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Biotoxicity Assessment of Pyrene in Soil Using a Battery of Biological Assays

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Abstract A test battery, composed of a range of biological assays, was applied to evaluate the ecological health of soil aged for 69 days and spiked with a range of pyrene levels (1.04, 8.99, 41.5, 72.6, 136, and 399 μg g^{-1} dry soil; Soxhlet-extracted concentrations after 69 days of aging). Chinese cabbage (*Brassica rapa*), earthworm (*Eisenia fetida*), and bacteria (*Vibrio fischeri*) were used as test organisms to represent different trophic levels. Among the acute ecotoxicity bioassays used, the *V. fischeri* luminescence inhibition assay was the most sensitive indicator of pyrene toxicity. We observed >8 % light inhibition at the lowest concentration (1.04 μg g^{-1}) pyrene, and this inhibition increased to 60 % at 72.6 μg g^{-1}. The sensitivity ranking for toxicity of the pyrene-contaminated soil in the present study was in the following decreasing order: root elongation of Chinese cabbage < earthworm mortality (14 days) < earthworm mortality (28 days) < luminescence inhibition (15 min) < luminescence inhibition (5 min). In addition, genotoxic effects of pyrene were also evaluated by using comet assay in *E. fetida*. The strong relationship between DNA damage and soil pyrene levels showed that comet assay is suitable for testing the genotoxicity of pyrene-polluted soil. In addition, tail moment was well correlated with soil pyrene levels ($r^2 = 0.99$). Thus, tail moment may be the most informative DNA-damage parameter representing the results of comet assay. Based on these results, the earthworm DNA damage assay and Microtox test are rapid and sensitive bioassays and can be used to assess the risk of soil with low to high levels of hydrocarbon pollution. Furthermore, an analysis of the toxic effects at several trophic levels is essential for a more comprehensive understanding of the damage caused by highly contaminated soil.

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Industrial and human activities lead to the discharge of a wide range of toxic chemicals in soils (Lors et al. 2010). Risk assessment of polluted soil samples are usually performed by means of chemical measurements, but these analyses alone may not be enough for biological assessment (Fernandez et al. 2005). A physicochemical analysis of all of the pollutants causing toxicity, in addition to their synergistic or antagonistic effects, is impossible. Hence, a biological method is practicable to integrate the effects of all of the bioavailable pollutants and their interaction (Eom et al. 2007; Tang et al. 2011).

Biological indicators have become important tools in hazard assessment and remediation for estimating clean-up end points. Studies concerned with determining the ecological effects of hydrocarbons in polluted soils have developed a range of bioindicators from microbial assays to flora and faunal organisms (Manzo et al. 2008; Juhasz et al. 2010; Gandolfi et al. 2010; Lors et al. 2010, 2011; Tang et al. 2011). For bioindicators to be effective, they must have key roles in the functioning of soil ecosystems;

integrate physico-chemical and biological properties; and have representative and reproducible responses to enable unambiguous comparisons between soils (Broos et al. 2005).

Seed germination and seedling growth tests have been used to monitor the toxicity of soils contaminated with organic contaminants during bioremediation processes (Chouychai et al. 2007; Lors et al. 2010; Tang et al. 2011). These plant bioassays in soil-pollution studies have gained considerable attention to assess hydrocarbon-contaminated soil health (Manzo et al. 2008). Indicator plants used to assess hydrocarbon-contaminated soils include perennial rye grass *Lolium perenne L.* (Cheema et al. 2009), Chinese cabbage *Brassica rapa L.* (Song et al. 2005; Labud et al. 2007), Lettuce *Lactuca sativa L.* (Lors et al. 2010, 2011), and white mustard *Sinapis alba L.* (Manzo et al. 2008). These soil toxicity-measuring plant assays serve as bioindicator response end points due to their simple methodology and potential use both in situ and *ex situ*.

Because they are bioindicators of soil pollution, earthworms have long been used as a key indicator organism of ecotoxicology diagnosis (Gandolfi et al. 2010; Tang et al. 2011; Amorim et al. 2011; Wu et al. 2012). Within the past 30 years, researchers around the world have created numerous earthworm ecotoxicology methods to test the presence of contaminants, and these have been nationally or internationally registered and tested (Organization for Economic Cooperation and Development [OECD] 1984; International Standard Organization 1998). The litter-dwelling earthworm, *Eisenia fetida* (Savigny), which commonly inhabits composts (Spurgeon et al. 2003), is the standard test species recommended by the OECD and has been used to assess hydrocarbon contamination (Eom et al. 2007; Lors et al. 2010, 2011; Tang et al. 2011; Khan et al. 2011, 2012). The single-cell gel electrophoresis (SCGE) assay introduced by Ostling and Johanson (1984) and developed by Singh et al. (1988) is the most promising bioassay in environmental monitoring and now plays a key role in many domains of science. In addition, known as the "comet assay," SCGE assay employing coelomocytes from earthworms has been recognized as one of the most sensitive methods available to asses various levels of genotoxic agents in contaminated soils by detecting DNA single-strain breaks and damaged alkali-sensitive sites (Cui et al. 2009; Gandolfi et al. 2010).

Among microorganisms, bacteria are commonly used to evaluate ecological health because they respond rapidly to the bioavailable fraction of pollutants (Hayat et al. 2002). Marker-based bacterial biosensors provide new and exciting opportunities to monitor contaminants in the environment due to their sensitivity, simplicity, and reproducibility (Tecon et al. 2006). Luminescence-based bacterial biosensors are widely used indicators of microbial response to contaminated soil porewaters (Eom et al. 2007; Manzo et al. 2008; Tang et al. 2011).

Polycyclic aromatic hydrocarbons (PAHs) are a group of the most ubiquitous organic contaminants that form from incomplete combustion of fossil fuel and are of environmental concern due to their high toxicity and carcinogenicity (Manzo et al. 2008; Khan et al. 2012). Pyrene is a tetracyclic aromatic hydrocarbon and one of the 16 PAHs on the priority list of the United States Environmental Protection Agency (USEPA; Khan et al. 2011).

Several previous studies have compared toxicity end points of PAH-polluted soils either using a single indicator organism (Ma et al. 1999; Reinecke and Reinecke 2004; Zheng et al. 2008; Amorim et al. 2011) or a single level of contamination (Eom et al. 2007). However, studies on the selection of suitable toxicity indicators according to level of soil contamination—and studies using a battery of soil acute, chronic, and geno-toxicity tests with varying levels of contamination—are clearly lacking. In addition, comparisons between tests commonly used for the biological assessment of soil quality are also scarce. Thus, the ultimate goal of this study was to assess acute, subchronic, and geno-toxicity of soil polluted with varying levels of pyrene using bioindicators representing different trophic levels; plants (e.g., *B. rapa*), microorganisms (e.g., *Vibrio fischeri*), and invertebrates (e.g., *E. fetida*) in the terrestrial compartments and to evaluate the comparative sensitivity and suitability of biotests for the assessment of biotoxicity of PAH-contaminated soil.

Materials and Methods

Chemicals

Pyrene of 99 % purity was purchased from Sigma Aldrich Co. Ltd., UK. All of the other chemicals used in the study were of analytical grade.

Soil Collection and Preparation

Uncontaminated soil with undetectable pyrene 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 air-dried and passed through a 2 mm sieve to remove stones and roots (Khan et al. 2011, 2012). The 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

Soil was spiked with six different concentrations of pyrene (i.e., 1.07, 9.72, 47.5, 88.4, 152, and 429 μg g^{-1} dry soil) in triplicate according to Khan et al. (2012). Spiked soil was stored at 20 ± 0.5 °C for aging (69 days) before extraction and analysis. Each sample, including the blank soil sample for background check, was prepared in triplicate. Soil moisture was maintained at 60 % water-holding capacity during storage. After 69 days of aging, Soxhlet-extracted concentrations of pyrene in soil were 1.04, 8.99, 41.5, 72.6, 136, and 399 μg g^{-1}. In the present study, various concentrations of pyrene were used to evaluate the potential and applicability of bioassays at varying levels of soil contamination (Chouychai et al. 2007).

Plant Assay

Chinese cabbage (*B. rapa*) was chosen to reflect the typical species found in the region. Uniform seeds of Chinese cabbage were surface sterilized in a 3 % H_2O_2 for 20 min and rinsed with distilled water 10 times (Ali et al. 2011). Fifty grams of the spiked and unspiked mixtures were placed in Petri plates (12 cm diameter) and made moist enough to sow the *B. rapa* seeds into. Twenty seeds per Petri plate of Chinese cabbage were sown, and moisture was maintained during germination. The Petri plates were kept in a controlled chamber with photoperiod of 16:8 h light-to-dark photoperiod intensity of 225 ± 25 μmol m^{-2} s^{-1}. The light-to-dark temperatures were set at 26/24 °C, and relative humidity was maintained at 85 %. The experiment was performed in three sets. The seeds were considered germinated with the emergence of radicals. The plant germination and growth assay was performed according to OECD guideline 208 (2003).

Animal Treatment

Earthworms (*E. fetida*) were raised at 22 ± 2 °C in soil in the laboratory and fed on pig manure. Healthy earthworms chosen for the assays weighed approximately 350 mg and had well-developed clitellum. For in vivo bioassay, selected worms were starved for 48 h on filter paper soaked with phosphate-buffered saline before exposure. Five earthworms were then exposed to 100 g freeze-dried soil moistened with distilled water in a glass jar. The jars were covered with aluminum foil that had 15 small holes for aeration, then put under lamplight to prevent worms from escaping the soil. During exposure tests, water was added every day to replenish water lost. Controls were also prepared in triplicate.

Earthworm Biomass and Survival Test

To assess lethality and sublethal effects, selected worms were divided into three groups and exposed to contaminated soil for different exposure times (i.e., 7, 14, and 28 days). All of the tests were performed in triplicate. All other conditions were the same as described previously. Worms were examined on days 7, 14, and 28 for lethality. Worms were again depurinated and reweighed on the following days (8, 15, and 29), and change in weight was calculated on both a fresh and a freeze-dried basis. In lethality tests (end point survival), results can be expressed as lower observed adverse effect concentrations (LOAEC values [ppm or μg g^{-1}]) compared with controls.

Comet Assay

After exposure, earthworm coelomocytes were obtained by noninvasive extrusion method (Rajaguru et al. 2003). Comet assay was performed according to Pandrangi et al. (1995) with slight modifications. All steps were conducted under dim red light and performed at 4 °C to prevent additional DNA damage. A portion of a 15 μL cell suspension was mixed with 85 μL 1 % low-melting agarose, and the obtained mixture was spread on a normal-melting agarose-precoated slide. After solidification of the agarose, the gel slide was covered with 85 μL 1 % low-melting agarose. The gel slide was then placed in freshly made, ice-cold lysis solution [2.5 mol l^{-1} NaCl, 100 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), and 10 mmol l^{-1} Tris (pH 10.0–10.5) with freshly added 1 % Triton X-100 and 10 % dimethyl sulfoxide] for 1 h. The gel slide was then put into an electrophoresis tank containing freshly made buffer [300 mmol l^{-1} NaOH and 1 mmol l^{-1} EDTA (pH = 13 ± 0.1)] for 20 min before electrophoresis in the same buffer for 20 min at 25 V and 300 mA. After electrophoresis, the gel slide was neutralized with 0.4 mol l^{-1} Tris solution (pH 7.5 ± 0.1) and dehydrated with pure ethanol. Before analysis by microscope, the dried gel was rehydrated and stained in 50 μl 0.01 % SYBR Gold solution (Molecular Probes, Taastrup, Denmark). At least 50 cells/gel slide were examined randomly. The slides were analyzed under a Leica DMLB florescence microscope at 400× magnification with a 450- to 490-nm emission filter and an LP515 excitation filter. For each parallel set of slides, 50 nonoverlapping comets were captured randomly.

Three slides were analyzed for each treatment and control. A Nikon digital camera captured images of the comets. Image-analysis system CASP, developed by Konca et al. (2003), was employed to measure comet parameters, and five parameters were recorded and calculated, includ-

ing 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). TM was measured by the computerized image-analysis system and encompassed both the length of DNA migration in the comet tail and the tail intensity. TM is considered to be one of the best indices of induced DNA damage among the various parameters (De Boeck et al. 2000).

Microtox Test

The marine Gram-negative luminescent bacterium *V. fischeri* (strain NRRLB-11177) was used throughout this bioassay. In brief, freeze-dried bacteria donated by the Research Center for EcoEnvironmental Sciences, Chinese Academy of Sciences, China, were revived and cultivated before testing. The culture and assay methods were similar to Ma et al. (1999). Briefly, each bacterium was inoculated from a stock culture, which is maintained on culture medium agar at 4 °C, to a fresh agar and cultured at 22 ± 1 °C for 24 h. We further grew the cells in liquid culture medium by shaking (120 rpm) at 22 ± 1 °C for 18 h and harvested them by centrifuge at 3,000 rpm for 10 min. The pellet was resuspended with test medium and centrifuged twice. Bacterial suspension was kept at 22 ± 1 °C for 30 min before testing. In this bioassay, pyrene solutions, prepared in ultra-pure water, were adjusted to the desired pH (approximately 6.5–7.5). This range of pH has been found to cause no changes in growth and metabolism under these experimental conditions (Fulladosa et al. 2004). The assay was conducted in triplicate. The assay was performed by adding 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. It was thoroughly mixed, and the initial light unit was recorded by Model Toxicity Analyzer DXY-2 (Nanjing Institute of Soil Science, Chinese Academy of Sciences) at 22 ± 1 °C.

The resulting decrease in bioluminescence was measured after 5 and 15 min at a constant temperature of 22 ± 1 °C. Toxicity was measured as percent inhibition of light emission from a treated aliquot corrected for loss of light in the control as:

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

where I^0 is the initial bacterial luminescence and I^t is the luminescence after introduction of the toxic compound in the bacterial suspension. Assay result can be expressed as effective concentration. The effective concentration (EC_{50}) is defined as the concentration that produces a 50 % decrease in light emission after 5- and 15-min exposures.

Statistical Analysis

All of the biological and chemical analyses were performed with Statistical Package for the Social Sciences 16.0 (SPSS, Chicago, IL) for windows (George and Mallery 2009). One-way analysis of variance was applied to detect significant differences ($P < 0.05$) between controls and treated samples. The correlation analysis was conducted using Pearson correlation.

Results and Discussion

Acute and Subchronic Toxicity Tests

Seed Germination and Root Elongation of Chinese Cabbage

Seeds of *B. rapa* were exposed to different levels of pyrene (i.e., 1.04, 8.99, 72.6, and 399 μg g^{-1}) to assess the adverse effects of pyrene on seed germination and root elongation. The results showed that pyrene, at all concentrations (1.04 to approximately 399 μg g^{-1}), had no significant inhibitory effect on the seed germination percentage of *B. rapa* compared with the control (Table 1). This suggests that seed-germination assay is not a good indicator of pyrene contamination. This is in agreement with the findings of other researchers, who also found seed germination to be a poor indicator of hydrocarbon pollution (Gong et al. 2001; Smith et al. 2006; Juhasz et al. 2010). However, our results are in contrary to the findings of Lors

Table 1 Germination and root elongation of Chinese cabbage (mean ± SE) grown in soil aged 69 days and spiked with varying concentrations of pyrene

Pyrene concentration in soil (μg g^{-1})	Germination (%)		Root elongation (cm)
	24 h	48 h	48 h
Ck	96.7 ± 2.9[a]	98.3 ± 2.9[a]	2.33 ± 0.03[a]
1.04	93.3 ± 7.6[a]	96.7 ± 2.9[a]	2.27 ± 0.15[a]
8.99	93.3 ± 2.8[a]	98.3 ± 2.9[a]	2.21 ± 0.08[a,b]
72.6	90.0 ± 5.0[a]	96.7 ± 2.9[a]	2.10 ± 0.05[b,c]
399	90.0 ± 0.0[a]	96.7 ± 2.9[a]	2.01 ± 0.01[c]

Variants possessing the same letters (columns) are not significantly different at $P < 0.05$

et al. (2011), who concluded that lettuce germination assay was more sensitive compared with other bioassays (i.e., lettuce growth inhibition, earthworm mortality). However, we used different pyrene concentrations and a different indicator plant (i.e., *B. rapa*) from those of Lors et al. (2011), who tested seed germination by exposing *L. sativa* seeds to different levels of PAHs.

Pyrene posed significant ($P < 0.05$) inhibitory effects on root elongation of *B. rapa* compared with the control. Root elongation in *B. rapa* decreased significantly with increasing pyrene levels in soil, with the exception of the 1.04 μg g^{-1} concentration (Table 1). In the present study, the results of the root elongation assay agree with those of other researchers (Song et al. 2005; Eom et al. 2007). Song et al. (2005) found a decreasing trend in root length with increasing pyrene concentrations and concluded that root-elongation assay of *B. rapa* was more sensitive than seed-germination assay. In addition, Eom et al. (2007) observed that tests based on plant growth were more sensitive for the detection of ecotoxicity in the soil than those based on seed germination.

The negative influence of pyrene on root elongation may be due to the disorder it causes in soil and plants (Ogboghodo et al. 2004). Due to its hydrophobic properties, pyrene may coat roots and thus prohibit or decrease gas and water exchange and nutrient absorption. It is known that pyrene damages cell membranes and blocks intercellular spaces, thus decreasing respiration rate and metabolic transport (Xu and Johnson 1995). The results obtained in the this study for pyrene toxicity are in agreement with findings of other studies performed on seed germination and toxicity using both the same or different plants species (Eom et al. 2007).

Earthworm Fresh/Dry Weight

In this study, *E. fetida* was chosen as a representative terrestrial invertebrate to evaluate the toxic effects of pyrene on animal physiology (growth and survival). Earthworm weight change can indicate contaminant stress and link contaminant effects to energy dynamics and ultimately growth inhibition (Wu et al. 2012). After 28 days of exposure to pyrene-contaminated soil, no significant ($P < 0.05$) difference in fresh and frozen dry-weight losses of *E. fetida* was found for different concentrations of pyrene (Table 2). However, fresh weight was relatively less at 136 μg g^{-1} (i.e., 0.185 ± 0.007 g) compared with the control (i.e., 0.219 ± 0.025 g), and the percent decrease in fresh weight after 28 days of exposure to 136 μg g^{-1} soil was 46.7 %, which is greater than that of the control (i.e., 37.3 %) levels. Furthermore, a high value of fresh weight loss (i.e., 37.3 % compared with initial fresh-weight value) in the control group was observed, which indicates that the decrease in weight of the worms was not only due to pyrene toxicity but might also be due to some other unknown factors (i.e., nutrition) (Spurgeon et al. 2003). In addition, this decrease in earthworm fresh weight may be related to earthworms' decreased food intake to avoid pollutants (Wu et al. 2012).

The LOAEC value (μg g^{-1}) of pyrene on earthworm weight loss was 136 after 28 days of exposure (Table 3). Moreover, no concentration-dependent response of weight change in *E. fetida* was noticed in the test performed. According to the present investigation, the effect of different concentrations of pyrene (ranging from 1.04 to 399 μg g^{-1}) on the change in fresh or dry weight of *E. fetida* was negligible. However, our results are contrary to the findings of Zheng et al. (2008), who found that the mean weight loss of earthworms was significantly greater in soil with 10 μg g^{-1} phenanthrene compared with the control. Similar results were reported by Tang et al. (2011) and by Wu et al. (2012), who both found that the inhibition rate of earthworm body weight or growth increased significantly with increasing concentrations of hydrocarbons. In the present study, however, the earthworm weight-loss bioassay showed insignificant sensitivity to pyrene in soil.

Table 2 Fresh weight, change in fresh weight, and freeze-dried weight of earthworms after 28 days of exposure to pyrene-contaminated soil

Pyrene concentration in soil (μg g^{-1})	Fresh weight (ew^{-1}) Before exposure (g)	Fresh weight (ew^{-1}) After exposure (g)	Fresh weight loss After exposure (%)	Dry weight (ew^{-1}) After exposure (g)
Ck	0.348 ± 0.019	0.219 ± 0.025[a]	37.3 ± 4.95[a]	0.036 ± 0.005[a]
1.04	0.350 ± 0.023	0.215 ± 0.034[a]	38.4 ± 11.5[a]	0.041 ± 0.007[a]
8.99	0.357 ± 0.028	0.215 ± 0.034[a]	40.1 ± 4.88[a]	0.040 ± 0.005[a]
72.6	0.364 ± 0.014	0.209 ± 0.020[a]	42.4 ± 7.05[a]	0.037 ± 0.005[a]
136	0.348 ± 0.018	0.185 ± 0.005[a]	46.7 ± 3.63[a]	0.034 ± 0.001[a]
399	0.349 ± 0.020	ND	ND	ND

Values are the means of three replications ± SE. Variants possessing the same letter in each column are not significantly different from each other at $P < 0.05$

ew^{-1} weight per earthworm, *ND* not detected (earthworms did not survive at this concentration)

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

LOAEC values and sensitivity	Luminescence inhibition (min)		Earthworm survival (days)			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})	1.04	1.04	136	136	136	136	136	399	339	8.99
Sensitivity (TU[a])	1.38	1.38	–	0.74	0.74	0.25	0.25	–	–	0.25

[a] TU (Toxic units): $100/EC_{50}$

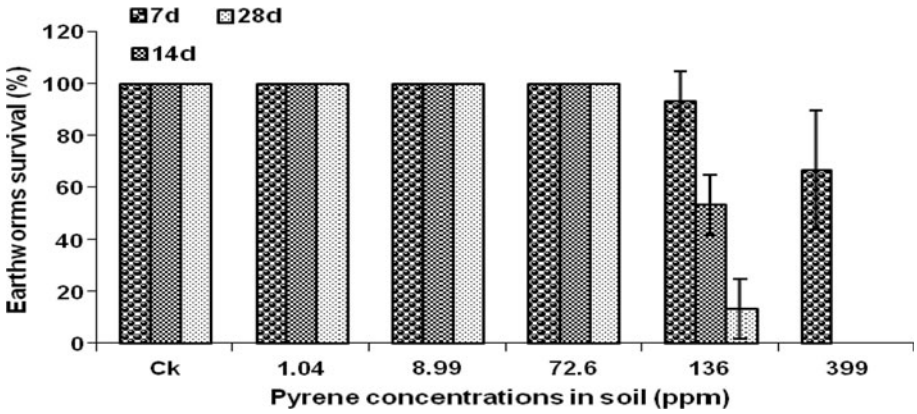

Fig. 1 Percent survival of earthworm (*E. fetida*) at different exposure times (7, 14, and 28 days) under varying concentrations of pyrene (i.e., 0 [Ck], 1.04, 8.99, 72.6, 136, and 399 ppm or μg g^{-1}). *Columns* and *error bars* representing means and SDs ($n = 3$), respectively. *Ck* means Control

Earthworm Survival

The earthworm mortality bioassay, as a bioindicator, is an important tool for ecotoxicological studies (Eom et al. 2007; Juhasz et al. 2010). In this study, no significant effect on earthworm survival was observed at the lower levels (i.e., 1.04, 8.99, and 72.6 μg g^{-1}) of pyrene throughout the experiment (Fig. 1). However, the time-dependent response of mortality in *E. fetida* was observed at higher concentrations. After 7 days of exposure, 6.7 and 32.7 % mortality was observed in 136 and 399 μg g^{-1} pyrene-amended soil, respectively (Fig. 1). A significant increase in mortality (46.7 %) was found after 14 days of exposure at 136 μg g^{-1}; later, after 28 days of exposure, mortality increased to 86.7 % at the same level of pyrene (i.e., 136 μg g^{-1}). Mortality was 100 % at 399 μg g^{-1} after 28 days of exposure to pyrene-amended soil. The LOAEC value (μg g^{-1}) of pyrene was 136 at all exposure times (7, 14, and 28 days) as listed in Table 3. The mean calculated LC_{50} values for pyrene after 28 days of exposure was 136 μg g^{-1}.

In the present study, we observed that the earthworm mortality assay was a more sensitive bioassay than earthworm weight loss (on fresh and dry basis). However, the earthworm survival assay was less sensitive compared with other bioassays (Microtox) employed in the present study. Our findings are in agreement with those of Eom et al. (2007) and Lors et al. (2011), who found that an earthworm survival assay was less sensitive than bioassays of other terrestrial organisms. In another study, earthworm survival was reported to be less sensitive than other end points (reproduction) and was not recommended for soil risk assessment (Van Gestel and Weeks 2004). However, most recently, Amorim et al. (2011) found that phenanthrene had significant effects on both survival and reproduction of *E. albidus*, with an EC_{50} of 33 μg g^{-1} dry soil.

Microtox Assay

Toxic responses to pyrene, using the Microtox acute test, were determined and are presented in Fig. 2. A range of pyrene levels (from 1.04 to 399 μg g^{-1}) in soil was used to assess the suitability and sensitivity of the test. A time- and concentration-dependent change in light levels and resulting change in the toxicity was observed in pyrene-polluted soils. Percent luminescence inhibition of *V. fischeri* increased as pyrene concentrations increased ≤72.6 μg g^{-1} and then decreased slightly at 136 and 399 μg g^{-1} compared with 72.6 μg g^{-1}. The percent luminescence inhibition was maximal (62.5 %) at the third highest concentrations (i.e., 72.6 μg g^{-1}) of pyrene, whereas lower inhibition (61 and 58 %) occurred at 136 and 399 μg g^{-1}, respectively. The reason for this anomaly is not known. The LOAEC value (μg g^{-1}) of pyrene on luminescence inhibition of *V. fischeri* was 1.04 at both 5 and 15 min as listed in Table 3. The mean calculated EC_{50} value for pyrene was 72.6 μg g^{-1}.

The Microtox assay of *V. fischeri* has been widely recognized as a sensitive test system, and in several recent studies it has proven to be the most sensitive assay used as

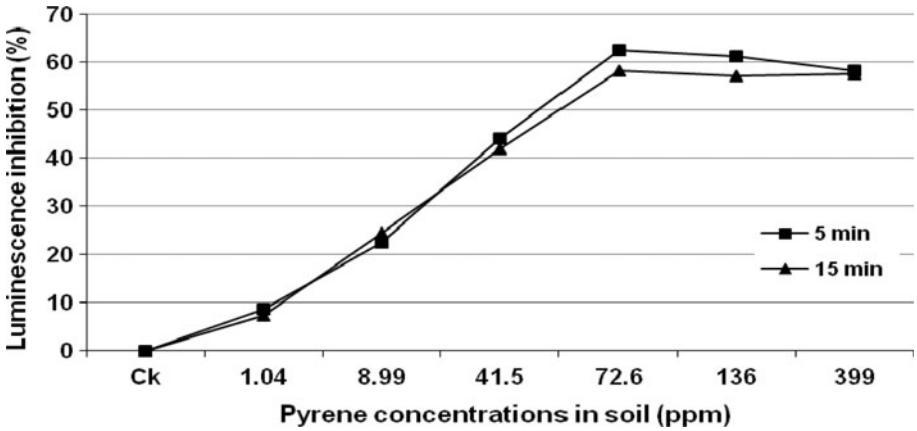

Fig. 2 Percent luminescence inhibition of *V. fischeri* under varying concentrations of pyrene (0 [Ck], 1.04, 8.99, 41.5, 72.6, 136, and 399 ppm or μg g^{-1}) after 5 (*black squares*) and 15 (*black triangle*) min of exposure. *Ck* means Control

part of a battery testing approach (Gandolfi et al. 2010; Tang et al. 2011). In the current study, we also found that the Microtox assay was the most sensitive test system among the tested bioassays. These results have been compared with those reported by other researchers (Macken et al. 2008; Matejczyk et al. 2011) who found the Microtox assay to be the least sensitive indicator to the toxicity of PAHs or mixed pollution. However, in these studies different PAHs levels were used and tests were performed under different experimental conditions.

We also found that the toxicity of pyrene using Microtox assay decreased with increasing exposure time (Fig. 2). These findings are in accordance with those of Macken et al. (2008) who found that toxicity of PAHs to *V. fischeri* decreased with lengthening exposure time. However, Salizzato et al. (1997) found that a 5 min EC_{50} value is adequate to report for organic contaminants. Our results are not in agreement with the findings of Salizzato et al. (1997) because the toxicity of pyrene to *V. fischeri* varied with exposure time (Fig. 2).

In short, the results of different bioassays showed their varying sensitivities to soils contaminated with pyrene. Although ecotoxicological significance of the various end points clearly differs, the responses of the different species used in testing can be compared. The acute-toxicity values, expressed in toxic units (TU = $100/EC_{50}$), indicated higher sensitivity of the *V. fischeri* acute test applied to soil water extracts compared with either the *E. fetida* survival or growth tests. The sensitivity ranking for the toxicity of pyrene-contaminated soil in the present study was in the following decreasing order: earthworm fresh-weight loss < earthworm dry-weight loss < earthworm mortality (14 days) < earthworm mortality (28 days) < luminescence inhibition (15 min) < luminescence inhibition (5 min) (Table 3). These results are consistent with previously published literature that underlines the sensitivity of acute and chronic end points (Mendonca and Picado 2002; Tang et al. 2011). From the sensitivity comparative results, we found that Microtox (5 min exposure luminescence inhibition) assay was the most sensitive parameter among all of the bioassays employed in the present study.

Genotoxicity Tests

Comet Assay

The DNA damage of earthworm coelomocytes was conventionally determined by comet TLs and damage classes (Reinecke and Reinecke 2004). In this study, we chose TL, TM, OTM, HD%, and TD% to express the final comet assay results. A significantly greater amount of DNA damage occurred in earthworms exposed to pyrene-contaminated soil than those exposed to control soil (Table 4).

The values of all of the parameters (TL, TM, OTM, and TD%) of DNA damage in the control soil remained low and consistent. A concentration-dependent response of DNA double-strand breaks (DSBs) in earthworms was observed with DNA damage increasing with increasing concentrations of pyrene in soil (Table 4). The greater DSBs, with mean TM ranging from 7.04 to 14.2 μm, were shown at the 41.5 and 72.6 μg g^{-1} concentrations of pyrene, respectively. At 1.04 μg g^{-1} pyrene, the mean TM value was still greater than that of the control. Similarly, the mean OTM values were 5, 10, 22, and 30 times greater at 1.04, 8.99, 41.5, and 72.6 (μg g^{-1}) pyrene, respectively, than the value of OTM at control. Likewise, TL values of DNA damage were also 4- to 5-fold greater at the higher pyrene concentrations (i.e., 41.5 and 72.6 μg g^{-1}) compared with the control. Briefly, values of all of the parameters taken in this assay were greater at all pyrene concentrations used in the experiment (ranging from 1.04 μg g^{-1} to 72.6 μg g^{-1}) compared with the control. This indicates the suitability and higher sensitivity of the comet assay. Therefore, a bioassay for genotoxicity should be taken into consideration as part of the battery of tests. The genotoxicity assessment of pollutants in terrestrial

Table 4 Distribution and mean levels of DSBs in coelomocytes of earthworms exposed to soil (aged 69 days and spiked with varying levels of pyrene) for 2-day in vivo bioassay

Pyrene (μg g^{-1})	TL (μm)	TM (μm)	OTM (μm)	HD (%)	TD (%)
Ck	14.5 ± 12.1^{e}	0.12 ± 0.24^{c}	0.47 ± 0.60^{d}	99.4 ± 0.87^{a}	0.59 ± 0.86^{e}
1.04	27.1 ± 23.6^{d}	1.36 ± 1.96^{c}	$2.89 \pm 2.94^{c,d}$	96.6 ± 3.18^{b}	3.42 ± 3.17^{d}
8.99	39.8 ± 21.6^{c}	3.09 ± 4.20^{c}	5.33 ± 4.17^{c}	93.8 ± 4.96^{c}	6.16 ± 4.95^{c}
41.5	60.1 ± 18.7^{b}	7.04 ± 5.46^{b}	11.7 ± 5.84^{b}	89.2 ± 5.56^{d}	10.8 ± 5.55^{b}
72.6	69.8 ± 32.9^{a}	14.2 ± 17.8^{a}	15.3 ± 13.3^{a}	84.1 ± 12.5^{e}	15.9 ± 12.4^{a}

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

ecosystems is challenging due to the complex nature of these environments (Donnelly et al. 2004). However, the comet assay is capable of examining DSBs in individual eukaryotic cells after in vivo or in vitro exposure and is conceived to be a sensitive biomarker for the identification and quantification of genotoxicity (Faust et al. 2004; Gandolfi et al. 2010).

Many studies showed that comet parameters tended to be heterogeneous and that the peaks of the distributions shifted upward with increasing levels of the contaminant (Pandrangi et al. 1995; Qiao et al. 2007). Similarly in our study, using in vivo bioassay, both nondamaged DNA and seriously fragmented DNA were found, and the distribution was not homogenous, even though the dominant ratio of DSBs shifted to the serious or less damaged population depending on pyrene concentrations (Table 4). The constant low DSB levels in the controls are assumed to be background values derived from endogenous and unavoidable exogenous sources (Qiao et al. 2007). However, DSBs with pyrene exposure showed significantly greater levels compared with the controls, which points to an exogenous genotoxicant as a major cause of the genetic damage.

A strong relationship was found between DNA damage parameters (i.e., TL, TM, OTM, HD%, and TD%) and pyrene levels in soil as listed in Table 5. The tail moment was strongly correlated ($r^2 = 0.99$) with pyrene levels compared with other parameters measured in the present study. These results agree with the findings of other researchers (Qiao et al. 2007; Gichner et al. 2007), who found a good relationship between TM and contaminants. Based on these results, DNA damage in earthworms is a sensitive and suitable bioassay and can be used to assess the risk of hydrocarbon soil pollution to invertebrates. In other words, the comet assay is a rapid, sensitive, and simple approach to detect DNA damage in *E. fetida* exposed to pyrene-contaminated soil (Singh et al. 1988; Cui et al. 2009; Gandolfi et al. 2010).

In the light of these results and after screening the published literature, we suggest that the comet assay (DNA damage) and Microtox test should be preferred as early assessment tools for PAH-polluted/-remediated soils when the level of pollution is expected to be feeble or when nothing is known at all. At higher levels of soil pollution, a battery of bioassays, combining acute, chronic and genotoxicity tests, should be preferred to monitor soil ecological health, and the results should be combined or compared according to one's needs.

Table 5 Relationship between pyrene levels in soil and earthworm DNA damage parameters

Parameters	Pearson correlation coefficients (r^2)				
	TL	TM	OTM	HD	TD
PYR	0.93*	0.99**	0.97**	−0.97**	0.97**
TL		0.93*	0.99**	−0.98**	0.98**
TM			0.96**	−0.98**	0.98**
OTM				−0.99**	0.99**
HD					−1.00**

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

Conclusion

In this study, we investigated the sensitivities of a battery of tests, using *B. rapa, E. fetida,* and *V. fischeri*, to pyrene polluted soil. Overall, the results showed that pyrene in soil ≤72.6 μg g^{-1} showed no significant acute toxicity to the germination and root elongation of Chinese cabbage or to the survival and growth of earthworms. Hence, these tests may not be suitable to be used as monitoring tools for soil with low-level PAH contamination. However, due to their high sensitivities, Microtox and *E. fetida* DNA damage assays may be suitable tests for the risk assessment of soil with low to high level of contamination. Furthermore, these bioassays could be applied to evaluate the efficacy of response to mitigative actions, mechanical removal, chemical cleaning, and "no treatment" options. Each of the selected toxicological tests is simple, ecologically relevant, and inexpensive to conduct and have good potential as

environmental monitoring tools. However, our findings show that a single test is not sufficient to evaluate soil ecological health. There is absolutely a need to employ an integrated approach to estimate ecological risk and use more relevant ecological test species for the development of environmentally acceptable toxicological end points for soil ecological health.

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