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Effects of Different Microbial Treatments on the Removal of Petroleum Hydrocarbons from Soil

Hong Liu*, Pengfei Gao*, Jing Fu* and Haroon Rashid**
*School of Resources and Environment Engineering, Jilin Institute of Chemical Technology, Jilin 132022, China
**Department of Civil Engineering, Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan, Pakistan

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ABSTRACT

In order to study the effect of different microbial treatment methods on the removal of petroleum hydrocarbons in soil, microbial remediation of Zichang oil-polluted soil in northern Shaanxi province was studied by means of bacterial inoculation and biological stimulation. By using infrared spectrophotometry to determine the removal effect of different treatment methods on petroleum hydrocarbon, the best scheme for remediation of oil-polluted soil in northern Shaanxi province was determined. In the restoration process, the maximum possible count method (MPN), PCR-agar-electrophoresis, and PCR-DGGE were used to determine the number of oil-hydrocarbon degrading bacteria, catalytic genes, and soil microbial diversity to study the ecological effects of soil microorganisms. The results showed that the remediation effects of different biological treatments on oil-contaminated soil were as follows: biological stimulation (N and P nutrients were added) > biological enhancement (degradation bacteria were added) > and others. There was a positive correlation between the degradation rate of petroleum hydrocarbon in soil and the catalytic gene content of degradable petroleum hydrocarbon. During the restoration process, the number of petroleum hydrocarbons and alkane degrading bacteria in soil was significantly higher than that of PAHs degrading bacteria. The addition of exogenous degrading bacteria sz-1 could significantly improve the diversity of soil bacterial community. The results are helpful to understand the change of microbial ecological effect in bioremediation of petroleum soil.

INTRODUCTION

Petroleum is mainly composed of alkanes, aromatic hydrocarbons and cycloalkanes, in addition to a small number of non-hydrocarbon compounds. In the process of oil exploitation, transportation and use, various kinds of environmental pollution are caused by leakage. The strong adhesion of petroleum will affect the composition and structure of microorganisms in the soil after it enters the soil. On the other hand, it will hinder the respiration of plant roots and the absorption of nutrients, leading to poor growth and even death of plants (Tariq et al. 2018, Razzak et al. 2018). Harmful substances in petroleum can enter the human body through the food chain and directly harm human health. In particular, polycyclic aromatic hydrocarbons (PAHs) have carcinogenic, teratogenic and mutagenic effects (Onwuka et al. 2019, Randhawa et al. 2019). Microbial remediation has become an important technique for remediation of soil oil pollution due to its advantages such as simple operation, low cost, little pollution to the surrounding environment and basically no secondary pollution. At present, many researchers are devoted to the research of bioremediation of oily soil by screening highly effective oil-degrading bacteria. In this paper, microbial remediation of Zichang oil-polluted soil in northern Shaanxi province was studied by means of microbial inoculation and biological stimulation (Sarwar et al. 2019, Man & Hwang 2017).

EARLIER STUDIES

There are many research methods for petroleum hydrocarbon in soil, such as soxhlet extraction, ultrasonic extraction and accelerated solvent extraction for pre-treatment of total petroleum hydrocarbon in soil (Ibrahim et al. 2018). Effects of different plant species, different soil conditioner and microbial agents and other conditions on the phytoremediation effect of petroleum hydrocarbon in soil and effects of complexation Fe^{2+}catalysed H_2O_2 on the removal rate of petroleum hydrocarbon (TPH) and organic matter (SOM) (Li et al. 2018). These methods are feasible, but the specific implementation is more complex and requires certain equipment, different raw materials, etc., for the application.

Microbial remediation is a new, economical, efficient and ecologically sustainable green and clean technology for treating oil-contaminated soil (Chen et al. 2017). A study has shown that immobilization techniques, particularly in harsh environments, can improve biodegradation rates (Partovinia & Rasekh 2018). Previous research carried out laboratory simulated remediation study on oil-polluted yellow soil by

using microbial method, and evaluated the remediation effect and influencing factors by measuring the contents of different hydrocarbon components, nitrogen and available phosphorus in different forms and the number of oil-hydrocarbon degrading bacteria in the remediation process (Alhawash et al. 2018). Alavi et al. (2017) studied the remediation effect of different bioremediation methods on long-term contaminated soil in oil field area and analysed the typical correlation between petroleum pollutants and microbial variables and soil physical and chemical properties variables. Liu et al. (2017) studied the response of microbial communities and selected petroleum hydrocarbon (pH) degradation genes in soil/sediments at different geographical locations to simulated pH overflow. A recent study used a variety of microbial binders to generate peroxidase and biosurfactant in the original position of SCR, promoting the enzymatic biodegradation of TPH and accelerating the biodegradation of petroleum hydrocarbon and COD removal (Moussavi et al. 2017). Maddela et al. (2017) conducted a field feasibility study on the total petroleum hydrocarbon (TPH) degradation potential of *Bacillus thuringiensis* B3, *Bacillus cereus* B6 and two fungi (*Geomyces pannulum* HR and *Geomyces* sp.).

In this paper, microbial remediation of Zichang oil-polluted soil in northern Shaanxi province was studied by means of microbial inoculation and biological stimulation. In the restoration process, the maximum possible count method (MPN), PCR-agar-electrophoresis, and PCR-DGGE were used to determine the number of oil-hydrocarbon degrading bacteria, catalytic genes, and soil microbial diversity to study the ecological effects of soil microorganisms.

MATERIALS AND METHODS

Tested Soil and Oil-Hydrocarbon Degrading Bacteria

Petroleum contaminated soil around an oil well in Zichang county, northern Shaanxi was selected, screened and mixed with 20-mesh sieve, and then used as soil for oil pollution degradation and remediation. Bush-hass medium was used to isolate and screen out the oil-hydrocarbon degrading bacteria SZ-1 (Acinetobacter) from the soil for remediation study.

Medium and Reagents

Bush-hass medium, 1.2 % agarose gel, 50×TAE buffer; the preparation method of PBS buffer solution is shown in literature [21]. Soil DNA extraction kit: PowerSoil DNA IsolationKit, MOBIO; Cloning kit: PMD19t, TAKARA;Mixed PAHs solution: phenanthrene (10g), anthracene (1g), pyrene (1g), dibenzothiophene (1g), dissolved with dichloromethane (1L). Iodonitrotetrazolium (INT) ($3g{\cdot}L^{-1}$) solution:3g of Iodonitrotetrazolium powder was accurately weighed, and the volume was fixed to 1L with sterilized pure water. 1.5% agarose gel:1×TAE buffer (50mL) was placed in a 250mL conical flask, and agar-agar powder (0.75g) was added to the buffer. The bottle was sealed with tin foil, and the agar-agar powder was completely dissolved after heating in the microwave oven for 2 minutes.

Experimental Scheme for Microbial Remediation of Oil-Contaminated Soil

500g of tested soil was respectively weighed and divided into a round porcelain basin. The soil was treated with bioremediation for 8 weeks according to the scheme shown in Table 1. During the restoration, the soil was turned evenly every day to maintain air permeability.

Methods for Determination of Petroleum Hydrocarbons in Soil Under Different Remediation Treatments

Soil samples of the above treatments were taken, dried and ground, and 1g of soil samples of each treatment were accurately weighed in a weighing flask, and 25mL carbon

Table 1: Microbial remediation treatment scheme for petroleum contaminated soil.

Repair treatment number	Soil remediation treatment program
1	Natural state test soil + sterilized pure water (soil moisture content 40%)
2	Sterilized test soil + sterilized pure water (soil moisture content 40%)
3	Natural state test soil + SZ-1 bacterial suspension + sterilized pure water (soil moisture content 40%)
4	Natural state test soil + nitrogen and phosphorus nutrient solution (soil C: N: P = 100:10:1) + sterilized pure water (soil moisture
5	Test soil in natural state + SZ-1 bacterial suspension + nitrogen and phosphorus nutrition (soil C: N: P = 100:10:1) + sterilized pure water (soil moisture content 40%)
6	Sterilized test soil + sz-1 suspension + sterilized pure water (soil moisture content 40%)

1) Sterilization soil using 0.103 MPa, 121°C, 1h pressure steam sterilization; SZ-1 bacterial suspension: inoculated bacterial suspension (20mL of D_{620}= 2.0) was placed in the test soil, and the number of degraded bacteria in the test soil was guaranteed to reach $10^9 \cdot g^{-1}$.Soil C: N: P =100:10:1 [add $(NH4)_2SO_4$ and KH_2PO_4 respectively as 19.6 $g{\cdot}kg^{-1}$ and 0.9 $g{\cdot}kg^{-1}$]; keep 40% soil water content (187.5mL sterilized pure water)

tetrachloride was added. Using ultrasonic cell crusher (JY92 - II, SONICS, USA) 15 min ultrasonic extraction with power 170W. The filtrate was collected in a 50mL volumetric flask with a constant volume. Three parallel samples were made for each sample. Petroleum hydrocarbon in soil was determined by OCMA-350 infrared oil detector.

Determination of Catalytic Genes in Soil

The DNA of soil samples 1, 3 and 4 was extracted with soil DNA extraction kit. The extracted soil DNA was diluted by 5-fold serial dilution method. The catalytic gene primers of degradable alkanes and degradable aromatics were obtained by referring to literatures. Two aromatics catalytic genes are included (1) (nahAcfor/nahAcrew) α-subunit naphthalene dioxygenase gene, (2) (AJ025 /AJ026) α-Subunit ring hydroxyl dioxygenase gene and two alkane catalytic genes, (3) (alk-3F/alk-3R) paraffin hydroxylase gene, (4) (alkFa / aklRa) paraffin monooxygenase gene.

Determination of the Number of Degrading Bacteria in Soil (MPN Method)

Weigh and take 1g of soil no. 1, 3 and 4 respectively, add 9mL PBS buffer solution, and shake well at 30°C,130 $r \cdot min^{-1}$. After shaking culture for 30min, the supernatant was taken for 10-fold serial dilution, and 101, 102 and... Soil diluent. The MPN substrates were n-hexadecane and mixed PAHs solution respectively. The number of oil-hydrocarbon degrading bacteria, alkane degrading bacteria and PAHs degrading bacteria were determined according to the method described in the literature.

Soil Microbial Community Analysis Method (PCR-DGGE)

Soil DNA extraction kit was used to extract DNA from soil samples. Universal primers for bacteria used were338F/518R (5'-ACTCCTACGGGAGGCAGCAG-3') / (5'-ATTACCG-CGGCTGCTGG-3') PCR amplification of soil DNA, 50μL PCR reaction system configuration: 22μL ddH_2O, 25μL 2 × Go Taqenzyme, 2μLPrimers F, 2μLPrimers R. PCR reaction heating procedure: 94°C modified 5 min, 94°C modified 30s, 30s, 57°C annealing,30s 72°C extending 30s, repeat cycle, the 72°C and then extended 7 min. PCR amplification products were detected by 1.5% agarose gel electrophoresis.

The detection bands were clear without trailing, indicating good amplification results. The PCR amplification products were analysed by denaturing gradient gel electrophoresis (DGGE). DGGE procedure: acrylamide gel concentration 10%, denaturation gradient range 40%-60%, the sample quantity of PCR product was10μL, 60°C, 60V, run it for 15 hours. Gelred staining was used for 1 h after electrophoresis, the DGGE images were captured using the gene imaging system and analysed using the Quantityone software (Quantity one-4.6.2), DGGE strips were cut and cloned and sequenced using PMD19T vector kit (PMD19T, TAKARA).

RESULTS AND DISCUSSION

Removal Effects of Petroleum Hydrocarbons in Soil under Different Remediation Treatments

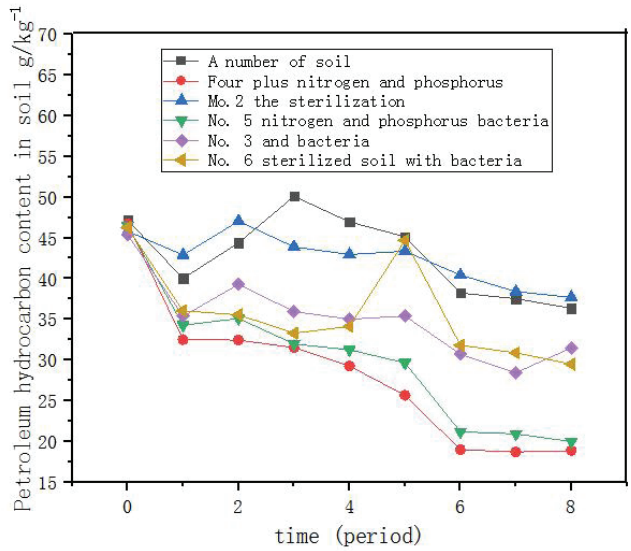

Fig. 1: Petroleum hydrocarbon contents in soil polluted by different remediation treatments.

The removal effects of different biological treatments on soil petroleum hydrocarbons are shown in Fig. 1. In this remediation experiment of petroleum contaminated soil around an oil well in Zichang, northern Shaanxi, biological stimulation (no. 4 and no. 5 treatment with nitrogen and phosphorus nutrition) has the best remediation effect on petroleum contaminated soil, the removal rate of petroleum hydrocarbon reached 59.7% and 57.5% respectively at 8 weeks of restoration. Secondly, for the soil treated with bio-enhanced treatment (no. 3 and no. 6 were added with exogenous bacteria), the removal rate of petroleum hydrocarbon reached about 33% after 8 weeks of treatment. However, under natural conditions, when the soil moisture content is 40% (no. 1 treatment) and sterilization (no. 2 treatment) have no obvious effect on soil remediation. From the above results, it can be seen that the native bacteria had poor degradation effect on petroleum hydrocarbon in this repair process, and when nitrogen and phosphorus nutrition were not added, the environmental adaptability of the foreign bacteria was stronger than that of the native bacteria, and the removal effect of petroleum hydrocarbon was more obvious. The results showed that the native bacteria could degrade petroleum hydrocarbon effectively only under the appropriate condition of carbon, nitrogen and phosphorus ratio in soil remediation.

Results of Catalytic Gene Determination of Degradable Petroleum Hydrocarbons in Soil

The catalytic genes of degradable alkanes and PAHs in No. 1, 3 and 4 soils were determined, as shown in Table 2. Before repair (week 0, when the bioremediation has not started), only AJ025/AJ026 primers and alkFa/aklRa primers-controlled genes were found in the oily soil. With the increase of repair time, the two primer control genes showed a trend of increasing first and then stabilizing or decreasing. Moreover, the number of AJ025/AJ026 primer genes in no. 3 treatment was significantly higher than that in the other two treatments. According to previous studies, the strain SZ-1 contains the gene controlled by AJ025 /AJ026 primer, it can be inferred that the increase of the primer gene in the no.3 treated soil was caused by the addition of exogenous bacteria SZ-1. In the early stage of microbial repair (week 1), AJ025 /AJ026 the content of catalytic genes controlling the degradation of aromatic hydrocarbons increased rapidly, moreover, a new catalytic gene for nahAcfor/nahAcrew degradable aromatics appeared in the treatment of 1 and 3.AlkFa/aklRa controlled the content of catalytic genes for alkane degradation increased rapidly during the repair period (2~7weeks),new alk-3f/alk-3r degradable alkanes catalytic genes were also detected. The results showed that in the process of microbial remediation, the number and types of catalytic genes for the degradation of aromatic hydrocarbons increased first and then decreased, while the number and types of catalytic genes for the degradation of alkanes increased significantly.

Determination results of the number of degradation bacteria of petroleum hydrocarbon, alkane and polycyclic aromatic hydrocarbon

Table 2: Results of bioremediation soil gene test.

Project	Target catalytic gene	Time per week				
		0	1	2	6	7
No. 1 treatment (soil)	(1) (nahAcfor/nahAcrew)	—	+ +	+ +	—	—
	(2) (AJ025 /AJ026)	+	+ + +	+ + +	+ +	+ +
	(3) (alk-3F/alk-3R)	—	—	+ +	+ + +	+ + + +
	(4) (alkFa/aklRa)	+	+	+ +	+ + +	+
No. 3 treatment (AcinetobacterAcinetobacter was added to the soil SZ-1	(1) (nahAcfor/nahAcrew)	—	+ +	—	—	—
	(2) (AJ025 /AJ026)	+ + ++	+ + ++	+ +	+ +	—
	(3) (alk-3F/alk-3R)	—	—	+	+ + +	+ +
	(4) (alkFa/aklRa)	+	+	+ + +	—	+ + +
No. 4 treatment (soil with N and P nutrition)	(1) (nahAcfor/nahAcrew)	—	—	—	—	—
	(2) (AJ025 /AJ026)	+	+ + +	+ + +	+	+ +
	(3) (alk-3F/alk-3R)	—	—	+ + +	+ + + +	+ + +
	(4) (alkFa/aklRa)	+	+ +	+ + + +	+ + +	+ + +

Note: "--" means the presence of the gene that does not control the primer, "+" means the presence of the gene controlled by the primer, and the number of "+" represents the dilution ratio of DNA. For example, "+" means the gene can be detected at the dilution ratio of 5:1, and "+ +" means the gene can still be detected at the dilution ratio of 5:2, and so on

Figs. 2-4 show the determination results of the degradation bacteria number of petroleum hydrocarbon, alkane and polycyclic aromatic hydrocarbon in no. 1, 3 and 4 soils. As can be seen from Figs. 2, 3 and 4, the number of degradation bacteria of petroleum hydrocarbon, alkane and PAHs in no. 4 treatment increased the most, moreover, the number of oil-hydrocarbon and alkane degrading bacteria can reach more than 10^5CFU·mL^{-1},the maximum number of PAHs degrading bacteria can reach 10^2CFU·mL^{-1}.In the process of remediation of petroleum contaminated soil, the number of degradation bacteria of petroleum hydrocarbon and alkane was significantly higher than that of PAHs. According to Fig. 5, the total number of biodegradable bacteria in the no. 4 treatment was far higher than that in the other two treat-

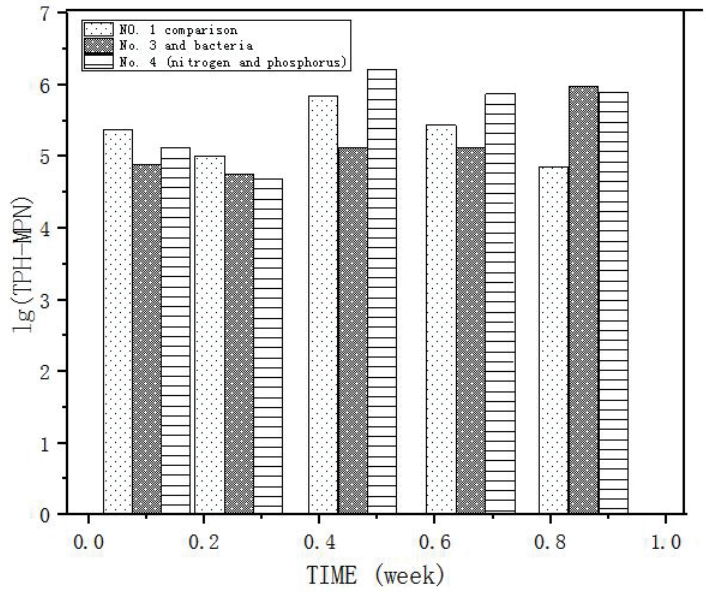

Fig. 2: Number of bacteria for petroleum hydrocarbon degradation in soil under different treatments.

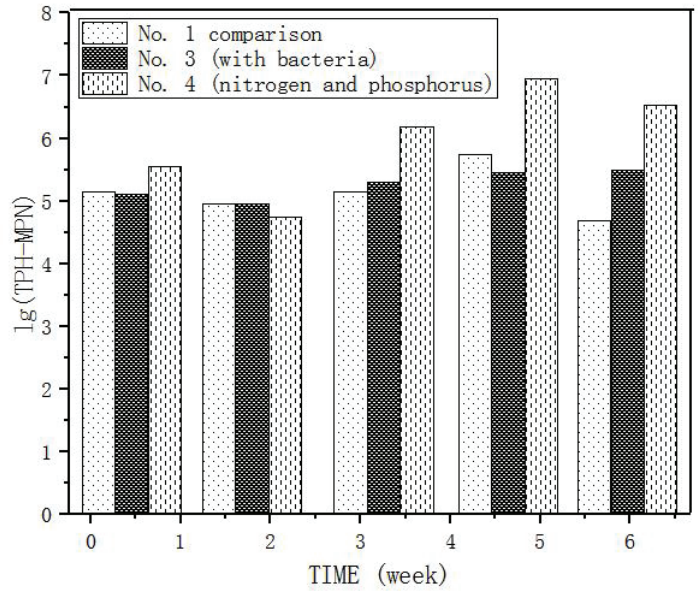

Fig. 3: Number of alkane-degrading bacteria in soil under different treatments.

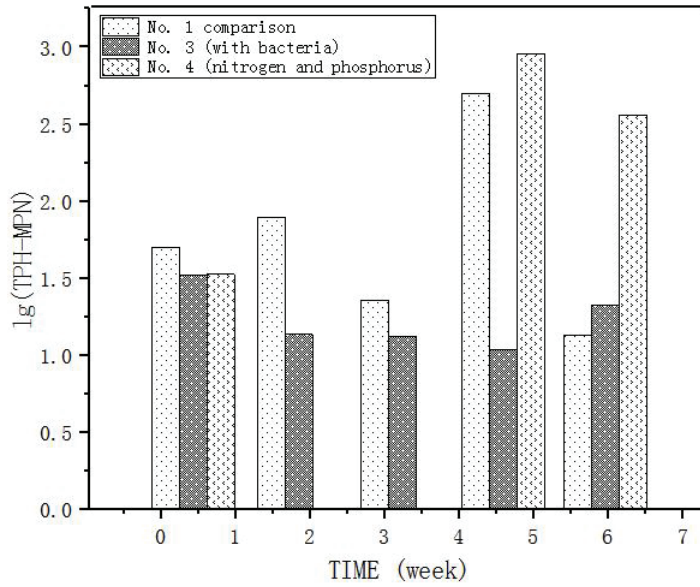

Fig. 4: Number of PAHs degrading bacteria in soil under different treatments.

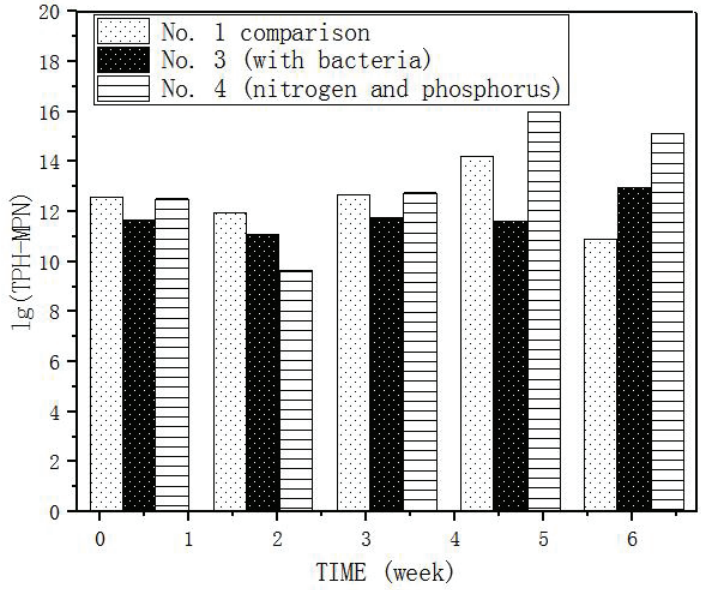

Fig. 5: Total number of degradation bacteria in soil under different treatments.

ments, in addition, the total number of degradable bacteria in different treatments showed a change pattern of decrease first and then increase in the repair period (week 0-6), and decrease in the late repair period (week 7).Soil change trend of total degradation bacteria number is consistent with the petroleum hydrocarbon in soil removal effect change rule. Thus it can be seen that different bioremediation process can promote the total number of degrading bacteria in the soil, the more degradation bacteria required material used in the synthesis, growth, accelerate the metabolism of biological process needed to use petroleum hydrocarbon as the only carbon source, so that to reduce the quantity of petroleum hydrocarbons in the soil and give better removal results.

Results of Soil Microbial Community Analysis

Soil DNA was extracted from no. 1, 3 and 4 for treatment,

PCR amplification of 338F/518R was performed using universal bacterial primers, results are shown in Fig. 6.The amplification bands were clear and bright, and there was no non-specific amplification, the amplification fragment was about 180 bp, and the amplification process was not contaminated. PCR-DGGE analysis was carried out on it, and the results are shown in Fig. 7.

As can be seen from Fig. 7 (PCR-DGGE spectrum),that the number of main strips in blank control (CK) was 7 ~ 9.Compared with the blank control (CK),the number of bands in the three treatments did not change much at week 1 (swimlane A, B, C),however, after restoration for 2 to 7 weeks, the number of strips in the restored soil increased significantly (swimlane D-L), and the structure of bacterial community changed greatly.

Lane CK: blank control, week 0 untreated oil contaminated soil, cutting strips: 4, 5, 10; Lane A, D, G, J: 1 (soil) soil remediation at week 1, 2, 6 and 7, cutting tape: 7, 9, 12,

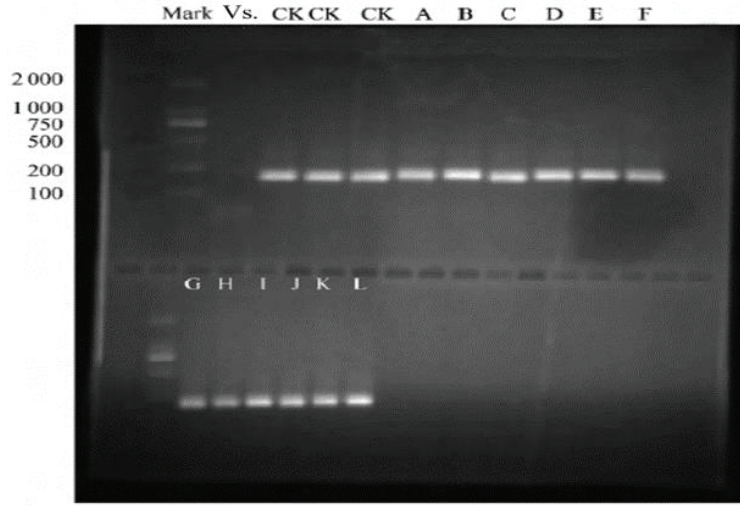

Fig. 6: Electrophoresis of PCR amplification products.

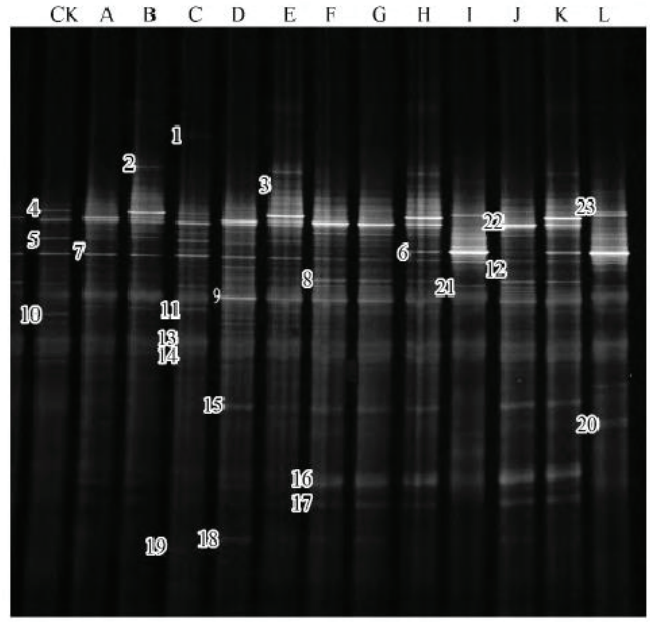

Fig. 7: Profiles of DGGE (denatured gradient gel electrophoresis) of PCR amplification products

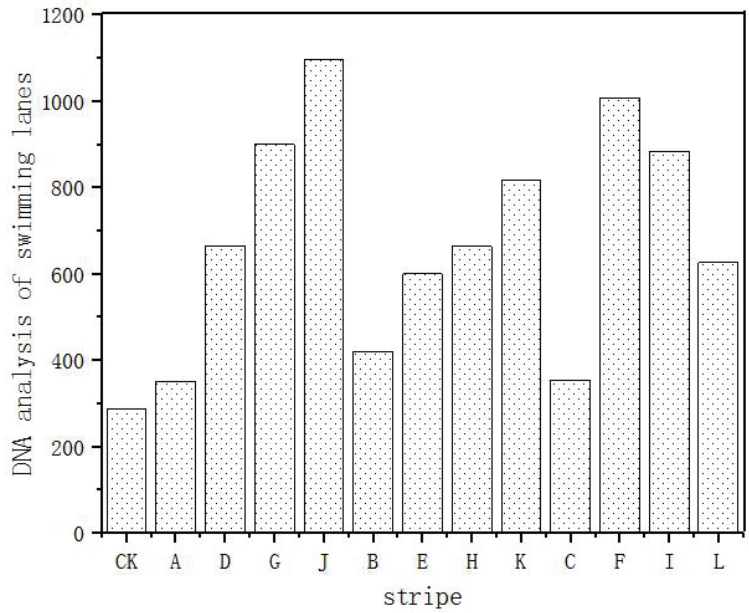

Fig. 8: Quantitative analysis results of DGGE swimlane strips.

15, 18, 22; Lane B, E, H, K: No. 3 (soil bacteria SZ-1) soil remediation at week 1, 2, 6 and 7, cutting tape: 2, 3, 6; Lane C, F, I, L: 4 (nitrogen and phosphorus added to soil) soil remediation at week 1, 2, 6 and 7, cutting tape: 1, 8, 11, 13, 14, 16, 17, 19, 20, 21, 23

Lane CK: blank control, week 0 untreated oil contaminated soil, cutting strips: 4, 5, 10;Lane A, D, G, J: 1 (soil) soil remediation at week 1, 2, 6 and 7,cutting tape: 7, 9, 12, 15, 18, 22;Lane B, E, H, K: no. 3 (soil bacteria SZ-1)soil remediation at week 1, 2, 6 and 7,cutting tape: 2, 3, 6;Lane C, F, I, L: 4 (nitrogen and phosphorus added to soil)soil remediation at week 1, 2, 6 and 7,cutting tape: 1, 8, 11, 13, 14, 16, 17, 19, 20, 21, 23

Strip 2 in week 1, 2, 6 and 7 (corresponding to swimming lanes B, E, H and K) of no. 3 treatment was cut and cloned and sequenced, it was found that this band belongs to Acinetobacter. Blast alignment results showed that the similarity between band 2 and degradation bacteria SZ-1 was more than 99%, therefore, it can be preliminarily concluded that this article may contain additional degradation bacteria SZ-1. Quantityone software quantitatively analysed the DNA concentration of the main strip of the DGGE swimming lane (trace),the results are shown in Fig. 8. At week 1 of restoration, compared with blank control (CK), the DNA concentration in soil samples with different treatments did not change much (swimlane A, B, C), but with the increase of restoration time, the total concentration of DNA in soil with different treatments basically showed an increasing trend.

CONCLUSION

In this paper, microbial remediation of Zichang oil-polluted soil in northern Shaanxi province was studied by means of microbial inoculation and biological stimulation. The conclusions are as follows:

(1) The order of restoration effect of oil contaminated soil samples in Zichang, northern Shaanxi is from good to bad: add N and P nutrients to the soil> add Acinetobacter SZ-1 > and other treatments to the soil. The native bacteria can degrade petroleum hydrocarbon effectively only under the suitable condition of carbon, nitrogen and phosphorus ratio. Exogenous bacteria SZ-1 has stronger environmental adaptability than native bacteria and can effectively degrade petroleum hydrocarbons in soil.

(2) The more the total number of catalytic genes and the total number of degrading bacteria in the soil, the better the removal effect of petroleum hydrocarbons in the soil. The number of PAHs degrading bacteria in the remediation soil was significantly higher than that of PAHs degrading bacteria.

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