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Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds: A laboratory experiment

LI Hui[1], ZHANG Ying[1,*], KRAVCHENKO Irina[2], XU Hui[1], ZHANG Cheng-gang[1]

1. Key Laboratory of Terrestrial Ecological Processes, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China. E-mail: huili@iae.ac.cn
2. Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow 117312, Russia

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Abstract

With 110-d incubation experiment in laboratory, the responses of microbial quantity, soil enzymatic activity, and bacterial community structure to different amounts of diesel fuel amendments were studied to reveal whether certain biological and biochemical characteristics could serve as reliable indicators of petroleum hydrocarbon contamination in meadow-brown soil, and use these indicators to evaluate the actual ecological impacts of 50-year petroleum-refining wastewater irrigation on soil function in Shenfu irrigation area. Results showed that amendments of ⩽1000 mg/kg diesel fuel stimulated the growth of aerobic heterotrophic bacteria, and increased the activity of soil dehydrogenase, hydrogenperoxidase, polyphenol oxidase and substrate-induced respiration. Soil bacterial diversity decreased slightly during the first 15 d of incubation and recovered to the control level on day 30. The significant decrease of the colony forming units of soil actinomyces and filamentous fungi can be taken as the sensitive biological indicators of petroleum contamination when soil was amended with ⩾5000 mg/kg diesel fuel. The sharp decrease in urease activity was recommended as the most sensitive biochemical indicator of heavy diesel fuel contamination. The shifts in community structure to a community documented by *Sphingomonadaceae* within α-subgroup of Proteobacteria could be served as a sensitive and precise indicator of diesel fuel contamination. Based on the results described in this paper, the soil function in Shenfu irrigation area was disturbed to some extent.

Key words: petroleum hydrocarbons; enzymatic activity; bacterial diversity; microbial community structure; DGGE

Introduction

Petroleum hydrocarbons are widespread industrial pollutants that are released into the environment through crude oil transporting, storing and accidental leaking, and petroleum refining and its wastewater irrigation. Ecological impacts of petroleum contamination on soil function were shown in the change in the composition and diversity of microbial community and the influence on the activity of microorganism and soil enzyme (Janke *et al*., 1992; Raeid *et al*., 2002). On the other hand, soil microbial activities and diversities are sensitive biological and biochemical indicators for the assessment of soil perturbation (Dick, 1997; Killham and Staddon, 2002).

Although many studies have been made on the impacts of petroleum hydrocarbons, especially polycyclic aromatic hydrocarbons (PAHs), on soil microbial activity and bacterial diversity (Kiss *et al*., 1998; Raeid *et al*., 2002; Wilfred *et al*., 2002; Steven *et al*., 2003), the soil enzymological and microbiological effects of oil contamination are still depend on the soil type, chemical composition and the amounts of oil contamination. The Shenfu irrigation area across Shenyang and Fushun, the two important heavy-industrial cities in Liaoning Province of Northeast China, is the largest area having been irrigated by petroleum-refining wastewater for more than 50 years in China. Our previous work (Li *et al*., 2005) examined the soil enzymatic activity and bacterial diversity at the sites along Shenfu irrigation channel, and found that the tested biological and biochemical characteristic had some correlations with soil petroleum hydrocarbons content.

In this study, a series of simulative laboratory experiments were performed with uncontaminated soil sample adjacent to the down-stream of Shenfu irrigation channel to examine the dynamic changes on soil microflora, soil enzymatic activity and bacterial community structure under different amounts of diesel fuel amendments, aiming to evaluate the impacts of petroleum hydrocarbon contamination on soil function of meadow brown soil, a widespread farmland soil in Liaoning Province, and reveal appropriate biological and biochemical characteristic which can serve as reliable indicators of different amounts of petroleum contamination. Simultaneously, use these indicators to evaluate the actual ecological impacts of 50-year petroleum-refining wastewater irrigation on soil

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function in Shenfu irrigation area.

1 Materials and methods

1.1 Soils samples

Soil samples of top soil (0–20 cm) are collected from Shenyang Ecology Experimental Station (41°32′N, 123°23′E). The soil was uncontaminated meadow brown soil with no pesticides and fertilizers applied for at least 20 years. The main physical and chemical properties are presented in Table 1. The soil was stored at 4°C before use.

1.2 Experimental design

Before incubation, the C:N:P ratio of the soil samples was adjusted to 120:10:1 as recommended by Sims *et al*. (1989). The diesel fuel provided by Fushun Second Refinery was selected as the pollutant. The chemical composition (approximately 30% aliphatic hydrocarbons and 65% aromatic hydrocarbons) of the diesel fuel was similar to that of petroleum wastewater from oil refining plants based on the data provided by Wu *et al*. (1985). The diesel fuel was emulsified by Tween-80 (300 g/kg diesel fuel) and added to the soil to a final concentration of 500, 1000, 5000, 10000, 30000 and 50000 mg/kg dry soil. Then, soil moisture content was adjusted to 25% by adding sterile distilled water. The control soil was only amended with Tween-80 and water. For the quantification of abiotic hydrocarbon losses, parallel amendments poisoned with 2% $HgCl_2$ were performed to inhibit biodegradation. All the amendments were triplicated. Each treatment was conducted with 500 g of treated soil in a 1000-ml glass jar and used for all the subsequent sampling. The glass jars were sealed with Parafilm and incubated in the dark at 25°C for 110 d. Water losses during the incubation were compensated regularly by adding sterile water. To avoid anaerobic condition, the contents of the glass jars were mixed thoroughly every second day. Soil samples of each treatment were taken on 5, 10, 15, 20, 30, 50, 70, 90 and 110 d of incubation and stored at 4°C before the analysis of residual total petroleum hydrocarbon (TPH), soil enzymatic activities, microbial numbers and community structures. Soil samples on day 5, 15, 30, 50 and 110 were selected for bacterial genetic diversity analysis.

1.3 Total petroleum hydrocarbon extraction and quantitation

Total petroleum hydrocarbon (TPH) from 10 g of soil was consecutively extracted with hexane, methylene chloride, and chloroform (20 ml each) as described by Sanjeet *et al*. (2001). All the three extracts were pooled and dried at room temperature by evaporating the solvents under a gentle nitrogen stream in a fume hood. After evaporation, the amount of residual TPH recovered was determined gravimetrically.

1.4 Enumeration of aerobic heterotrophic bacteria, actinomyces and fungi

Colony forming units (CFU) of aerobic heterotrophic bacteria (AHB), actinomycetes and filamentous fungi were determined by a modified plate dilution technique on meat-peptone agar, Gauses starch agar and Martin agar, respectively (Carter, 1993). The incubation temperature for all microorganisms was 28°C. The incubation durations were 2–3 d for AHB, 9–11 d for actinomycetes and 3–5 d for fungi.

1.5 Soil enzyme and SIR assays

Soil dehydrogenase activity was estimated as described by Casida *et al*. (1964) with a minor modification. Five grams soil was mixed with 10 ml 0.25% aqueous triphenyltetrazolium chloride (TTC) and incubated in a sealed tube at 30°C for 6 h. The absorbance at 485 nm of methanol extracts of the triphenylformazan (TPF) produced was then measured, using methanol as a blank. The activity of dehydrogenase was expressed as mg TPF/(g·h) dry soil. Soil hydrogen peroxidase activity was determined by the potassium permanganate titration method and was expressed as ml (0.1 mol/L $KMnO_4$)/g dry soil (Liu *et al*., 1996). Soil polyphenol oxidase activity was measured by colorimetric method based on the purpurogallin formation in the pyrogallicacid-ammended soil samples after 3 h incubation at 30°C, and was expressed as mg Purpurogallin/(g·3h) dry soil (Ma *et al*., 2003). Soil urease activity was determined by colorimetric method according to the NH_3-N formation in the urea-ammended soil samples after 48 h incubation at 37°C, and was expressed as mg NH_3-N/(g·24 h) dry soil (Nannipieri *et al*., 1980; Kandeler and Gerber, 1988). Substrate-induced respiration was determined as described by Alef and Nannipieri (1995) with a little modification. In brief, 15 g (dry weight) of soil was amended with 15 mg of glucose in solution (to adjust soil moisture to 120% WHC), the CO_2 released from the soil after 24 h incubation at 30°C was trapped in 20 ml of 0.1 mol/L NaOH and determined by titration with 0.1 mol/L HCl. Substrate induced respiration (SIR) was expressed as ml CO_2/kg dry soil.

1.6 Molecular methods and diversity calculation

Soil total bacterial DNA was extracted and purified by the procedure described in our previous work (Li *et al*., 2005). The variable region V_3 to V_5 of the 16S rDNA was amplified using eubacterial primers 341f-GC and 907r as previously described (Lyautey *et al*., 2003). 341f-GC had a 40-base GC-clamp linked to the 5' end of the primer to improve the separation of the DNA fragments. PCR

Table 1 Initial physical and chemical properties of soil

Soil type	Organic matter (g/kg)	Total N (g/kg)	Available P (mg/kg)	Available K (mg/kg)	pH (H_2O)	Bulk density (g/cm^3)	Sand (%)	Silt (%)	Clay (%)
Brown soil	16.17	0.76	10.92	88.94	6.4	1.4	57.9	27.7	14.4

was performed in a total volume of 50 μl containing 20 pmol/L of each primer, 200 μmol/L of deoxynucleoside triphosphates, 2.5 U of Taq DNA polymerase and the buffer supplied with the enzyme (Takara Bio. Inc., Shiga, Japan), and 1 μl of template DNA. Thermocycling was performed with a PTC-100™ programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) under the following conditions: 95°C for 1 min, 65°C for 1 min, 72°C for 3 min with a touchdown of 1°C per cycle for the first 20 cycles, followed by 10 cycles at the annealing temperature of 45°C. The last cycle was followed by a final elongation of 72°C for 10 min.

DGGE (denaturing gradient gel electrophoresis) was performed with the Bio-Rad D-Code Universal Detection Mutation system (Bio-Rad, Hercules, CA, USA) as described by Muyzer *et al*. (1993) with a minor modification. The PCR products were loaded onto 1-mm-thick 6% (w/v) polyacrylamide (37.5:1 acrylamide-bisacrylamide) gel with a denaturant gradient from 35% to 70% linear denaturing gradient. One hundred percent denaturant was 7 mol/L urea containing 40% (v/v) of deionized formamide. Gels were run in 1×TAE buffer at 60°C and 80 V for 16 h. The resulting gel was stained in 1×TAE buffer containing SYBR Green I (diluted 1:10000; Sigma), and digital image of the gel was obtained using Gel Doc 2000™ gel documentation systems (Bio-Rad, Hercules, CA, USA). Band patterns were analyzed using image analysis software (Quantity One 4.2.3, Bio-Rad). Lanes in the gel image were defined after background subtraction. One representative lane was selected as standard pattern, and the "maker pattern" on each DGGE gel was aligned to this standard pattern. The banding patterns of the samples were aligned gradually according to the alignment information provided by the closest neighboring "marker patterns". By aligning the bands of all markers and sample tracks from every gel to standard pattern, it became possible to compare patterns from different gels with each other. Profile similarity was calculated by determining Dice's coefficient for the total number of lane patterns and dendrograms were constructed by using the unweighted pair group method with mathematical averages (UPGMA). A densitometric curve was calculated for each gel track. Intensities of the bands as judged by peak areas in the densitometric curves were exported to EXCEL files and the Shannon-Wiener diversity index (H') calculated as:

$$H' = \sum_{i=1}^{s} P_i \times \lg P_i \quad (1)$$

where, P_i is the percentage of the total (s) intensity accounted for by the ith band (Shannon and Wiener, 1949).

Prominent bands in DGGE profiles were excised manually and placed in sterilized vials. Sterilized water 20 μl was added, and the DNA was allowed to passively diffuse into the water at 4°C overnight. Two microliters of the eluate was used as template DNA in a PCR with the primers and conditions described above. Following amplification, the PCR products were analyzed by DGGE to confirm their electrophoretic mobility relative to the fragment from which they were excised. The reamplified bands were purified by using the TaKaRa PCR purification kit (Takara Bio. Inc., Shiga, Japan) and sequenced by using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA) and the ABI PRISM 377XL automated fluorescence sequencer at Takara Biotechnology Co. Ltd. (Dalian, China). Sequences identifications were performed by using the BLAST database (National Center for Biotechnology Information (www.ncbi.nlm.nih.Gov)) and the Sequence Match Facility of the Ribosomal Database Project (www.cme.msu.edu/RDP).

1.7 Statistical analysis

All the data were the means of three triplicates. The data were analyzed by ANOVA and were compared by Fishers least significant difference (LSD) using SPSS for Windows (version 10.0, SPSS Inc., Chicago, Ill.). Linear correlation coefficients were determined between different biological and biochemical parameters. Significance of all statistical analysis was accepted at $p = 0.05$.

2 Results

2.1 Biodegradation of total petroleum hydrocarbon

After incubated for 110 d, the abiotic loss of TPH in the amendments with diesel fuel concentration of 500, 1000, 5000, 10000, 30000, and 50000 mg/kg was 23.5%, 26.3%, 24.4%, 18.7%, 10.2%, and 8.4%, respectively. Biotic degradation rate is presented in Fig.1. As for the samples with 500 and 1000 mg/kg diesel fuel, the biodegradation percentage reached 73.5% and 73.0%, respectively, by the end of the incubation. As for the samples with diesel fuel concentration of 5000 and 10000 mg/kg, the biodegradation percentage of TPH was relatively lower in the first days, but increased gradually with time, and reached 68.3% and 65.3%, respectively after 110 d incubation. In amendments amended with 30000 and 50000 mg/kg diesel fuel, the percentage of biotic oil removal increased with time after a lag period of 5 d, and was much lower by the end of the incubation (49.5% and 36.8%, respectively).

2.2 Total colony forming units of the main microbial groups

The colony forming units of AHB (Fig.2a) in control soil was low and had little variation during the whole

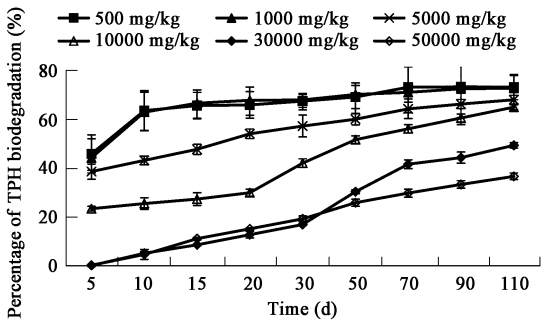

Fig. 1 Biodegradation rate of TPH in soil spiked with different amounts of diesel fuel during the incubation.

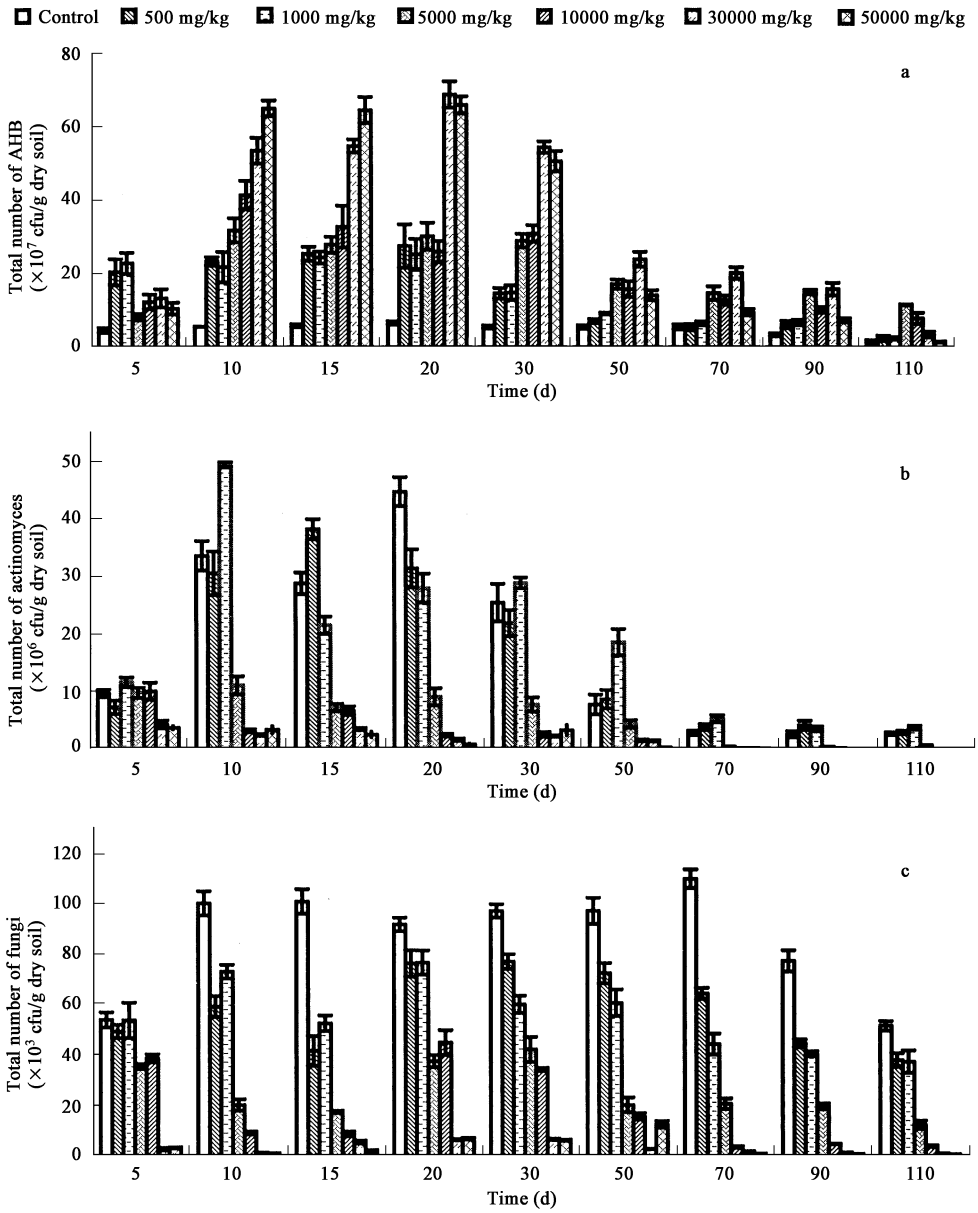

Fig. 2 Total CFU of the main microbial groups. (a) aerobic heterotrophic bacteria (AHB); (b) actinomyces; and (c) filamentous fungi.

period of incubation. The CFU of AHB in all spiked soils was significantly ($P < 0.05$) higher than that of control and correlated positively with soil residual TPH content during the first 30 d of the incubation ($r = 0.936$, $P < 0.05$). As for the samples with diesel fuel concentration of 500 and 1000 mg/kg, the CFU of AHB increased initially, peaked on day 15 (4–5 times higher than that of control), and then decreased to the control level on day 50. In four amendments with ⩾5000 mg/kg diesel fuel, the CFU of AHB increased sharply after a lag period of 5 d, reached the highest level on day 15, and decreased to the control level by the end of the experiment.

During the incubation, the CFU of actinomyces (Fig.2b) in control soil fluctuated between 2×10^6–45×10^6 CFU /g dry soil. It was similar ($P > 0.05$) to the samples with diesel fuel concentration of 500 and 1000 mg/kg. The CFU of actinomyce in four samples with diesel fuel concentration of ⩾5000 mg/kg was significantly ($P < 0.05$) lower than that of control, and there was no significant ($P > 0.05$) difference between these four samples.

The CFU of filamentous fungi (Fig.2c) varied between 5.1×10^4–11×10^4 CFU/ g dry soil in the control during the 110 d incubation. The CFU of fungi in all treated soils were significantly ($P < 0.05$) lower than that of control, and were correlated negatively with the residual TPH during the first 20 d of the incubation ($r = -0.873$, $P < 0.05$).

2.3 Soil enzymatic activities

In this study, dehydrogenase activity (Fig.3a) was changeless in the clean control soil throughout the study. Dehydrogenase activity in soil samples with diesel fuel concentration of 500 and 1000 mg/kg was significantly ($P < 0.05$) higher than that of control during the first 15 d of the incubation, and correlated positively with the CFU of AHB ($r = 0.771$, $P < 0.05$). However, the dehydrogenase activity was significantly ($P < 0.05$) inhibited in soils

treated with ⩾ 5000 mg/kg diesel fuel, and correlated negatively with the residual TPH concentration during day 50 to day 90 (r = –0.963, P > 0.05).

Hydrogen peroxidase activity (Fig.3b) in soil samples with diesel fuel concentration of ⩽ 5000 mg/kg showed a positive correlation with the residual TPH during day 20 to day 50 (r = 0.999, P < 0.05), and a positive correlation with the CFU of AHB (r = 0.647, P < 0.05). While, when

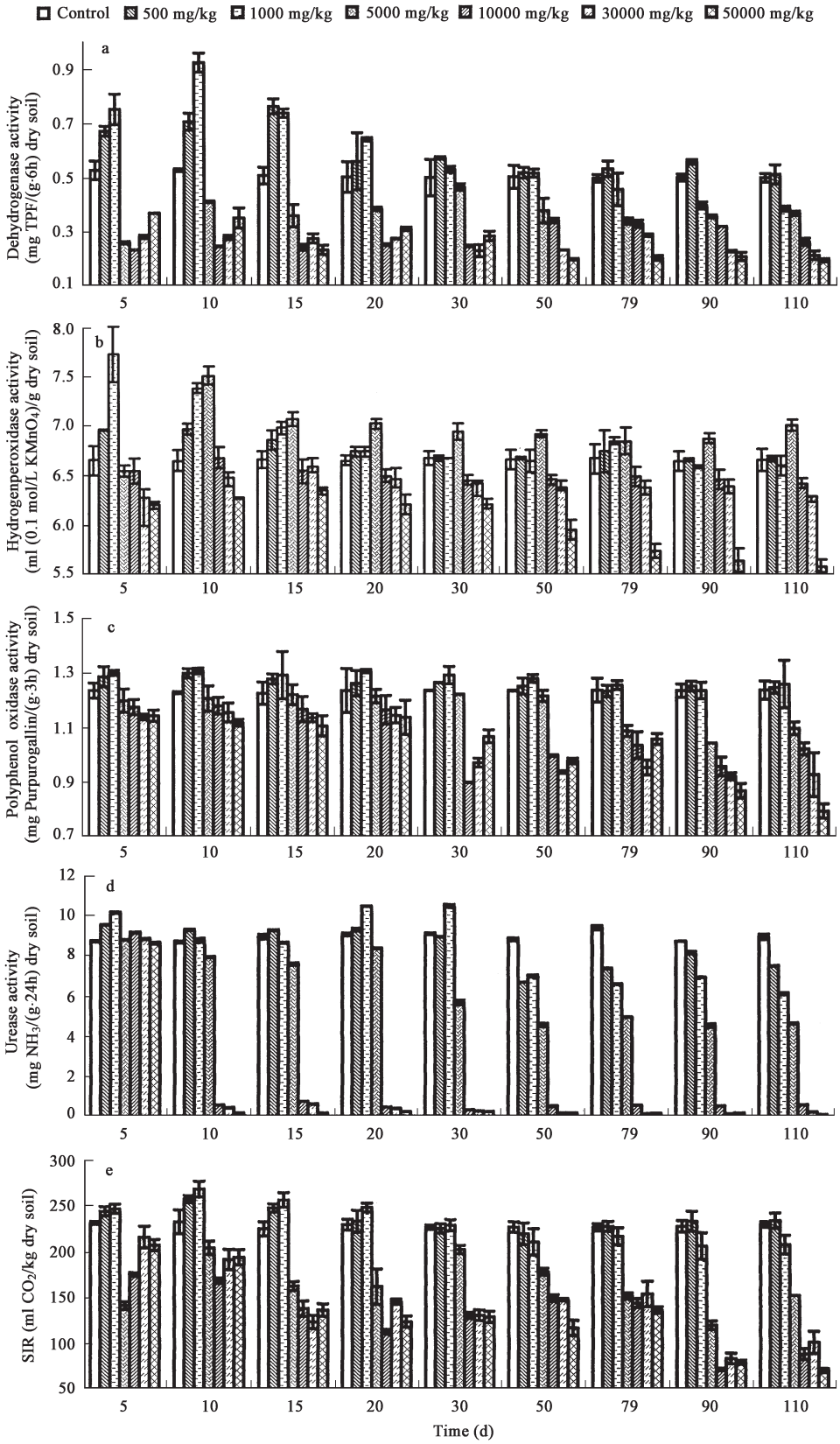

Fig. 3 Dynamic changes in soil enzymatic activities. (a) dehydrogenase; (b) hydrogenperoxidase; (c) polyphenol oxidase; (d) urease; (e) substrate-induced respiration (SIR).

the soils was amended with ⩾ 10000 mg/kg diesel fuel, the hydrogen peroxidase activity was significantly ($P < 0.05$) lower than the control level, and correlated negatively with the residual TPH at the beginning of the incubation ($r = –0.977$ on day 5, and $r = –1.000$ on day 10, $P < 0.05$).

Polyphenol oxidase activity (Fig.3c) in soil samples with diesel fuel concentration of 500 and 1000 mg/kg was significantly ($P < 0.05$) higher than that of control. As for the samples with diesel fuel concentration of ⩾ 5000 mg/kg, polyphenol oxidase activity was significant ($P < 0.05$) lower than the control level, and correlated negatively with the residual TPH by the end of the incubation ($r = –0.974$ on day 110, $P < 0.05$).

Urease activity (Fig.3d) in soil samples with diesel fuel concentration of 500 and 1000 mg/kg was similar to the control soil ($P > 0.05$). When the soil was spiked with 5000 mg/kg diesel fuel, urease activity decreased gradually after 10 d of incubation. The amendments of ⩾ 10000 mg/kg diesel fuel induced a sharp decrease in soil urease activity from origin level to a very low value after day 5, and then remained at this low level for the rest period of the incubation.

Soil substrate-induced respiration (Fig.3e) in soil samples with diesel fuel concentration of 500 and 1000 mg/kg was significantly ($P < 0.05$) higher than that of control during the first 30 d of the incubation. As for the samples with diesel fuel concentration of ⩾ 5000 mg/kg, SIR was significantly ($P < 0.05$) lower than that of control, but there were no significant ($P > 0.05$) differences between these four amendments.

2.4 PCR-DGGE analysis of bacterial community structure

DGGE profiles of soil samples on day 5, 15, 30, 50, and 110 are shown in Fig.4 and the cluster analysis of DGGE patterns in different sampling times were reported in Fig.6. After the amendments of diesel fuel, the DGGE band patterns revealed immediately dramatic changes in the structure of the microbial community on day 5. During this period, DGGE profiles of all amendments were less complex than that of control and established a new cluster far away from the control due to the selective pressure of oil contamination. There was a decrease in the number of bands with the increase of oil concentration. The number of DGGE bands decreased from 40 in the control soil to 25–29 in soils spiked with ⩽5000 mg/kg diesel fuel. Although small differences among the banding patterns of these three amendments were visible, they all clustered closely. As for the samples with diesel fuel concentration of ⩾10000 mg/kg, the number of DGGE bands decreased to 21–23, and these three amendments were grouped together. On day 15, the number of DGGE bands were further decreased to 23–26 in the samples with diesel fuel concentration of ⩽5000 mg/kg. Cluster analysis on day 15 revealed that there were three separate groups, corresponding to the clean control soil, two slight contaminations (500 and 1000 mg/kg), and four heavy contaminations (5000–50000 mg/kg). On day 30, the number of DGGE bands restored to about 33 in two low-level amendments (500 and 1000 mg/kg), indicating a rapid recovery of bacterial community structure. Accordingly, the most dramatic change in cluster analysis of day 30, compared with that of day 15, was that two slightly contaminated soils (500 and 1000 mg/kg) were grouped together with the clean control soil, separating them from four heavy contaminations (5000–50000 mg/kg). Relatively stable DGGE band patterns were observed between day 30 and 50, and no obvious changes in the composition of the predominant bands occurred during this period. By the end of the incubation, there was also a significant shift in microbial community structure. The number of bands had recovered to 36–37 in the samples with diesel fuel concentration of 500 and 1000 mg/kg, and to 23–28 in the other four amendments. Cluster analysis revealed that the DGGE profile of middle contamination (5000 mg/kg) was more similar to two low-level amendments, and grouped away from the other three heavily contaminated amendments (⩾10000 mg/kg), indicating a recovery of community structure.

Genetic diversity was calculated on the basis of signal quantification of each band performed by computerized image analysis. The genetic diversity (Fig.6) in the control soil was found to be more or less constant during the whole incubation. Bacterial genetic diversity in all amendments were significant ($P < 0.05$) lower than that of control,

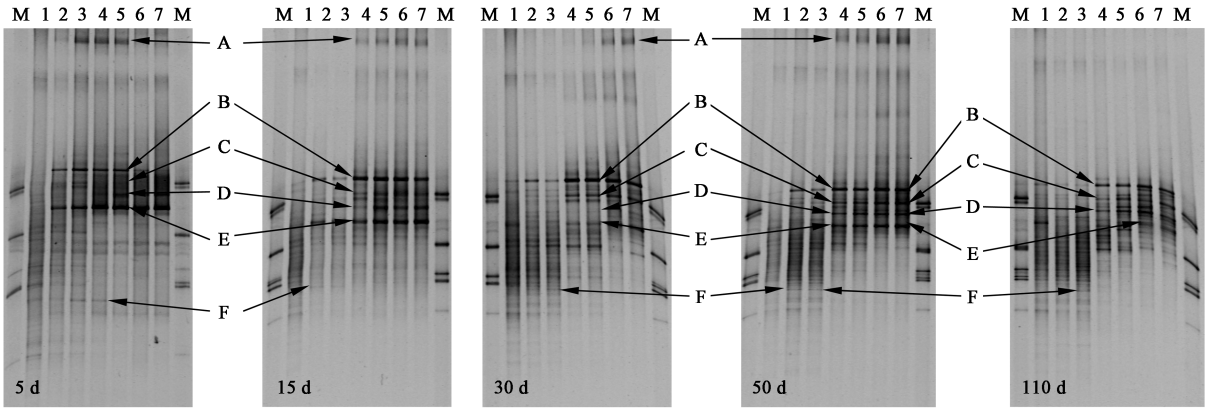

Fig. 4 DGGE fingerprints of 16S rDNA fragments in soil samples treated with different amounts of TPH. The labeled bands were excised from the gel, reamplified and subjected to sequence analysis. The numbers above the lanes refer to different amendments: (M) markers; (1) control soil; (2) 500 mg/kg; (3) 1000 mg/kg; (4) 5000 mg/kg; (5) 10000 mg/kg; (6) 30000 mg/kg; (7) 50000 mg/kg.

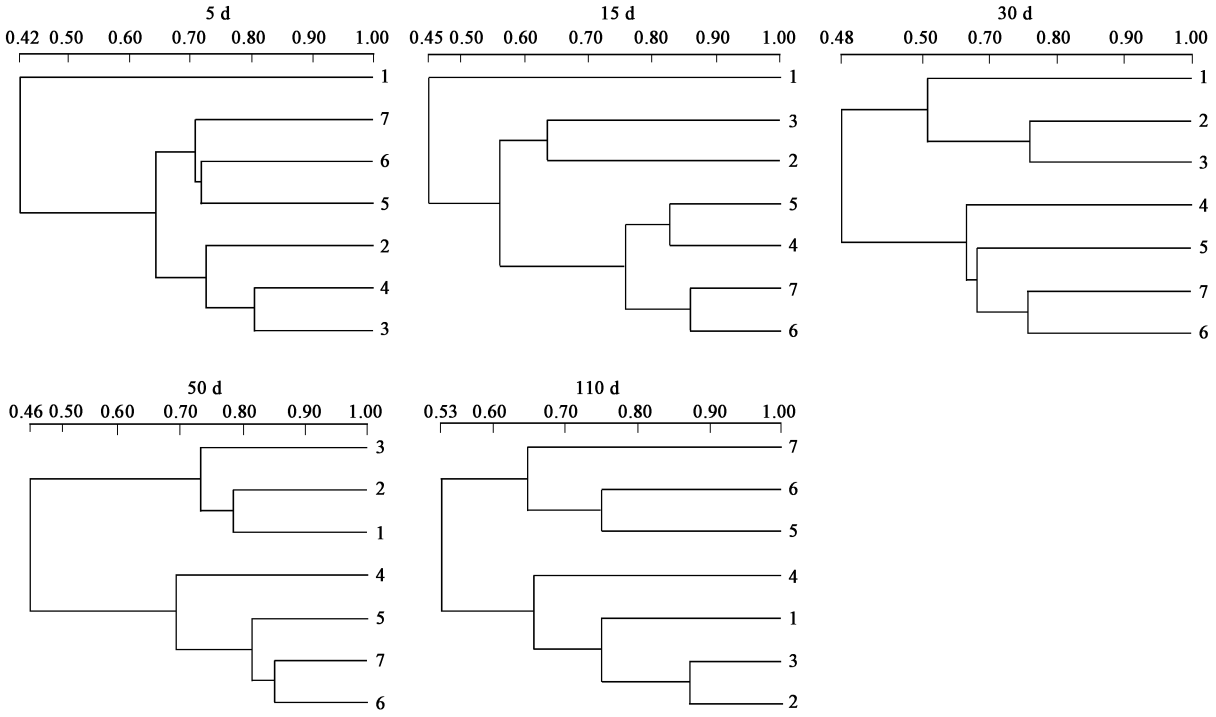

Fig. 5 A hierarchical cluster analysis of the DGGE profiles at different inoculation period. The numbers in the cluster trees refer to different amendments as described in the legend for Fig.4.

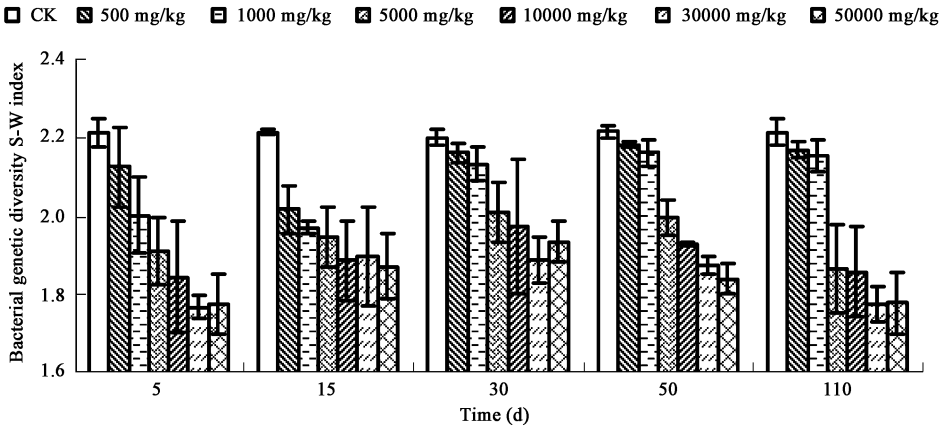

Fig. 6 Dynamic changes of bacterial genetic diversities based on DGGE profiles.

and showed a negative correlation with the residual TPH during the first 15 d (r = –0.749, P < 0.1). The H' value of bacterial community spiked with 500 mg/kg diesel fuel decreased from original level (H'=2.21) to 2.12 on day 5, and finally to 2.02 on day 15, while the H' value of soil samples with diesel fuel concentration of 1000 mg/kg decreased to 2.00 on day 5, and 1.97 on day 15. Subsequently, a rapid recovery of the bacterial community diversity to preoiling level occurred on day 30. The parameter H' decreased from 2.21 in the control to 1.92 in 5000 mg/kg amendment on day 110. Soils amended with ⩾10000 mg/kg diesel fuel exerted the most significant effects on the bacterial community structure diversity. The H' value decreased sharply to 1.77–1.84 on day 5 and remained in the range of 1.76–1.93 at the remaining time.

It was found that several dominant bands were present in DGGE profiles after the amendments of diesel fuel. Bands selected for analysis are shown in Fig.6. Prominent bands in DGGE gels were excised and sequenced, and then we conducted a BLAST search of the GenBank database to determine their phylogenentic type (Table 2). Band A showed 98% sequence similarity to a taxonomically unidentified bacterium clone D124, which was screened out from heavy metal contaminated soil (Nemergut *et al*., 2004). Band B exhibited 99% homology with *Sphingomonas* sp. HTCC 399, which was isolated from groundwater contaminated by trichloroethene (Connon *et al*., 2005). The 16S rDNA sequences of band C were 98% similar to the 16S rDNA sequences of PAH-degrading bacteria *Novosphingobium aromaticivorans* SMCC B0695 which was obtained previously from contaminated sediments in Southeast Coastal Plain, USA (Fredrickson *et al*., 1995). Band D was identical to strain F0917, an unidentified member of α-subclass of Proteobacteria. Band E had 100% identity

Table 2 Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rDNA fragments

Band no.	Bacterium with related bacterial sequence	Similarity (%)	Accession No.
A	Uncultured bacterium clone D124	98	AY274132
B	*Sphingomonas* sp. HTCC399	99	AY429693
C	*Novosphingobium aromaticivorans* SMCC B0695	98	U20755
D	Alpha proteobacterium F0917	99	AF235994
E	*Sphingomonas* sp. AC83	100	AJ717392
F	Uncultured *Nitrospirae* bacterium clone AKYG1809	99	AY921744

with *Sphingomonas* sp. AC83 which was isolated from a nonsaline alkaline environment (Tiago *et al*., 2004), and 100% identity with *Sphingomonas* sp. Ant17 (accession no. AF184222), an aromatic-hydrocarbon-degrading bacterium isolated from soil near Scott Base, Antarctica. The sensitive population for petroleum contamination, band F, was 99% similar to uncultured *Nitrospirae* bacterium clone AKYG1809.

3 Discussion

Fig.1 shows that in four treatments of ⩽10000 mg/kg diesel fuel, >40% of the added TPH was degraded within the first 5 d of incubation, suggesting that the degradation of petroleum hydrocarbons proceeded rapidly when the amount of petroleum hydrocarbons initially added was low. The initial chemical composition of the diesel fuel is about 30% aliphatic hydrocarbons and 65% aromatic hydrocarbons, while total degradation rate (include a biotic loss and biodegradation) of all amendments varied between 45.2% and 99.3%. It indicated that the aromatic hydrocarbons were degraded to some degree.

In the analysis of the total CFU of the main microbial groups, the CFU of AHB in all spiked soils was correlated positively with soil residual TPH content at the first 30 d of the incubation, this is in accordance with the conclusion made by Kaplan and Kitts (2004). While, in the samples with diesel fuel concentration of ⩾5000 mg/kg, the CFU of AHB increased sharply after a lag period of 5 d, indicating a potential toxicity of high petroleum concentration. However, during the later period, the effect of hydrocarbons as C and energy source was greater than their toxic effect on AHB. The CFU of actinomyces in control soil was similar to that of the samples with diesel fuel concentration of 500 and 1000 mg/kg, indicating that ⩽1000 mg/kg diesel fuel was not toxic to actinomyces in the soil we studied. However, the significant decrease of the CFU of soil actinomyces and filamentous fungi can be taken as the sensitive biological indicators of petroleum contamination when diesel fuel concentration was up to 5000 mg/kg or more.

Dehydrogenase is an intracellular enzyme involved in the energy transfer in respiration chain (García *et al*., 1997), and its activity typically occurs in all intact viable microbial cells. Thereby, its measurement is usually related to the presence of viable microorganisms and could be considered as an index of their oxidative capability (Trevors, 1984; García *et al*., 1994). The significant increase in dehydrogenase activity in soil samples with diesel fuel concentration of ⩽1000 mg/kg indicated that in brown soil we studied, low-level diesel fuel contamination was not toxic, but stimulated dehydrogenase activity, which was consistent with the findings described by Frankenberger *et al*. (1989). However, the dehydrogenase activity was significantly inhibited in soils treated with diesel fuel concentration of ⩾ 5000 mg/kg, indicating the toxicity of high contamination concentration to microbial activities. Soil polyphenol oxidase plays an important role in the oxidation of aromatic organic compounds and the formation of humic substances (Ma *et al*., 2003). Soil respiration, one of the most common measurements of soil microbial activity, was another important soil function (Prosser, 1997). The immediate respiration of a microbial community following a glucose addition is quantified in a manner avoiding a significant contribution of cell multiplication (Anderson and Domsch, 1978). Therefore, SIR can be taken as the measurement of the biomass of active microbes. Dynamic changes in polyphenol oxidase activities and SIR in soils spiked with different amounts of diesel fuel during the whole study were similar to the case of dehydrogenase. In addition, the polyphenol oxidase activity was correlated positively with the CFU of AHB ($r = 0.779$, $P < 0.05$), which in accordance with the findings described by Zhou *et al*. (1990). Also, SIR was correlated positively with the CFU of AHB ($r = 0.729$, $P < 0.05$) and the dehydrogenase activities ($r = 0.924$, $P < 0.05$), which was in accordance with the facts described by Tate (2000).

Hydrogen peroxidase is another key oxidoreductase associated with aerobic microbial activities (Rodríguez-Kábana and Turelove, 1982), which can decompose hydrogen peroxide into molecular oxygen and water, and thus, alleviate the toxicity of hydrogen peroxide to plants and soil organisms (Daniel *et al*., 1992). Hydrogen peroxidase activity in meadow brown soil was influenced to a lesser extent by the level of oil contamination than was dehydrogenase activity. When the soil was spiked with 5000 mg/kg diesel fuel, the dehydrogenase activity was inhibited, while the hydrogen peroxidase activity was stimulated at this level, suggesting that, to some extent, the addition of a small amount of petroleum hydrocarbons could stimulate hydrogen peroxidase activity in meadow brown soil. Similar phenomena have been reported in heating oil contaminated soil (Janke *et al*., 1992) and anthracene-contaminated soil (Ma *et al*., 2003). Urease plays a key role in transforming urea to ammonium and carbon dioxide (Dick, 1992; Gianfreda *et al*., 1994). It is susceptible for many disturbances (Gianfreda *et al*., 1994; Garciá-Gil *et al*., 2000), including petroleum hydrocarbons contamination (Li *et al*., 2005). The sharp decrease in urease activity in soil samples with diesel

fuel concentration of ⩾10000 mg/kg indicated that urease could be taken as one of the most sensitive biochemical indicator for petroleum contamination. This observation might have important implications in terms of the ability of microorganisms to use ammonium as a nitrogen source for biodegradation. This might explain the reason for the lower biodegradability of diesel in high concentrations.

As extensively reviewed by Kiss *et al*. (1998), even moderate levels of hydrocarbon contamination may cause a significant decline of several soil enzyme activities, showing each enzyme a different sensitivity to the presence of pollutants. In our study, the significant decrease in soil enzymatic activity and SIR at the concentration of ⩾10000 mg/kg diesel fuel, especially the sharp decrease in soil urease activity, could be taken as the sensitive biochemical indicators when the meadow brown soil was seriously polluted by petroleum hydrocarbons. Although the interpretation of soil enzyme activities is complex because both extracellular and intracellular enzyme activities contribute to the overall soil enzyme activity, some hypotheses might be advanced. In soil, non-polar organic compounds, such as hydrocarbons, may likely exert different effects on microbiological properties. Hydrocarbons may be toxic to soil microorganisms which may reflect in a consistent reduced enzymatic activity; and/or they my cover both organic-mineral and cell surfaces, thus hindering the interaction between enzyme active sites and soluble substrates with adverse effect on enzyme activity expression.

In this study, the Shannon-Wiener index of diversity was used in combination with the correspondence analysis of the DGGE banding patterns based on the similarity coefficient to monitor a range of community responses after the amendment of diesel fuel. The changes in community structure within the laboratory microcosms during the experiment were documented, and the major bands in DGGE profiles were identified. The results indicated that low-level contamination only had a slight and short time effect on community diversity. However, the significant decrease in bacterial diversity at the level of ⩾10000 mg/kg diesel fuel suggested that heavy petroleum contamination resulted in the dramatic decrease in community diversity in meadow brown soil. The bacterial community structures revealed by PCR-DGGE indicated that α-subgroup of Proteobacteria, especially the species in *sphingomonadaceae*, plays an important role in petroleum degradation in meadow brown soil. Species in *sphingomonadaceae* have also been isolated from other petroleum compounds contaminated sediments or aqueous environment and been identified as high-molecular-mass PAH-degraders (Kazunga and Aitken, 2000; Chung and King, 2001; Leys *et al*., 2004; Sohn *et al*., 2004). The appearance of such PAH-degraders verified that the aromatic fractions of the diesel fuel were degraded in a great measure. Results described above suggested that petroleum hydrocarbons pollution-induced community tolerance could be served as a sensitive and precise indicator of the effect of diesel fuel on soil microbial community. The shifts in community structure to a community documented by α-subgroup of Proteobacteria may be an indicator of how bacteria respond to oil amendments in the soil we studied.

In the analysis, the number of DGGE bands was taken as an indicator of species in each sample and the relative surface intensity of each band was used to estimate species abundance. However, some researchers (Eichner *et al*., 1999) noted that the number and intensities of bands do not equal the number and abundance of genotypes within the microbial community due to features of 16S rDNA-based phylogeny and bias inherent to PCR amplification from complex template mixtures. Whether the bands represent the most abundant species, the most easily extractable species, the most active species, or a combination of all these groups is uncertain. Nevertheless, DGGE might be a sensitive method for detecting differences in community diversity.

Soil TPH concentration in Shenfu irrigation area varied from 277 to 5213 mg/kg dry soil, and declined along the gradient of the irrigation channel from up- to downstream. Our previous investigation on this site indicated that the number of AHB, the activities of dehydrogenases, hydrogenperoxidases, polyphenol oxidases and SIR were significantly positively related with soil TPH concentration, while the urease activity showed a negative correlation with TPH concentration (Li *et al*., 2005). Another research work showed that bacterial genetic diversity was negatively correlated with TPH concentration ($r = -0.715$, $P < 0.05$) (Li *et al*., 2006). Most of the results above were in agreement with the findings obtained in this laboratory experiment. It was suggested that long-term petroleum wastewater irrigation did not caused a significant decrease in microbial activity and soil function in this area, which might due to the adaptation of microorganisms to this environmental stress and the biodegradation of petroleum pollutants by indigenous microorganisms. However, the significant decrease in bacterial genetic diversity and urease activity in Shenfu irrigation area indicated that the soil function was disturbed to some extent at current pollution level.

4 Conclusions

Results described in this paper showed that the petroleum hydrocarbons contamination had a variety of effects on microbial activity and community structure in meadow brown soil, and the biological and biochemical parameters tested had a close relationship with contamination concentration.

Amendments of ⩽1000 mg/kg diesel fuel (light contamination) had little or positive effects on microbial activities and community structures. The added oil can be degraded within approximately 30 d, and most of the effects of oil addition on microbial ecosystem occurred in this period. The added oil stimulated the growth of AHB, but result in a slight decrease in the CFU of fungi. There was no change in soil actinomyces number and urease activity. Dehydrogenase, hydrogenperoxidase, polyphenol oxidase activities and SIR also increased after oil addition, and significantly correlated with the CFU of AHB. Soil bacterial diversity slightly decreased during the first 15 d, and recovered to

control level on day 30. The shift of community structure to a community documented by *sphingomonadaceae* within α-subgroup of Proteobacteria followed by a rapid recovery to the control on day 50.

When the soil was spiked with 5000 mg/kg diesel fuel (middle contamination), the number of AHB increased significantly, and that of actinomyces and fungi decreased significantly. Soil dehydrogenase, polyphenol oxidase, urease activities and SIR were inhibited to some extent, while the hydrogen peroxidase activity was stimulated at this level. The bacterial diversity decreased significantly during the first 15 d and then gradually increased, but full recovery of the genetic diversity was not reached within the 110-d experiment. The bacterial community structure revealed by PCR-DGGE demonstrated a marked rise in the proportion of species belonging to the α-subgroup of Proteobacteria.

Amendments of ⩾10000 mg/kg diesel fuel (heavy contamination) could significantly affect the soil microbial activity and community structure. The number of AHB increased more significantly compared with the middle contamination, and that of fungi decreased more dramatically. Activities of all studied soil enzymes and SIR were strongly inhibited, especially soil urease. Soil bacterial diversity intensely decreased during the first 15 d and remained at a low level until the incubation was ended. Similar to middle contamination, the bacterial communities were dominated by α-subgroup of Proteobacteria.

Based on the parameters described above, the soil function in Shenfu irrigation area was disturbed to some extent, but long-term petroleum-refining wastewater irrigation did not cause a very serious perturbation at current pollution level.

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