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Enhanced Microbial Removal of Pyrene in Soils in the Presence of Earthworms

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Microbial degradation of pyrene was studied in soils in the presence and absence of earthworms (Eisenia foetida) to demonstrate an integrated innovative strategy for bioremediation of sites lightly polluted by polycyclic aromatic hydrocarbons. Desorption of pyrene and soil microbial respiration were measured to elucidate the mechanism of enhanced microbial degradation. The results showed that both soil properties and contact time could influence pyrene biodegradation. The introduction of E. foetida enhanced pyrene removal significantly both in freshly spiked and aged soils. The percentage pyrene removal in the presence of E. foetida was 45.5–91.0% after 14 d of incubation, which were 2.1 to 2.8 times greater than those without the worms. The enhanced pyrene removal is attributed to both enhanced microbial degradation and uptake by the worms. Microbial degradation of pyrene increased by 1.2 to 1.6 times in the presence of the worms. Overall, the introduction of live worms could improve both pyrene bioavailability and microbial activity, which leads to enhanced microbial degradation of pyrene.

Keywords Bioremediation, earthworm, PAHs, bioavailability, biodegradation

Introduction

The rapid increase of exploitation and utilization of petroleum, coal, and natural gas in modern society leads to an extensive contamination of polycyclic aromatic hydrocarbons (PAHs) in the environment. Except those severely contaminated sites aroused by industrial activities, PAHs are usually detected in farmland at several hundreds μg kg^{-1} to tens mg kg^{-1} due to the deposition of atmospheric pollutants (Jiang et al., 2009). Because of their mutagenic, teratogenic, and carcinogenic characteristics, these contaminants may pose significant risks to ecosystem and human health. Hence, a number of PAHs have been listed as the priority pollutants in China and other countries. Meanwhile the environmental fate and remediation strategies for PAHs in soils have aroused increasing public concern and scientific interest.

Bioremediation based on microbial degradation has been recognized as an economic and environment-friendly clean-up technology for organic pollutants, including PAHs,

compared to other chemical and physical methods, such as advanced oxidation processes and thermal desorption (Alexander, 1999; Potin et al., 2004; Li et al., 2008). However, the hydrophobicity of some hydrophobic organic chemicals (HOCs), like PAHs, makes them bind strongly to soil constitutes, being recalcitrant to microbial degradation (Kim et al., 2003; Gomez-Lahoz and Ortega-Calvo, 2005). The unavailability is thought to be the main reason for the persistent residue of HOCs in soils after bioremediation treatment. In addition, the low bioremediation efficiency of HOCs is, on the other hand, a result of microbial factors. It is generally accepted that *in-situ* bioremediation is limited, and even inhibited by the poor transport and uneven distribution of soil microbial inoculants (Elsas and Heijnen, 1990; Farenhorst et al., 2001), as well as by the absence of suitable nutrients and terminal electron acceptors in field soils (Boopathy, 2000; Romantschuck et al., 2000). The development of integrated innovative bioremediation approaches to further reduce the remaining threshold of HOCs is therefore of great importance.

Earthworms are the dominant invertebrate in temperate soils. It has been well documented that earthworms can not only exert significant impact on the distribution and activities of soil microflora (Binet et al., 1998; Mummey et al., 2006), but also modify soil structures (Martin, 1991; Kretzshmar, 2004), chemical transport (Bolan and Baskaran, 1996; Jégou et al., 2000), and moisture retention (Schaefer and Juliane, 2007) through ingesting, burrowing, and casting activities. These characteristics of earthworms have been successfully applied in the composting of agricultural and industrial wastes (Aira and Domínguez, 2009; Gupta and Garg, 2009; Suthar, 2009). As a consequence, earthworms are also expected to act as a promoter for the bioremediation of HOC-contaminated soils. However, research in this field is relatively limited (Hickman and Reid, 2008). Until now, studies have been mainly conducted on agrochemicals (atrazine and chlorinated pesticides) (Verma and Pillai, 1991; Farenhorst et al., 2001), crude oil hydrocarbons (Schaefer and Juliane, 2007), PAHs (Contreras-Ramos et al., 2008), and other HOCs, like PCBs (Singer et al., 2001; Tharakan et al., 2006). However, results are not all corroborative for the beneficial effect of earthworms on HOC bioremediation (Callaham et al., 2002; Binet et al., 2006). Although the amendment of earthworms is generally considered to be favorable for the reproduction and activity of soil microorganisms, a reduced amount of microorganisms was reported in the presence of earthworms both in constructed composting reactors (Aira et al., 2009) and in natural soils (Bohlen and Edwards, 1995), as earthworms might prey on microorganisms as their nutrient source. More recently, Monard et al. (2008) found that earthworms (*Lumbricus terrestris*) could promote atrazine degradation by changing the microbial community structure of indigenous microorganisms and bioaugmented species. An increase in the sequence copies number of a plasmid active in atrazine degrading was observed. In regard of pollutant mobility, both the release enhancement (Verma and Pillai, 1991; Eijsackers et al., 2001) and the sorption enhancement (Bolan and Baskaran, 1996; Binet et al., 1998) have been reported in the presence of earthworms or their casts. Moreover, Butenschoen et al. (2009) reported an enhanced degradation of catechol in soils, but only of minor importance in soils with high organic matter. Hence, a wide variability exists in the effect of earthworms upon the removal of HOCs due to the differences in compound properties, earthworm species, soil properties as well as experimental set-up.

The objectives of this study were: 1) to determine whether and to what extent the co-existing of live earthworms could enhance the dissipation of PAHs by microorganisms in aged and unaged soils using a four ring PAH, pyrene, as the target compound; and 2) to explore the mechanisms of earthworm's influence on pyrene biodegradation by examining the possible changes in pyrene bioavailability and microbial activities driven by earthworms.

Materials and Methods

Chemicals

Pyrene was purchased from Acros Corporation (New Jersey, USA), with a > 98% purity. Solvents used for extractions and preparation of standards were of HPLC grade, and other chemicals were of analytical grade or better. Tenax (60–80 mesh, 177–250 μm) used in desorption experiments was obtained from Buchem (Apeldoorn, the Netherlands). Before use, the Tenax pellets were cleaned with acetone, 80:20 (v/v) acetone and methanol, and acetone in sequence (each lasted for 48 h) with Soxhlet extraction.

Earthworms

Mature earthworms (*Eisenia foetida*) were collected from Jia Liming Earthworm Feedlot, Tianjin, China. Before use, the worms were allowed to acclimatize to the laboratory conditions for at least 20 d at room temperature ($24 \pm 3°C$). The worms (each with fresh weight of 0.6 ± 0.1 g) were selected for experiments, and the worms were placed on a moist filter paper for 24 h to let them purge their guts, and rinsed with water before use.

Bacterial Cultivation

A pyrene–degrading strain (*Bacillus subtilis*) was used in this study. The bacteria are able to grow with pyrene as their sole source for carbon and energy when the system does not contain another carbon source, such as glucose. The bacterial strain was inoculated to a shaken tube containing nutrient broth culture (5 g L^{-1} beef extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl, autoclaved, pH 7.0) and incubated at 37 °C for 12 h. The tube was then centrifuged at 3000 rpm, and the supernatant was decanted. Subsequently, the bacterial pellet was re-suspended using phosphate buffer (0.125 M K_2HPO_4 and 0.075 M KH_2PO_4, autoclaved, pH of 7.0) to OD_{600} of 1.0, which contained approximately 3.5×10^7 CFU mL^{-1}.

Soil Samples

Two different soils, sampled from a farmland around Shijiazhuang, Hebei Province (Red earth), and the campus garden of Nankai University, Tianjin (Aquic–fluvo soil), were employed in this study. The soils were air-dried, gently ground and screened through a 2–mm sieve, and then aliquots of the soil were placed into Erlenmeyer flasks with cotton stoppers. The soils in the flasks were sterilized by moist heating at 115°C for 20 min. The main physicochemical properties of the sterilized soils are summarized in Table 1.

The soil samples in the flasks were uniformly spiked with a specific amount of pyrene (1 g L^{-1} stock solution in methanol) under aseptic condition to get a final pyrene concentration of 9 mg kg^{-1} dry soil. To obtain freshly spiked and aged pyrene–contaminated soils, the flasks were sealed and placed in the dark at 25°C for 1 and 60 d, respectively.

Removal of Pyrene in the Presence and Absence of Earthworms

The following treatments were designed: (i) worm–worked bioaugmentation treatment, where 4 earthworms, 1.5 mL of the bacterial suspension with OD_{600} of 1.0, and 2 mL

Table 1
Selected physico–chemical properties of sterilized soils[a]

Soil	Organic Matter (%)	Sand (%)	Silt (%)	Clay (%)	pH
Red Earth	0.30	15	64	21	5.67
Aquic–Fluvo	3.56	27	39	34	6.73

[a]pH values of soil were determined in 0.01M $CaCl_2$ (soil: solution $= 2: 5$, w/w).

autoclaved mineralization medium (MM) were added into Petri dishes containing 10 g of the pyrene contaminated soils. A specific amount of sterilized deionized water was supplemented to obtain a moisture content of 60% of the water–holding capacity. The MM contained 14 g L^{-1} K_2HPO_4, 6 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 g L^{-1} NaCl, 2 g L^{-1} $(NH_4)_2SO_4$, and 1 mL L^{-1} of a microelements stock solution giving final concentrations of 5×10^{-3} μL L^{-1} H_2SO_4, 1 μg L^{-1} $FeSO_4$ and $MnSO_4 \cdot H_2O$, 0.1 μg L^{-1} each for the following: H_3BO_4, NH_4VO_3 and $NiSO_4 \cdot 6H_2O$, and 0.25 μg L^{-1} each for the following: $Na_2MoO_4 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, and $Co(NO_3)_2 \cdot 6H_2O$. The pH value of the MM was 7.0; (ii) worm–free bioaugmentation treatment, which contained the same components with the worm–worked treatment except that no earthworm was added; and (iii) control treatment, where 10 g of the pyrene contaminated soil was spiked with 1.5 mL phosphate buffer and 2 mL MM, and no organism was introduced. The Petri dishes were covered with caps and incubated in a dark and aerated incubator at 30°C. Water loss of the samples was daily compensated using autoclaved deionized water during the incubation. All the incubations were conducted in triplicate. The dishes were sacrificed at 0, 2, 4, 7, and 14 d, and both soils and earthworms were analyzed for pyrene concentration. After sampling, the worms were rinsed with water and placed on moist filter paper in a glass Petri dish for 24 h to allow for depuration. Then, the worms were sequentially washed with water, dried with paper towels, weighed, and freeze–dried. The worms were ground with a mortar and pestle and mixed with anhydrous Na_2SO_4, which was three times of their weight prior to Soxhlet extraction. The whole dish of soil and the shredded filter paper were mixed together to conduct the same treatment.

Soil Microbial Activity

Soil samples with compacted texture, obtained from a site adjacent to where the earthworms were collected, were used in this section. After sterilization, 2000 g of the soil sample was weighed into a porous pot. To check the effects of earthworms on soil microbial activity, two treatments were performed. For the worm–worked one, the worms and bacterial suspension were added into the soil sample in the pot at the same density and ratio as described above [approximately 800 earthworms (0.6 ± 0.1 g/each worm) and 300 mL of bacterial suspension with OD_{600} of 1.0 and 400 mL mineralization medium]. For the worm–free one, only bacterial suspension and mineralization medium but no worm was introduced into the soil. The pots were covered with a nylon sheet and incubated in a dark and aerated incubator at 30°C for one month. Water loss was compensated daily during the incubation period.

The two treatments were periodically subsampled, and the soil microbial activity was evaluated via soil microbial respiration using a self–made device (Fig. 1). Continuous air flow was eluted by a pool of 1 M NaOH solution to eliminate CO_2. Then, the CO_2–free
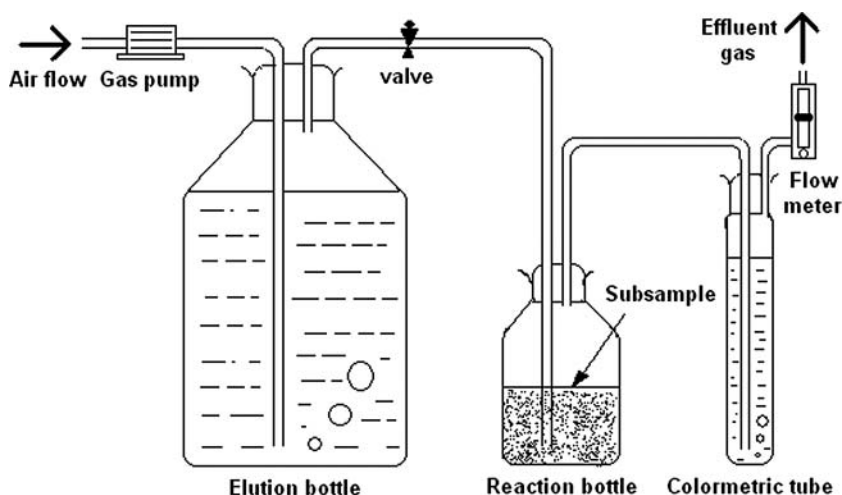
Figure 1. Schematic diagram of the device for measuring CO_2 produced by soil microbial (The replications are not shown).

air was transferred into a reaction pool sealed with Teflon–lined stoppers to contact with the soil samples (100 g soil), providing oxygen for microbial activity. The experiments were conducted in quadruplicate. During a period of 100 h, CO_2 generated by the aerobic respiration of the microflora in the soil samples was recovered using a trap containing 50 mL 1 M NaOH and sealed with a Teflon–lined stopper. A valve was located at the gas line between elution and reaction pools to control the flow rate of CO_2- free air, and a flow meter was linked to the outlet of the trap to record the flow rate. The amount of CO_2 incorporated in the trap solution was immediately measured using titration method with double indicators, methyl orange, and phenolphthalein.

Desorption

To obtain soil samples similar to those in worm–worked treatment, earthworms were introduced into the soils at the same density as described above. After 30 d of incubation at $25 \pm 1°C$, the soil was air–dried, gently ground, and thoroughly homogenized. Worm–free soil was treated in the same way but without the introduction of earthworms. Soil organic matter (SOM), dissolved organic carbon (DOC), and pH of the worm–worked and worm–free soils were measured. The DOC was determined by a TOC analyzer (5000 A, Shimadzu, Japan) using the supernatant of a soil slurry with solid/water ratio of 1/20 (w/v) after centrifugation.

A mixture of 1 g of the above dry soil samples, a specific amount of pyrene–methanol solution, and 20 mL milli–Q water containing 0.02% NaN_3 as a biocide was continuously shaken in a sealed 22–mL glass vial at $30 \pm 1°C$ for 48 h to allow pyrene to be sorbed on soil. Then, 0.2 g Tenax pellets were then weighed into the vials to initiate desorption. Preliminary experiments revealed that over 99.99% of the initial pyrene was sorbed onto the soil, and hence, initial pyrene concentration in soil was calculated to be 9 mg kg^{-1}. Sampling was conducted at 1, 3, 7, 14 and 21 d by refreshing Tenax pellets. Desorption kinetics were determined in triplicate.

At each sampling stage, the vials were centrifuged, while Tenax pellets were completely floated without disturbing the soil at the bottom. Pyrene sorbed on Tenax pellets was recovered by shaking the pellets in 20 mL acetone for 24 h with two successive extractions. The combined extracts were evaporated to 1 mL using a rotary evaporator, and sequentially to near dryness under a gentle stream of nitrogen. The residue was then re–dissolved in 10 mL methanol. Preliminary tests revealed that over 99% of the pyrene sorbed on Tenax could be recovered by this process.

Pyrene Analysis

Soil samples and ground worms were Soxhlet extracted with a mixture of dichloromethane (50 mL) and acetone (50 mL) for 24 h. The organic extract was concentrated as described above. This pretreatment method has been identified to recover approximately 95% of pyrene from both the soil and the worm samples. For the soil samples, there was no significant difference in recovery between unaged and aged ones. The concentration of pyrene in methanol was analyzed by a high performance liquid chromatographer (Waters 1525, USA) equipped with a Waters Symmetry C_{18} column (150 mm $\times$ 4.6 mm $\times$ 5 μm, Ireland). A mixture comprising 80% methanol and 20% water was used as the mobile phase with a flow rate of 1.0 mL min^{-1}. Pyrene was detected by a Waters 2475 Multi λ Fluorescence Detector (USA) at excitation and emission wavelengths of 333 and 390 nm, respectively.

Statistical Analysis

Statistical analysis was conducted with the software SPSS 13.0 for Windows (SPSS Inc., Chicago, USA). A paired–samples t–test analysis was performed to establish significant differences between treatments in the presence and absence of earthworms.

Results and Discussion

Biodegradation

In the absence of earthworms (worm−free treatment), pyrene concentration in unaged soils decreased with time (Fig. 2). The loss of pyrene $(1 - C_t/C_0)$ was ascribed to biodegradation since the loss caused by other ways, like evaporation and adsorption onto the wall of glass wares, was negligibly low, as indicated by the pre–experiment results. Pyrene was biodegraded to a greater extent in the Red earth compared to the Aquic–fluvo soil, with percentage loss of 41.8 $\pm$ 10.2 and 27.0 $\pm$ 1.4%, respectively, on the 14th day. The difference in biodegradation in the two soils could be mainly attributed to the difference in their SOM content (Table 1). Pyrene was degraded more rapidly and by a greater extent in the Red earth, which has less SOM than the Aquic–fluvo soil does. Li et al. (2007) have proposed that soils with greater SOM content possess more "difficultly desorbing sites" that could be easily accessed by pollutant molecules even without aging, and thus reducing their availability for bio–uptake.

Aging reduced pyrene biodegradation in the two soils, leading to a higher residual pyrene concentration (C_t/C_0) in soils, as compared with the freshly spiked (unaged) soils at each harvest time. After 14 d of incubation, only 36.5 $\pm$ 9.3 and 20.5 $\pm$ 5.3% of the initial pyrene were degraded in the aged Red earth and the Aquic–fluvo soil, respectively (Fig. 2). Aging allowed pyrene molecules bound with the "easily desorbing sites" to transfer to
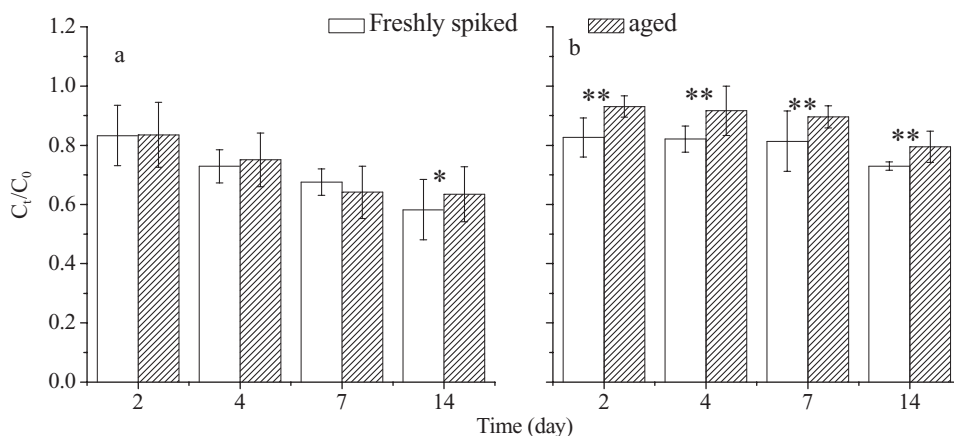
Figure 2. Residue of pyrene in freshly spiked and aged worm−free bioaugmented soils (a. Red earth, b. Aquic–fluvo soil. * and ** represent, respectively, $p < 0.05$ and $p < 0.01$ probability of significant difference between freshly spiked and aged soils).

the "difficultly desorbing sites," even to the "irreversible sites," and thus lowered pyrene bioavailability to microbes (Li et al., 2007; Sun and Li, 2005). The difference in the 14–d biodegradation percentage for the unaged and aged Aquic–fluvo soil was statistically significant ($p < 0.01$). Hence, even though a species (*Bacillus subtilis*), which could degrade 90% pyrene in solution, was introduced into soil, the degradation of pyrene in soils was generally low (20.5–41.8%). Hence, the limited PAH biodegradation during bioremediation could be generally attributed to their low availability in soil rather than the lack of active microorganisms capable of degradation.

Worm Assisted Bioremediation

In the worm–worked bioaugmentation treatment, no mortality of the worms was observed during the entire incubation period. In the presence of the worms, the removal of pyrene from the soils was enhanced markedly compared to those without the worms (Fig. 3). After 14 d of incubation, 89.2 ± 0.5 and $57.5 \pm 2.4\%$ of the initially spiked pyrene was removed from the unaged Red earth and Aquic–fluvo soil, respectively, enhanced by 2.1 to 2.8 times compared to those without the worms. As for the aged Red earth and Aquic–fluvo soil, up to 91.9 ± 8.5 and $45.5 \pm 6.7\%$ of the initial pyrene dissipated in the presence of earthworms, while only 36.5 ± 9.3 and $20.5 \pm 5.3\%$ of the initial pyrene was removed in the worm−free treatments. Similar results have been reported on the earthworm-assisted removal of PAHs in previous literature (Contreras-Ramos et al., 2008; Eijsackers et al., 2001; Alvarez-Bernal et al., 2006). However, the mechanism for the enhanced PAH removal is so far still unclear. Contreras-Ramos et al. (2008) reported that in the presence of earthworms (*Eisenia fetida*), the removal of PAHs, phenanthrene, anthracene and benzo[a]pyrene from the soil increased to 99, 91 and 16%, respectively, from 95, 42, and 3% when earthworms were absent. They ascribed this to the enhanced activity of soil indigenous microorganisms and the contribution of the microflora from the earthworms' guts. They did not attempt to differentiate the dissipation of PAHs into real degradation and accumulation by earthworms. Tharakan et al. (2006) developed a mass balance model to identify the various routes for PCB removal in
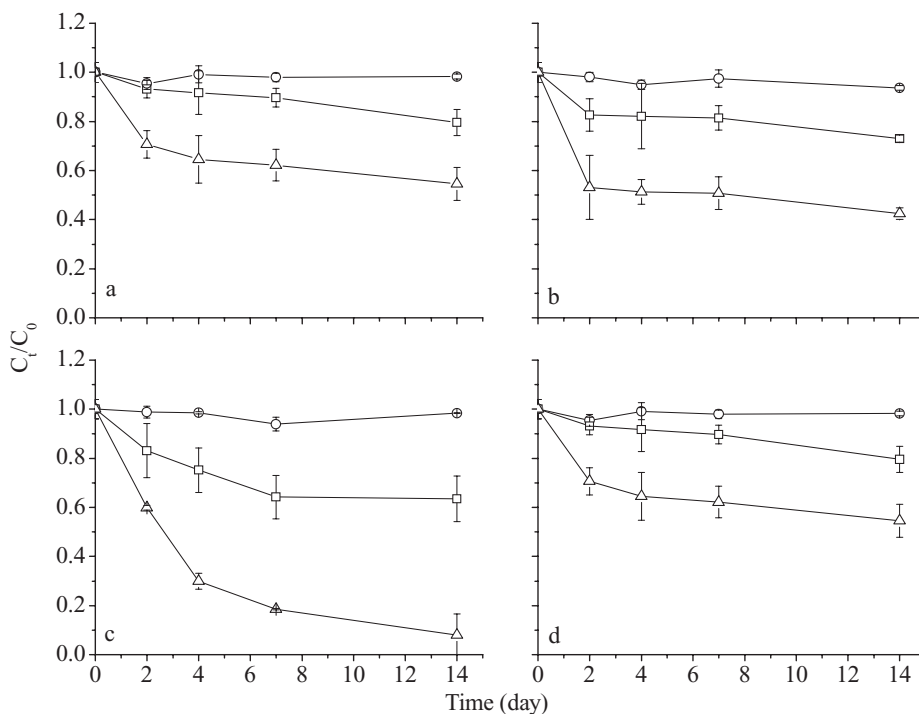
Figure 3. Residue of pyrene in the control (○), worm–free (□) and worm–worked bioaugmented soils (△) (a. freshly spiked Red earth, b. freshly spiked Aquic–fluvo soil, c. aged Red earth, d. aged Aquic–fluvo soil).

vermicomposting bioreactor. Earthworms in the vermicompost could bioaccumulate up to 313 mg/kg PCBs, while the earthworm-enhanced biodegradation was not affirmed, because similar PCB removal was observed in the presence and absence of earthworms.

Pyrene concentration in the earthworm bodies was measured (data not shown). At the end of the experiments, 37.8 ± 6.4 and $15.9 \pm 1.0\%$ of the initial pyrene in the unaged Red earth and Aquic–fluvo soil could be attributed to the uptake by the earthworms, and for the aged soils, 37.5 ± 1.3 and $12.6 \pm 0.0\%$ were accumulated by the worms. Slightly less bioaccumulation occurred in aged soils. A preliminary experiment showed that in addition to bioaccumulation, bodies of the living earthworms had no other effects (e.g. transformation, assisted volatilization, etc.) upon the removal of pyrene in soils. Hence, the loss of pyrene contributed by microbial degradation could be obtained by calculating the mass difference between those of the total removal and bioaccumulation (Fig. 4).

For both the unaged and aged soils, biodegradation of pyrene was significantly ($p < 0.01$ and $p < 0.05$) higher in the presence of earthworms than those in the absence of earthworms (Fig. 5). The enhancement in biodegradation was observed from the second day of the incubation. After 14 d of incubation, 51.5 ± 5.9 and $41.6 \pm 3.4\%$ of the original pyrene were degraded by microbes with the assistance of the worms in unaged Red earth and Aquic–fluvo soil, which was 9.7 ± 0.7 and $14.6 \pm 4.8\%$ higher than those without the worms, respectively. For aged Red earth and Aquic–fluvo soil, the biodegradation of pyrene was enhanced from 36.5 ± 6.3 and $20.5 \pm 5.3\%$ in the absence of the worms to
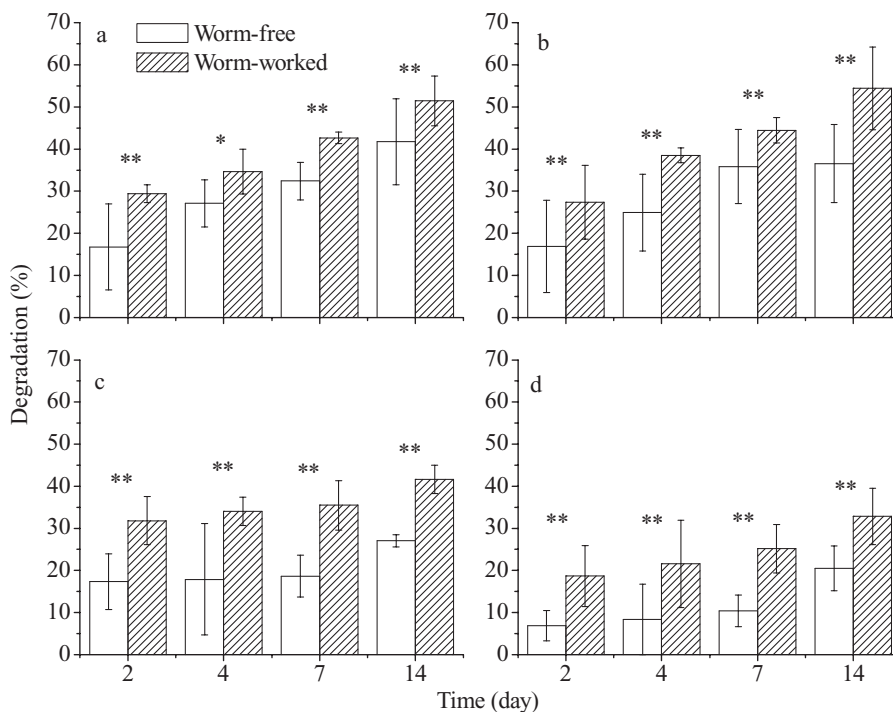
Figure 4. Biodegradation ratio in worm-free and worm-worked bioaugmented soils for freshly spiked (a) and aged (b) Red earth, and freshly spiked (c) and aged (d) Aquic–fluvo soil (* and ** represent, respectively, $p < 0.05$ and $p < 0.01$ probability of significant difference between worm-free and worm−worked treatments).
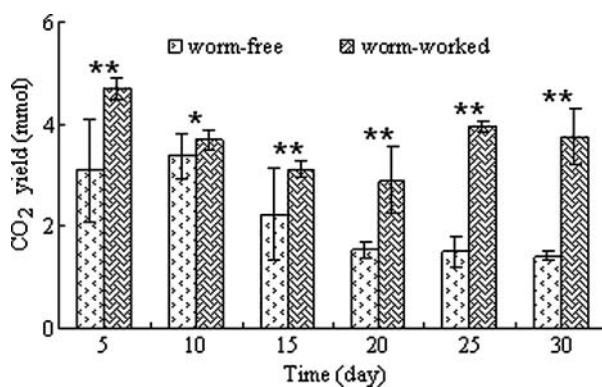

Figure 5. The effect of earthworms on microbial respiration in soil (* and ** represent, respectively, $p < 0.05$ and $p < 0.01$ probability of significant difference between worm–free and worm–worked treatments). The y-axis represents CO_2 production by one kilogram of bioaugmented soil samples in 100 h.

54.4 ± 9.8 and 32.8 ± 6.7% in the presence of the worms. Hence, in the presence of earthworms, biodegradation did increase, and the effect of earthworm amendment on the remediation efficiency of PAHs could not only be ascribed to the incorporation of PAHs into earthworm bodies but also to the reinforced biodegradation by microflora. The enhanced biodegradation of organic pollutants in the presence of earthworms has been reported in the literature (Contreras-Ramos *et al.*, 2008; Hickman and Reid, 2008, and literature therein; Butenschoen *et al.*, 2009). Contreras-Ramos et al. (2008) reported significantly enhanced biodegradation of anthracene and benzo[a]pyrene in the presence of *E. fetida*, and they ascribed this to the enhanced activities of soil indigenous microorganisms and the contribution of the microflora from the earthworms' guts. Butenschoen et al. (2009) found by microcosm experiments that the introduction of endogeic earthworms (*Octolasion tyrtaeum*) could increase the mineralization of C^{14}-labbelleld catechol in an arable soil but not in a forest soil with higher organic matter content. They attributed the enhanced mineralization of catechol to the elevation of labile fraction of the compound in arable soil. Degradation and mineralization are the most ideal way to remove organic hazards from the environment, and hence the enhanced biodegradation adds advantage to this integrated technology.

Soil Microbial Activity

It has been well documented that the presence of earthworms could prompt soil fertility and enhance the activity of soil microorganisms (Hickman and Reid, 2008). However, adverse effects were also observed (Aira et al., 2009; Bohlen and Edwards, 1995). Hence, the activity of microorganisms was checked through measuring CO_2 yield of the bioaugmented soil samples during a 30 d of incubation with and without the earthworms (Fig. 5). The introduction of the earthworms significantly enhanced the respiration of soil microorganisms, especially in the latter period. After incubated for 5 d, 4.7 ± 0.2 mmol of CO_2 was released per kilogram (fresh weight) of the worm−worked soil during 100 h, while 3.1 ± 1.0 mmol of CO_2 was released from the worm–free treatment. During the mid–period of incubation (from approximately Days 15 to 20), the microbial activity in both the worm–worked and the worm–free treatment decreased, but by a less degree in the worm–worked treatment. After the 20th day, the microbial activity in the worm–worked soil recovered with time such that the CO_2 evolution on Day 30 was similar to that on Day 10, whereas the microbial activity in the worm–free treatment remained continuously at a low level. On Day 30, a total of 3.8 ± 0.6 mmol of CO_2 was released per kilogram of worm–worked soil during 100 h, which was 2.6 times that of the worm–free treatment.

The enhanced microbial respiration in the worm–worked soil indicates strengthened microbial activity in the presence of earthworms. As has been well documented in the literature (Schack and Hildebrand, 1998; Toyota and Kimura, 2000; Wolters, 2000; Hickman and Reid, 2008), the enhanced microbial activity may be because the soil burrowed by the worms is more favorable for bacterial growth due to additional C and N resources and improved aeration, or because the bacteria are more widely dispersed and more likely to encounter the substrate to be degraded. During our experiments, we observed that the texture of the worm–worked soil gradually varied into a more homogeneous and finer one and the soil moisture remained constant, whereas the worm–free soil retained its original compact texture and its moisture content reduced rapidly. Similar results have been reported in the literature (Schaefer and Juliane, 2007; Barré et al., 2009). Moreover, DOC is the most labile fraction among soil total organic matter and can be used as a carbon or energy source by soil microflora (Caravaca and Roldán, 2003). After the introduction of earthworms, the

Table 2
Comparison of selected parameters of worm–free and worm–worked soils

	Red earth		Aquic–Fluvo Soil	
	Worm-Free	Worm-Worked	Worm-Free	Worm-Worked
DOC (mg L^{-1})	66.7	116.0	110.2	118.9
Organic Matter (%)	0.30	0.81	3.56	4.12
pH	5.67	6.85	6.73	7.18

DOC of the two soils both increased (Table 2), which is believed to provide soil microflora with a more available C source. As for other possible mechanisms for worm–assisted bioremediation, earthworms also have been shown to improve the dispersal and distribution of soil inoculants and the supply of suitable electron acceptors such as oxygen (Schack and Hildebrand, 1998; Singer et al., 1999).

Desorption

The possible change in the mobility of target pollutants driven by earthworms is seldom studied, and whether or not the existence of earthworms could enhance chemical mobility in soil is still controversial (Verma and Pillai, 1991; Binet et al., 2006). In this study, the desorption kinetics of pyrene from the worm–free and worm–worked soils were measured (Fig. 6). Pyrene desorption was strongly dependent on soil nature, and desorption proceeded faster and by a greater extent in the Red earth with less SOM content. For example, by the first day, approximately 21.6% of the sorbed pyrene was desorbed from the Red earth reached around, whereas only 2.8% from the Aquic–fluvo soil. At the end of desorption, $47.6 \pm 3.0\%$ of pyrene was desorbed from the Red earth, compared to the $31.6 \pm 4.7\%$ from the Aquic–fluvo soil. The percentage desorption represents the mobility of the contaminant, and it has been assumed in much of the literature that only contaminants dissolved in soil solution could be accessed by microorganisms (Cornelissen et al., 1998; Hawthorne et al., 2001; Kim et al., 2003; Gomez-Lahoz and Ortega-Calvo, 2005).
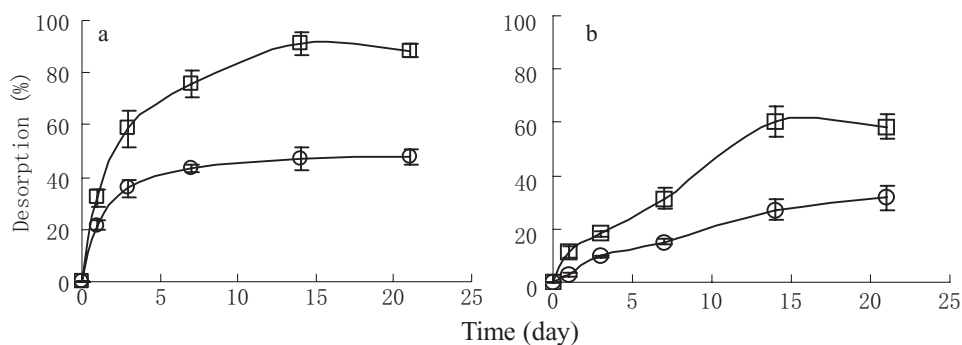

Figure 6. Pyrene desorption ratio in worm–free (○) and worm−worked soils (□) (a. Red earth, b. Aquic–fluvo soil).

The pretreatment by earthworms exerted a significant ($p < 0.01$) beneficial effect on pyrene desorption from soils. At the end of desorption, the percentage desorption reached $88.5 \pm 2.4\%$ and $58.5 \pm 4.5\%$ for the Red earth and the Aquic–fluvo soil, respectively, which represents a respective increase of 40.9% and 26.9% over the corresponding worm-free soils (Fig. 6). Soil parameters were determined after the activity of the worms (Table 2). The SOM increased as expected, but this cannot explain the increased release of pyrene since a higher SOM content is always linked with stronger binding and less mobility of HOCs. The DOC content is another key factor that should be taken into consideration for a better understanding of pollutant distribution between water and soil. Remarkably higher DOC content in the worm–worked soils was observed (Table 2), which is inconsistent with previous reports (Parkin and Berry, 1999; Wen et al., 2006). The increased DOC probably originated from dissolved components (e.g. mono and polysaccharides, proteins and certain organic acids) excreted by worms during burrowing. The DOC may act as surfactants for hydrophobic chemicals and is favorable for them to release into the aqueous phase (Chiou et al., 1986).

Conclusions

Our results strongly suggest that pyrene–degrading bacteria and earthworms could successfully cooperate to remove pyrene from soils in two ways: worm uptake and enhanced biodegradation. Further experiments concerning mechanisms of the actuated biodegradation showed that the introduction of worms led to increased microbial activity and an increased bioavailability of pyrene, which in turn acted synergistically to facilitate the microbial degradation of pyrene in soils.

The present study supports the application of earthworms, together with microbial remediation technologies, to generate an innovative comprehensive remediation strategy based on the integrated ecologic functions for the removal of organic pollutants. However, detailed studies may be required on a case by case basis to avoid any adverse effects.

Acknowledgments

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