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Pyrene removal and transformation by joint application of alfalfa and exogenous microorganisms and their influence on soil microbial community

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ABSTRACT

Phytoremediation is an attractive approach for the cleanup of polycyclic aromatic hydrocarbons-contaminated soil. The joint effect of alfalfa and microorganisms, including *Arthrobacter oxydans*, *Staphylococcus auricularis* and *Stenotrophomonas maltophilia*, on pyrene removal was investigated. The results showed that the joint effect primarily contributed to pyrene removal, and the concentration of residual pyrene in rhizosphere soil was lower than that in non-rhizosphere soil. After joint treatment for 45 d, pyrene in rhizosphere soils decreased from 11.3, 52.5 and 106.0 mg/kg to 2.0–3.0, 15.0–18.7, and 41.2–44.8 mg/kg, respectively. These bacteria significantly enhanced pyrene accumulation and microbial community diversity, and increased soil dehydrogenase and polyphenol oxidase activities. Pyrene was initially degraded through ring cleavage. One of the main metabolites 4-dihydroxy-phenanthrene was transformed into naphthol and 1,2-dihydroxynaphthalene, which were further degraded through salicylic acid pathway and phthalic acid pathway, separately.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have received significant environmental concerns due to their persistence, toxicity, bioaccumulation activity, and carcinogenic potential (Melymuk et al., 2014; Yu et al., 2011). So far, many studies have indicated that phytoremediation is an attractive alternative with environmental-friendly properties and low cost compared to traditional approaches to extract, sequester and detoxify existing PAHs for the cleanup of contaminated soil (Chigbo and Batty, 2013).

Some effective plants, such as *Lolium perenne* (Rentz et al., 2005), *Medicago sativa* (Rentz et al., 2005), *Helianthus annuus* (Tejeda-Agredano et al., 2013) and *Phragmites australis* (Toyama et al., 2011), with significantly large root surface area and good adaptability to different conditions of the soil have been selected to remove PAHs from the contaminated soils. However, inhibition of seed germination, root elongation, root exudation and plant growth in the presence of PAHs hampers the ability of these plants to decontaminate polluted soils efficiently. As a result, research focuses have been recently shifted to the synergistic interaction between plants and rhizosphere microorganisms for the better PAH accessibility and bioremediation (Khan et al., 2013; Toyama et al., 2011; Yousaf et al., 2011).

Some problems pertaining to the exposure of plants to PAHs can be moderated through the plant–microorganism interactions (Gerhardt et al., 2009). For example, root exudates can be utilized as a primary carbon and energy source in PAH degradation by microorganisms, producing dissolved metabolites of PAHs for plant transfer (Rentz et al., 2005; Wang et al., 2012). To further improve the associations of plant–microorganism and accelerate PAH removal in soil, effective microorganisms can be inoculated in this bioremediation process.

Alfalfa, a legume distributed extensively in the global scope, is a potential and valuable plant in restoring the soils contaminated by organic compounds and heavy metals. The removal of polychlorinated biphenyl and organochloride insecticide in soil by the combined use of alfalfa and microorganisms was investigated by some researchers (Kirk et al., 2005; Xu et al., 2010). However, the treatment of PAHs in soil by combination of alfalfa and effective microorganisms has rarely been reported, and among the few contributions, researches mainly focused on the enhancement of PAH dissipation and degradation by exogenous microorganisms,

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such as arbuscular mycorrhizal fungus (Teng et al., 2011). To further enhance PAH bioremoval, attention needs to be paid to the joint effect of plant and exogenous microorganisms, and the efficiency and pathways of PAH degradation.

The potentials of *Arthrobacter oxydans*, *Staphylococcus auricularis* and *Stenotrophomonas maltophilia* to enhance pyrene degradation with alfalfa were studied in the present work. The contributions of abiotic factors, plant accumulation, biological metabolism, and joint effects of alfalfa and these exogenous microorganisms on pyrene degradation in rhizosphere and non-rhizosphere soils were investigated. To determine the positive effects of these microorganisms on pyrene removal, soil dehydrogenase, polyphenol oxidase and microorganism activities, and microbial community diversity were analyzed. Pyrene metabolites were identified to reveal the pathways of pyrene biodegradation.

2. Materials and methods

2.1. Materials

Pyrene was purchased from American USDS Company with a purity of 97%. Alfalfa was obtained from Guangzhou Institute of Agricultural Science, China. *Arthrobacter oxydans*, *S. auricularis* and *S. maltophilia* were isolated from PAHs-contaminated sediments collected at an e-waste processing and recycling town Guiyu, Guangdong, China (Chen et al., 2014; Peng et al., 2012; Ye et al., 2013).

The paddy soil was collected from an experimental field (0–20 cm in depth) at South China Agricultural University, Guangzhou, China. The pH value, organic matter, cation exchange capacity, total nitrogen, total phosphorus and total potassium of soil were 5.86, 11.7 g/kg, 21.6 cmol/kg, 0.7 g/kg, 10.3 mg/kg and 89.1 mg/kg, respectively. There was no detected pyrene in this soil samples. These soil parameters were measured according to the farmland environmental quality evaluation standards of China for edible agricultural products (http://kjs.mep.gov.cn/hjbhbz/bzwb/stzl/%20200611/t20061122_96418.htm), published by Ministry of Environmental Protection of China.

2.2. Strain and plant culture

Strains were grown in medium containing (in g/L) 3 beef extract, 10 peptone and 5 NaCl at 30 °C on a rotary shaker at 120 r/min for 24 h. Subsequently, biomass was separated by centrifugation at 3500 *g* for 5 min, and washed three times with sterile distilled water (Chen et al., 2014). Seeds were surface sterilized in 10% H_2O_2 solution for 10 min and rinsed with sterile distilled water. Then the seeds were germinated in Hoagland solution at 25 °C for 14 d.

2.3. Pyrene degradation experiment

The soil samples were sterilized at 121 °C for 30 min. Pyrene dissolved in distilled acetone was added into the sterile soils at 0 (T0), 11.3 ± 1.0 (T1), 52.5 ± 1.7 (T2) and 106.0 ± 8.8 mg/kg (T3), respectively. After balancing for 14 d with soil moisture of 70%, and fertilizing with 60 mg/kg urea and 120 mg/kg $(NH_4)_2HPO_4$, 600 g soil was put into pot. Alfalfa seedlings were thinned out after 7 d of growth to leave 10 plants each pot. The exogenous bacteria suspended in sterile distilled water were added into the soil. Subsequently, the pots were put in greenhouse in which the day and night temperature, illumination time and intensity were set at 28 ± 2 °C, 24 ± 2 °C, 14 h/d and 5000–6500 lx, separately.

Treatments in this trial were set as follows: (1) alfalfa-planted soil without pyrene (CK); (2) pyrene-contaminated soil without alfalfa (Control); (3) pyrene-contaminated soil with alfalfa (B0); (4) pyrene-contaminated soil with alfalfa and *A. oxydans* (B1); (5) pyrene-contaminated soil with alfalfa and *S. auricularis* (B2); (6) pyrene-contaminated soil with alfalfa and *S. maltophilia* (B3). Biomass of exogenous bacteria was 50 mg/kg and all of the experiments were performed in triplicate.

The transfer coefficients (TCs) and biological accumulation coefficients (BACs) of pyrene are calculated as follows:

TCs = Pyrene concentration in shoot of alfalfa/Pyrene concentration in root of alfalfa.

BACs = Pyrene concentration in biomass/Residual pyrene concentration in soil.

2.4. Preparation of samples

After 45 d, the alfalfa roots and shoots were separated, washed with deionized water, dried by freeze-drying, and then milled. Rhizosphere soil was obtained by collecting soil adhering to root surface after shaking off the excess soil on it, and the non-rhizosphere soil was attained by sampling the mixture of pot soil. All soil samples were used to determine residual pyrene, metabolites, soil enzyme and microbial activity.

2.5. Determination of samples

Pyrene and its metabolites in samples were analyzed by gas chromatography–mass spectrometry (GC–MS) (QP2010, Shimadzu) equipped with a type Rxi-5MS GC column (30 m × 0.25 mm × 0.25 μm) (Teng et al., 2011). Briefly, 20 g freeze-dried samples were extracted by the mixture of 40 ml *n*-hexane, 40 ml dichloromethane and 20 ml acetone for 36 h. The organic mixture was collected, concentrated, dissolved and transferred to alumina-silica gel packed column. Subsequently, the eluent was dried, dissolved and analyzed.

BIOLOG ECO microplates were used to analyze substrate utilization patterns of microbial communities in rhizosphere soil of T0, T1, T2 and T3 treatments. These plates contained 96 wells with different carbon sources and a blank well with no substrate. Each well had the redox dye tetrazolium which was reduced by NADH produced by microbial metabolic pathways. The rate of color development in the wells correlated with the rate of cellular metabolism. Briefly, 10 g soil samples were mixed with 100 ml 0.85% sterilized NaCl solution, blended at 120 r/min for 15 min. Then samples of 150 μl were inoculated into each well of the microplate, and incubated at 25 °C in the dark. The optical density at 590 nm of each well was determined every 12 h. Diversity of microbial carbon utilization was studied by means of species richness, Shannon, Simpson and McIntosh (Kirk et al., 2005).

2.6. Statistical analysis

The mean values of triplicate samples were used in the calculations of pyrene degradation, soil enzyme and Biolog data by Origin 7.5 and SPSS 13.0 software. Principal component analysis (PCA) was performed to investigate the differences of the functional diversity in soil microbial communities.

3. Results and discussion

3.1. Residual pyrene in soil under different conditions

With increase in initial concentration of pyrene, the removal efficiencies decreased both in rhizosphere and non-rhizosphere

Table 1
Residual pyrene in soils under different treatments.

Soil samples		Rhizosphere soil				Non-rhizosphere soil			
Treatments		T0	T1	T2	T3	T0	T1	T2	T3
Residual pyrene (mg/kg)	CK	nd	–	–	–	nd	–	–	–
	Control	nd	10.0 ± 0.5[a]	47.5 ± 3.8[a]	98.3 ± 6.8[a]	nd	10.5 ± 1.2[a]	48.8 ± 5.2[a]	99.8 ± 8.5[a]
	B0	nd	6.9 ± 0.9[b]	33.8 ± 3.6[b]	71.3 ± 4.5[b]	nd	7.2 ± 0.2[b]	36.4 ± 2.9[b]	76.3 ± 7.0[b]
	B1	nd	2.0 ± 0.3[c]	15.0 ± 1.5[c]	41.2 ± 1.8[c]	nd	2.7 ± 0.1[c]	17.4 ± 1.1[c]	47.0 ± 2.4[c]
	B2	nd	3.0 ± 0.2[d]	18.7 ± 1.4[d]	44.8 ± 1.0[d]	nd	4.6 ± 0.3[d]	21.8 ± 1.7[d]	51.3 ± 3.7[d]
	B3	nd	2.2 ± 0.3[e]	16.1 ± 1.0[e]	42.3 ± 3.1[c]	nd	2.8 ± 0.2[c]	18.8 ± 1.2[c]	48.2 ± 2.9[c]

[a,b,c,d] Dancan examination was used to determine the difference between data ($P < 0.05$). Data marked with different superscripts mean significant difference, those marked with same superscripts mean non-significant difference. Pyrene concentrations of T0, T1, T2 and T3 were 0, 11.3 ± 1.0, 52.5 ± 1.7 and 106.0 ± 8.8 mg/kg, respectively. CK, Control, B0, B1, B2 and B3 represented alfalfa-planted soil without pyrene, soil without alfalfa and microbes, alfalfa treatment, alfalfa and *A. oxydans* treatment, alfalfa and *S. auricularis* treatment, alfalfa and *S. maltophilia* treatment, separately.

soils (Table 1). The residual pyrene in rhizosphere soil was around 9% lower than that in non-rhizosphere soil, which was primarily attributed to permeability, soil aeration, microbial growth stimulation or the selection of effective microbes in rhizosphere. Even though pyrene was set at the same initial concentration, there was some difference in the residual concentration of pyrene under different treatments. Compared with the control, the removal efficiencies in rhizosphere soil were 27%, 62%, 56% and 61%, and those in non-rhizosphere soil were 25%, 60%, 50% and 58%, under treatments B0, B1, B2 and B3, respectively. This indicated that pyrene was effectively removed by alfalfa, and the removal was significantly enhanced by *A. oxydans*, *S. auricularis* and *S. maltophilia*. Microorganism-assisted phytoremediation is an effective method to degrade organic contaminants in soils by plants and their associated microbes. Gao et al. (2011) also found a significant enhancement of PAH degradation when arbuscular mycorrhizal fungi were present in alfalfa-planted soils, with more than 88.1% pyrene being degraded within 70 d. After degradation by alfalfa and *Rhizobium meliloti* for 90 d, the concentrations of PAHs in an agricultural soil were also significantly declined in comparison with alfalfa or microbe treatment (Teng et al., 2011). Although hybrid poplar acted as the main effective approach for PAH dissipation, *Burkholderia fungorum* led to the highest PAH abatement (Andreolli et al., 2013).

3.2. Pyrene accumulation in alfalfa

With the rise of pyrene concentration in B0 treatment, pyrene accumulation in both shoot and root of alfalfa increased significantly (Table 2). Although pyrene in shoot was remarkably higher than in root, the total accumulated one in shoot was lower than that in root because of the small biomass. BACs in shoot and root were 3.2–9.8 and 1.1–4.5, separately. Both the TCs and BACs of pyrene in alfalfa decreased along with the increasing initial concentration of pyrene in soil. These findings indicated that translocation of the tested PAHs across different parts of alfalfa selected in the current experiments was easy. BACs of B1, B2 and B3 treatments also significantly rose as compared with B0 ($P<0.01$), which were 6.7 – 3.1, 6.3 – 2.6 and 6.4 – 3.0, respectively. Pyrene accumulation by root increased 114%, 80% and 109%, separately. TCs under three strains treatments were 0.46 – 0.37, 0.46 – 0.37 and 0.44 – 0.37, respectively. These findings demonstrated that the strains used in the current experiments all enhanced alfalfa's ability to accumulate pyrene.

Along with the increase of initial pyrene concentration, pyrene accumulation both in shoot and root ascended significantly ($P<0.01$) under treatments B1, B2 and B3, compared with B0. In comparison with *Glomus mosseae* and *Glomus etunicatum*, the enhanced function of our strains was greater than that of those fungi, which only increased pyrene accumulation in alfalfa roots but decreased in shoots (Gao et al., 2011). Phytoremediation of PAH-contaminated soils primarily involves PAH absorption by plants, plant transport and volatilization, plant secretion and enzyme decomposition, and strengthened absorption and degradation by rhizosphere microbes. For example, the spore of *Rhizophagus custos* could absorb and store up anthracene (Aranda et al., 2013). The absorption of hydrophobic organic pollutants in soils by plants is considered to be the rate-limiting process, which contains several steps: from soil to soil pore water, from water to root, and from xylem water to shoot (Gao and Collins, 2009). Exogenous microbes in rhizosphere soils are the connection between water and root for pollutant transport accelerating the absorption reaction.

3.3. Contributions of biotic and abiotic factors on pyrene removal

Pyrene removal involves abiotic losses (percolation, adsorption, photolysis and volatilization), biological effects (plant enrichment and degradation, and microbial metabolism), and combined effect of plant and microorganism. On the premise of considering no interactive effects among different factors, pyrene removal efficiencies could be calculated in Eqs.(1)–(3).

$$R_{control} = Ta \quad (1)$$

$$R_{B0} = Ta + Tpc + Tpa \quad (2)$$

$$R_{B1,2,3} = Ta + Tpc + Tpa + Tpm \quad (3)$$

where $R_{control}$ and R_{B0} represent the control and *B*0, individually. $R_{B1,2,3}$ is pyrene removal efficiencies under treatments *B*1, *B*2 and *B*3. *Ta, Tpc, Tpa* and *Tpm* are abiotic loss, plant catabolism, plant accumulation and joint effect of plant and microorganism, respectively.

Table 3 revealed that abiotic loss was not the main route of removing pyrene, nor the plant accumulation and metabolism. Although some related researches showed that the effects of plants and bacteria on PAH degradation were not necessarily cumulative (Jouanneau et al., 2005), the major contribution of Tpm to pyrene removal under treatments B1, B2 and B3 in the current experiments clearly revealed that the joint action was the main pathway for pyrene-contaminated soil restoration. The positive joint effects of these strains with alfalfa were ranked as

Table 2
Pyrene concentration in shoot and root of alfalfa under different treatments.

Parts of alfalfa		Shoot				Root			
Treatments		T0	T1	T2	T3	T0	T1	T2	T3
CK	Pyrene (mg/kg)	nd	–	–	–	nd	–	–	–
	Biomass (g)	0.11 ± 0.01^{A}	–	–	–	0.91 ± 0.04^{A}	–	–	–
B0	Pyrene (mg/kg)	nd	0.11 ± 0.01^{a}	0.28 ± 0.02^{a}	0.33 ± 0.03^{a}	nd	0.05 ± 0.01^{a}	0.10 ± 0.01^{a}	0.12 ± 0.01^{a}
	Biomass (g)	0.11 ± 0.01^{A}	0.11 ± 0.01^{A}	0.11 ± 0.01^{A}	0.11 ± 0.01^{A}	0.91 ± 0.03^{A}	0.92 ± 0.03^{A}	0.91 ± 0.02^{A}	0.90 ± 0.02^{A}
B1	Pyrene (mg/kg)	nd	0.16 ± 0.02^{b}	0.66 ± 0.06^{b}	0.88 ± 0.08^{b}	nd	0.07 ± 0.00^{b}	0.26 ± 0.02^{b}	0.33 ± 0.04^{b}
	Biomass (g)	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}	0.11 ± 0.01^{B}	0.91 ± 0.06^{B}	0.92 ± 0.02^{B}	0.91 ± 0.06^{B}	0.91 ± 0.04^{B}
B2	Pyrene (mg/kg)	nd	0.15 ± 0.01^{c}	0.52 ± 0.05^{c}	0.73 ± 0.07^{c}	nd	0.07 ± 0.01^{c}	0.20 ± 0.01^{c}	0.27 ± 0.03^{c}
	Biomass (g)	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}	0.11 ± 0.01^{A}	0.91 ± 0.05^{B}	0.92 ± 0.03^{B}	0.91 ± 0.05^{B}	0.90 ± 0.04^{B}
B3	Pyrene (mg/kg)	nd	0.16 ± 0.01^{b}	0.64 ± 0.08^{b}	0.85 ± 0.09^{b}	nd	0.07 ± 0.00^{b}	0.25 ± 0.02^{b}	0.32 ± 0.03^{b}
	Biomass (g)	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}	0.12 ± 0.01^{C}	0.11 ± 0.01^{B}	0.91 ± 0.06^{B}	0.92 ± 0.04^{B}	0.91 ± 0.02^{B}	0.90 ± 0.05^{B}

^{a,b,c,d,A,B,C,D} Dancan examination was used to determine the difference between data ($P<0.05$). Data marked with different superscripts mean significant difference, those marked with same superscripts mean non-significant difference.

B1 > B3 > B2. The structural complexity, low water solubility and low vapor pressure of PAHs result in their persistence in natural environments. Therefore, abiotic disappearance is not a major pathway for PAHs removal. The fact that less than 15% of PAHs removed through the mangrove plant uptake was also consistent with the present study that the prevailing route for PAHs removal relied on the synergistic metabolism of plant and microbes (Tam and Wong, 2008).

3.4. Activities of soil dehydrogenase and polyphenol oxidase

Dehydrogenase is involved in various biological processes, and has been considered as an indicator of microbial activities (Wang et al., 2014). Compared with the control, dehydrogenase activities in soils under treatments B0, B1, B2 and B3 all increased significantly ($P < 0.01$, Fig. 1A and B), but decreased remarkably when pyrene concentration increased. This suggested that alfalfa contributed to the expression of soil dehydrogenase, which was significantly enhanced by three exogenous strains. The upregulation expression of dehydrogenase induced by alfalfa was primarily related to root exudates. Similar results found by Wang et al. (2014) confirmed that the removal of 4- and 5-ring PAHs, dehydrogenase activity and six types of low-molecular-weight organic acids released by *Bruguiera gymnorrhiza* exhibited significantly positive correlation. In addition, moderate or low concentrations of pyrene stimulated plant roots and rhizosphere microorganisms to secrete more dehydrogenase, which in turn promoted pyrene decomposition. However, high concentration of pyrene inhibited

Table 3
Contributions of biotic and abiotic factors to pyrene degradation under different treatments.

Contribution (%)	Control			B0			B1			B2			B3		
Treatments	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
T_a	8.0	7.4	6.0	8.0	7.4	6.0	8.0	7.4	6.0	8.0	7.4	6.0	8.0	7.4	6.0
T_{pa}	—	—	—	8. 7	4.0	2.3	13.3	10.3	6.3	12.5	8.0	5.1	12.9	10.0	6.1
T_{pc}	—	—	—	19.8	20.0	20.5	15.2	13.8	16.4	16.1	16.0	17.6	15.6	14.0	16.6
T_{pm}	—	—	—	—	—	—	40.7	36.1	27.7	24.9	28.0	23.8	39.3	33.5	26.6
Total	8.0	7.4	6.0	36.5	31.4	28.8	77.2	67.6	56.4	61.5	59.4	52.5	75.8	64.9	55.3

Ta, Tpc, Tpa and Tpm are abiotic loss, plant catabolism, plant accumulation and joint effect of plant and microorganism, respectively.

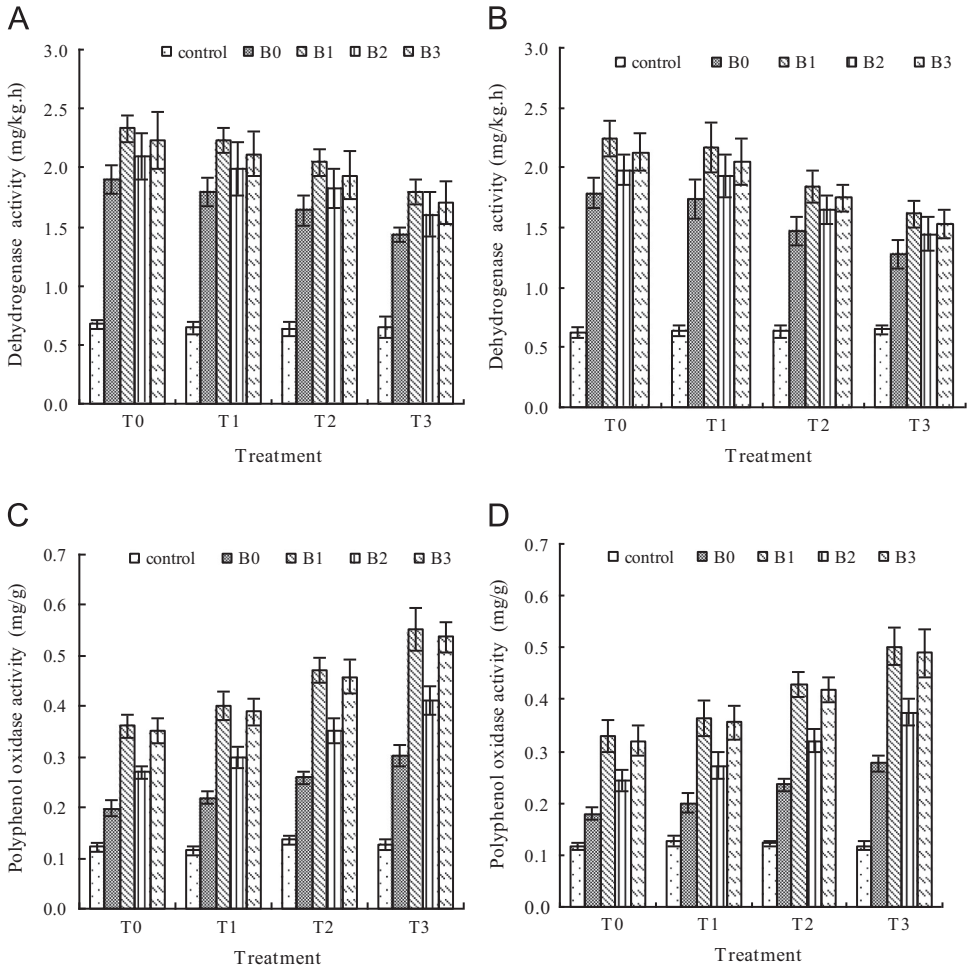

Fig. 1. Dehydrogenase ((A) rhizosphere and (B) non-rhizosphere) and polyphenol oxidase ((C) rhizosphere and (D) non-rhizosphere) activities in soils under different treatments.

alfalfa growth and microbe reproduction, resulting in the reduction in dehydrogenase activity.

As an important enzyme in soil, the activity of polyphenol oxidase embodied the ability of microorganisms and plants to degrade organic substances (Lee et al., 2008). Compared with the control, polyphenol oxidase activities in rhizosphere soils under the treatments B0, B1, B2 and B3 also showed significant enhancement from 61% to 143%, 194% to 341%, 119% to 230%, and 186% to 330%, respectively ($P<0.01$). Sun et al. (2010) found a similar significant increase of the polyphenol oxidase activities in planted soil, which suggested that the polyphenol oxidase was primarily originated from the plant root. Fig. 1C and D indicated that polyphenol oxidase activities in both rhizosphere and non-rhizosphere soils ascended with the increase of pyrene concentration. This improvement was partially responsible for the enhanced removal of pyrene by plant and microorganism association, as shown in Section 3.1.

3.5. Soil microorganism activity and carbon utilization diversity

AWCDs under different treatments all fell down obviously as pyrene concentration increased (Fig. 2A), which demonstrated that pyrene exerted suppressive effect on the metabolism of microbial communities. As time extended, the ability of microbial communities using carbons gradually increased. There were significant differences in the AWCD of B0, B1, B2 and B3 compared with the control ($P<0.01$). These results were mainly attributed to the following reasons: (1) All soils used in the current study were sterilized, resulting in low microbe activity in the control. (2) Alfalfa significantly improved the activity of soil microorganisms ($P<0.01$). For example, perennial ryegrass and alfalfa have been found to increase the number of rhizosphere bacteria in the hydrocarbon-contaminated soil (Kirk et al., 2005). Meanwhile, the metabolic activity and biomass of soil microorganisms could be greatly enhanced by root exudates (Lee et al., 2008; Sun et al., 2010). During the microbe-assisted phytoremediation process, alfalfa clearly increased the biomass of PAH-degrading bacteria, microbial activities and carbon assimilation in the soil microbial community (Teng et al., 2011). (3) Exogenous strains used in the current experiments notably improved the soil microbial diversity ($P<0.01$), enhancing pyrene degradation. Zhong et al. (2011) verified that *Rhizobium meliloti* significantly lowered PAH concentration in soil, increased the soil microbial activity, and enhanced carbon utilization by soil microbial community.

Carbon substrates in the Biolog plates were classified as polymer, carbohydrate, amphiphilic product, carboxylic acid, amino acid and amine to investigate the differences of the functional diversity in soil microbial communities through PCA. PC1 and PC2 were separated from these carbons, and they accounted for 47% and 27% of the used variable variance,

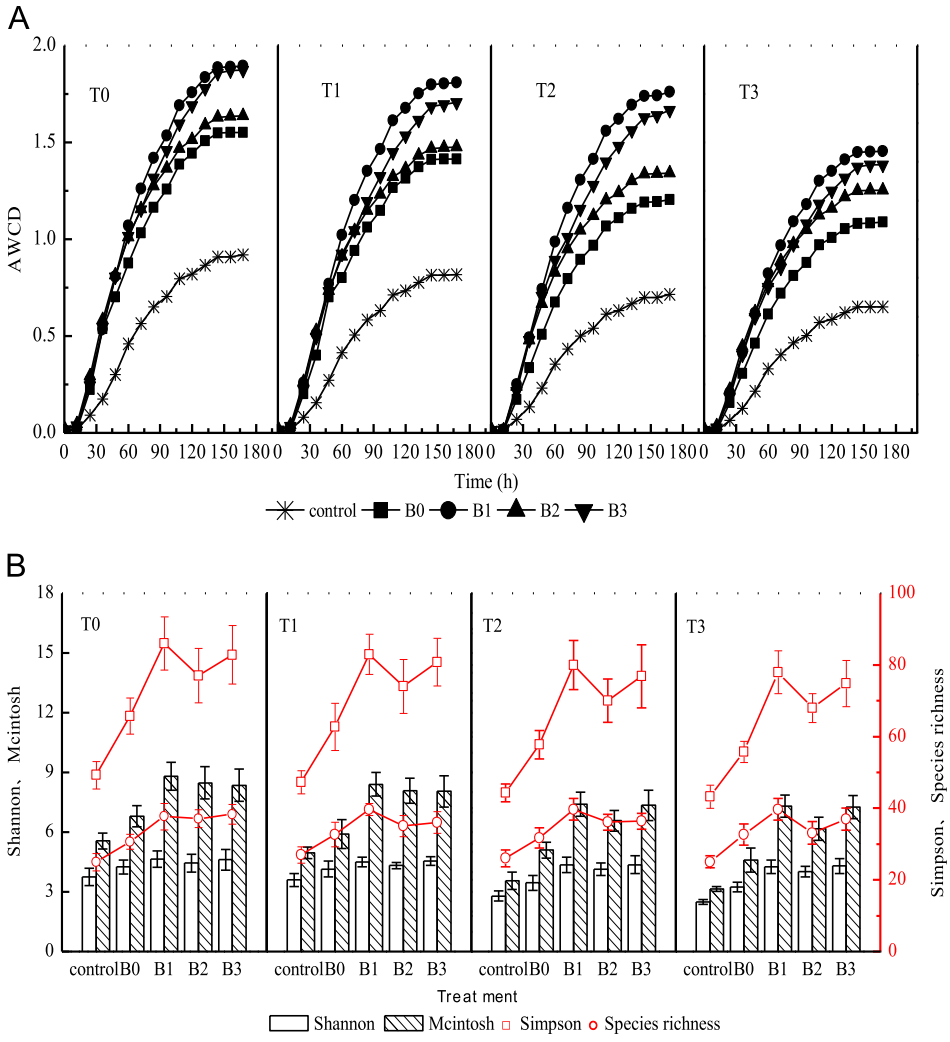

Fig. 2. (A) AWCD of microorganisms in rhizosphere soil of T0, T1, T2 and T3 treatments with incubation time. (B) Functional diversity of microbial community in different treatments.

respectively. PC1 basically reflected the information related with polymer, carbohydrate and amphiphilic product, while PC2 essentially represented one pertaining to carboxylic acid, amino acid and amine. These findings illustrated that polymer, carbohydrate and amphiphilic product were assimilated by a group of microbes, whereas carboxylic acid, amino acid and amine were used by other kind of microbes.

Species richness increased in the presence of alfalfa, and was further enhanced with the addition of exogenous strains (Fig. 2B). The similar variation trends of Shannon, Simpson and McIntosh were also observed. In comparison to the control, these indexes under the alfalfa-planted treatments at the same level of pyrene all increased greatly ($P < 0.01$), which was due to the function of alfalfa roots. Furthermore, the indexes in treatments B1, B2 and B3 were all greater than those in B0 ($P < 0.01$), suggesting that the joint treatment further enhanced the function diversity of soil microbial community. It was clear that these indexes could reflect the influence of plant and exogenous microbes on the diversity of soil microbial communities.

3.6. Pyrene transformation

Pyrocatechol, phthalic acid, 1-naphthol, 2-naphthol, 1,2-naphthalenedione, 1,2-dihydroxynaphthalene, 4-dihydroxy-phenanthrene, 3-methylcoumarin and 9,10-phenanthrenedione were detected in treatments B1, B2 and B3 (Fig. 3A). The identification of 4-dihydroxy-phenanthrene and 9,10-phenanthrenedione revealed

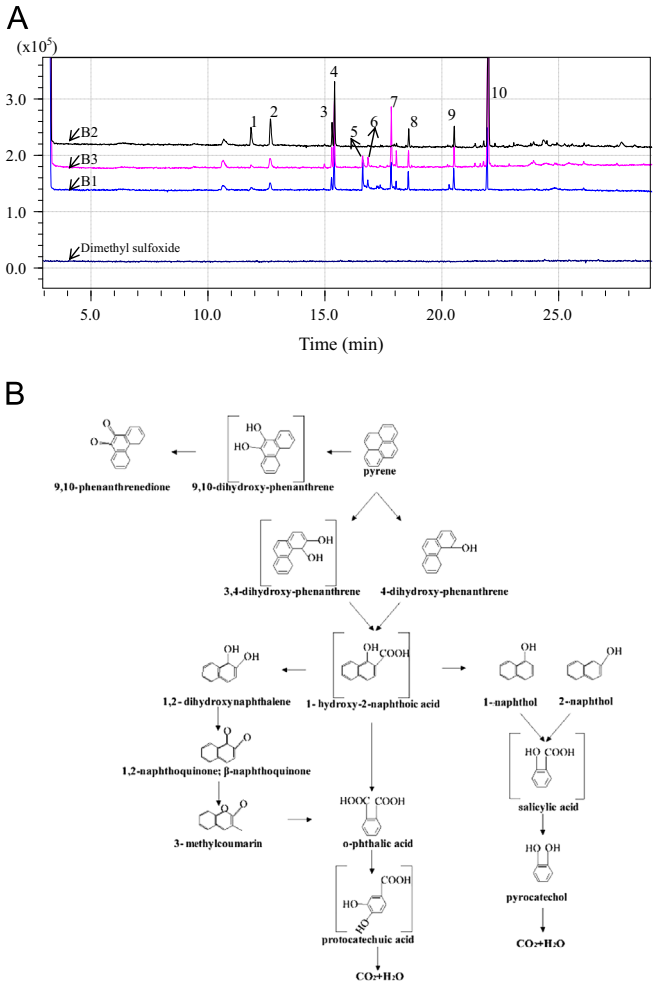

Fig. 3. (A) GC–MS analysis of pyrene metabolites produced in different treatments. (B) Pathways of pyrene degradation by exogenous microorganisms and alfalfa.

that under the joint action of bacteria and alfalfa, pyrene was initially degraded through ring cleavage, which was an efficiency controlling step for PAH biodegradation. Subsequently, 4-dihydroxy-phenanthrene was further broken down through monooxygenase-induced degradation process (Seo et al., 2012). With the catalysis of oxygenase, it could be degraded into *cis*-diol via 3,4-C dioxygenation (Seo et al., 2006), then dihydroxyl compounds were created. After further ring cleavage, 1-hydroxy-2-naphthoic acid was formed (Zhong et al., 2011), and transformed to 1-naphthol, 2-naphthol and other bicyclic compounds. The metabolites of 1-naphthol and 2-naphthol could be further degraded through salicylic acid pathway, producing salicylic acid and pyrocatechol.

There were two pathways for hydroxy phenanthrene degradation after it was transformed into 1-hydroxy-2-naphthoic acid (Fig. 3B). One was phthalic acid pathway, the other was salicylic acid pathway. In our experiment, salicylic acid was not detected, probably due to its quick utilization by microbes, nevertheless, pyrocatechol and phthalic acid were proven to be presented in all samples. Considering that pyrocatechol was the metabolite of salicylic acid, it was inferred that after pyrene was degraded into phenanthrene, the product was further metabolized through the combination of phthalic acid and salicylic acid pathways (Ye et al., 2011).

4. Conclusions

Although alfalfa uptake and accumulation had a certain effect on pyrene removal, the biological metabolism and joint effects of alfalfa and microorganisms mainly contributed to pyrene removal. *Arthrobacter oxydans*, *S. auricularis* and *S. maltophilia* significantly enhanced soil dehydrogenase, polyphenol oxidase, microbial activities, community diversity, pyrene degradation and uptake. However, these exogenous bacteria did not change the pathway of pyrene degradation, which was initiated through ring cleavage. The produced 4-hydroxy-phenanthrene was transformed into naphthol and 1,2-dihydroxy-naphthalene, which were further degraded through salicylic acid pathway and phthalic acid pathway, separately.

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