Journal of Biodiversity Management & Forestry

Research Article A SCITECHNOL JOURNAL

Exploitation of Native Plants and Microbial Diversity Profiling for Phytoremediation of Petroleum Hydrocarbon Contaminated Coastal Wetland Soil

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Received: 26 October, 2020, Manuscript No. JBMF-20-21207;

Editor assigned: 29 October, 2020, Manuscript No. PreQC No. JBMF-20-21207;

Reviewed: 12 November, 2020, QC No. JBMF-20-21207;

Revised: 01 September, 2022, Manuscript No. QI No. Q-21207; Manuscript No. JBMF-20-21207;

Published: 29 September, 2022, DOI: 10.4172/ jbmf 27-4417.10029

Abstract

Soil pollution by Petroleum Hydrocarbon (PHC) in the coastal wetland of Yellow River Delta, China has seriously threatened ecosystem health and function. Phytoremediation is an innovative and cost-effective option to remediate the contaminated soil. However selection of the suitable plant species is an important step for successful bioremediation of the PHC contaminated soils. The biodegradation abilities of five plant species including Susana, Seep weed, Sea-lavender, and Central Asia Saltbush grown in the PHC contaminated soil was investigated using a 90-day pot experiment. The removal of PHC in the rhizosphere soils was more efficient compared to the without planted soils (64.8% vs 20.2%) With reference to plant properties, the biomass surface area and volume of the roots were negatively correlated with PHC concentration ($r=-0.816,-0.869$ and -0.90, $P<0.05$, $n=10$) respectively, confirming that plant with higher biomass and larger root resulted in more PHC remediation. The Bio Log community profile illustrated that Sesbania rhizo sphere was the most dynamic micro zone. In addition the rhizosphere soil pH declined from 7.94 to 7.58 during the incubation period. Overall Sesbania with higher biomass and larger root system and active microbial diversity (Shannon diversity index 3.2 on 90 day) was an ideal plant for on-site remediation of the petroleum polluted coastal wetland soils.

Keywords: Petroleum hydrocarbon; Microbial diversity; Rhizoremediation; Root biomass; Contaminated soil

Introduction

Globally wetland ecosystem is always considered as vulnerable to pollution, and in the last few years these sites have been critically degraded specially due to the lack of wetland ecosystem protection by legal authorities. The Yellow River Delta (YRD), China, one of the oldest and largest coastal wetland ecosystem that was formed less than 150 years ago. However, this YRD has progressed economic development due to industrialization and population growth. Shengli oil field is second-largest oil production basin in China, at Yellow River Delta, greatly contribute to the economic growth for the government. However, the wetland soils have been seriously contaminated by Petroleum Hydrocarbons (PHC) due to oil exploitation, refining, transportation and utilization. The deterioration of soil quality and fertility resulted from the PHC pollution has become a serious environmental issue in these wetland soils. Therefore, development of an environment-friendly technique to remediate the PHC contaminated soils is an extremely important task. Phytoremediation has been used to enhance the degradation of PHC in soils. Plants used in phytoremediation could promote the dissipation of PHC in soils through phytostabilisation, phytoextraction, phytovolatilisation, phytodegradation,enhancement of microbial activities and diversity, improvement of physical and chemical conditions in rhizosphere soils . The planted soils harbored more oil-utilizing bacteria than the non-planted soils. Several plants e.g., *S. persica* ahani, S. iranica Akhani and alfalfa have been used in phytoremediation which results in enhanced degradation of petroleum hydrocarbon in the contaminated soils. Different species of plants have different tolerance to the nutrient-deficient polluted soil including the Napier grass, which was used for cadmium phytoremediation and proved as a potential candidate plant for cadmium phytostabilization. Therefore, re-construction of contaminated soil texture before phytoremediation was a good solution to the nutrients deficient soils.

Natural biodegradation of the contaminants was found insufficient with indigenous soil microorganisms [1,2]. The use of plants and their related microbes to degrade hydrocarbon pollutants in soils was referred to as rhizoremediation where mutual cooperation had effectively removed pollutants from the soil environment. Therefore, the selection of appropriate plant was an important step for the successful bioremediation. In this study, Petroleum Hydrocarbon (PHC) contaminated soil was taken from the Shengli Oilfield which is situated near the coast of YRD, Dongying city of China, and the soil has high salinity (and the ability of the native plants to tolerate these conditions for rhizoremediation were the main concern in the present research. The main purposes of this study were to:

- Assess the potential of five plants to degrade PHC in the contaminated soil using a pot experiment.
- Explore the relationships between PHC removal efficiency and plant root morphologies, and microbial activity.
- To identify the functional diversity and community abundance of microorganism for PHC degradation in the rhizosphere. Information gained from this study was useful in the constructing the *in-situ* rhizoremediation for the petroleum contaminated soils [3-5].

Materials and Methods

Pot experiment

Contaminated soil was initially collected from area adjacent to oil wells in Shengli Oilfield of Dongying, China. The coordinates of the Plates sites were: Latitude 38.01°N and longitude 118.92°E. The soil sample was treated by adding 30% (w/w) sand to increase porosity and drainage of soil. The soil was fertilized with NH4HCO3 to adjust the ratio of C/N to 15/1. The physicochemical properties of the contaminated soil were shown in Table 1.

Soil	PHC (mg kg^{-1})	pH	Organic matter (g kg^{-1})	Organic C(g kg^{-1})	Total N (g kg^{-1})	C/N	Total P (g kg^{-1})
Initiala	2200 ± 40	-	17.6 ± 0.5	10.2 ± 0.3	0.3 ± 0.0	32/1	0.5 ± 0.0
Finalb	780 ± 60	8.0 ± 0.2	15.3 ± 0.6	8.9 ±0.3	0.6 ± 0.0	44576	0.5 ± 0.0

Table 1: The selected physical and chemical properties of the soil samples.

Four dominant indigenous plants including Sesbania (Sesbania cannabina (Retz.) Poir.), Seepweed (Suaeda glauca), Sea-lavender (Limonium), and Central Asia Saltbush (Atriplex Centralasiatica Iljin), and one conventional phytoremediation plant ryegrass (Lolium Linn.) were selected for the pot experiments. All the seeds were obtained from the Dongying Institute of Agricultural Sciences, China. The pot experiments were conducted to investigate the potential of these five plants to promote biodegradation of PHC. The greenhouse experiment was carried out in average temperature of 23°C (14 h) and 17°C (10 h) during the day and night, respectively [6-8].

Soil moisture was maintained at 60% of maximum Water Holding Capacity (WHC). Twenty seeds of each plant species were sow in each pot and plants were analyzed after 60 and 90 days of plant growth. The rhizosphere soils (that attached to the roots) were collected by hand-shaking method and the soil in the pot without any plant growth was labeled as non-planted soils. The roots of all the plants were scanned and analyzed by Epson Scanning and WinRHIZO Pro. 2005 to obtain the root morphological parameters.

Soil analysis for PHC

The PHC contents in the soils were determined as described by Khan. Briefly, 1 g soil samples that had been pre-sieved through a 120-mesh sieve were extracted three times in 10 mL hexane by ultra sonication for 60 min each time. Then the extractions were analyzed according to the method reported by Phillips [9].

Enumeration of hydrocarbon degraders

Hydrocarbon-degrading microorganisms were enumerated by 96-well micro titre plate by the Most-Probable-Number (MPN) procedure as described by Wang and incubated at 30°C for 14 days. The well color change was used to calculate the MPN of hydrocarbon degraders which was expressed as Colony-Forming Units (CFU) g^{-1} dry soil. The composition of the MSM medium used for MPN procedure per liter was: KH2PO4 1.0 g, K_2HPO_4 1.0 g (NH_4) $2SO_4$ 1.0 g, NaCl 15 g, $MgSO_4$ 0.2 g, $FeCl_3$ 0.05 g and $CaCl_2$•H_2O 0.02 g.

Microbial diversity profiling

BIOLOG ECO plates were used to investigate the soil microbial functional diversity. Briefly, 10 g of the fresh soil samples was placed into an autoclaved flask which contained 100 mL of 0.85% (w/v) NaCl solution and shaken for 30 min, and then the 150 µL of a 10-3 dilution of soil suspension were poured into the ECO plates. were incubated at 28°C for 7 days and then were analyzed by the Microplate Reader every 24 h at 590 nm.

Average Well Color Development (AWCD) was estimated according to the method reported by Tanase. The Shannon-Weiner (H) diversity index, Simpson index for bacterial species richness and Evenness index were calculated. The multivariate statistical modelling tool Principal Component Analysis (PCA) was applied to further investigate the relationships between different samples's carbon substrate utilization [10,11].

Statistical analysis

Each experiment was performed in triplicate. Statistically significant differences between the treatments were evaluated by one-way Analysis of Variance (ANOVA) along with LSD test using SPSS (version 18.0). The significance levels were quoted at a 95% confidence level ($P<0.05$).

Results and Discussion

Degradation of PHC in the rhizosphere and non-planted soil

PHC degradation in the soils after 60 days and 90 days of incubation are depicted in Figure 1.

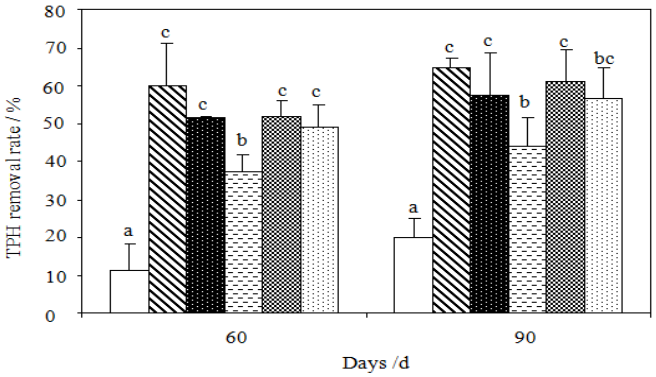

Figure 1: Removal rate of total Petroleum Hydrocarbon (PHC) in the rhizosphere and non-planted soils grown with five different plants. PHC remove rate values are means ± standard deviations of triplicate determinations. Mean values with the same letter for the same batch of samples indicate no significant differences analyzed by LSD test at 5% level using SPSS (18.0). Note: □: Non-planted; ▨ : Sesbania ; ■ : Seepweed; , ▩ : Ryegrass; ▩ : Sea-lavender ; and □ : Central Asia Saltbush.

All plants promoted remediation of PHC from the contaminated soil. The rates of PHC degradation in the rhizosphere soils of Sesbania, Seepweed, Sea-lavender and Central Asia Saltbush (49.0%-60.0%) were significantly higher ($P<0.05$) than that in the ryegrass rhizosphere (37.5%) during the initial 60 days. The removal rate of PHC in the non-planted soil was 11.5% [12].

The trends of hydrocarbon degradation during plant growth showed that the initial stage (first 60 days) was more efficient for PHC removal than the later stage. This could be due to the easily degradable hydrocarbons (e.g., alkanes), which were preferred carbon sources to rhizospheric microorganisms capable of utilizing both alkanes and aromatics at the early incubation. After 90 d of plant growth, the trend of PHC removal was similar to the first 60 days, and the ryegrass (43.9%) was the least efficient plant in PHC removal compared with other four plants (56.7%-64.8%).

Thus, Sesbania, Seepweed, Sea-lavender and Central Asia Saltbush performed better in degrading petroleum hydrocarbon than the ryegrass. These results were disagreement with the previous researches, which described that ryegrass was the most efficient plant to enhance biodegradation of PAHs or diesel oil in the soils Kaimi. It could be concluded that the degradation of PHC jointly affected by the characteristics of the polluted soil, plant root system and activity of rhizosphere microbial diversity.

Plant growth characteristics and root morphology

The plant biomass (Table 2) and root morphology (Figure 2).

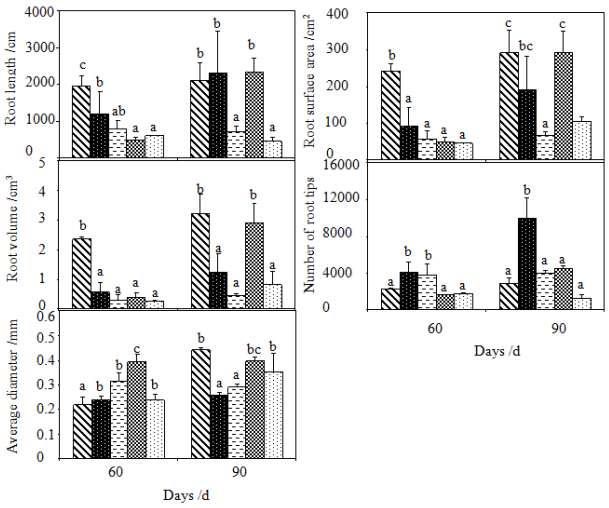

Figure 2: The parameters of root morphology of the five plants. The values are means ± standard deviations of triplicate determinations. Mean values with the same letter for the same batch of samples indicate no significant differences analyzed by LSD test at 5% level using SPSS (18.0). **Note:** ▨: Sesbania; ■: Seepweed; ▩: Ryegrass; ▩: Sea-lavender; and □: Central Asia Saltbush.

Were measured, which could be used to evaluate the remediation efficiency. After 90 days, the root biomass of Sesbania (0.52 g) and Sea-lavender (0.56 g) was significantly larger than other plants. The ratio of root to shoot biomass of these plants showed the same order to the root biomass (Table 2).

Days (d)	Plant species	Root biomass (g)	Shoot biomass (g)	Ratio of root to shoot
60	Sesbania	0.26	1.62	0.17
	Seepweed	0.07	2.10	0.03
	Ryegrass	0.07	0.49	0.14
	Sea-lavender	0.10	0.76	0.13
	Central Asia Saltbush	0.05	1.51	0.03
90	Sesbania	0.52	4.30	0.12
	Seepweed	0.15	6.28	0.02
	Ryegrass	0.11	0.80	0.14
	Sea-lavender	0.56	4.63	0.12
	Central Asia Saltbush	0.11	4.26	0.02

Table 2: Root and shoot biomass and the ratio of root to shoot of the five different plants harvested at 60 and 90 days of plant incubation.

Data followed by the same letters in the same column for the same batch of samples indicate no significant differences at $P<0.05$.

Sesbania is a large bushy plant with high biomass production, and it has fast-growing biomass which is easy to be harvested.Fast growth rate of this plant leads to reduce the time required for optimum root density, stem height and root biomass which enhances the tolerance of plants to the toxic compounds (e.g., metals and organic compounds) and subsequently improved the pollutants removal efficiency (Figure1). Therefore, the Sesbania is a promising candidate that efficiently degraded petroleum hydrocarbon [13-16].

The parameters of root morphology including length, surface area, volume, tips and diameter were shown in Figure 2. Generally, the trend of these root parameters was similar to the root biomass at 90 d. Sesbania had the deepest root system, high surface area and volume, about 2-7 times as large as those of ryegrass, while there was no significant difference in the number of root tips between Sesbania and ryegrass. The root distribution and development was influenced by the spatial distribution of contaminants due to this reason that root growth was especially necessary for remediation, and the larger root system had stimulated the degradation of TPH [17].

As shown in Figure 3, root biomass (r=-0.816, P<0.05, n=10), surface area (r=-0.869, P<0.05, n=10) and volume (r=-0.900, P<0.05, n=10) were negatively correlated with the PHC concentrations in the soil. Regression analyses between the root parameters and PHC concentration showed a strong association.

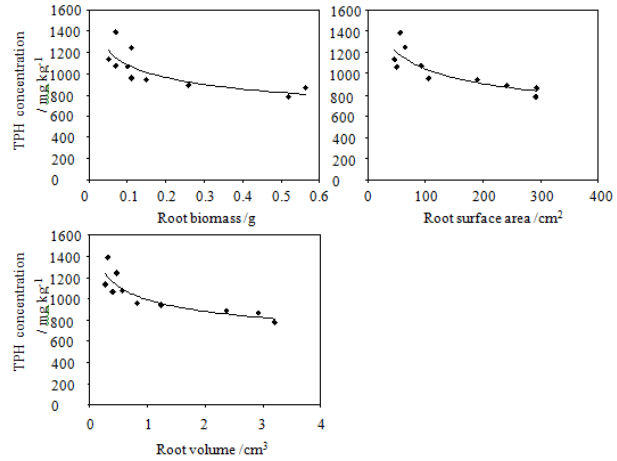

Figure 3: Regression relationships between total petroleum hydrocarbon (PHC) concentration. **Note:** (a): Root biomass; (b): Surface area; (c): Volume, respectively.

With the augmentation in root biomass, surface area and volume, the PHC concentrations tended to decline (Figure 3). The roots with larger biomass, surface area, and volume could explore more soil volume, and thus enhanced the possibility to contact with contaminants, which lead to a higher PHC degradation rates. Roots create pores that improve water, nutrients and oxygen flow in the soil, removal of Chemical Oxygen Demand (COD), total and available P and ammonium nitrogen (NH4-N) had different correlations with the root exudates. Roots also carry the degrading microorganisms through the soil and increase the contact between degraders and the contaminants. Consequently, the plants with dense root system (e.g., Sesbania) can significantly enhance the PHC degradation [18-20].

Hydrocarbon degrading microbial count

The total hydrocarbon degrading microorganisms (MPN cfu kg^{-1} dry soil) in the soils (rhizosphere and non-planted) were shown in Figure 4.

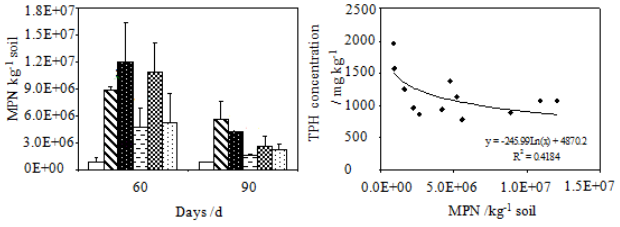

Figure 4: Most-probable-number (MPN) of hydrocarbon-degrading bacteria in the rhizosphere and non-planted soils and the relationships of MPN of hydrocarbon degrading microorganisms with total Petroleum Hydrocarbon (PHC) concentration.

After 60 days of plant growth, there was a significant increase of degraders in the rhizosphere soils compared to those in the non-planted soils (P<0.05). Total hydrocarbon degraders count in the rhizosphere soils grown with Seepweed (1.2×10^7 cfu kg^{-1} dry soil), Sea-lavender (1.1×10^7 cfu kg^{-1} dry soil) and Sesbania (8.9×10^6 cfu kg^{-1} dry soil) were about 10-14 times larger than non-planted (8.7×10^5 cfu kg^{-1} dry soil). After 90 days of plant growth, the highest number of degrading microorganisms presented in the Sesbania rhizosphere was 5.6×10^6 cfu kg^{-1} dry soil, about 6 times higher than the lowest number measured in the bulk soil (9.0×10^5 cfu kg^{-1} dry soil).

The rhizosphere soils were more enriched with the degraders in response to the soil contaminated with crude oil. For the five plants, the number of the hydrocarbon degraders in the non-planted were 8.7×10^5 cfu kg^{-1} dry soil and in the rhizosphere were 4.7×10^6 to 1.2×10^7 cfu kg^{-1} dry soil, which were significantly negatively correlated with PHC concentration (r=-0.647, P<0.05, n=12).

This indicated that the higher bacterial load was responsible for higher PHC biodegradation. Regression analyses between the number of degraders and PHC concentration showed that the values are best fitted in regression line (Figure 5).

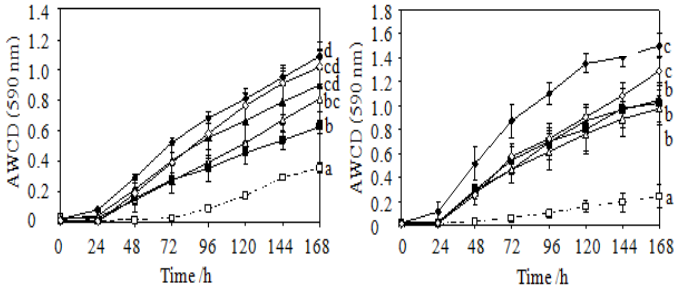

Figure 5: Bacterial numbers are means ± standard deviations of triplicate samples. Mean values with the same letter for the same batch of samples indicate no significant differences among treatments by LSD test at 5% level. **Note:** □: Symbols stand for Non-planted; ▨: Sesbania; ■:Seepweed; ▩: Ryegrass; ▩: Sea-lavender; and ▩: Central Asia Saltbush respectively. Regression equation, line of best fit and R2 values were shown using SPSS (18.0).

Plants acted as a vector, enabling associated microbes on roots to reach their targets contaminants and to metabolized or transform them. These microorganisms together with root exudates of plants might help in the removal of PHC from the selected soil, and plant-microbe interaction should be exploited which may enhance the degradation of persistent organic pollutants [21,22].

Functional diversity of soil microbial communities

The AWCD was analyzed using BIOLOG data to detect microbial catabolic activity and utilization ability with respect to 31 different Carbon substrates. The AWCD values at 60 and 90 d were varied significantly for the different plant rhizosphere, and the order was: Sesbania>Central Asia Saltbush>ryegrass>Seepweed>Sea-lavender>non-planted (Figure. 6).

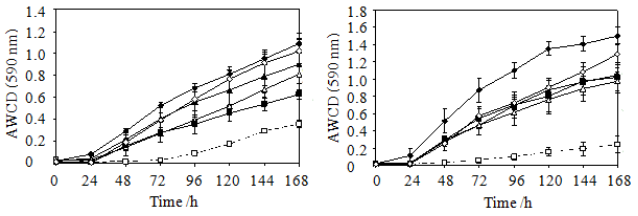

Figure 6: Average well color development (AWCD) in BIOLOG ECO plates for the microbial diversity in the rhizosphere soils collected at (a) 60 d and (b) 90 d. AWCD values are means ± standard deviations of triplicate determinations. Mean values with the same letter indicate no significant differences among treatments by LSD test at 5% level. **Note:** □: Non-planted; ◆ : Sesbania; △ : Seepweed; ▲ : Ryegrass; ■ : Sea-lavender; and ◇ : Central Asia Saltbush; respectively.

Data followed by the same letters in the same column for the same batch of samples indicate no significant differences at $P<0.05$.

Respectively which showed similar trends to the AWCD values. Shannon (species diversity) and Simpson indices (reflected the most common species) both showed significant increases ($P<0.05$) in the plant treatments compared with the control pots (non-rhizosphere), while the Evenness indices (a measure of uniformity) decreased.

Some of the differences in rhizosphere degradation was related with the microbial population diversity and the stimulated density and activity of microbes.

Days (d)	Plants	Shannon (H')	Evenness (E)	Simpson (D)
60	Non-planted	2.24	1.06	0.87
	Sesbania	3.06	0.93	0.94
	Seepweed	2.64	0.91	0.91
	Ryegrass	2.74	0.94	0.92
	Sea-lavender	2.42	0.84	0.92
	Central Asia Saltbush	3.58	1.17	0.84
90	Non-planted	1.82	0.96	0.71
	Sesbania	3.22	0.95	0.96
	Seepweed	2.76	0.92	0.92
	Ryegrass	2.84	0.94	0.93
	Sea-lavender	2.87	0.93	0.93
	Central Asia Saltbush	2.97	0.92	0.94

Table 3: Diversity indices of the rhizosphere microorganisms in the five different plants treatments at 60 and 90 days.

Principal Components Analysis (PCA) of the responses confirmed that catabolic diversity differed between the rhizosphere and non-planted soils (Figure 7).

The AWCD data at both 60 d and 90 d illustrated that Sesbania rhizosphere was the most dynamic microzone, where the activities of soil microbial communities remarkably increased compared with other plant treatments and the bulk soil (non-planted) which was mainly associated with the plant-specific factors [23-26].

Sesbania is a leguminous shrub with greater biomass productivity microbial populations in its rhizosphere had much more accesses to the organic substrates derived from the root and became more reactive which subsequently led to higher rhizodegradation of PHC.

The mean Shannon indices were 3.22, 2.97, 2.87, 2.84, 2.76 and 1.82 in the rhizospheres for Sesbania, Central Asia Saltbush, Sea-lavender, ryegrass, Seepweed and non-planted at 90 d (Table 3).

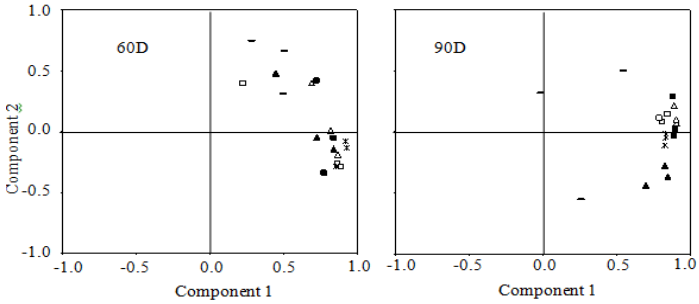

Figure 7: Comparison of the culturable bacterial community in (a) the rhizosphere and (b) non-planted soils. Orthogonal plot of principal components 1 and 2 for carbon substrate utilization analysis with plant species represented as Non-planted (-), ▲ : Sesbania; ■ :Seepweed; △ : Ryegrass; □ : Sea-lavender; and ✳ : Central Asia Saltbush; respectively.

The principal component 1 (PC1) and 2 (PC2) explained 67.0% of the variables for the soil samples collected at 60 d, while the first and second principal components (PC1 and PC2) explained 62.3% and 7.0% of the variance in the data for the samples collected at 90 d, respectively. Principal components differences were mainly associated with the rhizosphere process (e.g., increased microbial number and activity). After 90 d of incubation, in PC1, all the plant rhizospheres

had progressive influence on microbial diversity compared to the data of non-planted control, probably due to the modifications of the rhizosphere microbial diversity by the incorporation of huge amount of freely available organic substrates in the form of root. The concentration of pollutants and trophic condition can also altered the physiological structure of the microbial community. Moreover, "pollution-induced community tolerance" was able to lead the tolerant microbes to replace the sensitive ones which has increased the catabolic potential of rhizosphere soil through the plant-microorganism bioremediation system [27-30].

PHvariationoftherhizospheresoil

Soil pH together with organic matter quality and redox potential are consider as the important factors for organic pollutant solubility and bioavailability. During plant cultivation, soil pH declined from 7.94 to 7.58 (Figure 8).

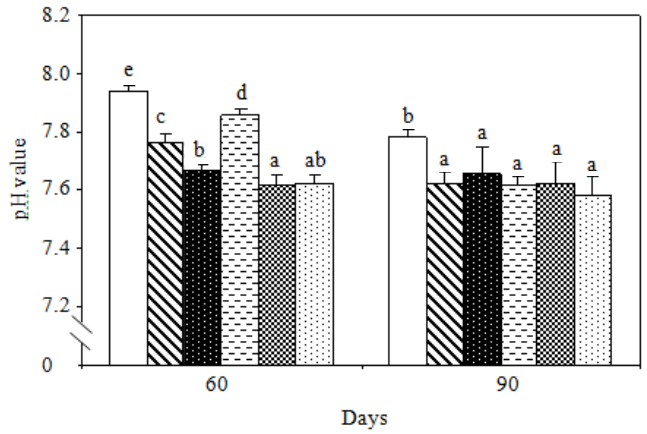

Figure 8: The pH values of the rhizosphere and non-planted soils in the five plant treatments. pH values are means ± standard deviations of triplicate determinations. Mean values with the same letter for the same batch of samples indicate no significant differences among treatments by LSD test at 5% level. **Note:** □ : Symbols stand for Non-planted; ▨: Sesbania; ■: Seepweed; ▩: Ryegrass; ▩ : Sea-lavender; and □ : Central Asia Saltbush; respectively.

The pH values in the rhizosphere soils at 60 d were all significantly decreased compared with the non-planted soils, and varied from the plant species ($P<0.05$). However, the values had no significant differences between these five plants after 90 d of incubation. Changes of the pH in the rhizosphere soil was caused by the released of acidic metabolites by microbes during hydrocarbon biodegradation [31]. In addition, the exudation of root organic acids induced pH decrease in the rhizosphere soil, which also increases the mobility of less available soil phosphates and substantially promoted plant growth. In the Yellow River delta, the soil was severely alkaline (Table 1), the response of rhizosphere to neutral conditions was also very important to enhance the rhizoremediation efficiency and soil quality improvement [32-34].

It is well known that pollutant's bioavailability is one of the key limiting factors for the bioremediation of recalcitrant organic pollutants in soils described that the incorporation of root exudates accelerated the desorption of phenanthrene and pyrene in soils and increased their bioavailability. During the long-term chronic organic contamination of soil, some recalcitrant pollutants were probably locked in soil particle pores, especially in the micro-pores [35].

Conclusion

The PHC degradation was more efficient in the rhizosphere soils than in the non-planted soils. The plants with larger biomass, greater root surface area, and stronger root volume explored more soil volume and enhanced the possibility for rhizosphere microorganisms to contact with the pollutants, then subsequently resulted in the higher rate of PHC removal. The increased microbial activity and metabolic diversity of microbial communities in the rhizosphere soils also enhanced rhizodegradation process. As Sesbania cannabina with higher biomass and rhizosphere effects showed higher PHC degradation than other plants, it can be a promising plant for rhizoremediation of petroleum hydrocarbon contaminated coastal wetland soils.

Acknowledgment

This research project was funded by Foundation of key program of the Education Ministry of China, project number 308016, in National Major technological program.

ConflictofInterest

The authors declare no conflict of interest.

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