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Influence of *Rhizobium meliloti* on phytoremediation of polycyclic aromatic hydrocarbons by alfalfa in an aged contaminated soil

Ying Teng[a], Yuanyuan Shen[a], Yongming Luo[a,*], Xianghui Sun[a], Mingming Sun[a], Dengqiang Fu[a], Zhengao Li[a], Peter Christie[b]

[a] Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[b] Agri-Environment Branch, Agri-Food and Biosciences Institute, Newforge Lane, Belfast BT9 5PX, United Kingdom

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ABSTRACT

Microbe-assisted phytoremediation is emerging as one of the most effective means by which plants and their associated rhizosphere microbes degrade organic contaminants in soils. A pot study was conducted to examine the effects of inoculation with *Rhizobium meliloti* on phytoremediation by alfalfa grown for 90 days in an agricultural soil contaminated with weathered polycyclic aromatic hydrocarbons (PAHs). Planting with uninoculated alfalfa (P) and alfalfa inoculated with *R. meliloti* (PR) significantly lowered the initial soil PAH concentrations by 37.2 and 51.4% respectively compared with unplanted control soil. Inoculation with *R. meliloti* significantly increased the counts of culturable PAH-degrading bacteria, soil microbial activity and the carbon utilization ability of the soil microbial community. The results suggest that the symbiotic association between alfalfa and *Rhizobium* can stimulate the rhizosphere microflora to degrade PAHs and its application may be a promising bioremediation strategy for aged PAH-contaminated soils.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants that are widely distributed in soils [1]. Soils have become an important environmental sink for PAHs because of their large holding capacity for pollutants and remediation of PAH-contaminated soils has to be undertaken. Bioremediation, i.e. the use of living organisms to remediate polluted soils, is an emerging technology [2,3]. In the past two decades numerous studies have indicated that microbe-assisted phytoremediation may have some potential as an effective and inexpensive means to clean up polluted soils [4–10]. The successful application of these remediation techniques is largely dependent on the capacity of contaminant degraders or plant growth promoting microbes to efficiently colonize growing roots of plants of various species and nutrient status [10].

It is well known that symbiotic nitrogen-fixing bacteria are commonly associated with the roots of leguminous plants. Rhizobia colonize the roots of legumes where they fix atmospheric N_2, some of which can be utilized for plant growth [11]. Rhizobia are found in contaminated environments where various toxic chemicals are present [12,13]. Several bacterial species in the genus *Rhizobium* are able to utilize PAHs, polychlorinated biphenyls (PCBs), or heterocyclic aromatic compounds [14–17] but little is known about the ability of *Rhizobium* to further degrade PAHs in soils. Rhizobial bacteria have been studied in relation to phytoremediation because of their potential to form symbiotic associations with leguminous plants [18–20]. Alfalfa (*Medicago sativa* L.), the most widely grown perennial legume in the world, is a deep-rooted perennial species that may have strong potential for the remediation of a number of organic contaminants [21–24]. Studies have therefore begun on the potential of the alfalfa–*Rhizobium* symbiosis to remediate soils contaminated with persistent organic pollutants such as polychlorinated biphenyls (PCBs) and petroleum hydrocarbon compounds (PHC) [21,25,26]. However, little is known about the effects of *Rhizobium* on phytoremediation by alfalfa of aged PAH-contaminated soils. A recent study found that total concentrations of 16 PAHs in some surface soils in the Yangtze River Delta region of east China reached about 10,000 $\mu g\ kg^{-1}$ dry soil, with 3-ring, 4-ring and 5(+6)-ring PAH congeners accounting for about 6, 55 and 39% of the total PAH contents. The bioremediation of PAH-contaminated agricultural soils has therefore become a major environmental interest in this region.

The two main aims of the present study were to evaluate the potential of the alfalfa–*Rhizobium* association to remediate PAH-

* Corresponding author at: Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, No. 71 East Beijing Road, Nanjing 210008, Jiangsu, China.
Tel.: +86 25 86881101; fax: +86 25 86881128.
E-mail address: ymluo@issas.ac.cn (Y. Luo).

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contaminated soil and to examine the associated changes in soil PAH-degrading bacterial counts and in microbial activity.

2. Materials and methods

2.1. Soil

The soil used was collected from the top 15 cm of the soil profile of PAH-contaminated agricultural land in Anzhen town, Wuxi district, Jiangsu province, in the Yangtze Delta region of east China. The contaminated site was adjacent to an iron and steel foundry. Uncontrolled emissions of gases and dust from the factory have resulted in PAH pollution over a 30-year period. The soil type is classified as a Hortic Anthrosol according to the FAO soil classification system [27]. Stones and plant root residues in the soil sample were discarded and the soil was air-dried, sieved through a 2-mm mesh, and stored at 4 °C in darkness for 3 days prior to use. Physico-chemical analysis [28] shows that the soil is a silt loam with 11.1 g kg^{-1} total organic carbon, a pH (in water) of 6.4, 1.0 g kg^{-1} total nitrogen, 14.7 g kg^{-1} total potassium, and 78.4 mg kg^{-1} hydrolysable nitrogen on a dry weight basis. The concentration of 16 individual PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene) was 10124 μg kg^{-1} dry soil, with concentrations of the 3-, 4-,5- and 6-ring PAHs of 661, 5547, 2656 and 1260 μg kg^{-1}, respectively. According to the Canadian Environmental Quality Guidelines released by the Canadian Council of Ministers of the Environment [29], this heavily PAH-contaminated soil would not be suitable for agricultural use because of the high concentration of PAHs present (more than 1000 μg kg^{-1} dry soil).

2.2. Microbial inocula and host plants

The rhizobial strain used was *Rhizobium meliloti* (strain ACCC 17519) from the Agricultural Culture Collection of China. *Rhizobium* cultures were prepared by inoculating 100 ml of yeast extract mannitol broth with a loopful of cells from a stock culture and growing on a rotary shaker (200 rev min^{-1}) at 28 °C for 48 h. The cultures contained approximately 1.8×10^8 colony forming units per ml (CFU ml^{-1}). The cultures were absorbed in pre-sterilized peat in a 2:1 ratio (wt/vol) of peat and *Rhizobium* culture and thoroughly mixed to produce the inoculum (a population of 1.5×10^9 CFU g^{-1} of inoculum). Alfalfa seeds (*Medicago sativa* L.) were purchased from Jiangsu Academy of Agricultural Sciences, China. Before sowing, the seeds were surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min, rinsed with sterile distilled water and germinated on moist filter paper overnight.

2.3. Experimental design and sample collection

The four treatments were set up in a fully randomized layout of greenhouse pots with four replicates of each treatment. The treatments were: (1) unplanted control soil with sterilized *R. meliloti* inoculum (CK); (2) unplanted soil inoculated with *R. meliloti* inoculum (R); (3) soil planted with alfalfa and sterilized *R. meliloti* inoculum (P); and (4) soil with alfalfa and inoculated with *R. meliloti* inoculum (PR). Surface sterilized and germinated seeds were sown in porcelain pots (15 cm high and 20 cm in diameter) containing 1.5 kg air-dried soil inoculated with 15 g *R. meliloti* inoculum per pot. Uninoculated treatments received 15 g of sterilized inoculum. The soil moisture content of all pots was adjusted to 60% of water holding capacity (WHC). After germination the seedlings were thinned to four per pot and the plants grew for 90 days. Throughout the growing period the plants were monitored daily and watered as necessary. At the end of the experiment the soil was removed manually and transferred to a paper envelope. Each soil sample was divided into two parts, one of which was placed in a small plastic bag at 4 °C for subsequent analysis of microbial activities and the other was freeze-dried and passed through a 60-mesh sieve prior to analysis for PAHs. In addition, four plants were harvested from each pot, separated into roots and shoots and carefully rinsed with distilled water to remove any remaining soil.

2.4. Extraction and analysis of PAHs

PAHs in bulk soil samples were extracted using Soxhlet extraction. In brief, 5 g of freeze-dried sample with filter paper was placed in a porous cellulose thimble (25 mm × 70 mm) and placed in a Soxhlet extractor. The extractor was then fitted to a 100 ml round bottom flask containing 60 ml dichloromethane and the extraction was performed for 24 h. All the extracts in the round bottom flasks were dried by rotary evaporation. The residues were dissolved in 2 ml of cyclohexane and 0.5 ml of the solute was transferred and purified with a silica gel column (8 mm × 220 mm) and washed with a mixture of hexane and dichloromethane (1:1). The first 1 ml of eluate was discarded because it contained non-polar saturated hydrocarbons and was less retained than PAHs by silica gel. The second 2-ml aliquot of eluate was collected, dried by sparging with N_2 and then re-dissolved in 1 ml acetonitrile for HPLC determination.

PAHs in plant samples were extracted by ultrasonication. In brief, 2.0 g of freeze-dried plant sample was extracted with 10 ml methanol for 15 min using an ultrasonic bath (Model KQ-600DB, Jinan Labtek Biological Co. Ltd., Jinan, China), then centrifuged at 2236 × *g* for 5 min and the supernatant was collected. This extraction process was repeated three times. The extracts were collected in 100 ml glass vials and dried by rotary evaporation. The subsequent analytical steps were the same as described above for the analysis of the soil extracts.

The determination of 16 EPA PAHs was carried out according to the method described by Ni et al. [30]. Briefly, analysis was conducted on a Shimadzu Class-VP HPLC system (Shimadzu, Japan) with a fluorescence detector (RF-10AXL). A reversed phase column C18 (VP-ODS 150 mm × 4.6 mm I.D., particle size 5 mm) using a mobile phase of water and acetonitrile mixture (1:9, v/v) at a constant solvent flow rate of 0.5 ml min^{-1} was used to separate the 16 PAHs. The excitation and emission wavelengths for individual PAHs were set separately.

An external standard mixture was used for quantification of the 16 PAHs. The detection limit of the HPLC method for the 16 PAHs in soil was in the range of 0.12–1.57 μg kg^{-1}. Method blanks (solvent) and spiked blanks (soil spiked with standards of EPA610 PAH mixture LA 96245, Supelco, Bellefonte, PA) were extracted and analyzed by the methods described above. The recoveries and the relative standard deviations of this method for 16 PAHs were in the ranges of 74–110% and 0.53–3.57%, respectively. Results of blanks extracted under the same conditions were below detection limits and sample results are presented without recovery ratio correction.

2.5. Enumeration of PAH-degrading soil bacteria

After incubation the PAH-degrading soil bacteria were counted using a miniaturized most probable number (MPN) method in 96-well microplates with five replicates per dilution [31]. Briefly, phenanthrene, anthracene, fluorene, and dibenzothiophene were dissolved in acetone and added as the sole carbon sources to support the proliferation of aromatics-degrading bacteria. Serially diluted samples were inoculated into the wells and the microplates were incubated at room temperature for 3 weeks. Wells turning yellow or brown owing to the accumulation of partial oxidation

products of aromatic substrates were regarded as positive. Published MPN tables were used to determine the MPN value.

2.6. Soil microbial biomass C and enzyme activities

Soil microbial biomass C was determined by the fumigation–extraction method [32]. Chloroform fumigation was carried out with ethanol-free $CHCl_3$ for 24 h at 25 °C in the dark, the $CHCl_3$ was removed and the soil samples were extracted by shaking with 50 ml 0.5 mmol l^{-1} K_2SO_4 for 30 min on a rotary shaker. The suspensions were then filtered through Whatman No. 42 filter paper. Triplicate subsamples of unfumigated control soils were placed in a vacuum desiccator for 24 h at 25 °C in the dark and were extracted in the same way and at the same time as the fumigated samples. Organic C was measured with an automated TOC Analyzer (Shimadzu TOC-500, Kyoto, Japan). Biomass C (Bc) was calculated from the expression Bc = 2.22 × Ec, where Ec = [(organic C extracted from fumigated soil) − (organic C extracted from non-fumigated soil)]. The factor 2.22 is a proportionality constant, accounting for the observation that approximately 45% of biomass C is extracted after fumigation.

Soil dehydrogenase activity (DHA) was assessed by a modification of the method described by Singh and Singh [33]. Weighed 5.0-g soil subsamples were placed in 50-ml polypropylene centrifuge tubes and mixed with 5 ml 0.5% 1,3,5-triphenyltetrazolium chloride (TTC) solution. Tubes were incubated for 6 h at 30 °C in the dark. After incubation, triphenylformazan (TPF) formed by the reduction of TTC was extracted with three batches of 100 ml methanol. Tubes were shaken in an orbital shaker at 300 rev min^{-1} for 1 h, centrifuged (1744 × *g*, 5 min), and the supernatant was filtered with filter paper. Blanks without the addition of TTC were processed in the same manner. The concentration of TPF was determined by spectrophotometry at 485 nm and the results are expressed as g TPF g^{-1} soil.

2.7. Physiological profiles of the soil microbial community

Soil microbial community level physiological profiles were performed as described by Yao et al. [34]. Briefly, 10 g of fresh soil was added to 100 ml of distilled water in a 250 ml flask and shaken for 10 min. Ten-fold serial dilutions were made and the 10^{-3} dilution was used to inoculate BIOLOG® ECO plates (BIOLOG, Hayward, CA). The plates were incubated at 25 °C for 7 days and color development in each well was recorded as optical density (OD) at 590 nm with a plate reader at regular 12-h intervals. Microbial activity in each microplate, expressed as average well-color development (AWCD), was determined using the expression $AWCD = \sum OD_i/31$, where OD_i is the optical density value from each well [35].

2.8. Statistical analysis

The percentage of PAH removal (%) was given by the formula removal (%) = 100 × [(Mi − Ms)/Mi], where Ms was the concentration of PAHs in each treatment and Mi was the initial PAH concentration present in soil. Statistical analysis was carried out using the SPSS 13.0 for Windows software package. Data were analyzed by one-way analysis of variance. Mean values were compared by Duncan's new multiple range test at the 5% level using the SPSS package.

3. Results

3.1. Soil PAH removal

Soil target PAH concentrations after planting with alfalfa and inoculation with *Rhizobium* are presented in Fig. 1. After 90 days

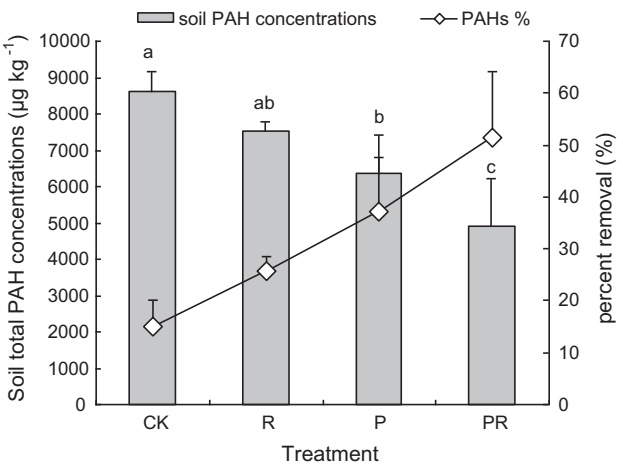

Fig. 1. Soil concentrations and percent removal of target PAHs in the different treatments. CK, unplanted control soil with sterilized *R. meliloti* inoculum; R, unplanted soil inoculated with *R. meliloti* inoculum; P, soil planted with alfalfa and sterilized *R. meliloti* inoculum. PR, soil with alfalfa and inoculated with *R. meliloti* inoculum. Data are displayed as mean and standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ by Duncan's multiple range test ($n = 4$).

the final PAH concentrations across all treatments ranged from 4916 to 8620 μg kg^{-1} dry soil and there were significant differences between planted and unplanted soils ($p < 0.05$). At the end of the experiment the lowest concentrations of PAHs were observed in soil with alfalfa and inoculated with *Rhizobium* (PR), which was significantly lower than in all other treatments ($p < 0.05$). The inoculated *Rhizobium* treatment (R) also slightly lowered PAH concentrations in soil but did not differ significantly from control (CK) soil. The PAH concentration in soil with alfalfa inoculated with *Rhizobium* (PR) was degraded to 51.4% of the initial concentration after 90 days and approximately 37% of PAHs was degraded in the planted soil. The percentage PAH removal increased significantly in the planted treatment that received *Rhizobium* (PR) compared to the planted treatment without *Rhizobium* (P) ($p < 0.05$) (Fig. 1).

The concentrations of 3-, 4-, 5- and 6-ring PAHs in soil under the different treatments are shown in Fig. 2. In treatment P the residual levels of 3-, 4-, 5- and 6-ring PAHs were 217.1 ± 46.5, 3510.5 ± 759.6, 1317.3 ± 203.9 and 1035.5 ± 180.9 μg kg^{-1} dry soil,

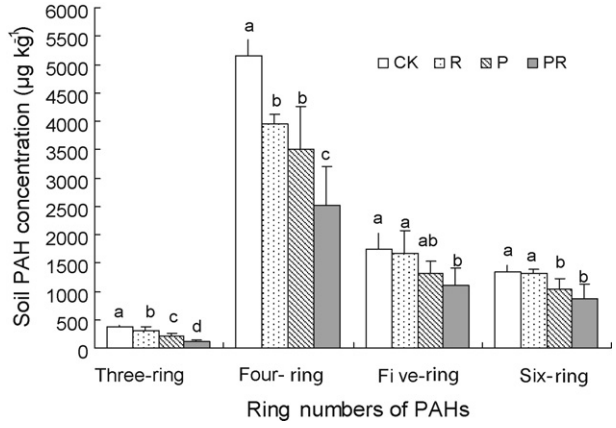

Fig. 2. Concentrations of different ring PAH compounds in the soil under the different treatments. CK, unplanted control soil with sterilized *R. meliloti* inoculum; R, unplanted soil inoculated with *R. meliloti* inoculum; P, soil planted with alfalfa and sterilized *R. meliloti* inoculum. PR, soil with alfalfa and inoculated with *R. meliloti* inoculum. Data are displayed as mean and standard deviation. Different letters in the same ring compounds indicate a significant difference at $p < 0.05$ by Duncan's multiple range test ($n = 4$).

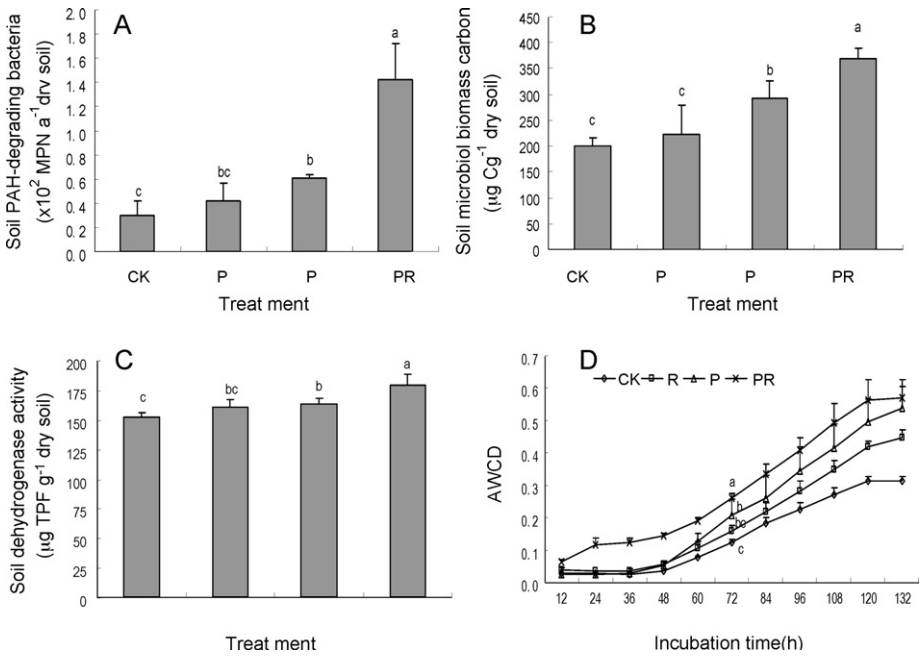

Fig. 3. Soil PAH-degrading bacteria and microbial activities under the different treatments. CK, unplanted control soil with sterilized *R. meliloti* inoculum; R, unplanted soil inoculated with *R. meliloti* inoculum; P, soil planted with alfalfa and sterilized *R. meliloti* inoculum. PR, soil with alfalfa and inoculated with *R. meliloti* inoculum. Data are displayed as mean and standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ by Duncan's multiple range test ($n = 4$).

respectively. In treatment PR the residual levels of 3-, 4-, 5- and 6-ring PAHs were 122.7 ± 28.2, 2522.2 ± 676.3, 1104.8 ± 296 and $866.4 \pm 257.5\ \mu g\ kg^{-1}$ dry soil, respectively. Compared with the control, significant degradation ($p < 0.05$) was observed of 3-, 4- and 5(+6)-ring PAHs in both treatments. Inoculation with *Rhizobium* markedly enhanced the depletion of 3- and 4-ring PAHs, and there were significant differences between PR and P treatments and between R and CK treatments (both $p < 0.05$).

3.2. Soil PAH-degrading bacterial counts

Soil PAH-degrading bacterial counts in the different treatments after 90 days of bioremediation are presented in Fig. 3A. Compared with the controls, significantly higher counts ($p < 0.05$) of PAH-degrading bacteria were observed in the planted treatment inoculated with rhizobium (PR). Furthermore, bacterial counts in the PR treatment ($1.42 \pm 0.30 \times 10^3$ MPN g^{-1} dry soil on average) were 2.33 times higher than in the uninoculated planted treatment ($0.61 \pm 0.03 \times 10^3$ MPN g^{-1} dry soil) and the difference between the two treatments was significant ($p < 0.05$).

3.3. Soil microbial biomass, enzyme activities and microbial community level physiological profiles

After 90 days of growth both the PR and P treatments showed higher soil microbial biomass C ($p < 0.05$) than the control (Fig. 3B). Soil microbial biomass C values in the PR and P treatments were 368.5 ± 21.6 and $291.4 \pm 34.2\ \mu g\ C\ g^{-1}$ dry soil, respectively, increases of 85% and 46% compared with control soil. Furthermore, soil microbial biomass C was significantly higher ($p < 0.05$) in the PR treatment than in the P treatment. Higher dehydrogenase activities (DHA) were observed in the soil after 90 days, with significant enhancement ($p < 0.05$) in both the PR and P treatments (179.5 ± 9.1 and $163.5 \pm 4.8\ \mu g\ TPF\ g^{-1}$ dry soil, respectively) (Fig. 3C). Variation in average well color development (AWCD) of soil samples from the different treatments after 90 days of bioremediation is shown in Fig. 3D. Soil bacterial community metabolic profiles from the PR treatment also showed significantly greater carbon utilization than those from the P treatment ($p < 0.05$).

4. Discussion

Phytoremediation has now emerged as a promising strategy for removal of a variety of soil contaminants [36–39]. The efficiency of phytoremediation relies on the establishment of active plants with sufficient biomass growth, active root proliferation and/or root activities that can support a flourishing microbial consortium assisting phytoremediation in the rhizosphere [3,40,41]. Legumes, including alfalfa, have been identified as strong candidates for remediation of contaminated sites [22,23,42,43]. In the present study planted soil did show significantly lower concentrations of PAHs suggests that alfalfa in this experiment played a role in the remediation of PAH-contaminated soil. The formation of bound residues and the biodegradation of PAHs in soils are two important processes for end-point assessment during phytoremediation. Bound residue formation in soil was assessed using the methyl-isobutyl-ketone (MIBK) fractionation method [44] and using ^{13}C-PAHs combined with GC–MS isotopic ratio determination [45] and ^{13}C NMR analysis [46]. Richnow et al. [45] found that soil-bound anthracene residues increased in ^{13}C content with biological activity, and alkaline hydrolysis of samples containing bound residues released ester-bound metabolic products. The formation of bound residues is mainly attributed to the interactions between the SOM and the PAH metabolites rather than the parent PAH compounds [47]. The mineralization of parent compounds and their metabolites usually indicate the biodegradation of PAHs. Chen et al. [48] reported that 37.7 and 30.4% of ^{14}C-pyrene was mineralized in soil planted with tall fescue and switchgrass, respectively, while 4.3% mineralization was observed in unplanted controls after 190 days of incubation. Plants may therefore be able to stabilize contaminants in the soil and also enhance microbial degradation in the rhizosphere.

Furthermore, alfalfa had substantial PAH concentrations in the shoots and roots (Table 1). The average PAH concentrations in

Table 1
Influence of *Rhizobium meliloti* on root and shoot yields and PAH concentrations and percent phytoextraction of PAHs from soil.

Item	P	PR
Shoot dry weight (g pot^{-1})	3.59 ± 0.54b	5.95 ± 1.24a
Root dry weight (g pot^{-1})	4.08 ± 0.95b	6.18 ± 1.43a
PAH concentration in shoots (μg kg^{-1} dw)	105.4 ± 18.2a	127.5 ± 9.9a
PAH concentration in roots (μg kg^{-1} dw)	482.2 ± 77.2b	699.0 ± 60.0a
Percentage of PAHs phytoextracted (%)	0.015 ± 0.004b	0.034 ± 0.008a

N.B. Percentage of PAHs phytoextracted is the ratio of the total mass of PAHs in the plant to that present in the initial soil. P, soil planted with alfalfa and sterilized *R. meliloti* inoculum. PR, soil with alfalfa and inoculated with *R. meliloti* inoculum. Data are displayed as mean and standard deviation. Different letters in the same row indicate a significant difference at $p < 0.05$ by Duncan's multiple range test ($n = 4$).

shoots and roots of the P and PR treatments were 105.4 ± 18.2 and 127.5 ± 9.9 μg kg^{-1} and 482.2 ± 77.2 and 699.0 ± 60.0 μg kg^{-1} dry biomass, respectively. The percent removal rates of target PAHs by the uninoculated alfalfa and alfalfa inoculated with Rhizobium were only 0.015 ± 0.004 and 0.034 ± 0.008%, respectively. However, alfalfa clearly enhanced the total number of culturable PAH-degrading bacteria and microbial activities and the carbon utilization ability of the soil microbial community in comparison with unplanted soil. Hence, the PAH concentrations in the soil decreased, perhaps due to the rhizosphere micro-organisms, or alternatively by modification through the excretion of root exudates during the process of phytoremediation or rhizodegradation. Lee et al. [49] also found that plant-enhanced dissipation of PAHs in planted soils might be derived from increased microbial activity and enzymes released from plants. A recent study showed that PAH degradation in planted soil can be attributed primarily to the presence of degrading microorganisms in the soil [50]. A specific type of phytoremediation, namely microbe-assisted remediation, has therefore become increasingly recognized as a potentially effective method for the removal and/or degradation of organic contaminants from aged soils. To further develop this remediation technique it is likely to be necessary to employ suitable combinations of plant species and their associated rhizosphere microbes such as plant growth promoting rhizobacteria or contaminant degraders.

Rhizobial associations, and particularly the naturally symbiotic types, can be applied to enhance the phytoremediation of contaminated soils [19,20,43,51], and the legume–*Rhizobium* symbiosis may be crucial for plant establishment on contaminated sites [52]. In the present study there were significantly lower concentrations of soil PAHs in the planted treatment inoculated with *R. meliloti* than in the uninoculated planted treatment. After inoculation with *R. meliloti* the dissipation of soil PAHs may be mainly due to rhizodegradation by alfalfa. Indeed, a significant increase in counts of PAH-degrading bacteria was observed in the planted treatment inoculated with *Rhizobium* after 90 days and this may have resulted in substantial degradation of PAHs in the soil. Previous studies have indicated that rhizobia can increase exudation from host plant roots and secondary plant metabolites such as luteolin (a plant flavone) may serve to control *nodABC* expression during nodule development [53,54]. Thus, increased amounts of exudates may in turn support the growth of microbial degraders or influence pollutant availability. Johnson et al. [19,51] also found that symbiotic association with *R. leguminosarum* bv. *trifolii* enhanced plant vigour and growth in inoculated planted treatments, and that rhizobia played an important role in the rhizoremediation of high-molecular-weight PAHs. It therefore follows that enhancement of plant metabolic activities by inoculation with rhizobia may lead to stimulation of the growth of PAH-degrading bacteria. Some studies have indicated that rhizobia, either as free living cells or in symbiosis with host plants, have the ability to transform PCBs and PAHs [12,14,16,25]. In the present study, inoculation with *Rhizobium* significantly enhanced depletion of 3- and 4-ring PAHs in the soil. Moreover, we also found that microbial biomass C, soil dehydrogenase activity (DHA) and the carbon utilization ability of the soil microbial community were significantly higher with rhizobial inoculum. Soil dehydrogenase activity (DHA) is usually related to the presence of viable microorganisms and their oxidative capability [55]. Soil microbial functional diversity can be determined through the utilization of community level physiological profiles (CLPPs) which reflect the potential of the cultivable portion of the heterotrophic microbial community to respond to carbon substrates [56]. A study by Yoshitomi and Shann [57] found that pyrene degradation in the rhizosphere can result from changes in the microbial community. PAHs or their metabolites were likely utilized as substrates to increase the soil microbial biomass and community level physiological profiles which in turn increased the enzymatic activities. The higher microbiological activity in the treatment that had received a rhizobial inoculum further confirms that the alfalfa–*Rhizobium* symbiotic association may offer a good rhizoremediation strategy for PAH-contaminated soils.

5. Conclusions

The present study indicates that alfalfa can play a significant role in the dissipation of PAHs in soil and inoculation with *R. meliloti* enhanced PAH degradation in the soil. The alfalfa–*Rhizobium* symbiotic association can significantly increase PAH-degrading bacterial counts and microbial activities in soils and restore soil microbiological functioning of PAH-contaminated soil. The alfalfa–*Rhizobium* symbiosis may be a suitable plant–microbe partnership for phytoremediation of PAH-contaminated soil. However, further studies are required to elucidate the metabolic pathway of PAH degradation in plant-microbial associations and the molecular feedback mechanisms that lead to PAH degradation and transformation in the rhizosphere.

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