Table 5, a strong correlation was observed between DNA damage parameters (viz. TL, TM, OTM, HD%, and TD%) and phenanthrene concentrations in soil. TM was strongly correlated ($r^2 = 0.98$) with phenanthrene concentrations compared with other parameters taken in this study. Our results are in agreement with those of Qiao et al. (2007) and Gichner et al. (2007), who found a good correlation between TM and genotoxicants. In the light of these results, DNA damage in earthworms is a sensitive and appropriate biotest and can be employed to evaluate the risk of PAH-pollution to invertebrates. In other words, the DNA damage assay in *E. fetida* is a simple, robust, and highly sensitive method to detect genotoxicity of phenanthrene-polluted soil (Singh et al. 1988; Cui et al. 2009; Gandolfi et al. 2010; Khan et al. 2012b).

Based on these findings and after screening the published scientific work, we propose that Microtox test and comet assay (DNA damage) should be preferably used as early assessment tools for PAHs-contaminated/-remediated soil when the level of pollution is expected to be low. However, at greater levels of soil contamination, a battery of biological assays (containing a combination of acute toxicity, chronic toxicity, and genotoxicity tests) should be preferably employed to assess ecotoxicological status of soil.

Conclusion

In the present study, a battery of bioassays, using *B. rapa, E. fetida,* and *V. fischeri,* was employed to evaluate the ecological health of soil artificially contaminated with varying levels of phenanthrene. Results are consistent with our previously reported data of pyrene biotoxicity. The

results indicated that seed germination of *B. rapa* and survival of *E. fetida* may not be appropriate bioassays to be used as monitoring tools for soil with low-level PAH pollution. Nevertheless, comet assay and Microtox test are sensitive and could be appropriate monitoring tools for the risk evaluation of low to highly PAH-contaminated soil. In general, each of the selected bioassays is ecological relevant, inexpensive, easy to conduct, and suitable to be used for toxicological studies. Furthermore, our findings indicate that a single biological test does not sufficiently evaluate soil quality. Hence, the application of an integrated approach consisting of multiple ecological-relevant test species is needed to evaluate ecological health of polluted environment.

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