

Removal of pyrene in simulated wetland by joint application of *Kyllinga brevifolia* Rottb. and immobilized microbes

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ABSTRACT

The objectives of this study were to evaluate the effects of plant and immobilized microbes on pyrene removal and soil microbial functional diversity of co-contaminated soil. The results indicated that the dissipation ratios of pyrene in the soil with plant (P), soil with immobilized microbes (IM) and soil with both of them (PIM) were $51.5 \pm 0.69\%$, $55.2 \pm 3.8\%$, $63.2 \pm 1.29\%$ respectively, and were higher than CK ($31.2 \pm 1.5\%$) with neither of them. Soil microbial functional diversity was assessed by the community-level physiological profiles (CLPP) using Biolog Eco-plates. The Biolog results revealed that the metabolic intensity in the rhizospheric soil marked P-R and PIM-R was significantly higher than that of non-rhizospheric soil and the CK during the incubation period. A similar variation in the diversity indexes (Shannon, Simpson and McIntosh) was observed. Principal component analysis (PCA) of the distribution of carbon substrate utilization for all treatments suggests that the microbial community catabolic diversity is different. Among the carbon utilization profile, carbohydrates were the main carbon source of the sample soil and IM increased the potential utilization of two substrate groups (phenols and carbohydrates) more strongly than that of the other groups.

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1. Introduction

Mixed pollution caused by simultaneous contamination of organic (herbicides, plastics, tannins, polyphenols, pesticides, etc.) and inorganic compounds (As, Cd, Cu, Pb, Cr, Hg, etc.) was a widespread global problem (Politi et al., 2014; Kang et al., 2015). Co-pollution was a very important issue because more than one third of contaminated sites were found to have more than one type of pollutant (Politi et al., 2014). Remediation of environments co-contaminated with metals and organic compounds were difficult (Bacosa and Inoue, 2015; Efsun et al., 2015; Joanna et al., 2016). The widespread industrial uses of chromium or its compounds and mining activities resulted the release of chromium (Cr)-containing wastes into the environment that contaminated the soils and sediments (Mar-Yam et al., 2014; Wan et al., 2015). As a transition metal, chromium existed in different valence states. Cr (VI) and Cr

(III) were the dominant species in the environment (Thatoi et al., 2014). Cr(VI) was a harmful pollutant characterized by its chronic toxicity, neurotoxicity, genotoxicity, carcinogenicity, mutagenic and immunotoxicity (Xiao et al., 2015), and Cr(VI) compounds were approximately 1000 times more toxic and mutagenic than Cr(III) compounds (Kang et al., 2015). However, Cr (VI) compounds had several uses in industry (Politi et al., 2007) and chromium contamination by these compounds in soil and water had been detected in and around a wide variety of industrial sites. It is worth mentioning that chromium contamination is serious in Wenzhou (Chen et al., 2014).

Polycyclic aromatic hydrocarbons (PAHs) are widespread soil contaminants. They have received significant environmental concerns due to their strong carcinogenicity, teratogenicity (Lu et al., 2013), persistence, toxicity, and bioaccumulation activity (Ye et al., 2014). Pyrene (4-rings) is a representative compound belonging to the group of PAHs and is a recalcitrant contaminant in the environment (Wang et al., 2012). It was studied as a representative PAHs because it is among the commonly found PAHs in contaminated sites in the environment and it has been extensively characterized in prior studies.

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Heavy metals (HM) and polycyclic aromatic hydrocarbons (PAHs) are two of the most abundant and potentially harmful pollutants found in most polluted soil (Shen et al., 2005). Increasing attention has been paid to the treatment of environment associated with either Cr or pyrene in recent years (Su et al., 2008; Hale et al., 2011). Rhizoremediation of phenol and chromium was researched by Ontañón et al. (2014). However, remediation of co-contaminated soil by Cr and pyrene has been rarely studied.

There was an increasing focus on bioremediation technologies as a result of their unique advantages, including low cost, lack of secondary pollution (Xiao et al., 2015). Phytoremediation has increasing potential to become cost-efficient and effective ways of removing organic contaminants from the environment (Zhang et al., 2012; Cheung and Gu, 2003, 2005, 2007). Some effective plants, such as ryegrass (Wang et al., 2012; Liu et al., 2013), Alfalfa (Kirk et al., 2005; Ye et al., 2014), *Brassica juncea* (Chigbo et al., 2013), Switchgrass and Timothy grass (Balsamo et al., 2015), *Scirpus triquetus* (Liu et al., 2011), and *Phragmites australis* (Ahmad et al., 2014; Hechmi et al., 2014) with significantly large root surface area and good adaptability to different conditions of the soil have been selected to remediate contaminated soils. Hao et al. (2015) investigated cadmium accumulation capacity and subcellular distribution of *Kyllinga brevifolia* Rottb. (KBR). Metals and metalloids can also be accumulated by plants (Yu and Gu, 2007a,b; Yu et al. 2007; Yu and Gu, 2008a,b,c & Yu et al. 2008). Our test plant KBR grow in Oujiang river delta of Wenzhou, China, being in the same sedge family as *S. triquetus*, was not found in many reports in bioremediation.

Several studies specified the critical roles of the rhizosphere microflora as well as the plants (Zhang et al., 2011; Moubasher et al., 2015). Bacteria – assisted phytoremediation was a successful approach to remediate contaminants. Rhizoremediation can be optimized by using suitable plant-microbe partnerships (Ontañón et al., 2014). Both stimulating indigenous microorganisms and adding exogenous microorganisms were proved helpful to enhance degradation effectiveness. Immobilized microbes can be used for bio augmentation technique. The immobilized carriers are intended to offer a protective niche for inoculated microbes and hence reduce competition with indigenous microorganisms (Chen et al., 2012).

In recent years, the intense search for a solution to co-contamination has led to the development of remediation technologies that can address treatment of not just a single compound, but which can simultaneously deal with multiple contaminants (Wei et al., 2010).

Our research has conducted pot experiments aiming to investigate the effects of KBR combined with immobilized microbes on Cr and pyrene co-contaminated soil remediation. This paper dealt with the effects on pyrene removal and microbial functional diversity.

2. Materials and methods

2.1. Materials

Pyrene was purchased from Aladdin Reagent Corporation (Shanghai, China) with a purity of 98%. Wetland perennial *K. brevifolia* Rottb. (KBR) and microbes used in the study were obtained from PAHs-contaminated sediments in the intertidal area of Oujiang river delta of Wenzhou, China. The microbes named as *Bacillus* sp.W1 were characterized with 16S rDNA and their sequence data were submitted to GenBank resulting with accession numbers KT444619. The microbes were immobilized in matrices of alginate including biochar as carrier. The entrapment of the cells was performed according to the method of Chen et al. (2012).

Biochar derived from pineapple peel was pyrolyzed at 500 °C for 2 h in a muffle furnace.

Test soil for pot experiments were collected from the nursery garden on the campus of Wenzhou University in the southern suburb of Wenzhou City, China (120°42'E, 27°54'N). There was no detected pyrene and Cr (VI) in this soil samples.

2.2. Experimental design

The soil was air-dried, sieved (1 mm) and contaminated with pyrene dissolved in acetone and spiked with $K_2Cr_2O_7$ solution to produce a final concentration of 100 mg/kg pyrene and 80 mg/kg Cr(VI). After balancing for 30 d, 5% organic fertilizer was added into the soils and stirred evenly, then the soils (1.5 kg dry weight per pot) were packed into plastic buckets (18 cm in diameter and 20 cm in height) which were sealed at the bottom. Immobilized microbes spherules were added into soils at 0 (IM 0), 5% (spherules-soil mass ratio, the same to followings, IM 1), 10% (IM 2) and 20% (IM 3), respectively.

Treatments in this trial were set as follows: (1) co-contaminated soil with neither KBR nor immobilized microbes (CK), (2) co-contaminated soil with KBR only (P), (3) co-contaminated soil with 10% immobilized microbes only (IM), (4) co-contaminated soil with both KBR and immobilized microbes (PIM) (marked PIM 1, PIM 2, PIM 3 at 5%, 10%, 20% respectively), (5) control treatment is uncontaminated soil with KBR (NPP). KBR seedlings (5.0 g per pot) were put into bags (5 cm in width and 7 cm in height), filled with test soils, and planted into their corresponding pots. They were randomly placed outdoor in the open air incubated 50 d (from Late October to Early December, air temperature about 5–25 degree centigrade, sufficient sunlight). All treatments were irrigated with running water to ensure water was kept at 2–3 cm above the surface of the soil. NCBI deposition number of the test microbes named as *Bacillus* sp. W1 was KT444619. All of the experiments were performed in triplicate.

The transfer coefficients (TCs) and biological accumulation coefficients (BACs) of pyrene are calculated as follows (Ye et al., 2014):

$TCs = \text{Pyrene concentration in shoot of KBR} / \text{Pyrene concentration in root of KBR}.$

$BACs = \text{Pyrene concentration in biomass} / \text{Residual pyrene concentration in soil}.$

2.3. Analysis of soil pyrene

The soils in root bags were named as rhizospheric soil (R) and residuals were non-rhizospheric soil (NR) by sampling the mixture of pot soil. All soil samples were used to determine residual pyrene and Biolog Eco-plates data.

The analysis of soil pyrene was according to the method of Zhang et al. (2011). Two grams of soil sample was ultrasonically extracted (45 kHz, 300 W) in 10 ml of 1:1 (v/v) mixture of dichloromethane and acetone for 30 min followed by centrifugation. This process was repeated two times. The solvent fractions were then evaporated and exchanged to 2 ml hexane, followed by filtration through silica gel with 5 ml of 1:1 (v/v) elution of hexane and dichloromethane. The samples were then evaporated and dissolved by methanol with a final volume of 1.0 ml. The extract was stored in glass vials at 4 °C until analysis. Extracts were quantified using high-performance liquid chromatograph (HPLC, Waters e2695, fluorescence detector 2475 USA) equipped with a reverse-phase C_{18} column (150 mm × 4.6 mm). The mobile phase was methanol and water (90:10, v/v), delivered at 1 ml/min. The column temperature was 30 °C and the injection volume was 1 µl.

The pyrene concentration was obtained by the external standard method.

2.4. Biomass and pyrene accumulation in KBR

The roots and shoots were separated, washed with deionized water and fresh biomass was gained. One gram fresh roots and shoots sample were cut into pieces, respectively, and were ground together with anhydrous sodium sulfate in mortar washed with dichloromethane in advance. The sample was ultrasonically extracted (45 kHz, 300 W) in 15 ml of 1:1 (v/v) mixture of hexane and acetone for 30 min followed by centrifugation. This process was repeated two times. The following steps were same to that of soil noted earlier.

2.5. Microbial functional diversity

Biolog Eco-plates were used to analyze substrate utilization patterns of microbial communities in every treatment. It was more sensitive to environmental changes than phospholipid fatty acid method. 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. The community-level physiological profile (CLPP) of each wetland was assessed using substrate utilization patterns gathered via Biolog Eco-plates. The procedure is as follows: the whole microbial community was extracted from different soil samples and inoculated into Biolog Eco-plates. Triplicate 3 g fresh samples were suspended in 30 ml sterile saline solution (NaCl, 0.85%) on a rotary shaker at $200 \text{ r} \cdot \text{min}^{-1}$ for 30 min at 25°C . The suspensions were allowed to settle for 5 min. Tenfold serial dilutions were made for three times and then the 10^{-3} dilution was prepared. Then $150 \mu\text{l}$ of the 10^{-3} dilution was added to each plate well. The absorbance of the wells at 590 nm and 750 nm was read using Multimode Reader (CYtation3, USA). The plates were then sealed inside a plastic bag and incubated at 25°C in darkness, and read every 24 h over seven days (Yu et al., 2015). The average well color development (AWCD) was calculated using equation of $\text{AWCD} = \sum(C - R)/31$, where C and R are absorbency in each C-source well and the control, respectively. Diversity of microbial carbon utilization was studied by means of species richness, Shannon, Simpson and McIntosh (Kirk et al., 2005).

Since the AWCD value at 72 h was often used to describe the difference of rhizospheric microbial activities among the different treatments, thus we used the absorbance at 72 h to quantify C source utilization (Zhao et al., 2010).

Three different indices which indicate different aspects of functional diversity of microorganisms were used to evaluate biodiversity. Shannon index is greatly affected by the species richness of community, Simpson index shows the most common species in the community and the McIntosh is an index measuring the homogeneity of species in the community.

$$\text{Shannon index, } H' = - \sum P_i \ln P_i \quad (1)$$

$$\text{Simpson index, } D = 1 - \sum P_i \times P_i \quad (2)$$

$$\text{McIntosh index, } U = \sqrt{\sum n_i \times n_i} \quad (3)$$

where P_i was calculated by subtracting the blank from each substrate absorbance and then dividing this value by the total color

change, n_i was calculated by subtracting the blank from each substrate absorbance.

2.6. Statistical analysis

All data reported were mean values of three replicates. The differences of pyrene dissipation ratios were analyzed by Duncan's test. Some indexes, such as AWCD, Shannon, Simpson and McIntosh were investigated by analysis of variance (ANOVA). Principal component analysis (PCA) was performed to investigate the differences of the soil microbial community structure. The data were standardized by the average well color development in each microplate to remove inoculum density effects (Gryta et al., 2014). The above analyses were performed using the Statistical Package for Social Sciences (SPSS 20.0 for Windows) (SPSS Inc., USA) at 0.05 significance levels.

3. Results and discussion

3.1. Residual pyrene in soil under different conditions

There was some difference in the residual concentration of pyrene under different treatments. The removal efficiencies in soils of IM, P and PIM were $55.18 \pm 3.76\%$, $53.17 \pm 3\%$, $63.17 \pm 1.29\%$, respectively, and were higher than CK ($31.20 \pm 1.5\%$) significantly. Fig. 1 shows the synergic effect of KBR and IM on the pyrene dissipation. As shown, the pyrene removal in PIM was significantly higher ($p < 0.05$) in compared to the case of the IM and P. These results showed that either KBR or IM had the potential to remove pyrene from soil, and more significant influence exhibited when both of them were applied together.

3.2. Pyrene accumulation in KBR

With the rise of IM amount added from 5% of IM-soil ratio, pyrene accumulations in both shoot and root of KBR show a rule of declined first then ascended. Even, when it reached 20% the accumulation in root of PIM 3 was higher than that of P. Biomass curve shows the similar variation (Fig. 2). No significant biomass between in shoots of NPP and P ($P > 0.05$), indicating pyrene did not have observable effects on the growth of KBR. Meanwhile, BACs and TCs

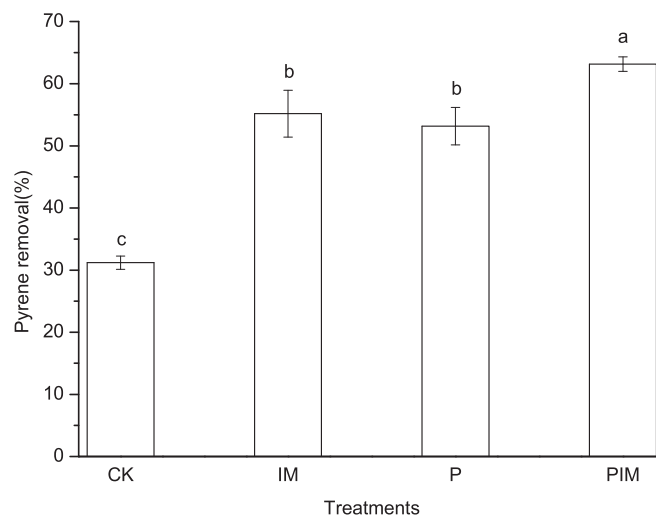


Fig. 1. Percentage of pyrene removal from soils. CK, IM, P, PIM indicate unplanted soils without immobilized microbe, soils with immobilized microbe, planted soils, planted soils with immobilized microbe, respectively. The error bars indicate the standard deviation of the means ($n = 3$). Letters a, b and c indicate significant differences between data ($P < 0.05$).

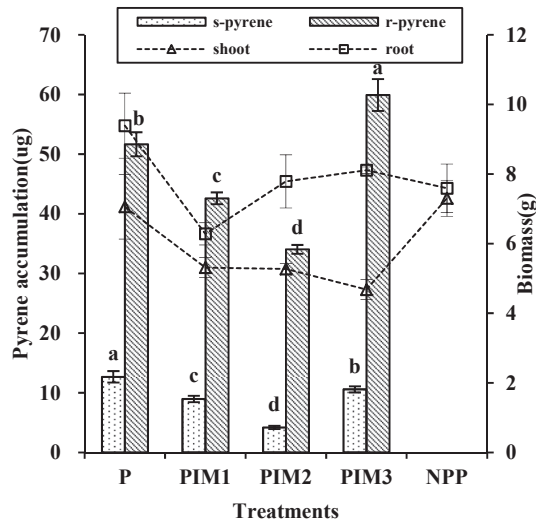


Fig. 2. Pyrene accumulation in shoot and root of *Kyllinga brevifolia* Rottb. P, PIM1, PIM2, PIM3, NPP indicate planted soils with 0, 5%, 10%, 20% immobilized microbe, uncontaminated planted soil, respectively. s-pyrene, r-pyrene indicates pyrene accumulation in shoots and roots, respectively. The error bars indicate the standard deviation of the means (n = 3). Letters a, b and c indicate significant differences between data (P < 0.05).

of pyrene in KBR were 0.005–0.008 and 0.08–0.18 respectively. As we know, phytoremediation of PAH-contaminated soils primarily involves PAH absorption by plants, plant transport and volatilization, plant secretion, enzyme decomposition, strengthened absorption and degradation by rhizosphere microbes. For example, the spore of *Rhizo-phagus custos* could absorb and store up anthracene (Ye et al., 2014). The results indicated that IM inhibited pyrene translocation across KBR because of its possible adsorptive property. Phytoextraction plays little role on dissipation of pyrene in soil but rhizospheric effects and IM play the predominant role.

3.3. Microbial functional diversity

The shape of the curves are conform to the general trend of microorganism using substance, there were increased logarithmic phase, stable phase and attenuation phase (Qin et al., 2014). Microbial activity, as measured by AWCD, increased rapidly from 24 to 72 h incubation period (Fig. 3A). As time extended, the ability of

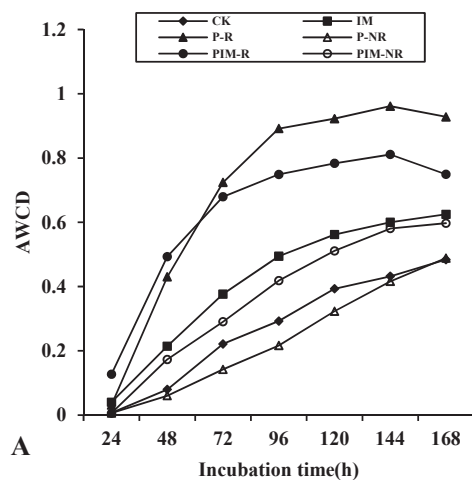


Fig. 3. (A) AWCD of the Biolog Eco-plates for different treatments. (B) Functional diversity of microbial community in different treatments. CK, IM, P-R, P-NR, PIM-R, PIM-NR indicate unplanted soils without immobilized microbe, soils with immobilized microbe, rhizosphere of planted soils, non-rhizosphere of planted soils, rhizosphere of planted soils with immobilized microbe, non-rhizosphere of planted soils with immobilized microbe.

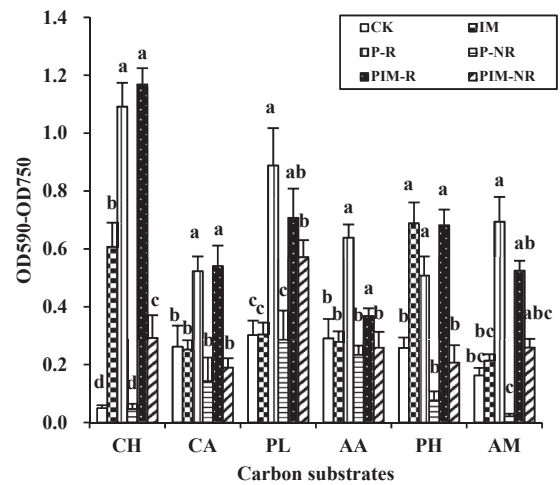
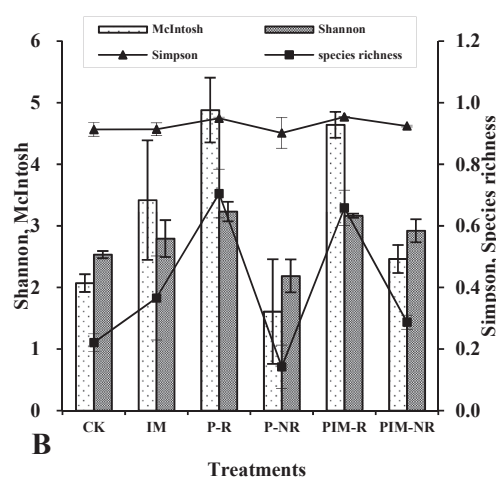


Fig. 4. Average utilization of carbon substrates from different substrate groups by soil microorganisms of different treatment. The error bars indicate the standard deviation of the means (n = 3). Letters a, b and c indicate significant differences between data (P < 0.05). Abbreviations: CH: carbohydrates, CA: carboxylic acids, AM: amines/amides, AA: amino acids, PL: polymers, PH: phenols. Acronyms for treatments are the same as that of Fig. 3.

microbial communities using carbons gradually increased. There were significant differences in the AWCD of IM, P, PIM compared with CK (P < 0.01). These results were mainly attributed to the following reasons: (1) KBR significantly improved the activity of soil microorganisms (P < 0.01). For example, *S. triquetra* has been found to increase the number of rhizosphere bacteria in the pyrene-contaminated soil (Zhang et al., 2012). Plant had a significant effect on AWCD parameter, since the planted wetlands usually had a higher total microbial activity than the unplanted over the study period (Zhao et al., 2010; Duman and Koca, 2014). Meanwhile, the metabolic activity and biomass of soil microorganisms could be greatly enhanced by root exudates (Sun et al., 2010; Zou et al., 2013). (2) IM used in current experiments notably improved the soil microbial diversity (P < 0.05), enhancing pyrene degradation. Chen et al. (2012) verified the dissipation of 15 PAHs in soil was significantly enhanced by all immobilized unidentified indigenous bacterium which was isolated from PAH-contaminated soil.

To further compare the catabolic diversity among different treatments, Shannon index, Simpson index and McIntosh index in the incubation time of 72 h are shown (Fig. 3B). The similar



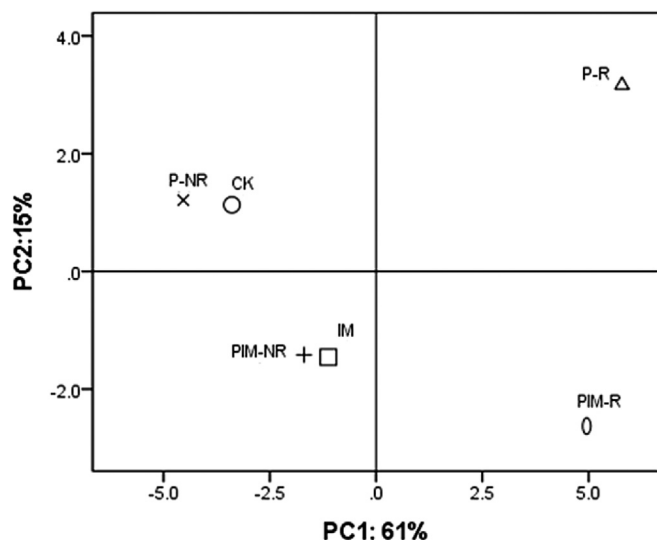


Fig. 5. PCA-ordination biplot of the first two principal component axes (PC1 and PC2) based on average utilization data of carbon substrate groups at 72 h in Biolog Eco-plates for six treatments. Acronyms for treatments are the same as that of Fig. 3.

variation trends of Shannon, Simpson and McIntosh were observed. In comparison to CK, these indexes under the KBR-planted treatments increased greatly, especially rhizospheric environment (P-R, PIM-R) ($P < 0.05$), which was due to the function of KBR Root exudates. Furthermore, the indexes in treatments PIM-R and PIM-NR were all greater than those in CK ($P < 0.05$), suggesting that the joint treatment further enhanced the function diversity of soil microbial community. But the joint action was not distinct because there was no significant difference ($P > 0.05$) between corresponding index of P-R and PIM-R. It was clear that these indexes could reflect the influence of plant and IM on the diversity of soil microbial communities.

3.4. Soil microbial functional structure

Carbon substrates in the Biolog Eco-plates were classified as amines; amino acids; carbohydrates; carboxylic acids; phenols; polymers. Principle component analysis (PCA) illustrated the microbial functional structure based on their carbon substrate utilization patterns (Fig. 4). The highest utilization of all carbon sources

(except phenols) occurred for soils from P-R and PIM-R. Mean carbohydrate and amines utilization of soil from the P-R and PIM-R were significantly greater than those in the other treatments. Amino acids and polymers utilization from P-R were also detected to be the highest. Phenols were the most strongly utilized from IM. In contrast, soil from P-NR had the lowest utilization of amines, phenols, carbohydrates and carboxylic acids, meanwhile soil from PIM-NR had the same trend. There was no significant difference in the utilization of amino acids among all treatments except P-R.

Principal components analysis (PCA) based on AWCD data after 72 h of incubation differentiated the samples from treatments with and without IM on canonical variate axis 2 (Axis 2), rhizospheric and non-rhizospheric environment on canonical variate axis 1 (Axis 1). Samples from P-R and P-NR clustered to the upper end of axis 1, and samples from PIM-R, IM and PIM-NR clustered below axis 1 (Axis 1). Similarly, P-R and PIM-NR clustered to the right of axis 1, P-NR and PIM-NR to the left. Treatments were distinctly divided by the two axes, and showed 61% of the variance between rhizospheric and non-rhizospheric environment accounted for on the first axis, and an additional 15% of the variation accounted for addition of IM on the second axis (Fig. 5).

Factor loadings for PC1 (>0.8) and PC2 (>0.6) are shown in the Table 1. Further analysis of the carbon source loadings on the PCA axes indicated that carbohydrates (α -D-Lactose, D-Xylose, N-Acetyl-D-Glucosamine, D-Galacturonic Acid, D-Cellobiose, L-Erythritol, β -Methyl-D-Glucoside), polymers (α -Cyclodextrin, Glycogen, Tween 40), carboxylic acids (α -Ketobutyric acid, Itaconic acid, γ -Hydroxybutyric acid), amino acids (L-Serine, L-Threonine, L-Arginine, L-Asparagine) had greater influences than the other substrates. These carbon resource components were most responsible for the separation between rhizospheric and non-rhizospheric environment along axis1 (Table 1). Similarly, phenols (4-Hydroxy Benzoic), polymers (D-Galactonic Acid, Tween 80), carbohydrates (D, L-a-Glycerol) were most responsible for addition of IM along axis 2.

4. Conclusions

This study clearly shows that the joint application of KBR, a kind of wetland plant, and immobilized microbes is an effective way to remove pyrene and improve the soil microbial diversity in simulated wetland. Although KBR had a certain effect on pyrene removal by uptake, accumulation and biological metabolism, the joint effects of KBR and immobilized microbes mainly contributed to

Table 1

Correlations between carbon substrate utilization in Biolog Eco-plates and the first two principal components (PC 1 and PC 2).

Carbon substrate	R _{PC1}	Substrate class	Carbon substrate	R _{PC2}	Substrate class
α -D-Lactose	0.969	CH	4-Hydroxy Benzoic	0.751	PH
D-Xylose	0.962	CH	D-Galactonic Acid γ -Lactone	0.66	PL
α -Cyclodextrin	0.96	PL	D,L-a-Glycerol	0.646	CH
α -Ketobutyric Acid	0.948	CA	Pyruvic Acid Methyl Ester	0.628	CA
Glycogen	0.926	PL	Tween 80	0.613	PL
N-Acetyl-D-Glucosamine	0.913	CH			
Phenylethyl-amine	0.906	AM			
D-Galacturonic Acid	0.903	CH			
L-Serine	0.892	AA			
Itaconic Acid	0.883	CA			
L-Threonine	0.882	AA			
Tween 40	0.873	PL			
γ -Hydroxybutyric Acid	0.872	CA			
L-Arginine	0.857	AA			
D-Cellobiose	0.851	CH			
L-Asparagine	0.842	AA			
L-Erythritol	0.824	CH			
β -Methyl-D-Glucoside	0.804	CH			
D-Malic Acid	0.802	CA			

Acronyms for carbon substrate as for Fig. 4. Only substrates with factor loadings >0.80 , >0.60 in PC1 and PC2 respectively are presented.

pyrene removal. Soil microbial functional diversity varied between PIM and CK is closely related to the quality of carbon sources that input to the soil through KBR and IM. Meanwhile, the results suggest the changes in microbial activities under chromium stress were favorable to the dissipation of pyrene. The joint application of KBR and IM has better effect than single of them.

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