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Spacial characteristics of pyrene degradation and soil microbial activity with the distance from the ryegrass (*Lolium perenne* L.) root surface in a multi-interlayer rhizobox

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ABSTRACT

To investigate rhizosphere effects on the biodegradation of pyrene with the distance away from root surface in the rhizosphere of ryegrass (*Lolium perenne* L.), a glasshouse experiment was conducted using a multi-interlayer rhizobox where ryegrass were grown in a soil spiked with pyrene. The largest and most rapid dissipation of pyrene in planted soil appeared at 2 mm zone from the root zone. The pyrene degradation gradient followed the order: near-rhizosphere > root compartment > far-rhizosphere soil zones. In contrast, there was no difference in pyrene concentration with distance in the unplanted soil. Dynamic changes of soil microbial biomass carbon (C_{mic}) and the activities of both soil polyphenol oxidase and dehydrogenase were to some extent coincident with the degradation of pyrene with distance away from the root compartment in planted soils, which indicated the changes of soil microorganisms in different soil zones of rhizosphere were mainly responsible for the observed pyrene degradation. The largest C_{mic} and activities of both soil polyphenol oxidase and dehydrogenase also occurred in near-rhizosphere, especially in 2 mm zone from the root surface. The above results suggest that the effect of root proximity is important in the degradation of pyrene in ryegrass growing soil.

1. Introduction

Soil contamination by polycyclic aromatic hydrocarbons (PAHs) poses a great threat worldwide to the agricultural food quality and human health and calls for an immediate action to remedy the contaminated sites [1,2]. Among PAHs, *pyrene* has been attracting great concern because it is a relatively immobile organic compound with moderate to high soil sorption coefficients and represents common industrial and agricultural pollutants [3]. Pyrene degrades relatively slow in soil and is a persistent pollutant in agricultural soil. However, there is a lack of data to date on the reliable and cost-effective remediation of PAHs in soils [1].

Vegetation has a decisive effect on the fate of soil PAHs and organic contaminants often disappear quickly from the planted soil compared to unplanted soil [4–8]. However, the mechanisms involved are still not well elucidated. The large-scale application of the phytoremediation as an effective and reliable remediation option requires a reasonable understanding of how plants contributing to the dissipation of PAHs in contaminated soils. The principal contribution of plants to phytoremediation is either stimulation of soil microbial activity in contaminant degradation, or direct uptake and accumulation of the contaminants by plant [9]. Actually, microbial transformation/mineralization might be the most important route for pesticide degradation in soils. The size and the activity of the soil microbial biomass influence the rate of organic contaminant degradation. Plants sustain large microbial populations in the rhizosphere by secreting substances, such as carbohydrates and amino acids, through root cells and by sloughing root epidermal cells. The magnitude of rhizodeposition by plants can be quite large. Root cap cells may be lost into soil at a rate of 10,000 cells per plant per day. In addition, root cells secrete mucilage, a gelatinous substance that is a lubricant for root penetration through the soil during growth [10,11]. This mucigel produces root exudates. Soluble exudate includes aliphatic and aromatic hydrocarbons, amino acids and sugars. Root cap cells and exudates provide important sources of nutrients for microorganisms in the rhizosphere. Larger microbial populations can therefore exist in rhizospheric soil than in the bulk soil. These larger populations have been shown to enhance the degradation of organic chemicals including pesticides [5,12,13]. But the root exudates diffuse

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into the soil where they gradually disappear due to the increasing radius from root surface and microbial consumption. Therefore, a hypothesis is put forward, namely, along with substrate gradient gradually disappearing, which may result in corresponding degradation gradients of chemical molecules for which phytoremediation is effective.

To investigate the above hypothesized degradation gradients of xenobiotics in the rhizosphere, an experiment was conducted in environmental chambers using a specially designed a multi-interlayer rhizobox where ryegrass seedlings were grown for 60 days in a soil spiked with pyrene at a concentration of 30 mg/kg. The soil in the multi-interlayer rhizobox was divided into six separate compartments at various distances from the root surface. Changes in pyrene concentrations, soil microbial biomass, endoenzyme-dehydrogenase and polyphenol oxidase activity with increasing distance from the root surface were then analyzed. The aim of such work was to reveal and interpret the rhizosphere effect on the removal process of pyrene with substrate gradient gradually disappearing due to radius away from root surface increase in a multi-interlayer rhizobox.

2. Material and methods

2.1. Soil

Soil was collected from agricultural fields at Huajia Campus, Zhejiang University, China. The soil was analyzed using standard methods and found to have the following physical properties: organic matter, 19.92 g/kg, pH 5.62, cation exchange capacity, 14.59 cmol/kg, and sand, silt, and clay content of 65, 29 and 6%, respectively. The soil was identified to be free of pyrene.

2.2. Experimental design

A multi-interlayer rhizobox was designed as described by Wang et al. [14]. The dimension of the rhizobox was 140 mm × 140 mm × 240 mm (length × width × height). The rhizobox was divided into three sections: a root compartment (20 mm in width), and left and right soil compartments (60 mm in width). Each soil compartment was further separated into several sub-zones (1 mm thick) by nylon mesh (<25 μm), sandwiching layers of soil in sub-zones were named as the 1–5 mm near-rhizosphere zones and the >5 mm far-rhizosphere zone according to the distance from the central zone. Such multi-interlayer rhizobox successfully prevents root hairs from entering the adjacent soil zones as well as keeping the soil zones separated, while permitting the transfer of soil microfauna and root exudates between the compartments. Weight equal to five kilograms air-dried soil mixed thoroughly with pyrene were used to fill the central and rhizosphere compartments, the concentration of pyrene was 30 mg/kg in soil, the spiked soils were equilibrated in the greenhouse for 7 days at 50% water holding capacity before seeding with ryegrass. Seeds of ryegrass (*Lolium multiflorum* L.) were thoroughly rinsed with water, and germinated on a cotton gauze. After seeds were germinated for 24 h at 28 °C in the dark, germinated seeds (80 seeds per root compartment) were sown in soil. Seedlings were subsequently thinned to 50 plants after emergence. The plants were grown under greenhouse conditions. Soils were fertilized fortnightly with an inorganic salt solution including a total of 35 mg N/kg, 25 mg P/kg and 30 mg K/kg (dry soil) [5]. Another set of rhizoboxes without plants were included as controls. 60 days later after sowing, dismantling each rhizobox, separating the layers of each soil zone and removing the plants from the root compartment. Roots and shoots were manually separated from soils washed with deionized water, and then blotted dry with filter paper, while the soil samples from different soil zones were homogenized before analysis. Then, the pyrene residues in soil and plant, soil microbial biomass, endoenzyme-dehydrogenase and urease activity were analyzed.

2.3. Analytical methods

Pyrene residues in 2 g soil samples (planted and unplanted) were mixed with anhydrous Na_2SO_4, and extracted by ultrasonication with dichloromethane. The soil extracts were filtrated through a silica gel column with elution of 1:1 (v/v) hexane and dichloromethane. The solvents were evaporated using a rotary evaporator, and exchanged to HPLC grade methanol with a final volume of 2 ml. The residues of pyrene were estimated using HPLC equipped with a UV detector (LC-6A, Shimadzu Corporation, Japan). Pyrene was separated on a Lichrospher 100 RP-8 150 × 4.6 mm 5 μm column. The operating condition was: mobile phase, acetonitrile–water (80:20, v/v); wavelength, 254 nm; flow rate 1 ml/min. The detection limits was 0.02 ng/kg for pyrene in soil and the recovery rate was 89.49% with RSD (relative standard deviation) 4.05% (n = 4).

The analysis of pyrene residue in plant was used the same method as soil except that the plant tissue was crushed prior to extraction.

Soil microbial biomass carbon (C_{mic}) in different soil zones was quantified using the fumigation extraction technique [15]. The dissolved organic carbon in the resulting 0.5 M K_2SO_4 soil extracts was measured using an automated total organic carbon analyzer (TOC-500, Shimadzum, Japan).

Polyphenol oxidase activity was estimated by colorimetric determination of the pyrogallic acid which formed during the soil sample incubation with 1,2,3-trihydroxybenzene at 30 °C for 3 h, expressed as mg pyrogallic acid per g soil per h [16]. Dehydrogenase activity (DHA) assay was based on the use of 2,3,5-triphenyltetrazolium chloride (TTC), a water-soluble compound, which was reduced to triphenyl formazan (TPF), a red-color compound [17].

2.4. Statistical analysis

All experimental data were processed by Microsoft Excel 2000. The multivariate regression and the stepwise linear regression were conducted using the statistical programs of SPSS (V10.1). The least significant difference (LSD) was used to test the significance between means.

3. Results

3.1. Degradation characteristics of pyrene with respect to distance from the root surface

In the present study, pyrene was used as a model contaminant. The residual concentrations of pyrene in individual samples were plotted against the distance to the root compartment (Fig. 1), the lines represented the rhizospheric gradient characteristics of pyrene concentrations at harvest in soils initially treated with 30 mg/kg pyrene. The concentration of pyrene residue in different soil compartment was affected by proximity to the roots after growing ryegrass (Fig. 1). The general trend in the degradation of pyrene was typically near-rhizosphere > root compartment > far-rhizosphere, which indicated the degradation of pyrene was not gradient gradually disappearing due to radius away from root surface increase. The degradation of pyrene in different soil compartment was as following order: 2 mm > 3 mm > 1 mm > 4 mm > 5 mm > root compartment > far-rhizosphere (>5 mm), while the degradation of

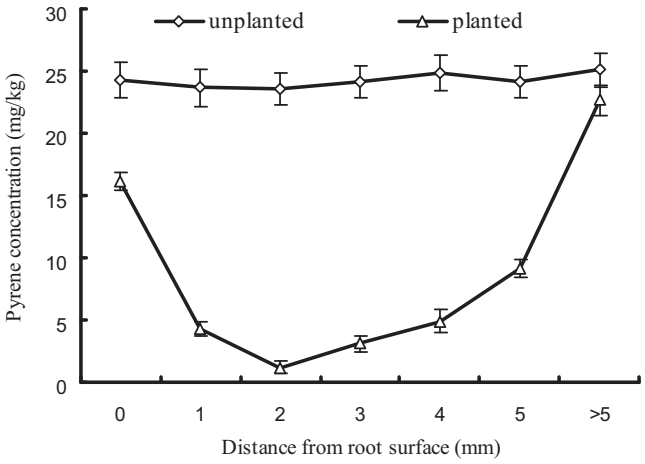

Fig. 1. Pyrene concentration in various sampling soil zones as a function of proximity to root compartment with ryegrass growth. Bars are the standard error of means of three replicates.

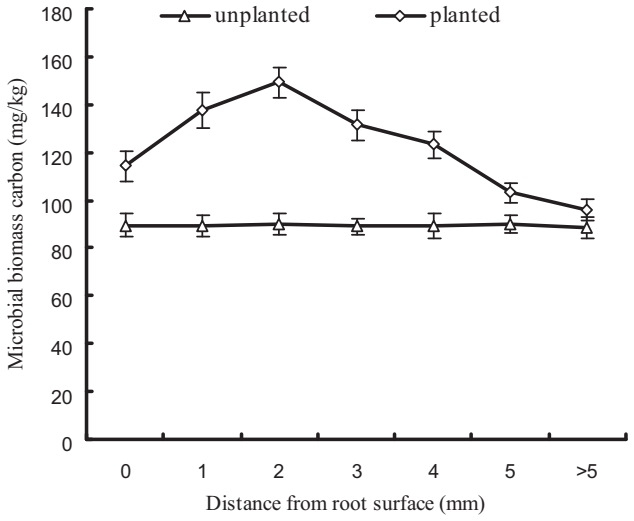

Fig. 2. The amount of microbial biomass carbon (C_{mic}) in various sampling soil zones as a function of proximity to root compartment with ryegrass growth. Bars are the standard error of means of three replicates.

pyrene in near rhizosphere zone had statistically significant differences comparing with root compartment and far-rhizosphere ($p < 0.05$). Comparing with unplanted soil, the differences of most soil compartment in multi-interlayer rhizobox with ryegrass growth were statistically significant ($p < 0.05$), which implied enhancement of degradation in the rhizosphere was potentially important as far as the microcosmic characteristics of the rhizosphere was concerned. The largest and most rapid dissipation of pyrene in planted soil was at 2 mm soil zone from the root compartment zone where total pyrene decreased to 1.2 mg/kg. The above results indicated rhizosphere effects on pyrene degradation were restricted in some extent around plant root.

The degradation dynamics of pyrene in unplanted soils were consistent as expected (Fig. 1). There was no perceptible difference in pyrene concentration with increasing distance from the root compartment of the rhizobox. The unplanted microcosms removed an average of 19% of the initial pyrene in 60 days, which was much smaller than in the soils growing ryegrass (an average of 71%). Furthermore, the amounts of pyrene remaining in unplanted soils were larger than in planted soils at all distances. However, comparing with more distant zones and in the unplanted soils at all distances, the smaller pyrene concentrations in near rhizosphere zones in multi-interlayer rhizobox with ryegrass growing, indicated that the near rhizosphere zone was the most effective at degrading pyrene, which might be due to an interaction with root exudates and the soil microbial community.

3.2. The dynamic responses of C_{mic} and corresponding soil enzyme activities in various soil zones with respect to distance from the root surface

The concentration of soil microbial biomass carbon and activities of soil polyphenol oxidase and dehydrogenase were determined to further demonstrate the gradient effect with respect to distance from the root surface in the rhizosphere on pyrene degradation (Fig. 2). In the unplanted soil, microbial biomass measured as total C_{mic} was almost the same in various soil zones. But, in the planted soil, C_{mic} was larger than in unplanted soil in various soil zones and affected by distance from the root surface in the rhizosphere. C_{mic} was 8.6–65.7% greater with, than without plants. Dynamic changes in C_{mic} were to some extent coincident with the degradation of pyrene (Table 1). The largest C_{mic} concentration was found in the near-rhizosphere soils, especially at the 2 mm sampling zones (Fig. 2), which had statistically significant differences comparing with root compartment and far-rhizosphere ($p < 0.05$). It indicated that rhizosphere effects caused the increased response characteristics in the soil with plants compared to unplanted one.

Table 1
The linear regression and correlation analysis between pyrene concentrations (y) and C_{mic} concentrations, polyphenol oxidase, dehydrogenase activities ($y = ax + b$).

Indexes (x)	a	b	r^2
C_{mic}	−0.3502	51.578	0.714**
Polyphenol oxidase	−189.06	98.788	0.727**
Dehydrogenase	−0.3032	93.051	0.718**

** Correlation is significant at 0.001 probability level.

Soil polyphenol oxidase and dehydrogenase are two kind of enzymes which can degrade PAHs, such as pyrene [18–20]. Soil polyphenol oxidase and dehydrogenase are always secreted from soil microorganism, which is supported by the linear regression analysis in the present study between C_{mic} concentrations and enzyme activities of polyphenol oxidase and dehydrogenase (Table 2). The correlation coefficients for polyphenol oxidase and dehydrogenase had better values ($r_{\text{polyphenol oxidase}}{}^2 = 0.967^{**}$, $r_{\text{dehydrogenase}}{}^2 = 0.963^{**}$). The activities of soil polyphenol oxidase and dehydrogenase can also reflect the PAHs degradation capacity by soil microorganism. The activities of both soil polyphenol oxidase and dehydrogenase were shown in Fig. 3. In the unplanted soil, the activities of soil polyphenol oxidase and dehydrogenase were also almost the same in various soil zones. But in the planted soil, activities of both soil polyphenol oxidase and dehydrogenase were larger than in unplanted soil in various soil zones and also affected by distance from the root surface in the rhizosphere. The activities of both soil polyphenol oxidase and dehydrogenase were 14.7–48.5% and 10.9–35.2% greater with than without plants, respectively. Dynamic changes in the activities of both soil polyphenol oxidase and dehydrogenase were as well to some extent coincident with the degradation of pyrene (Table 1). The

Table 2
The linear regression and correlation analysis between C_{mic} concentrations (y) and polyphenol oxidase, dehydrogenase activities ($y = ax + b$).

Indexes (x)	a	b	r^2
Polyphenol oxidase	1.1366	157.98	0.963**
Dehydrogenase	−0.0018	0.2212	0.967**

** Correlation is significant at 0.001 probability level.

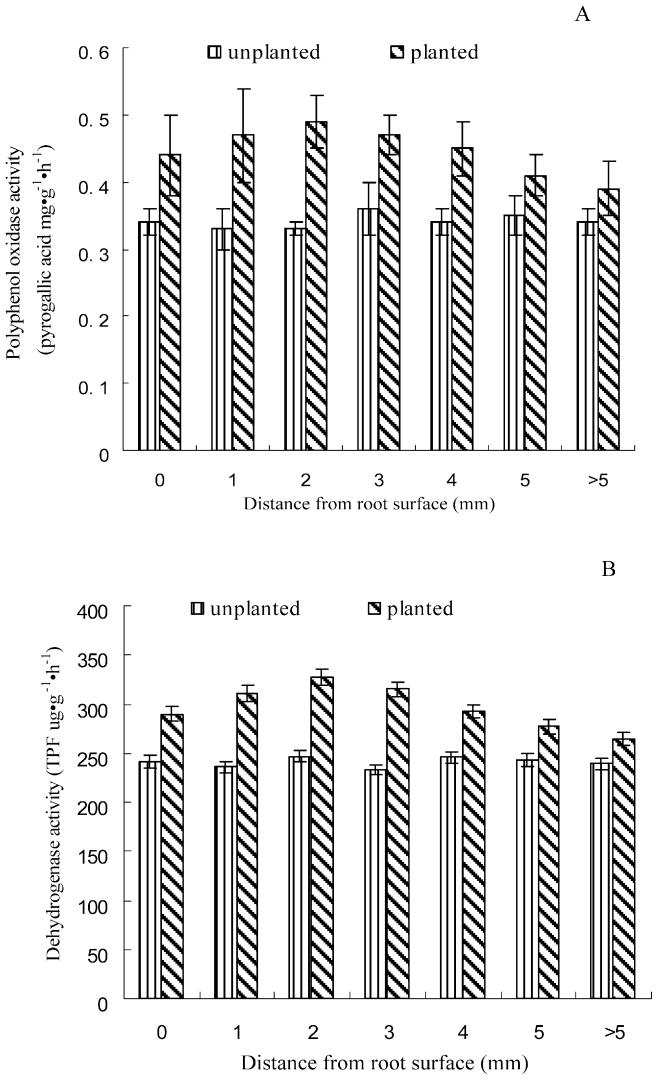

Fig. 3. The activities of soil polyphenol oxidase (A) and dehydrogenase (B) in various sampling soil zones as a function of proximity to root compartment with ryegrass growth. Bars are the standard error of means of three replicates.

largest activities of both soil polyphenol oxidase and dehydrogenase were also found in the near-rhizosphere soils, especially at the 2 mm sampling zones, which had statistically significant differences comparing with root compartment and far-rhizosphere ($p < 0.05$).

The linear regression and correlation analysis were performed between pyrene concentrations and C_{mic} concentrations and enzyme activities of polyphenol oxidase and dehydrogenase (Table 1). The correlation coefficients for C_{mic}, polyphenol oxidase and dehydrogenase had better values ($r_{C_{mic}}{}^2 = 0.714^{**}$, $r_{\text{polyphenol oxidase}}{}^2 = 0.727^{**}$, $r_{\text{dehydrogenase}}{}^2 = 0.718^{**}$). It indicated that rhizosphere effects might be caused by increasing microbial response characteristics in the soil with plants compared to unplanted one.

3.3. Ryegrass uptake of pyrene from the soil

In order to acquire a comprehensive understanding about the mechanisms of pyrene degradation, the uptake of pyrene by plants was measured. Total accumulation of pyrene in the shoots and roots were 0.024 and 0.071 mg/pot, respectively. Total accumulation of pyrene in ryegrass for each pot was 0.095 mg. With total concentrations of 150 mg pyrene applied to the soil, such accumulation represents <0.64‰ of the initial amounts of pyrene added.

4. Discussion

In the present study, there were the lowest concentrations of pyrene, the largest C_{mic} concentrations and generally the highest activities of polyphenol oxidase and dehydrogenase in the near-rhizosphere zone after 60 days of ryegrass growth. Such results implicated the role of root exudates in pyrene degradation. Vegetation would result in beneficial effects on degradation of pyrene by associating roots with microbial activity, in which a multitude of changes occur in soil in the presence of roots. Such changes included soil microbial composition, soil microbial activity and soil enzyme activity. Due to the large quantities of readily available organic substrates in the form of root exudates in rhizosphere soil, the change of root exudates would result in soil microbial characteristics around roots occurring a multitude of changes, root exudates have been the most important factor for microbial changes around roots [21]. These exudates diffuse into the soil where they gradually disappear due to radial dilution and microbial consumption. Along with substrate gradient change, which would result in other gradients change, such as resulting in corresponding degradation gradients of organic compounds for which phytoremediation was effective. The results of present study demonstrated that such gradients exist for pyrene.

Further more, the isolation of soils intimately associated with roots, such as near-rhizosphere soils, demonstrated a high pyrene degradation potential in the rhizospheric soil, which would not have been detected if they had been diluted with non-rhizosphere soil, as in bulked samples from planted soil. In the planted soil, pyrene degradation was enhanced in around rhizosphere soils, which might be due to a higher density and activity of microorganisms in the rhizosphere soils than the unplanted soils and the enhanced pyrene bioavailability via root exudates [6,7,22]. Root exudates also provide the substrates for co-metabolic degradation and modify the soil environment to be more suitable for microbial transformation [5,23].

In the multi-interlayer rhizobox with ryegrass growth, the pyrene degradation gradients observed appeared to be highest at 2 mm, then in 3 mm, 1 mm, 4 mm, 5 mm, root compartment, and far-rhizosphere (>5 mm) sequentially. The degradation of pyrene was typically near-rhizosphere > rootcompartment > far-rhizosphere. It was an interesting conclusion, which was not consistent with the gradients in root exudates and the depletion zones of the most diffusion limited mineral nutrients which was gradually down with the increasing distance from root surface [21]. Similar results were found in the degradation of phenanthrene and dibenzo[*a*,*h*]anthracene in plant rhizosphere [24,25]. However, the pyrene degradation gradients were consistent with the gradients in C_{mic} concentrations and the activities of polyphenol oxidase and dehydrogenase. It might be the result of most optimum biodegradation environment caused by the root exudates, especially in the effects of the concentrations of root exudates on the biomass and activites of soil microbe [26]. To our knowledge, changes in quality and quantity of root exudates can influence the degradation of organic contaminant, appropriate concentrations of root exudates can stimulate the biomass and activites of soil microbe [26]. The phenomena in present study are a microbial adapting and mediating process, which was presumably linked with decreased root exudates availability. The growth of roots, resulting in the exudation of soluble organic and inorganic compounds, may influence the degradation of pyrene in three ways. First, the root exudates provide substrates for microbial growth to accelerate pyrene degradation. Second, these compounds act as a non-pyrene source of available carbon and energy, which are likely to be more favored

by the microbial biomass as a substrate than pyrene, and by that pyrene can therefore tend to remain less decomposed. Third, higher concentrations of root exudates will inhibit the biomass and activities of soil microbe. Therefore, the 2 mm zone represents the zone of optimum substrate availabilities. And the highest extent of removal of pyrene could be achieved here because the soil microbial activities would be maximal (Figs. 2 and 3, Table 2), giving the highest potential for pyrene degradation. Otherwise, the degradation of pyrene would be diminished both in the case of root exudates being deficient and in excess. In contrast, if the readily available root exudates were deficient (e.g. in 3, 4 and 5 mm zones), the activities of soil microbes would not be maximized. While if these growth substrates were surplus (e.g. in root compartment and 1 mm zones), the competition effect for pyrene degradation would be enhanced. In both of the above cases, the extent of pyrene degradation would be diminished. Possibly, therefore, the significance of the rhizosphere in a phytoremediation context differs significantly between the present study and that of previous studies of plant physiology and agronomy. But, the exact mechanisms still need to be confirmed by further experiments, in which different plant species should be involved [5,6,21].

In the present study, soil microbial biomass, activities of polyphenol oxidase and dehydrogenase and removal of the pyrene by plant uptaking data supported the hypothesis that soil microorganisms were mainly responsible for the observed pyrene degradation. The concentration of soil microbial biomass carbon and activities of soil polyphenol oxidase and dehydrogenase were determined to further demonstrate the gradient effect with respect to distance from the root surface in the rhizosphere on pyrene degradation (Figs. 2 and 3, Table 2). Planted treatments, especially near-rhizosphere, contained a significantly increased and large microbial biomass and activity of polyphenol oxidase and dehydrogenase that could mediate the enhanced degradation of pyrene. The differences observed between soil with and without plants, as well as among various sampling zones in proximity to roots of the planted soils, were expected on the basis of microbial growth and community structure modified by both pyrene and root exudates.

In addition, removal of the pyrene by plant uptaking was not a significant mechanism in the present study. The total pyrene accumulation represented <0.64‰ of the initial amounts of pyrene added. Such results were also supported by higher residual pyrene concentration in the root compartment than in the 1–5 mm soil zones. This phenomenon happened probably due to the physicochemical characteristics of pyrene, such as the octanol–water partition coefficient. Organic compounds most likely to be taken up by plants are moderately hydrophobic compounds with $\log K_{ow}$ ranging from 0.5 to 3 [27]. With $\log K_{ow}$ of 6.50, pyrene may be difficult to transferred within the soil–plant system.

5. Conclusion

Plant rhizosphere was important zone to the removal process of pyrene, meanwhile, which was obviously dependent on the distance from the root surface. The higher pyrene degradation gradients observed appeared to be at near-rhizosphere, then to be at root compartment and far-rhizosphere (>5 mm). Dynamic changes of C_{mic} and the activities of both soil polyphenol oxidase and dehydrogenase were to some extent coincident with the degradation of pyrene with distance from the root compartment in planted soils, which indicated the changes of soil microorganisms in different soil zones of rhizosphere were mainly responsible for the observed pyrene degradation. The near-rhizosphere environment increased the soil microorganisms' number and ability to remove organic contaminant. As a result, the corresponding degradation gradients of pyrene in rhizosphere occurred. But the mechanisms involved in the pyrene degradation that dependent on the distance from the root surface in plant rhizosphere remained uncertain, the present findings cannot be simply interpreted based upon one single factor of soil microbe. More effort should be made to identify the composition of root exudates components and reveal how root exudates affect soil microbe with the distance from the root surface in the plant rhizosphere in the future.

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