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Uptake and accumulation of phenanthrene and pyrene in spiked soils by Ryegrass(*Lolium perenne* L.)

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Abstract: Phytoremediation has long been recognized as a cost-effective method for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil. A study was conducted to investigate the uptake and accumulation of PAHs in root and shoot of *Lolium perenne* L. Pot experiments were conducted with series of concentrations of 3.31—378.37 mg/kg for phenanthrene and those of 4.22—365.38 mg/kg for pyrene in a greenhouse. The results showed that both ryegrass roots and shoots did take up PAHs from spiked soils, and generally increased with increasing concentrations of PAH in soil. Bioconcentration factors(BCFs) of phenanthrene by shoots and roots were 0.24—4.25 and 0.17—2.12 for the same treatment. BCFs of pyrene by shoots were 0.20—1.5, except for 4.06 in 4.32 mg/kg treatment, much lower than BCFs of pyrene by roots (0.58—2.28). BCFs of phenanthrene and pyrene tended to decrease with increasing concentrations of phenanthrene and pyrene in soil. Direct uptake and accumulation of these compounds by *Lolium perenne* L. was very low compared with the other loss pathways, which meant that plant-promoted microbial biodegradation might be the main contribution to plant-enhanced removal of phenanthrene and pyrene in soil. However, the presence of *Lolium perenne* L. significantly enhanced the removal of phenanthrene and pyrene in spiked soil. At the end of 60 d experiment, the extractable concentrations of phenanthrene and pyrene were lower in planted soil than in non-planted soil, about 83.24%—91.98% of phenanthrene and 68.53%—84.10% of pyrene were removed from soils, respectively. The results indicated that the removal of PAHs in contaminated soils was a feasible approach by using *Lolium perenne* L.

Keywords: phytoremediation; polycyclic aromatic hydrocarbons(PAHs); phenanthrene and pyrene; uptake and accumulation

Introduction

Polycyclic aromatic hydrocarbons(PAHs) are widespread pollutants that attract concern because of their recalcitrance and mutagenic/carcinogenic properties (Guerin, 1988; Gevao, 1998; Haeseler, 1999; Joner, 2001; Jian, 2004). PAHs consist of two or more fused benzene rings in linear, angular, or cluster arrangements. They are formed and released into the environment through natural and man-made sources. Natural sources include volcanoes and forest fires, while the man-made sources come mainly from oil processing, accidental spilling of processed hydrocarbons and oils, coal liquefaction and organic oil seepage (Heitkamp, 1988; Freeman, 1990). These pollutants have a low biodegradability and high persistence in the environment (Banks, 1999; Binet, 2000). Persistence of PAHs may constitute a significant ecological risk, these compounds are on the United States Environmental Protection Agency (USEPA)'s priority pollutant list(Kipopoulou, 1999; Binet, 2001; Jian, 2004).

Phytoremediation has long been recognized as a cost-effective method for removal of organic pollutants from soil. It also appears to have great potential for the treatment of soils contaminated with residual levels of polynuclear aromatic hydrocarbons(PAHs) (Joner, 2001; Itziar, 2001; Chen, 2003; Xu, 2005). Plants provide a robust, solar-powered system that has little or no maintenance requirements. With their copious root systems, plants can scavenge large areas and volumes of soil PAHs. The rhizosphere soil(soil near plant roots) has microbial population orders of magnitude greater than bulk soil(non root soil). Laboratory and pots experiments have demonstrated that plant can enhance dissipation of PAHs when compared to unplanted controls (Banks, 1999; Yoshitomi, 2001; Joner, 2003; Xu, 2005). Phytoremediation field trials have resulted in accelerated reduction of PAHs and other petroleum hydrocarbons in the rhizosphere(Chen, 2003; Glick, 2003). Recently, Liste and Alexander reported that the degradation of pyrene can be promoted by nine different plant species, including three field crops, three horticultural plants, and three pine seedlings (Liste, 2000). Howsam reported that there were significant differences among oak, ash and hazel leaves in their PAH concentrations(sum of 23 PAHs), and in the relative contribution of individual PAHs to the sum with the leaves of three deciduous tree species(Howsam, 2000; 2001). Binet founded that ryegrass rhizosphere potentially enhanced dissipation or biotransformation of a large range of PAHs including 5- and 6-ring PAHs(Binet, 2000). In a word, during the last decades, there were many studies on investigating the uptake of PAHs by plants (Kipopoulou, 1999; Howsam, 2001; Mattina, 2003; Vervaeke, 2003), and contamination of PAHs was often found in various wood and grass categories (Liste, 2000; Binet, 2001; Gao, 2004), but results are not usually identical. Some reports indicated that there were direct relationships between soil and plant concentrations, while others found that no such relationship exists (Wild, 1992; Kipopoulou, 1999). Translocations of phenanthrene and pyrene from roots to shoots were still ambiguous, the impact of these processes had not been clearly elucidated. The information on the contributions of plant uptake and accumulation of PAHs in plant-promoted removal on a quantitative scale was also scant. Therefore, information about PAH distribution and concentration in plants was important in predicting the effectiveness of phytoremediation operation.

The aim of the present work was to make a detailed evaluation on the phytoremediation of PAHs in spiked soil by ryegrass in terms of concentrations in roots and shoots and

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their residues in soil. Phenanthrene and pyrene were selected as the target compounds, and the ryegrass (*Lolium perenne* L.) was selected to represente a wide range of grass plant. It aims to obtain basic information about plant contributions to the promoted removal of PAHs in soils on a quantitative scale. The results from this work may advance our understanding of the phytoremediation mechanisms of PAHs.

1 Materials and methods

1.1 Soil preparation and experimental design

Phenanthrene and pyrene were obtained from Sigma Chemical Co. with a purity of 99.9%. A loam soil(pH 6.12 and 2.36% organic matter) with no detectable PAHs was used in this study. The agricultural soils were sampled in the upper horizon (0—20 cm) near Hangzhou City, air-dried, and passed through a 2-mm sieve. A part of the soils was then spiked with a mixture of high purity phenanthrene and pyrene in acetone(6% total quantity of soil to be spiked). When acetone was evaporated off, the spiked soils were mixed with uncontaminated soil and thoroughly mixed. The final concentrations of phenanthrene and pyrene in treated soils were measured by HPLC and data are shown in Table 1. In here, T0—T5 was the No. of treated soils with different initial concentrations of phenanthrene and pyrene. Treated soils(500 g dry weight soil per pot) were then packed into 15 cm diameter plastic pots by lining with 0.1 mm sieve, placing in the pot bottom to aid drainage and avoid soil loss. Then these pots were packed into a greenhouse, and equilibrated for 7 d at field moisture before the introduction of plants.

Table 1 Initial concentrations of phenanthrene and pyrene in treated soils(mg/kg dw)

	T0	T1	T2	T3	T4	T5
Phenanthrene	ND	3.310 ± 0.02	20.49 ± 6.37	79.88 ± 6.03	169.5 ± 1.09	378.4 ± 19.31
Pyrene	ND	4.320 ± 2.56	24.02 ± 0.52	117.89 ± 9.98	169.1 ± 3.24	365.4 ± 12.37

Notes: Control uncontaminated soil; ND. not detectable; $n = 3$

Seeds of *Lolium perenne* L. were obtained from the grasses cooperation stock center. Seeds were grown for 15 d in vermiculite before transferring to the growth chambers, and seedlings were transplanted to the designated greenhouse pots 5 to 10 d after emergence. Twelve seedlings of ryegrass per pot were used. Controls with spiked soil were treated at the same time including unplanted pots and unplanted microbe-inhibited pots(T0—T5; 0.2% NaN_3 was used to inhibit the microbial activity). The seeding date was considered as 0 d. Pots containing planted and controls were transferred to a growth chamber maintained at 25℃ during a 16-hour day and at 19℃ during an 8-hour night. The soil was watered as needed and fertilized once a week with an inorganic salts solution. Each test was made in triplicate, and the plants were randomized in the greenhouse. At the end of 60 d experiment, the soils from vegetated or non-vegetated pots were carefully collected. Ryegrass was also harvested, rinsed with tap water and distilled water, and separated into shoot and root components. All samples(soils, ryegrass shoots and roots) were freeze-dried, bagged and stored at 4℃ before analytical treatment. Both shoots and roots were weighed for the determination of fresh weight in the same time.

1.2 Analysis of phenanthrene and pyrene in soils and plants

The procedure used to extract PAHs from soils was a modification of those of Kipopoulou and Tomaniova (Kipopoulou, 1999; Tomaniova, 1998). Soil samples analysed were spiked with known phenanthrene and pyrene. Two gram of freeze-dried soil samples were transferred into Erlenmeyer flask with 10 ml of dichloromethane, and the flask was placed into an ultrasonic bath with ultrasonication for 1 h followed by centrifugation. Then 3 ml of supernatant was filtered through 2 g of silica gel column with 10 ml 1∶1 (v/v) elution of hexane and dichloromethane. The solvent fractions were then evaporated to dryness, and dissolved in 2 ml methanol.

Phenanthrene and pyrene in roots and shoots were extracted in the same method as used for soil extraction except a saponification step was included. 0.5 g of plant freeze-dried samples were transferred into Erlenmeyer flask with enough 3∶2 (v/v) solution of hexane and acetone extraction solvent. Erlenmeyer flask was placed into an ultrasonic bath by ultrasonication for 30 min. The solvent was then decanted, collected and replenished. This process was repeated three times. The solvents were then evaporated to dryness, and exchanged to 2 ml methanol. The next step was the same step as used for soil.

All methanol extracts were filtered prior to analysis with 0.45 μm teflon syringe filter and then analyzed by high performance liquid chromatography (Agilent 1100 series, USA) with ultraviolet (UV) detection and an automatic injector, and fitted with 4.6 mm × 150 mm reverse phase C_{18} (Zorbax XDB-C_{18}, Agilent, USA) column using methanol/water 90/10(v/v) as the mobile phase at a flow rate of 1 ml/min. Phenanthrene and pyrene were detected by the absorbance at 250 and 235 nm, respectively. Replicate analyses gave an error in the range of ± 5 to 10%.

1.3 Quality controls and statistical analyses

All data were subject to strict quality control procedures. Each set of samples analysed(soil and plant) was spiked with known phenanthrene and pyrene, then analysed. The reproducibility and recovery of the extraction method for the spiked soil samples were satisfactory, with a recovery averaged 94.91% ($n = 7$, relative standard deviation(*RSD*) less than 8.98%) for phenanthrene and 97.18% ($n = 7$, *RSD* less than 9.49%) for pyrene, respectively. The recoveries of pyrene and phenanthrene in spiked plant samples were (90.45 ± 5.26)% ($n = 7$) and (98.10 ± 3.53)% ($n = 7$) in the entire procedure, respectively. The method detection limits for phenanthrene and pyrene in all samples were 5—10 ng/g. Statistical significance was evaluated using SPSS version 10.0 with one-way ANOVA and least significant difference (*LSD*) for comparison of treatment means with $p < 0.05$.

2 Results and discussion

2.1 Plant biomass

The root and shoot yields of *Lolium perenne* L. on a

fresh weight basis are shown in Fig.1. The results indicated that growing plant showed no signs of stress and produced a similar biomass between pots with all the spiked soils and those with T0. At the end of harvest, the shoots fresh weight was tended to slightly decrease with the increment of high contaminant levels, however, the differences were not statistically significant. Weights of shoots were higher in T2 treatment than control T0. The roots in spiked pots were the same as roots in the control pots. Ryegrass formed a dense fibrous root system in all soils irrespective of treatment. So the yields of plant shoots and roots growing in spiked soils were not affected, plants in spiked pots germinated and grew normally, like those in the control pots.

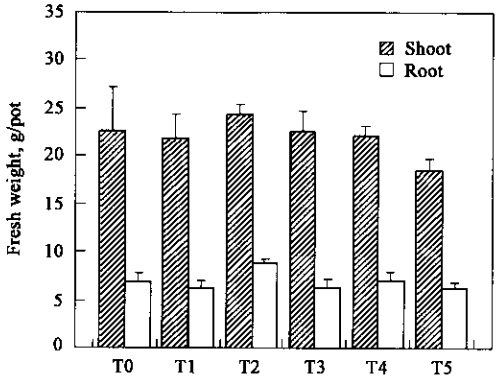

Fig.1 Shoot and root biomass of ryegrass after 60 d in variously spiked soils and in an un-spiked soil. Values above columns for the same plant part(root or shoot). T0—T5 are No. of the treated soils

2.2 Uptake and accumulations of phenanthrene and pyrene in roots and shoots

Concentrations of phenanthrene and pyrene in ryegrass shoots and roots are shown in Fig.2. With the increment of soil phenanthrene and pyrene concentrations, root contents of these compounds significantly increased, and more of phenanthrene and pyrene would be transfered to the above parts, which eventually led to increase accumulation of these compounds in shoots. It was observed that concentrations of pyrene in root were higher than in shoot(Fig.2b), however, root concentrations of phenanthrene were lower than their shoot concentrations (Fig. 2a). A general tendency also showed that concentrations of pyrene in root were higher than ones of phenanthrene in the same treatment (Fig. 2). It means that accumulation of pyrene may be easier than that of phenanthrene in roots. According to the research of Kipopoulou (Kipopoulou, 1999), a lipophilic organic compound entering plant's roots from contaminated soil depends on the K_{ow}. Generally, more lipophilicity results in the higher concentrations in plant. K_{ow} of pyrene is relatively higher than that of phenanthrene ($\log K_{ow}$ exhibits 4.57 for phenanthrene and 5.18 for pyrene), therefore, translocation of phenanthrene was more significant than pyrene, and transport of pyrene was hindered. Beside, it was notable that root concentrations of phenanthrene in unspiked soils (T0) were 0.18 mg/kg. However, root concentrations of pyrene in same treatment(T0) were undetectable. Shoot concentrations of these chemicals in T0 were 2.23 (phenanthrene) and 1.02(pyrene) mg/kg, implying that shoots uptake of PAHs from the ambient air, possibly originally volatilized from the soils, was an important pathway for the PAHs taken up into the ryegrass above-ground parts. Gao found that shoots of 12 plant species grown in unspiked control soils apparently accumulated phenanthrene and pyrene from air (Gao, 2004). Fig.2 suggests that there were no PAHs in the soil, there might still be some PAHs in the plant, also supporting the atmospheric contamination route. However, Gao also found that the air sampler concentrations of phenanthrene and pyrene located 5 or 15 cm above the surface of greenhouse pots showed no difference, volatilized concentrations of phenanthrene and pyrene were very low(Gao, 2004). Our result suggestes that shoot concentrations of PAHs in spiked soils have a positive correlation with their respective soil concentrations (Fig. 2). The average 74.33% of phenanthrene and 90.15% of pyrene in shoots were translocated from root uptake, only average 25.67% of phenanthrene and 9.85% of pyrene in shoots comes from the atmosphere(Fig.3), which indicates that the concentrations of phenanthrene or pyrene in shoots grown in spiked soils were much larger than shoot uptake and accumulation of these compounds from atmosphere, the translocation of phenanthrene or pyrene from roots to shoots was also significant. Similar results have been reported by several authors(Wang, 1994; Schroll, 1994; Gao, 2004). Wang and Jones reported that root or shoot accumulation of phenanthrene and pyrene in contaminated soils was elevated with the increase of their soil concentrations(Wang, 1994). Schroll's results showed that about 22%—95% of phenanthrene and 32%—96% of pyrene in shoots were translocated from root uptake. Besides, roots uptake of PAHs was related to several environment factors, such as compound property, diffusion rate, temperature, soil and water. Simonich found that roots uptake was restricted by various conditions, including compound diffusion rate in soil, sorption efficiency on root epidermis surfaces, and penetration in the roots(Simonich, 1994). Trapp *et al*. also reported that water solubility gives an indication of compound mobility in soil(Trapp, 1990). Relationships between plant uptake and environment factors should be deepened to research in the future.

2.3 Promoted removal of phenanthrene and pyrene in spiked soils by ryegrass

In this experiment, the removal of phenanthrene and pyrene in unplanted spiked soils adding NaN_3 indicated the abiotic removal of these compounds, including chemical degradation and physical sorption. The disappearance of these chemicals in unplanted spiked pots and planted spiked pots may be considered as the microbial degradation and plant promoted removal, respectively. Fig.4 gives the measured concentrations of phenanthrene and pyrene remaining in the soils at the time of harvesting ryegrass. The results showed that phenanthrene and pyrene removal in vegetated soils was significantly greater than that in non-vegetable soils. The removal ratios (R) of phenanthrene and pyrene in series of treated pots were calculated as: $R = (C_0 - C_t) \times 100/C_t$, where C_0 is the initial soil concentrations of phenanthrene and pyrene; and C_t is the residual concentrations of these compounds. The average removal ratios of phenanthrene in planted soils were 84.34%, which was 14.27%(denoted as the removal promotion of phenanthrene in planted as compared to unplanted soils) larger than those in

corresponding unplanted soils. Average removal ratio of pyrene in series of spiked soils (T1—T5) with *Lolium perenne* L. was 78.00%, which is 30.75% higher than those with unplanted soil (Fig. 5). Abiotic removal average ratios of phenanthrene and pyrene in spiked soils was 20.17% and 14.69%, respectively (Fig. 5). The results indicated that removal of phenanthrene and pyrene in spiked soils was obviously promoted by *Lolium perenne* L. The removal promotion of pyrene was generally more evident than that of phenanthrene in the presence of *Lolium perenne* L. It was remarkable that the removal ratios of the pyrene in planted or unplanted soils for the same treatment were consistently much lower than those of the phenanthrene, implying that 4-ring pyrene was more persistent than 3-ring phenanthrene in soils.

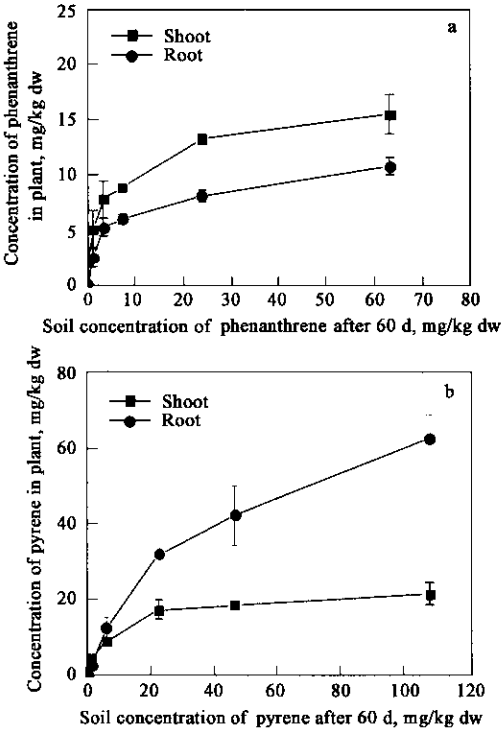

Fig. 2 Concentrations of phenanthrene and pyrene in roots and shoots of ryegrass
a. phenanthrene; b. pyrene

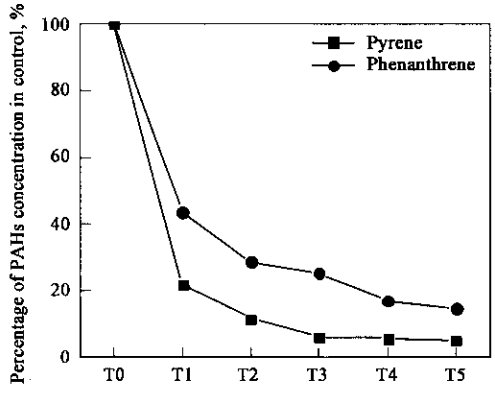

Fig. 3 Concentrations of phenanthrene and pyrene (mg/kg) in control (T0) shoots of ryegrass as a percentage of concentrations of phenanthrene and pyrene in PAHs treatments (T0—T5) after 60 d

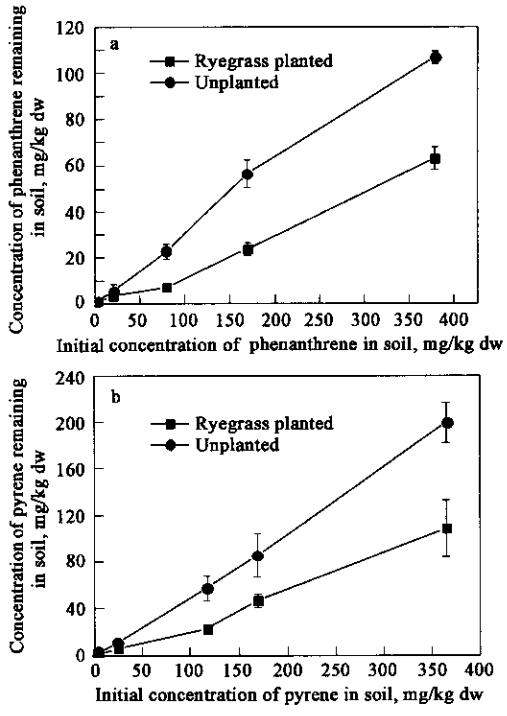

Fig. 4 Residual concentrations of phenanthrene and pyrene in unplanted and planted soils
a. phenanthrene; b. pyrene

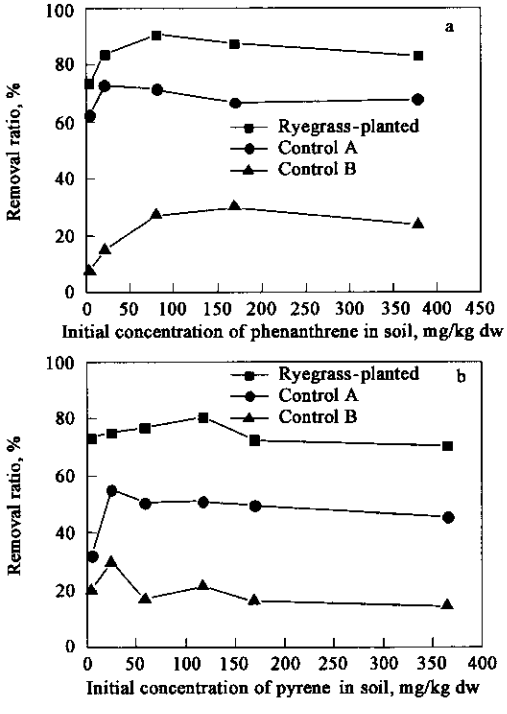

Fig. 5 Removal ratios of pheneanthrene and pyrene in variously spiked soils after 60 d
a. phenanthrene; b pyrene; the initial concentrations of soil phenanthrene and pyrene greatly enlarged from T1 to T5

2.4 Relationships between concentrations of phenanthrene and pyrene in ryegrass and their residues in soil

Bioconcentration factors (BCFs) (defined as the ratio of phenanthrene and pyrene concentrations in the root and shoot tissues of ryegrass and the soils) are shown in Fig. 6. The

average of phenanthrene and pyrene concentrations in the soils after ryegrass harvest was used for the estimation of these BCFs. Although many uncertainties should be acknowledged, the calculation results implied that phenanthrene and pyrene of the ryegrass were just taken up from the corresponding pot in which the *Lolium perenne* L. grew. BCFs of phenanthrene by shoots were 0.24—4.25, higher than BCFs of phenanthrene by roots(0.17—2.12) for the same treatment. More than half of the BCFs in shoots were > 1, showing that the shoots samples had a higher content of the phenanthrene than the soils. BCFs of pyrene by shoots were 0.20—1.50, except for 4.06 in T1 treatment, much lower than BCFs of pyrene by roots(0.58—2.28). BCFs of phenanthrene and pyrene generally tended to decrease with the increase of their soil concentrations (Fig.6). This indicated that the transfer of tested PAHs from root to shoot was considerably restricted. Results are similar to those of Gao and Zhu(Gao, 2004). They observed that BCFs of phenanthrene and pyrene in roots for plants grown in contaminated soils were 0.05—0.67 and 0.23—4.44, respectively. This phenomenon was also reported by Petersen(Petersen, 2002), they found that the BCFs of PAHs in potato were 0.020—0.100, and in most cases, BCFs values decreased with increasing concentrations of PAHs in soils.

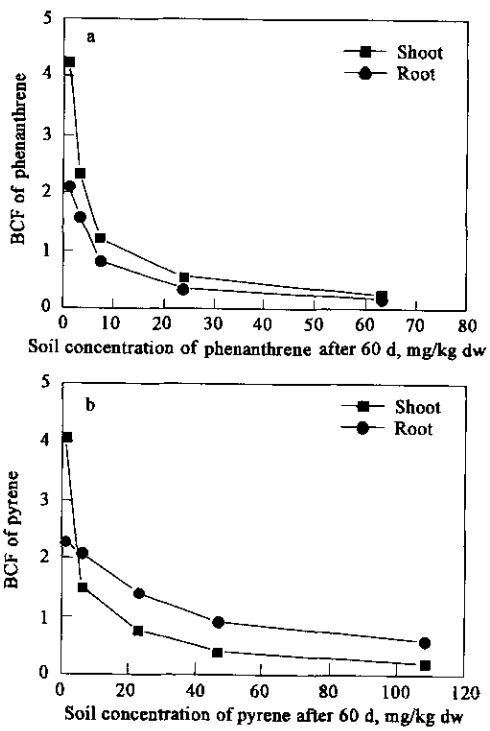

Fig.6 Bioconcentration factors(BCFs) of phenanthrene and pyrene by roots and shoots of ryegrass
a. phenanthrene; b. pyrene

2.5 Ryegrass contributions to the removal enhancement

Both ryegrass shoots and roots did take up the phenanthrene and pyrene (Fig. 5). Ryegrass made an insignificant contribution to the total loss of PAHs added to the soils. During this experiment, around 83.24%—91.98% of phenanthrene and 68.52%—84.10% of pyrene were removed from soils planted with ryegrass. However, only 0.025%—0.62% of the phenanthrene and 0.11%—0.60% of pyrene were taken up by the plants in each pot. The degradation of these compounds in the ryegrass was about 5.39%—20.60% of the phenanthrene and 23.18%—46.41% of pyrene. Contribution of plant-promoted microbial degradation was considered more than 50%—90% of the total dissipation enhancement for both phenanthrene and pyrene. Obviously, as a result, plant may contribute to the dissipation of PAHs by an increase in microbial numbers. Binet speculated that when a chemical stress was present in soil, a plant may respond by increasing or changing exudation to the rhizosphere which modifies rhizospheric microflora composition or activity (Binet, 2000). In sum, enhanced removal of soil phenanthrene and pyrene by *Lolium perenne* L. was the results of plant degradation and plant-promoted microbial degradation. As compared to the other loss pathways, the plant uptake and accumulation of these compounds was negligible. This was consistent with the report that contributions of plant off-take of PAHs to the total remediation enhancement in the presence of vegetation was less than 0.01% for phenanthrene and 0.24% for pyrene. By contrast, plant-promoted biodegradation was the predominant contribution to the remediation enhancement for soil phenanthrene and pyrene(Gao, 2004).

3 Conclusions

Both roots and shoot of ryegrass did take up PAHs from spiked soils. At the end of the experiment(60 d), the loss of phenanthrene and pyrene in spiked soils with *Lolium perenne* L. was 83.24%—91.98% and 68.52%—84.10% of the soil with these chemicals, which were 5.39%—20.60% and 23.18%—46.41% larger than the loss in soils without *Lolium perenne* L., respectively. Although plant accumulations of phenanthrene and pyrene were obvious, and generally increased with increasing soil concentrations, the plant-enhanced dissipation of soil phenanthrene and pyrene predominantly was the result of degradation in plants and plant-promoted microbial degradation. Direct uptake and accumulation of these compounds by *Lolium perenne* L. were very low (only 0.025%—0.62% of the phenanthrene and 0.11%—0.60% of pyrene in the total amount added to the soils) compared with the other loss pathways. The presence of ryegrass markedly promoted the removal of phenanthrene and pyrene in soils, and the healthy growth of the plant in variously spiked soils indicated that the removal of PAHs in contaminated soils was a feasible approach by using *Lolium perenne* L.

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