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# Adaptability of microbial inoculators and their contribution to degradation of mineral oil and PAHs

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**Abstract:** Five dominant bacteria strains(*Acetobacter* sp., *Alcaligenes* sp., *Micrococcus* sp., *Arthrobacter* sp. and *Bacillus* sp.) and five fungi strains (*Cephalosporium* sp. I, *Cephalosporium* sp. II, *Aspergillus* sp. I, *Aspergillus* sp. II and *Fusarium* sp.) isolated from petroleum-contaminated soil were used to assess the potential capability of mineral oil and PAH enhanced degradation separately and jointly using the batch liquid medium cultivation with diesel oil spiked at 1000 mg/L. The experiment was performed on a reciprocal shaker in the darkness at 25℃ to 30℃ for 100 d. The dynamic variation in the activity of microbial inoculators in each treatment and the degradation of the target pollutants during the period of experiment were monitored. Results showed a more rapid biodegradation of mineral oil and PAHs at the beginning of the experiment (about 20 d) by dominant bacteria, fungi and their mixture than that of the indigenous microorganisms, however, thereafter an opposite trend was exhibited that the removal ratio by indigenous microorganisms was superior to any other dominant treatments and the tendency lasted till the end of the experiment, indicating the limited competitive capability of dominant microorganisms to degrade the contaminants, and the natural selection of indigenous microorganisms for use in the removal of the contaminants. At the end of the experiment, the removal ratio of mineral oil ranged from 56.8 % to 79.2 % and PAHs ranged from 96.8 % to 99.1 % in each treatment by microbial inoculators.

**Keywords:** dominant microorganism; indigenous microorganism; mineral oil; PAHs; biodegradation

## Introduction

Soil contamination by petroleum occurs in the processes of production, refining, utilization and transportation of petroleum and its products (Ren and Huang, 2000). There is an increasing concern about the remediation of petroleum-contaminated soils because of the hazard effect of the pollutants existing in the petroleum-contaminated soil on the ecosystem and human health (Won *et al.*, 2001), such as the carcinogenic, mutagenic, and other toxic effects (Readman *et al.*, 1987; Joel *et al.*, 2002; Viguri *et al.*, 2002; Basheer, 2003). As a complex mixture of petroleum hydrocarbons, diesel oil consists of various components including the volatile, low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, phenathrene, and pyrene (Facundo *et al.*, 2001; Zhu *et al.*, 2005) which are relatively persistent in the soil environment, and the complexity of diesel oil makes it more difficult to deal with by chemical and physical treatment due to the high cost and low efficiency as well as the side-effect on the physiochemical properties of soil (Wang *et al.*, 1990; Sun *et al.*, 1999; Song *et al.*, 2001, 2005).

Based mainly on the ability of soil microorganisms to metabolize contaminants partially or completely, bioremediation techniques have been developed and regarded as a useful and cost-effective tool in the degradation and detoxification of organic pollutants (Fredrickson, 1993; Gong, 2002; Zhou and Song, 2004). In order to enhance the biodegradation, many investigations have been conducted on the enhanced capability of dominant microorganisms isolated from contaminated soils on the degradation of mineral oil and PAHs for individual and/or mixtures of pure chemicals spiked in the laboratory conditions with careful control (Won *et al.*, 2001; Song *et al.*, 2001; Vaclav *et al.*, 2003). Unfortunately, up to now no successful case has been reported to extend the laboratory results into the practical field conditions, and data obtained were also contrary to the expectation on the dominant microorganisms given in the laboratory conditions. For instance, in the former study on the phytoremediation of mineral oil and PAHs in diesel-contaminated soils we found that the dominant bacterial and fungi inoculators isolated from petroleum-contaminated soil could not show any enhanced effect ($P<0.01$) on the removing of target contaminants than the indigenous microorganisms during the process of phytoremediation under the 20-week outdoor experiment (Song *et al.*, 2001). The possible reasons for the unexpected biodegradation ability of so called "dominant microorganisms" with the indigenous microorganisms might be the severe challenge of survival adaptability and competing under the field conditions. However, data on these aspects are still limited. Thus, it is necessary to carry out a further experiment to conform the situation

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mentioned. For this purpose, a study was set up on the adaptability of dominant microorganisms and their contribution to degradation of minerals oil and PAHs as a chemical mixture. The same dominant bacterial and fungi inoculators from our outdoor experiment were used, and the experiment was performed under the laboratory conditions in order to avoid the complex interferences.

## 1 Materials and methods

### 1.1 Tested contaminant and preparation of microorganism strains

The 20# heavy diesel oil(with density of 820 g/L) supplied by the Fushun Refinery Plant, Liaoning Province, China was used as the tested petroleum.

Dominant bacteria and fungal strains: 5 bacteria (*Acetobacter* sp., *Alcaligenes* sp., *Micrococcus* sp., *Arthrobacter* sp. and *Bacillus* sp.,) and 5 fungi (*Cephalosporium* sp. I, *Cephalosporium* sp. II, *Aspergillus* sp. I, *Aspergillus* sp. II and *Fusarium* sp.) isolated from a long-term petroleum-contaminated site were identified as hydrocarbon-degrading microorganisms. The microorganisms were activated for 2 d before they were used.

The preparation of indigenous microbial inoculants: meadow brown soil (0—20 cm) collected from the Shenyang Ecological Station of Chinese Academy of Sciences was used for the preparation of indigenous soil microorganisms. 50.0 g of fresh soil was weighed into a 250-ml flask, mixed with 100 ml of deionized water sterilized in the autoclave at 120℃, 1 kPa for 30 min, and shaken on the reciprocal shaker at 150 r/min in dark room at 25—30℃ for 30 min. The suspension was collected as indigenous microorganism inoculants.

### 1.2 Culture medium

The liquid mineral medium in per liter water was prepared as follows: 0.20 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.40 g/L $KH_2PO_4$, 0.02 g/L $MnSO_4 \cdot H_2O$, 1.00 g/L $NH_4NO_3$, and 0.60 g/L $Na_2HPO_4 \cdot 12H_2O$ were added in per liter of culture medium. In addition, 2.00 g glucose and 0.50 g yeast were added to maintain the long-term growth of microorganisms, and the pH value was 7.0. All the reagents used were of analytical grade.

### 1.3 Experiment design

Diesel oil (50.0 mg) was spiked by a minim injector into a 150-ml flask with 49 ml autoclaved mineral medium (1 kPa, 30 min). Then it was inoculated with 1.0 ml inoculants (the initial count of bacteria and fungi referred to the result at 0 h in Fig.1a and 2a). The flasks were put on the reciprocal shaker at 150 r/min in the dark, and kept at a temperature ranging from 25℃ to 30℃ for 100 d. There are 4 treatments, namely B (5 selected bacteria mixtures), F (5 selected fungi mixtures), M (B+F), and I (soil indigenous microorganism suspension).

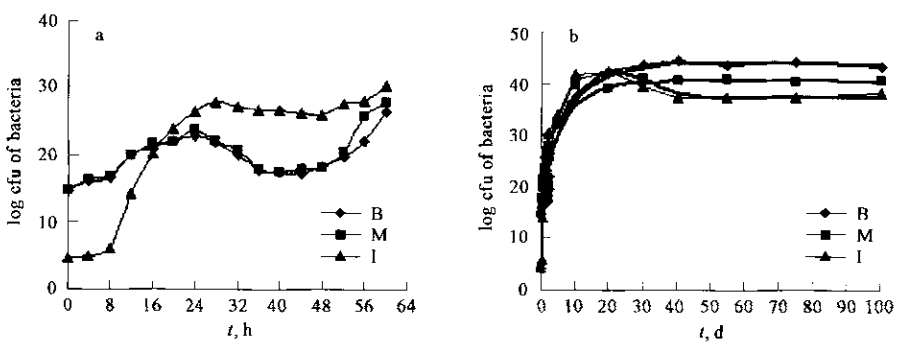


Fig.1 Logarithmic growth curve of bacteria based on sample B, M and I
a. 0—60 h; b. 0—100 d

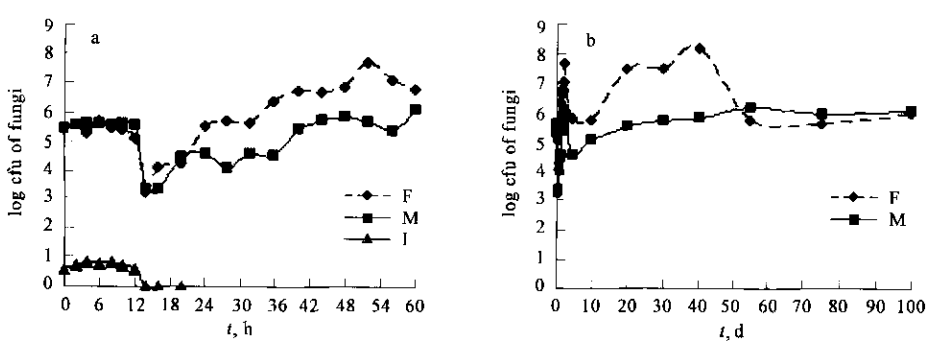


Fig.2 Logarithmic growth curve of fungi based on sample F, M and I.
a. 0—60 h; b. 0—100 d

Dynamics of microbial growth were monitored continuously in the former 60 h, then flasks were removed periodically for the determination of the growth of microbes and dynamic changes in mineral oil and PAH contents from the day 5 to 100. All the experiments were carried out triply.

### 1.4 Soil-microbial counts

Microbial cell number was estimated by the plate-count on nutrient agar. The 1.0 ml suspension after appropriate serial dilutions was spread over the surface of duplicate agar plates with media. Bacteria and fungi plates were incubated at 28℃ for 48 and 72 h, respectively, with the lids down to estimate the total colony forming units(cfu).

### 1.5 Mineral oil extraction and determination

The liquid medium in the flask was sampled periodically for mineral oil extraction and UV spectrometer determination. It was transferred into 125 ml of tap funnel with addition of 30 ml *n*-hexane for liquid-liquid extraction by a shaker machine for 20 min. The extract was filtered and collected into a flask equipped with 5.0 g of sodium sulfate (heated up at 400℃ for 2 h in oven) for dryness. This step was repeated twice and the supernatant was incorporated. Then the volume was fixed to 50 ml and it was detected referring to UV-spectroscopy (2050-spectrophotometer, Japan). The concentration of mineral oil was detected by the absorbency value at 254 nm taking *n*-hexane as the control, and calculated according to the standard curve. The removal ratio of mineral oil was calculated according to the following equation:

$$R=(C_0-C_t)/C_0\times 100\% \quad (1)$$

Where $R$ is the removal ratio of mineral oil, $C_0$ is equal to 1000 mg/L, $C_t$ is the concentration of mineral oil at time $t$. In the experiment the content of mineral oil at the day 5, 10, 20, 30, 40, 55, 75, and 100 were determined.

### 1.6 PAHs extraction and determination

The extract after determination of mineral oil, was reduced to dryness in a rotary evaporator(EYELA N-3NW, Japan) and was made up to 1.0 ml with dichlorobenzene for cleanup in a column filled with 1.0 g of silica gel(70—230 mesh, Sigma). The column was eluted with 1:1 (volume ratio) mixture of dichlorobenzene and *n*-hexane, and the elution was reduced to dryness again by nitrogen stream and made up to 1.0 ml with methanol for analysis.

PAHs identification and quantification were performed by HPLC(Hewlett Packard HP-1090 Series II) with a fluorescence detector (Hewlett Packard HP 1046). The column temperature was programmed as initial temperature of 40℃ held for 40 min. A 15.0 μl extract was injected and separated using a RP-C18 sphersorb(octadecylsilane) column(25 cm×2.1 mm, 5 μm). A mobile phase acetonitrile/water flow rate of 0.800 ml/min was used with the following linear gradient elution program, acetonitrile:water (v/v) elution consisted in a linear gradient to 50:50 at 0 min, to 100:0 in 40 min. An external standard 1647d SRM NBS mixture was used for quantification of 16 PAHs. Fluorescence data were qualified on a VG minichroma data handling system by manual integration. Identification of PAHs was carried out on the basis of retention time with further confirmation on a photodiode array detector (SP spectra monitor 3100, USA) if necessary. In the experiment the PAH content at day 20, 30, 40, 75, and 100 was determined. The removal ratio of total PAHs was calculated by the ratio of the total concentration of USEPA 16 PAHs to the initial PAH concentration in the liquid media.

Dichloromethane and *n*-hexane were of p.a. grade or residue analysis grade. Acetonitrile and ultra pure water were of HPLC purity grade (as HPLC mobile phase). Silica gel (70—230 mesh) and sodium sulfate were residue analysis grade and obtained from Sigma. PAHs standard SRM 1647C was purchased from Promochem, Germany.

### 1.7 Data analysis

The means, standard deviation, and analysis of variance (ANOVA) were performed on the data obtained from the experiment. The multiple comparison procedure (LSD) was used to examine significant differences among the means of the results of mineral oil and PAH removal ratios in different treatments by the SPSS 12.0 package.

## 2 Results and discussion

### 2.1 Logarithm growth of bacteria inoculators

The logarithm growth of bacteria for the sample B, M, and I was depicted in Fig.1a and 1b at the intervals of 0—60 h at the beginning of the experiment and among 0—100 d, respectively. The characteristics of the bacteria growth were found and identified as three periods including the lag stage, the logarithm stage, and the stable stage. By comparison among the sample B, M, and I(Fig.1a), an obvious lag stage of growth as well as the logarithm stage was found for sample I. For example, the bacteria growth in sample I at the previous 8 h was at the lag stage, among the period of 8—24 h the bacteria growth was more rapidly and the growth changed into the logarithm stage. Then from 24 to 48 h there was a relatively stable stage of the bacteria growth, and thereafter(48—60 h) the amount of bacteria showed a gentle increase indicating that a new rising stage of bacteria growth appeared. The new increase period of bacteria growth was mainly responsible for the bacteria adapted to the contaminated environment and began to utilize the easily degraded hydrocarbons as carbon sources to complement the culture media in the

diesel oil. The logarithm growth curve showed that the nutrient and environmental conditions in the experiment were appropriate to the growth and reproduction of soil bacteria(William, 1995).

As shown in Fig.1a, the characteristic of logarithmic growth of bacteria in sample B and M was quite similar(0—60 h), which was relative to the same inoculum volume and the same bacteria strains. The cardinal number of inoculums in sample B and M was at a higher amount compared with that in sample I, which caused the lag stage of B and M appeared in advance, therefore, the microbial logarithmic growth characteristic at the lag stage and the logarithmic stage got more ambiguous. In addition, the bacteria count in sample M was always a little superior to that in sample B at the initial time of 0—60 h, which was probably due to the co-existence of fungi, which might play a role in stimulating the growth of bacteria in sample M in the case of abundant nutrients supplied.

Fig.1b described the logarithmic growth of bacteria for sample B, M, and I during the whole period of 0—100 d. As assessed by the plate counts on agar, the bacteria counts maintained increasing in all treatments since 2.5 d(60 h). For instance, the bacteria counts in sample I had reached at the peak value($10^{42}$ cfu/ml) at the day 20 and thereafter decreased gently, the counts remained almost stable after 40 d, and was kept till the end of the experiment ($10^{37}$ cfu/ml). In comparison, the maximum value of bacteria counts appeared at the day 40 in B and M (20 d delay than that in sample I), then the counts remained almost unchanged till the end of experiment with the range of $10^{43}$—$10^{44}$ cfu/ml for B and $10^{40}$—$10^{41}$ cfu/ml for M, respectively. The fact that the maximum value of bacteria counts in the three treatments appeared in different time was mainly correlated with the environmental conditions of the microcosmic and the growth status of bacteria in the early time. During the whole incubation period of 100 d, the bacteria counts in sample B, M, and I were high and no contabescence period was observed, indicating the adaptability of bacteria to contaminated media as well as their ability to utilize diesel oil as a carbon source for growth was enhanced. Although the bacteria count in sample I was low at the beginning, it reached up to the same level as other treatments after 10 d, which sufficiently exhibited the adaptability and potential of indigenous bacteria to the contaminants in soil.

### 2.2 Logarithm growth of fungi inoculators

The logarithm curves of fungi growth for samples F, M, and I were given in Fig.2a (0—60 h) and Fig.2b (0—100 d). In Fig.2a, it could find that, from 0 to16 h, the counts of fungi colonies in sample I were much less than those in other treatments, and no fungi colonies were detected after 20 h incubation. For sample M, the fungi colonies maintained at the original level from 0 to 12 h, slightly decreased from 12 to 16 h, and gradually increased since 16 h and then ascended fluctuantly; as for F, the fungi count was higher than that in sample M at all time. The lower fungi count in sample M (fungi mixed with bacteria) indicated the competition of nutrients in the mixed cultivation. The difficult growth of fungi in sample I might be attributed to the reasons: (1) there were too low initial cardinal number to maintain the increase of fungal population; (2) the predominant population in the indigenous culture (as shown in Fig. 1a), which should be responsible for the inhibition of indigenous fungi growth, even caused their extinction under the competition environment. In common sense, the growth and the adaptability of fungi were inferior to those of bacteria(Cao, 2002).

Fig.2b shows the growth situation of fungi in sample F and M during the whole period of experiment from 0 to 100 d. The fungi count in sample M increased gradually since 20 d after a short time fluctuation at the beginning, and the fungi count remained almost unchanged at the rest period of incubation. The fungi colony in sample F had a different pattern from that in sample M, for instance, great increase of fungi count was found in sample F at the beginning of the incubation, and then the reducing trend was followed.

Sample F was a mixture of 5 selected fungi, and sample M was a mixture of 5 selected fungi combined with 5 selected bacteria at the ratio of 1:1, so the total amount of fungi in sample F was much greater than that in sample M, which was also corresponded with the initial experimental design as indicated in Fig.2b. The count of fungi increased continuously with time till the day 40 when it reached the peak value, thereafter, the count of fungi began to reduce till the day 60. The obvious decrease of fungi count in F reflected that the easily utilized nutrients in the liquid culture were exhausted. From 60 to 100 d the count of fungi in F maintained stable, and the growth curve was in superposition with that in M basically, which indicated that the counts in the two samples were nearly equal. On the whole, the activity of fungi in F was superior to that in M. The fact that the count of fungi decreased in F with time was obviously correlated with the nutrient application in the culture media. Fungi species was much bigger than bacteria, so the nutrient content needed was higher to support its growth. The nutrient consumption by fungi leaded to the deficiency of nutrient supplication so that its growth was confined and a decreasing inflexion appeared in Fig.2b. For sample M, the count of fungi was inferior to that in F and the growth curve of fungi in M kept a stable with a little ascending state under the designed concentration of inoculation in the experiment, which indicated that proper supplement

of nutrients was well correlated with the growth of fungi.

### 2.3 Biodegradation of mineral oil

The removal ratio of mineral oil in the control samples (sterilized sample) was measured in order to define the contribution of abiotic degradation to the total removal of mineral oil by the microbial treatments. Results indicated that the removal ratio of mineral oil was 25.1% at the day 5 and 26.7% at the day 40 (Table 1). The microbial counting did not detect any bacteria or fungi colony, meaning that the loss of mineral oil in the samples was responsible for the physical loss including volatilization and adsorption. No further results were obtained after 40 d due to the inbreak of microorganisms in the process of sampling.

**Table 1 A biotic removal ratio of mineral oil in sterilized control**

| Time, d | 5 | 10 | 20 | 40 |
|---|---|---|---|---|
| Removal ratio, % | 25.1±0.5 | 26.2±1.1 | 26.6±0.5 | 26.7±0.4 |

The content of mineral oil are in sample B, M and F was measured at different time during the experiment, and the concentration dynamics and the removal ratio of mineral oil are listed in Table 2. Results show that the concentration range of mineral oil in the 4 treatments was 670—752 mg/L at 5 d, higher removal of mineral oil in sample B and M were found compared with the control (Table 2 and Fig.3), indicating the activity of dominant bacteria and fungi spiked into the samples and showing the better adaptability of bacteria over fungi to the contaminants. At the same time, the loss of mineral oil in samples F and I was not significantly different from that of the sterilized control, which suggested that the loss of mineral oil was mainly attributed to the non-biodegradation, e.g. volatilization and sorption. The removal ratio in sample F was nearly the same as the control, which was related to the poorer adaptability of fungi. The removal ratio of mineral oil in the samples followed the order: B>M>F>I.

**Table 2 Degradation of mineral oil in samples B, F and M at different time intervals**

| Time, d | Average concentration of mineral oil, mg/L(removal ratio, %) | | | |
|---|---|---|---|---|
| | B | F | M | I |
| 5 | 670(±6)(33.0)* | 743(±33)(25.7) | 689(±43)(31.1) | 752(±63)(24.8) |
| 10 | 657(±37)(34.3) | 648(±19)(35.2) | 655(±39)(34.5) | 627(±25)(37.3) |
| 20 | 635(±50)(36.5) | 607(±21)(39.3) | 613(±58)(38.7) | 624(±12)(37.6) |
| 30 | 629(±11)(37.1) | 593(±57)(40.7) | 596(±47)(40.4) | 530(±56)(47.0) |
| 40 | 604(±24)(39.6) | 502(±26)(49.8) | 590(±54)(41.0) | 417(±21)(58.3) |
| 55 | 519(±19)(48.1) | 479(±44)(52.1) | 534(±9)(46.6) | 343(±38)(65.7) |
| 75 | 377(±10)(62.3) | 365(±46)(63.5) | 486(±38)(51.4) | 222(±32)(76.2) |
| 100 | 326(±23)(67.4) | 335(±28)(66.5) | 432(±36)(56.8) | 208(±21)(79.2) |

Notes: * Data behind ± in the bracket represent the standard deviation(*SD*), $n = 3$; and the data in the last bracket are removal ratios of mineral oil

Statistically, the removal ratio of mineral oil in samples B and M was significantly higher than that of the sterilized one($P<0.05$).

From 5 d to 10 d of the experiment, the removal ratio of mineral oil in samples F and I increased greatly (9.5 % for sample F, and 12.5 % for sample I, but only 1.3 % and 3.4 % for sample B and M, respectively). The rapid improvement in the degradation of mineral oil for sample F and I might come from the recovery of the suitability of microbiological growth.

High removal ratio of mineral oil in sample F was dominated from 10 d (35.2%) to 40 d (49.8%) of experiment compared with that in sample B (34.3%, 39.6%) and M(34.5%, 41.0%). However, the trend did not maintain to the end. From Table 2 it can be seen that the residue concentration of mineral oil measured at 100 d for samples B and F were nearly the same (326 and 335 mg/L). The highest content (432 mg/L) of mineral oil was found in sample M and the lowest one (208 mg/L) was given from sample I, which indicated the potential ability of mixed indigenous microorganisms for the degradation of mineral oil when undergone a tame process to achieve the adaptability, indicating their advantage over the introduced ones in the process of long-term bioremediation of organic pollutants, the similar role was found by some researchers when they studied bioremediation of petroleum hydrocarbon contaminated soil with composting (Jorgensen *et al.*, 2000; Namkoong, 2002). The inoculated microbes always need a period of time to adapt to the contaminated environment (Cerniglia, 1992). The adaptability phenomenon of microorganisms was similar to that described by Spain and Heitkamp, which played an important role in decomposing contaminants and decided the initial removal rate of contaminants (Spain, 1980; Heitkamp and Cerniglia, 1988).

It was worth the whistle that the removal ratio of mineral oil in M (with 5 bacteria and 5 fungi) at the end of the experiment was significantly inferior to any other treatments. This proved the drawback of the artificial combination of microorganisms at random on the enhancement of contaminant decomposition. Up to

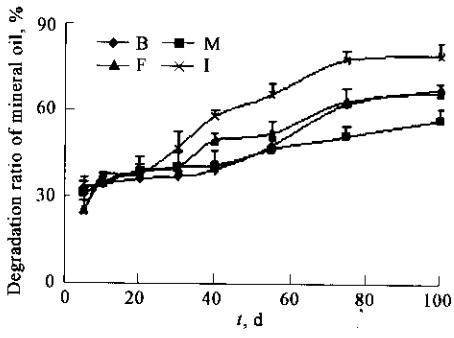


**Fig.3 Degradation ratio of mineral oil in different treatments**

now, the selection process of microbial consortia cooperative in metabolizing contaminants was absolutely at random and the mechanism of function was still not clear. So the amendment of dominant strains in practical engineering should be cautiously and deeply investigated(Song *et al.*, 2004).

### 2.4 Biodegradation of PAHs

Nine PAHs listed in the USEPA were detected in the tested diesel oil. They were 2-, 3-, and 4-ring PAHs, namely, naphthalene (Nap), acenaphthylene (Aceph), acenaphthene (Ace) /fluorene (Fl), phenathrene(Phe), anthracene(An), pyrene(Pyr), and benz[a] anthracene (BaA)/ chrysene (Ch). The sum of PAH

**Table 3 Concentrations(μg/L) and removal ratios(%) of PAHs in different treatments of bioremediation**

| Treatment | Time, d | ΣPAH | TR* | Nap | Aceph | Ace/Fl | Phe | An | Pyr | BaA/Ch | BbF** | BaP** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5447.1 | 0 | 1057.1 | 114.8 | 1498.1 | 2081.2 | 214.2 | 438.7 | 43.1 | Nd | Nd |
| B | 20 | 876.6 | 83.9 | Nd***(100) | 11.1(90.3) | 303.2(79.8) | 424.1(79.6) | 18.9(91.2) | 73.4(83.3) | 26.5(38.6) | 19.6 | Nd |
| | 30 | 764.6 | 86.0 | Nd(100) | 11.6(89.9) | 255.8(82.9) | 373.3(82.1) | 18.8(91.2) | 68.1(84.5) | 22.9(46.8) | 14.0 | Nd |
| | 40 | 728.5 | 86.6 | Nd(100) | 13.5(88.2) | 187.9(87.5) | 406.5(80.5) | 10.1(95.0) | 67.9(84.5) | 23.3(45.9) | 18.7 | Nd |
| | 75 | 302.5 | 94.5 | Nd(100) | 4.9(95.7) | 65.8(95.6) | 155.1(92.5) | 8.3(96.1) | 46.8(89.3) | 14.4(66.5) | 7.2 | Nd |
| | 100 | 176.8 | 96.8 | Nd(100) | 6.1(94.7) | Nd(100) | 108.0(94.8) | 4.1(98.1) | 39.7(90.9) | 13.3(69.2) | 5.5 | Nd |
| F | 20 | 916.3 | 83.2 | 49.6(95.3) | 13.8(88.0) | 305.2(79.6) | 433.5(79.2) | 20.8(90.3) | 68.4(84.4) | 25.1(41.8) | Nd | Nd |
| | 30 | 835.4 | 84.7 | Nd(100) | 12.7(89.0) | 232.8(84.5) | 442.3(78.7) | 22.6(89.5) | 75.5(82.8) | 26.2(39.1) | 14.7 | 8.6 |
| | 40 | 510.5 | 90.6 | Nd(100) | 12.0(89.6) | 103.2(93.1) | 310.6(85.1) | 9.8(95.4) | 56.2(87.2) | 15.6(63.7) | Nd | 2.9 |
| | 75 | 274.2 | 95.0 | Nd(100) | 3.0(96.7) | 52.0(96.5) | 132.3(93.6) | 7.9(96.3) | 40.6(90.7) | 10.8(75.0) | 11.7 | 15.9 |
| | 100 | 149.0 | 97.3 | Nd(100) | 12.1(89.5) | 38.5(97.4) | 34.2(98.4) | 1.5 (99.3) | 23.7(94.6) | 14.3(66.8) | 12.7 | 12.1 |
| M | 20 | 1072.1 | 80.3 | 28.4(97.3) | 17.6(84.6) | 314.9(79.0) | 533.0(74.4) | 17.5(91.9) | 92.2(79.0) | 32.6(24.3) | 36.0 | Nd |
| | 30 | 634.6 | 86.6 | Nd(100) | 11.0(90.4) | 184.3(87.7) | 344.6(83.4) | 14.5(93.2) | 59.6(86.4) | 20.6(52.2) | Nd | Nd |
| | 40 | 732.5 | 88.4 | Nd(100) | 12.1(89.5) | 234.1(84.4) | 373.1(82.1) | 17.5(91.8) | 64.4(85.3) | 21.0(51.2) | Nd | 10.2 |
| | 75 | 341.4 | 93.7 | Nd(100) | 9.5(91.7) | 101.0(93.3) | 289.5(86.1) | 11.5(94.6) | 54.9(87.5) | 13.1(69.7) | 13.9 | 14.0 |
| | 100 | 93.7 | 98.3 | Nd(100) | 3.1(97.3) | 31.9(97.9) | 35.0(98.3) | 1.0(99.5) | 12.1(97.2) | 1.1(97.5) | Nd | 9.6 |
| I | 20 | 1218.5 | 77.6 | 15.2(98.6) | 11.9(89.6) | 431.9(71.2) | 593.4(71.5) | 30.4(85.8) | 101.8(76.8) | 34.1(20.9) | Nd | Nd |
| | 30 | 308.6 | 94.3 | Nd(100) | 3.8(96.7) | 108.3(92.8) | 144.7(93.0) | 4.3(98.0) | 32.6(92.6) | 15.1(65.0) | Nd | Nd |
| | 40 | 250.3 | 95.4 | Nd(100) | 4.8(95.8) | 82.0(94.5) | 113.2(94.6) | 11.6(94.6) | 25.8(94.1) | 12.9(70.1) | Nd | Nd |
| | 75 | 73.3 | 98.7 | Nd(100) | Nd(100) | 33.8(97.7) | 4.5(99.8) | 6.3(97.0) | 12.2(97.2) | 11.0(74.4) | Nd | 5.5 |
| | 100 | 48.8 | 99.1 | Nd(100) | Nd(100) | 33.6(97.8) | 4.0(99.8) | Nd(100) | Nd(100) | 3.3(92.4) | Nd | 7.9 |

Notes: * TR, total PAH removal ratio (%); BbF, benz[b]floranthene; BaP, benz[a]pyrene; Nd, no detection

concentration(ΣPAHs) was 5447.1 μg/L, in which the 2-, and 3-ring PAH were dominant(in the second line of Table 3). The dynamics of concentrations and removal ratios of ΣPAHs and individual PAH (at the day 20, 30, 40, 75, and 100) are listed in Table 3.

Removal ratios of individual PAH in four treatments were correlated with the number of aromatic rings and molecular weight of PAHs(Table 3). For example, at the day 20, Nap had the highest removal ratio ranging from 95.3% to 100% in all treatments, and for Aceph, Ace/Fl, and Phe, the removal ratios were from 79.6% to 90.3% in treatment B. The lowest removal ratio was obtained for 4-ring PAHs, Pyr and BaA/Ch (83.3% and 38.6% ) in treatment B, which was corresponded with the higher molecular weight and the lower bioavailability. For the higher removal ratios of Nap and Aceph might also be explained in the same way. The 2-ring PAH, Nap, was under the detectable levels in all treatments after 30 d incubation.

The introduced microorganisms or the indigenous ones were all effective in removing most PAHs. At the end of the experiment, all 2- and 3-ring PAHs were either below detectable levels or at very

low levels, even the removal ratio of 4-ring Pyr was also exceeding 90% . However, removal ratios of BaA/Ch in the end (at the day 100) were markedly different among treatments, in B and F it was 69.2% and 66.8%, respectively, while in M and I was 97.5% and 92.4%, respectively. This difference indicated that the co-culture of bacteria and fungi had advantages over the purely bacteria or fungi culture in the metabolisms of high-ring PAH, BaA/Ch. In fact, the degradation of many high-ring PAHs ($n \geqslant 4$) was complex and very difficult, and the cooperation of several metabolism colonies could always be more effective(Cerniglia, 1992).

From Fig.4 we could find that indigenous microorganisms played an important role in the long-term degradation of PAHs over introduced microbial inoculators. For example, the total removal ratio of total PAHs was 99.1% for sample I (indigenous microbes), and 96.8%, 97.3%, and 98.3% for sample B, F, and M, respectively, at the end of experiment. However, the introduced microbial inoculators had a predomination in the degradation of PAHs over the indigenous ones in short term(about 20 to 30 d) for that they had been domesticated in advance in the contaminated environment and had achieved a stronger adaptability. In fact, soil as a natural culture medium lived with abundant microorganisms, which had been proved by the microbial counts and the preliminary identification of the indigenous micro- organisms in treatment I. However, it took time for the indigenous microorganisms to adapt to the contaminated environment. Once the indigenous microorganisms obtain the adaptability, they could stimulate a great deal the decomposition of the contaminants by the cooperation of different strains(Song *et al.*, 2004).

Benz [b]floranthene (BbF) and benz[a]pyrene (BaP) that did not exist in the tested diesel oil were detected after 20 d incubation. Some secondary contaminants or by-products were obtained in the degradation process of diesel oil as a chemical mixture. The similar report of secondary contaminants was given by Sun *et al.* in the study of bioremediation of diesel-contaminated soil(Sun *et al.*, 1999; Song and Sun, 1999), which indicated the complexity of biodegradation of organic pollutants.

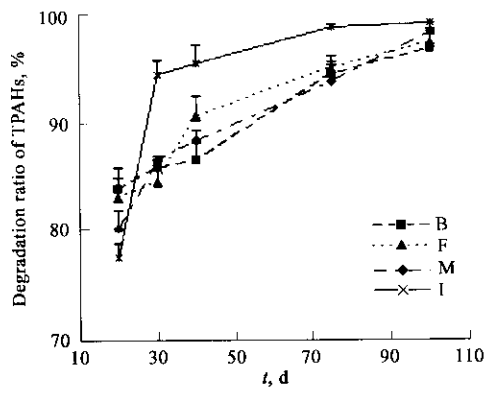


**Fig.4 Removal ratios of total PAHs(TPAHs) in samples**

### 2.5 Relationships between microbial growth and mineral oil and PAH degradation

By comparing the results of the degradation of mineral oil and PAHs in Fig.3 and Fig.4 and the growth status of microorganisms in Fig.1 and Fig.2, we found obvious relativity between the removal ratio of the contaminants and the microbial growth. The rapid degradation of mineral oil and PAHs happened in the treatments B, F and M (introduced microbes) in the initial 40 d, which coincided with the high microbial counts and activity in each treatment. However, the highest removal ratio for both mineral oil and PAHs was given in treatment I, which dominated by significant amount of indigenous bacteria due to its quick adaptability to the contaminated media. Thus the indigenous bacteria were considered as the most important role in biodegradation of the contaminants under the condition of sufficient nutrient supplement and other appropriate conditions. The population of indigenous microorganisms should be given an increasing attention on the cleaning up of pollutants both *in situ* and *on situ* bioremediation(Jansson, 2000).

## 3 Conclusions

Enhanced function of dominant microorganisms was exhibited on the degradation of mineral oil and PAHs. However, in comparison with indigenous soil microorganisms the biodegradation advantage of dominant microorganisms exhibited mainly in short term (about 20 d). For the long-term degradation, the indigenous microorganisms later had an obvious degradation advantage over the introduced ones either bacteria or fungi or their mixtures. Consequently, in practical engineering the addition of dominant microorganisms should be cautious according to the situation of a contaminated site, for instance, the dominant microorganisms were expected to be inoculated when soil was newly contaminated and needed urgent bioremediation, and when the seriously contaminated soil without effectively degradative microorganisms was to be biodegraded.

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## References:

Basheer C, Obbard J P, Lee H K, 2003. Persistent organic pollutants in Singapore's coastal marine environment: Part II. Sediments[J]. Water Air and Soil Pollution, 149: 315—325.

Cao J W, 2002. Microbial Engineering[M]. Beijing: Science Press. 62—78.

Cerniglia C E, 1992. Biodegradation of polycyclic aromatic hydro-

carbons[J]. Biodegradation, 3: 351--368.

Chang S W, Hyman M R, Williamson K J *et al.*, 2002. Cooxidation of naphthalene and other polycyclic aromatic hydrocarbons by the nitrifying bacterium, *Nitrosomonas europaea* [J]. Biodegradation, 13: 373—381.

Facundo J, Marquez R, Lamela M T, 2001. Biodegradation of diesel oil in soil by a microbial consortium [J]. Water, Air, and Soils Pollution, 128: 313--320.

Fredrickson J K, 1993. *In situ* and *on site* bioremediation [J]. Environmental Science and Technology, 27(9): 1711—1716.

Gong P, Siciliano S D, Srivastava S *et al.*, 2002. Assessing pollution-induced microbial community tolerance to heavy metals in soil using ammonia-oxidizing bacteria and Biolog assay [J]. Human and Ecological Risk Assessment, 8(5): 1067—1081.

Heitkamp M A, Cerniglia C E, 1988. Mineralization of polycyclic aromatic hydrocarbon by a bacterium isolated from sediment below an oil field [J]. Applied and Environmental Microbiology, 54: 1612—1614.

Jansson J K, Bjorklof K, Elvang A M *et al.*, 2000. Biomarkers for monitoring efficacy of bioremediation by microbial inoculants[J]. Environmental Pollution, 107: 217—223.

Joel R, Arturo T, Luz E, 2002. Determination of the temperature dependence of water solubilities of polycyclic aromatic hydrocarbons by a generator column-on-line solid-phase extraction-liquid chromatographic method [J]. Chemosphere, 47: 933—945.

Jorgensen K S, Puustinen J, Suortti A M, 2000. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles[J]. Environmental Pollution, 107(2): 245— 254.

Readman J, Mantoura R, Rhead M, 1987. A record of polycyclic aromatic hydrocarbons (PAH) pollution obtained from accrediting sediments of the Tanar Estuary UK: evidence for nonequilibrium behaviour of polycyclic aromatic hydrocarbons [J]. Science of the Total Environment, 66: 73- -94.

Ren L, Huang T L, 2000. Contamination of soil by petroleum [J]. Agro-environmental Protection, 19(6): 360—363.

Song Y F, Gong P, Zhou Q X *et al.*, 2005. Phytotoxicity assessment of phenanthrene, pyrene and their mixtures by a soil-based seedling emergence test [J]. Journal of Environmental Sciences, 17(4): 580 --583.

Song Y F, Song X Y, Zhou Q X *et al.*, 2004. Issues concerned with the bioremediation of contaminated soils[J]. Environmental Science, 25(2): 129— 133.

Song Y F, Sun T H, 1999. Effect of surfactant TW-80 on the biodegradation of PAHs in soil [J]. Chinese Journal of Applied Ecology, 10(2): 230—232.

Song Y F, Xu H X, Ren L P, 2001. Bioremediation of mineral oil and polycyclic aromatic hydrocarbons (PAHs) in soils with two plant species [J]. Chinese Journal of Applied Ecology, 12 (1): 108—112.

Spain J C, 1980. Effects of adaptation on biodegradation ratios in sediment/water cores from estuarine and freshwater environments [J]. Applied Environmental Microbiology, 40: 726—734.

Sun T H, Song Y F, Xu H X, *et al.*, 1999. Plant bioremediation of PAHs and mineral oil contaminated soil [J]. Chinese Journal of Applied Ecology, 10(2): 225—229.

Vaclav S, Tomas C, Manish B, 2003. Use of fungal technology in soil remediation: a case study[J]. Water Air and Soil Pollution, 3: 5--14.

Viguri J, Verde J, Irabien A, 2002. Environmental assessment of polycyclic aromatic hydrocarbons(PAHs) in surface sediments of the Santander Bay, Northern Spain [J]. Chemosphere, 48: 157—165.

Namkoong W, Hwang E Y, Park J S *et al.*, 2002. Bioremediation of diesel-contaminated soil with composting [J]. Environmental Pollution, 119(1): 23— 31.

Wang X, Yu X, Bartha R, 1990. Effect of bioremediation on PAHs residues in soil [J]. Environmental Science and Technology, 24: 1086 -1089.

William C, 1995. Bioremediation [M]. Alexandria: Water Environment Federation. 7 —10.

Won S S, Pardue J H, Jackson W A *et al.*, 2001. Nutrient enhanced bioremediation of mineral oil in tropical salt marshes [J]. Water Air and Soil Pollution, 131: 135—152.

Zhou Q X, Song Y F, 2004. Principles and methods of contaminated soil remediation[M]. Beijing: Science Press. 206—262.

Zhou Q X, Sun F H, Liu R, 2005. Joint chemical flushing of soils contaminated with petroleum hydrocarbons [J]. Environment International, 31(6): 835—839.

Zhu L Z, Cai X F, Wang J, 2005. PAHs in aquatic sediment in Hangzhou, China: Analytical methods, pollution pattern, risk assessment and sources [J]. Journal of Environmental Sciences, 17(5): 748 —755.

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