Chapter 7

Characterizing Microbial Activity and Diversity of Hydrocarbon-Contaminated Sites

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1. Introduction

Hydrocarbons, one of the major petroleum constituents, mainly include saturated alkanes and cycloalkanes, unsaturated alkenes, alkynes and aromatic hydrocarbons. The usual composition of light crude oil is 78% saturates, 18% aromatics, 4% resins and <2% asphaltenes (Olah and Molnar 2003). The hydrocarbon fractions in the order of decreasing volatility are C_6- C_{10}; C_{10} – C_{16}; C_{16} - C_{34} and C_{34} - C_{50}, in which C_x is referred to the number of carbon molecules in the alkane backbone.

Polycyclic aromatic hydrocarbons (PAH) are compounds consisted of two or more fused benzene rings in linear, angular, of cluster arrangements (Johnson et al, 1985), and they are mainly associated with industrial processes, though they also occur as natural constituents of unaltered fossil fuels. By definition, only C and H atoms are shown in PAH structures, but nitrogen, sulfur and oxygen atoms may readily substitute in the benzene ring to form herterocyclic aromatic compounds, commonly grouped with PAHs. In general, petroleum, coal, by-products of industrial processing, the products of incomplete combustion of organic compounds are considered to be the major sources of PAHs. Anthropogenic sources, particularly the combusting of fossil fuels, are the most significant sources of PAHs entering to the environment (Lim, et al. 1999). Sites where refining petroleum have occurred are frequently contaminated with PAHs and other aromatic hydrocarbons (Smith, 1990). Spills and leak from the petroleum storage tanks also cause significant PAH contamination.

Exposure to PAHs constitutes a significant health risk for people living in the industrialized areas of the world. PAHs are suspected human carcinogens, and they have been linked to genotoxic, reproductive, and mutagenic effects in humans. Most PAHs have long environmental persistence, their hydrophobic nature and corresponding limited water solubility lead to be recalcitrant to biodegradation and remain as environmental

contaminants for extremely long time periods. The chemical properties are related to molecule size and the pattern of ring linkage. Persistence increases as PAH molecular weight increases. Low-molecular-weight PAHs (those containing less than four benzene rings) are acutely toxic, with some having effects on the reproduction and mortality rates in aquatic animals. Most high-molecular –weight PAHs (containing four or more benzene rings) are mutagenic and carcinogenic (Boonchan et al., 2000). Therefore, the US Environmental protection Agency (USEPA) has listed 16 PAHs as priority pollutants to be monitored in industrial waste effluents.

Sites contaminated with hydrocarbons are difficult to remediate. The type of petroleum product discharged will influence the terrestrial migration; in general, the more viscous the product, the slower it will migrate. Smaller more volatile fractions will move quickly, or evaporate, while larger fractions will take more time to flow through soil. The spill profile, or spatial area directly contacted with discharged hydrocarbons, depends on the time, amount, extent, and type of petroleum product, in addition, the soil particle size of the spill site also is the important factor. Hydrocarbons move through the soil and larger soil particles generally allow greater migration. The state of the ground on which the spill is discharged affects the vertical and horizontal spill profile.

When the hydrocarbons flow down through the soil, some of the organic carbon will be removed from the system by abiotic uptake. Some of the hydrocarbons such as PAHs, can be adsorbed to humic substances. The sorption characteristics of hydrocarbons to soil can further depend on the soil matrix; in a marsh environment with multiple soil types, a greater reduction of hydrocarbons was observed in sandy soils than in mineral soils (Lin et al. 1999).

The loss of hydrocarbons by abiotic processes in a system is finite, reaching a saturation point that, unless conditions change, will prevent further removal (Ping et al. 2006). This is apparent in aged spills where the residual hydrocarbons, especially for PAHs, can remain in the soil for long time without any apparent removal by abiotic processes. Volitization of hydrocarbons can remove hydrocarbons, mostly in the C6- C10 fraction, from hydrocarbon contaminated sites, but the cold temperatures will greatly reduce this volitization.

Bioremediation that uses microorganisms to remove toxic substances from the environment in an attempt to return the environment to pre-contaminated conditions has been developing for some 40 years. Bioremediation of PAHs contaminated soil and sediment is believed to be a promising cleanup process in comparison to other remediation processes such as chemical or physical ones. Therefore, many investigations have focused attention on bioremediation process that showed higher efficiency to clean up the contaminated sites. The approaches for the bioremediation of PAHs contaminated sites have included the three major processes: monitored natural attenuation; bioaugmentation and biostimulation (Stallwood et al. 2005). Environmental conditions determine which bioremediation approach, or combination, is most appropriate. Bioaugmentation, the addition of specialized microorganisms to enhance the biodegradation efficiency of contaminants in soil, has proved to be a feasible and economic method compared with other treatment techniques, such as chemical or physical ones, and has been received increasing attention in

recent years (Vogel 1996). Monitored natural attenuation is considered the simplest bioremediation approach and comprises checking the intrinsic degradation of contaminants in an environment. Contaminated sites that have remained unchanged for long periods of time represent situations not suited for monitored natural attenuation because there is no evidence that once monitoring of the site starts, the contamination level would decrease without anthropogenic intervention. Biostimulation addresses the deficiencies of the environment, providing the optimal conditions for microbial growth, activity and thus biodegradation. Common biostimulation processes include supplementation with necessary or additional nutrients, water or air. More site-specific treatments may include chelating agents to detoxify metals or surfactants to increase hydrocarbon bioavailability. The application of biostimulants to a contaminated site can be an important factor that should account for the environmental conditions and other biodegradation limitations of the system.

Biodegradation efficiency and extent of PAHs in contaminated sites depend upon a multitude of factors, including bioavailability of the PAH to microorganisms, the characteristics of the environment (pH, temperature, concentration and availability of suitable electron acceptors), the extent of sorption of the PAH, identity of the sorbent, mass transfer rates, and microbial factors (buildup of toxic intermediates, kinetics, or the presence or absence of organisms capable of degrading a given compound). The bioavailability of PAHs is of paramount importance, which is a compound-specific phenomenon, as sorption, desorption. Physical-chemical factors affecting bioavailability include sorption, nonaqueous phase identity, the length of time the PAH has been in contact with the soil, and microbial factors. These factors are interrelated, complex, and compound-specific. The sum of these processes determine the bioavailability of a PAH to an organism. The type of soil to which PAHs are bound can have a profound influence upon the bioavailability of PAHs.

Four steps can be put forward during the biodegradation of soil-sorbed PAH. Firstly, the PAH must be desorbed from the soil into particle-associated pore water. Secondly, the dissolved PAH must travel through the pore water to the bulk aqueous fluid. Thirdly, bulk fluid movement must transport the PAH to the microbial cell. Lastly, the microbial cell must transport the PAH molecule across its cell membranes to the cell interior where metabolism occurs. Each of these steps is subject to equilibrium and kinetic effects, and each is capable of being the rate-limiting step for a particular PAHs contaminated sites.

Mass transfer between the site where a PAH is sorbed and the microbial cell is a prerequisite to biodegradation. Both mass transfer factors and biological factors are relevant. Mass transfer concerns include desorption of the PAH molecule and arrival of the PAH and the organism at the same location, either by mixing, PAH diffusion, or cell locomotion. The cell must possess the necessary enzymes to degrade the PAH, and those enzymes must be expressed at a sufficient level of activity.

The maximum potential rate of PAH biodegradation is determined by the rate of mass transfer of the PAH to the microbial cell and the intrinsic metabolic activity of the cell. The bioavailability of a compound reflects the balance between these two processes. Inherent

properties such as the octanol:water partitioning coefficient (Kow) and molar volume affect the behavior of a PAH in processes such as sorption/desorption and diffusion. The hydrophobic nature of PAHs results in their partitioning onto soil matrix and their strong adsorption onto the soil organic matter (Manilal and Alexander 1991; Weissenfiels *et al*. 1992). Because of their hydrophobicity and low solubility, PAHs in a soil or sediment matrix will tend to associate with the organic matter coating of the particle matrix rather than the hydrated mineral surface. The sorption of organic contaminants by natural organic matter often limits the bioavailability of substrates, and is an important factor affecting microbial degradation rates in soils and sediments (Grosser et al. 2000). Sorption behavior of PAHs is complicated by the physical complexity and heterogeneity of the soil matrix (Luthy et al. 1997; Stokes et al. 2005). It is generally believed that adsorbed hydrophobic pollutants are not directly available to the microbial population. A well-designed bioremediation process should consider methods to mobilize these contaminants from soil surface and make them available to the microorganism. Variable results have been shown concerning the utility of using surfactants or solvents in hydrocarbon solubilization and biodegradation. It is to be expected that some of these flushing agents will remain in the treated soil after the flushing process. The potential impact of residual flushing agents on microbial processes is a question of concern.

Soil is thought to contain the greatest biodiversity of any environment on Earth. Different investigation strategies into the biodiversity in soil exist that involve of environmental sampling and extraction of target molecules and include analysis of key biogenic molecules like membrane lipid and/ or respiratory quinone profiles. The most commonly used method for microbial classification is by sequencing the 16S rRNA gene. The 16S rRNA gene provides a highly conserved marker, with a slow and constant mutational rate that can be used to measure taxonomic distances between species based on differences in the DNA sequence. Many molecular phylogenetic environmental studies using 16S gene analyses have uncovered numerous, potentially new microbial species, genera and even domains lurking, with no cultured, laboratory strain representative for comparison. Speculation of the order of magnitude concerning the total number of bacterial species is debated by microbiologists (Hong et al. 2006), making it impossible to precisely quantify the significance of the cultured laboratory stains, which may only represent ~ 1 % of the total number of species on the planet (Amann et al. 1995).

16S rRNA gene analysis introduces biases and limits the practicality of basing community profiles solely on DNA isolation, amplification and sequencing. The process can be divided into three major stages, each of which can introduce bias; DNA extraction; polymerase chain reaction (PCR); DNA sequencing and bioinformatic analyses. Various chemical and mechanical techniques exist that are designed to extract DNA from within cells and the surrounding physical matrix, and purify this separated DNA (Sambrook and Russell 2001). The efficiency for DNA extraction depends on the methods used, the physical matrix, and the cell type (Greer 2005). Although extraction methods are designed to deal with distinct matrices and cell types, for instance Gram-positive cells are generally more resistant than Gram-negative cells to lysis, no method is considered infallible (Krsek and Wellington 1999; Martin-Laurent et al. 2001).

2. Background of the contaminated site

The Beijing Coking and Chemistry Plant was constructed in 1959, and was totally suspended in July 2006 and relocated to Tangshan City, Hebei Province. The plant was located in the east of Beijing city, was adjacent to Beijing Dye Processing Plant in the west, 5th Ring Road in the east, Huagong Road in the south, old Boluoying Village in the north and Huagong Bridge in the southeast corner, as the starting point of another Jing-jin-tang Expressway and also a major part of Beijing (Fig.1).

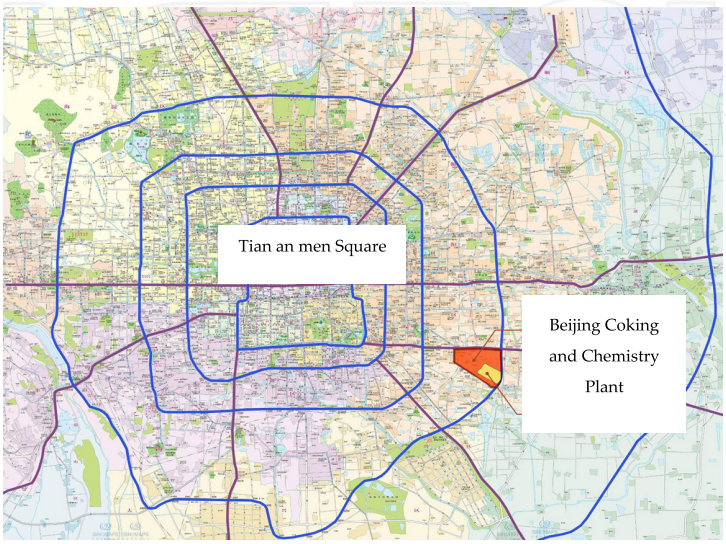

Figure 1. Location of Beijing Coking and Chemistry Plant

The identification of appropriate remediation technologies will be conditioned by knowledge of the soil conditions that exist, and of water movement and contaminant transport in the unsaturated zone. Field investigations are conducted to initially determine fundamental soil and contaminant properties, and to pilot test and evaluate remediation technologies. In addition to standard sampling at various depths, and laboratory determinations of soil organic carbon, total P, total N, total K etc., it is intended to make use of more sophisticated geophysical techniques to map shallow water tables and contamination distribution. All of the results were shown in Table1 and Table 2.

Parameter	Value
Soil organic carbon (dry weight.%)	3.32
Total P (mg P/kg soil)	0.29
Total N (mg N/kg soil)	0.81
Total K (mg K/kg soil)	14.91
Soil pH (in reagent grade water)	8.13

Table 1. The characteristics of the soil.

PAHs	Soil depth		
	0.5m	1.2m	3.0m
Acenaphthylene	11.37	10.70	7.70
Acenaphthene+ Fluorene	2.83	1.43	5.51
Anthracene	2.17	0.49	6.18
Fluoranthene	14.0	3.93	1.81
Phenanthrene	16.32	5.76	2.98
Pyrene	6.14	1.54	0.84
Benzo(a)anthracene + Chrysene	18.14	8.60	21.94
Benzo(b)fluoranthe	24.05	8.73	17.79
Benzo(k)fluoranthene	10.14	3.81	4.04
Benzo(a)pyrene	9.88	4.06	8.72
Dibenzo(a,h)anthracene	23.43	3.17	6.10
Indeno(1,2,3-cd)pyrenene	21.21	7.38	12.96
Total amount	159.68	59.59	96.57

Table 2. PAH concentration in soil of different depth (mg/kg)

3. Sampling and analytical methods

3.1. Soil and groundwater sampling and analysis

Collection of representative soil samples groundwater samples for contaminants of concern are crucial for the whole analysis process. The sampling programs would employ a judgemental sampling approach with borehole and monitoring wells being targeted to assess known areas/depths of contamination. Soil samples were taken by bores located on the site. Surface soil samples were collected from the bores and Selected distances, up to 12.74 m; in depth samples were collected at 1 m intervals. Control samples were collected from points located far away from the disposal sites, where soil contamination is practically nonexistent. The soil samples used in the experiment were collected in brown glass bottles and stored at 4°C until analysed. The samples were dried and then sieved with 2 mm diameter before experiment. The groundwater was sampled after purging the wells using a submersible pump and a Teflon hose. The samples were stored in brown glass bottles at 4°C until analyses.

Soil analysis was carried out using standard methodologies (Page, 1982). Particle size distribution was carried out using the Bouyoukos method (Bouyoucos, 1962); pH and electrical conductivity were measured in the paste extract using pH/EC meter equipped with glass electrode; Field moisture, water holding capacity (WHC)and degree of saturation were determined for each homogenized sample upon return to the laboratory. Organic matter was determined by dichromate oxidation; carbonates by using Bernard calcimeter; total N by the Kjeldahl method; available phosphorous using sodium hydrogen carbonate extraction; exchangeable K, Ca and Mg using BaCl2 extraction, while available Mn, Fe, Cu and Zn using DTPA extraction. Determination of NH_4^+, NO_3^-, Cl^-, PO_4^{3-}, and SO_4^{2-} was performed in

1:10 water extracts using Dionex-100 Ionic Chromatography. Soil was extracted with boiling water using the azomethine-H method. Methanol extractable phenol compounds were quantified by means of the Folin–Ciocalteu colorimetric method (Box, 1983).

3.2. Microbial parameters analysis

3.2.1. Counting of the total bacterial community and PAH-degraders

The total bacteria cultured from the soil samples were quantified by mixing 10 g of soil with 50 mL of sterile Ringer solution in a Waring Blender for 1.5 min at high speed. From this soil suspension, successive 1/10 dilutions were made by adding 1 mL of the soil suspension to 9 mL of sterile Ringer's solution. An aliquot (0.1 mL) of each diluted soil suspension was spread on media constituted by Nutrient Agar (Difco, Detroit, USA). Colonies were counted after 7 d of incubation at 30°C. Total cultivated bacteria were expressed in colony-forming units per gram of dry soil (CFU g-1 of dry soil). Bacterial counts were carried out in triplicate.

Bacterial PAH-degraders were quantified in sterile polypropylene microplates (Nunc, Germany) according to Stieber et al. (1994). The wells were filled with 250μL of mineral salts medium. One of the PAHs that was most representative of the studied soil, phenanthrene or fluoranthene, was added, according to the selected test. The wells were inoculated with 25 μL of previously diluted soil suspension (from 10^{-1} to 10^{-8}). Inoculation was done in triplicate. The microplates were incubated at 30°C for 20–30 d and then were evaluated for colored products. Enumeration of bacterial PAH degraders was carried out using the Most Probable Number (MPN) method (De Man, 1977).

3.2.2. Polymerase chain reaction (PCR) amplification technique

The screening and cultivation of PAHs-degrading microorganisms usually precede the other steps in studies of PAHs microbial degradation. Once such microorganisms were obtained, identification and categorization of related microorganisms is a frequently confused technical problem. Categorization and identification of newly isolated above mentioned microorganisms traditionally depend on phenotypic characteristics such as colony and cell morphology as well as biochemical and serological characteristics such as protein and fatty acid pattern profiles. However, it is often time-consuming and highly experience-reliance. With the rapid development of molecular-biology, modern taxonomy prefers sequencing technologies of molecular markers such as 16S rRNA or 18S rRNA. These technologies allow the identification of colonies isolated from microbial consortia and the establishment of phylogenetic relationships between them (Molina et al., 2009). In addition to taxonomy, PCR combined with other approaches could also be used to estimate in situ how pollution affects the bacterial community structure and composition of sediments. Several PCR techniques such as random amplified polymorphic DNA (RAPD-PCR), arbitrarily primed-PCR (AP-PCR) could be used to identify species. Besides, bacterial 16S rRNA has become the most commonly used molecular index for its evolutionary distinctive sequence. PCR approach (16S rRNA) did make categorization and identification of interested strains convenient.

3.2.3. Fingerprinting techniques based on 16S rRNA

The applications of molecular biological techniques to detect and identify microorganisms have shown enormous wealth of microbial diversity, and at the same time the limitations of traditional cultivation techniques to retrieve this diversity. However, although successful, these studies have only focused on the exploration of microbial diversity, they have not given any information on the complex dynamics in which microbial communities can undergo by PAHs changes and seasonal fluctuations or after environmental perturbations. It is now widely accepted that it is the whole community, but not only one or several microbes that could fulfill the complex degradation of the PAHs. According to widely accepted opinion, about 99% of the microorganisms in natural environment are unculturable through traditional methods until now. In this sense, molecular technique is a preferable approach avoiding this insurmountable obstacle to discover the whole community of certain microorganisms because the DNA of majority microorganisms in certain environment could be obtained. However, the cloning approach exclusively is not efficient, because it is time-consuming and labour intensive, and hence impractical for multiple sample analysis. Fingerprinting techniques based on 16S rRNA such as DGGE（denaturing gradient gel electrophoresis）, SSCP (Single-Strand Conformation Polymor-phism), T-RFLP（terminal restriction fragment length polymorphism） and RISA（Ribosomal intergenic spacer analysis）could analysis the diversity and dynamics of the whole community at molecular level.

A single nucleotide change in a particular sequence, as seen in a double-stranded DNA, cannot be distinguished by electrophoresis, because the physical properties of the double strands are almost identical for both alleles. After denaturation, single-stranded DNA undergoes a 3-dimensional folding and may assume a unique conformational state based on its DNA sequence. The difference in shape between two single-stranded DNA strands with different sequences can cause them to migrate differently on an electrophoresis gel. SSCP used to be applicable as a diagnostic tool in molecular biology but was first introduced into environmental ecology in 1996 (Lee, 1996). The shortfall of SSCP include low reproducibility, short effective sequence (about 150–400 bp) and susceptibility of gel concentration and electrophoresis temperature. T-RFLP aims to generate a fingerprint of an unknown microbial community for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The major advantage of T-RFLP is the use of an automated sequencer which gives highly reproducible results for repeated samples. However, the fact that only the terminal fragments are being read means that any two distinct sequences which share a terminal restriction site will result in one peak only on the electropherogram and will be indistinguishable.

Although above mentioned several molecular methods were widely adapted in microbial ecology researches, the most commonly used method in PAHs biodegradation was DGGE. The technique is based on the electrophoretic separation of PCR-generated double-stranded DNA in an acrylamide gel containing a gradient of a denaturant. As the DNA encounters an appropriate denaturant concentration, a sequence-dependent partial separation of the

double strands occurs. This conformational change in the DNA tertiary structure causes a reduced migration rate and results in a DNA band pattern representative of the sampled microbial community. The interested strands could be punched out from the DGGE gel and the enclosured DNA could be released and amplified. Thereafter, nucleotide sequence of the target DNA could be obtained and then compared with those available in GenBank to identify the closest relatives using the BLAST algorithm. On the other hand, it is widely accepted that only a pretty small part of the microorganisms community (0.1–10%) present in any environmental sample could be cultivated in general laboratory media (Amman et al. 1995) and the appliance of new developments in culture-independent methods such as DGGE/TGGE has greatly increased the understanding of more members of microbial consortia than culture-dependent approaches. Although several PAHs degrading bacterial species have been isolated, it is not expected that a single isolate would exhibit the ability to degrade completely all PAHs. A consortium composed of different microorganisms can better achieve this. PCR–DGGE of 16S rRNA gene sequences was used to monitor the bacterial population changes during PAHs degradation of the consortium when pyrene, chrysene, and benzo[a]pyrene were provided together or separately in the TLP cultures (Lafortune et al. 2009). Nevertheless, from the application point of view, cultivated microorganisms are more interested and necessary for the further extraction and purification of the enzymes involved in PAHs degradation as well as large-scale cultivation for engineering input.

3.3. Extracting DNA and enzymes from soil samples

3.3.1. DNA extractions

In the initial efforts to extract DNA from sediments and soils workers used either cell extraction (recovery of cells from the soil matrix prior to cell lysis) or direct lysis within the soil matrix (Holben et al. 1988). Direct lysis techniques, however, have been used more because they yield more DNA and presumably a less biased sample of the microbial community diversity than cell extraction techniques yield. A major drawback of direct lysis methods is that more PCR-inhibitory substances are extracted along with the DNA (Ogram et al. 1987). In addition, the number and diversity of the direct lysis DNA extraction protocols used for soils and sediments are daunting (Herrick et al. 1993), but each protocol usually includes from one to all three of the following basic elements: physical disruption, chemical lysis, and enzymatic lysis.

Four extraction procedures, method 1 to method 4 (M1–M4), were employed; all were based on the direct lysis of cells in the sample, with subsequent recovery and purification of nucleic acids. Before extraction, all solutions were rendered DNase-free by treatment with 0.1% diethyl pyrocarbonate (DEPC). Method 1 (M1) was modified from DeLong et al. (1993).

Method 1 (M1): liquid nitrogen (approx. 10 ml) was mixed with 250 mg of each biomass sample (wet weightfrom pellet) in a mortar, ground and transferred to a micro-centrifuge tube (Eppendorf, Germany), and 1 ml of cetyl trimethylammonium bromide (CTAB)

extraction buffer (Griffiths et al. 2000) was added, followed by vortexing for 30 s. After the addition of 500 µl of lysis buffer (50 µM Tris–HCl [pH=8]; 40 µM ethylene diamine tetraacetic acid [EDTA; pH=8]; 750 µM filter-sterilised sucrose) and 20 µl of lysozyme (10 mg ml^{-1}; Sigma-Aldrich, Germany), mixtures were briefly vor-texed (30 s) and incubated at 37°C for 30 min. Sodium dodecyl sulphate was added to a final concentration of 2%; the samples were again vortexed and then incubated at 70°C for 1 h. After this, 6 µl of proteinase K (Sigma-Aldrich) were added. Samples were then vortexed and incubated at 50°C for a further 30 min followed by centrifugation for 15 min (10,000×g). The supernatants were transferred to fresh micro-centrifuge tubes, and the aqueous phase was extracted by mixing an equal volume of chloroform–isoamyl alcohol (24:1) followed by centrifugation (10,000×g) for 10 min.. Total nucleic acids were then precipitated from the extracted aqueous layer with 0.6 vol of isopropanol overnight, at room temperature, followed by centrifugation (10,000×g) for 15 min. The pelleted nucleic acids were washed in 70% (v/v) ice-cold ethanol and air dried before re-suspension in 50 µl DEPC-treated water.

Method 2 (M2): Soil or sediment of 250 mg and 1 ml of 1% CTAB were beaten for 2 min with 250 mg of zirconia/silica beads (1.0, 0.5 and 0.1 mm; Biospec Products, USA), in the Mini Beadbeater-8 (Biospec Products) at the median speed setting. A 500-µl aliquot of lysis buffer was added to the mixture, and the remainder of the extraction protocol was continued as described for M1.

Method 3 (M3): Briefly, 500 mg of the soil or sediment samples were added to 0.5 ml of CTAB extraction buffer and 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1; pH 8.0) and lysed for 30 s with 250 mg of zirconia/silica beads (1.0, 0.5 and 0.1 mm), in the Mini Beadbeater-8 at the median speed setting. The aqueous phase, containing the nucleic acids, was separated by centrifugation at 10,000×g for 5 min and removed to respective fresh micro-centrifuge tubes. The aqueous phase was extracted, and phenol was removed by addition of an equal volume of chloroform–isoamyl alcohol (24:1) followed by centrifugation for 5 min (10,000×g). Two volumes of polyethelene glycol (PEG)–1.6 M NaCl (30% w/v) were used to precipitate total nucleic acids at room temperature, which were then washed with ice-cold 70% (v/v) ethanol and air dried before re-suspension in 50 µl of DEPC-treated water (Griffiths et al. 2000).

Method 4 (M4): The MoBio Ultraclean™ soil DNA kit (Cambio, Cambridge, UK). DNA was extracted from 250 mg of soil or sediment according to the manufacturer's instructions.

To inspect the quality of extracted DNA, 5-µl aliquots of crude extract were run on Tris–acetate–EDTA (TAE) agarose gels (1%) containing ethidium bromide (1 ng ml^{-1}; Maniatis et al. 1982) for DNA staining and visualisation, with Lambda DNA/HindIII molecular size marker (Promega, USA). Gel images were captured using a UV transillumination table and the AlphaDigiDoc 1201 system (Alpha Innotech, USA).

All four methods successfully extracted DNA, which was visible on agarose gels from the three soils tested. Many studies of DNA extraction techniques have reported lysis efficiencies that concur with the range observed in this study. M2 lysed most cells (93.8%), while M1 was

least successful (87.7%). M3 and M4 were very similar with regards to their lysis efficiencies. M2 achieved, in almost every case, significantly higher cell lysis than the other three methods; however, there was considerable variation in the lysis efficiencies between different soil types, indicating that soil texture has a substantial impact on these measurements.

The choice of the DNA extraction method significantly influenced the bacterial community profiles generated. Greater variations were, however, observed between replicate DGGE profiles generated with M1 and M4, demonstrating that a lesser degree of reproducibility was achieved with these methods.

The results of this comprehensive evaluation of nucleic acid extraction methods suggested that M2 and M3 were both suitable for use in a large-scale study involving the direct comparative analysis of multiple soil types. The application of M2—in almost all cases—resulted in the resolution of greater diversity and employed in the research case on Beijing Coking and Chemisitry Plant.

3.3.2. Enzymes analysis

The microbial activities, in terms of soil enzymes, in long-term contaminated site soils were measured using the following methods. Dehydrogenase activity was determined as described by Casida et al. (1964). Soil Catalase activity was determined by the method of Cohen et al. (1970). Catalase is an iron porphyrin enzyme which catalyses very rapid decomposition of hydrogen peroxide to water and oxygen (Nelson and Cox, 2000). The enzyme is widely present in nature, which accounts for its diverse activities in soil. Catalase activity alongside with the dehydrogenase activity is used to give information on the microbial activities in soil.

Dehydrogenases (DHA) are enzymes which catalyse the removal of hydrogen atom from different metabolites (Nelson and Cox, 2000). Dehydrogenases reflect physiologically active microorganisms and thus provide correlative information on biological activities and microbial populations in soils (Rossel et al., 1997). This parameter is considered to be highly relevant to ecotoxicological testing and sensitive to PAH contaminants. DHA activity in soils was carried as following process. Six gram of soil were placed in a test tube, mixed with 0.2 g $CaCO_3$, 1 mL TTC (2,3,5-triphenyltetrazolium chloride – as an electron acceptor) and 2.5 mL distilled water. The tubes were corked and incubated for 24hr at 37°C. The triphenylformazan (TPF) was extracted with ethanol and the intensity of the reddish colour was measured on a Beckman DU-68 spectrophotometer at a wavelength of 485 nm with ethanol as a blank. All dehydrogenases activity determinations were done in triplicate and the results were expressed as an arithmetic mean.

Soil catalase activity can be an indicator of the metabolic activities of aerobic microorganisms and correlated to the population of aerobic microorganisms and soil fertility. Catalase activity was determined by the method of Cohen where decomposed hydrogen peroxide is measured by reacting it with excess of $KMnO_4$ and residual $KMnO_4$ is measured spectrophotometrically at 480 nm. One tenth ml of the supernatant was

introduced into differently labelled test tubes containing 0.5 ml of 2 mMol hydrogen peroxide and a blank containing 0.5ml of distilled water. Enzymatic reactions were initiated by adding sequentially, at the same fixed interval, 1ml of 6 N H_2SO_4 to each of the labelled test tubes containing different concentrations of spent engine oil ranging from 0.25 to 2%and to the blank sample. Also, 7 ml of 0.1N $KMnO_4$ was added within 30 s and thoroughly mixed. Spectrophotometer standard was prepared by adding 7 ml of 0.1 N $KMnO_4$ to a mixture of 5.5 ml of 0.05 N phosphate buffer, pH 7 and 1 ml of $6NH_2SO_4$, the spectro-phosphotometer was then zeroed with distilled water before taking absorbance readings.

3.4. Analyzing microbial diversity using PCR-DGGE

Bacterial diversity was analysed using the culture independent molecular techniques 16S rDNA gene PCR following with denaturing gradient gel electrophoresis (DGGE) . This technique proved to be a powerful tool to monitor the changes in total and PAH-degrading bacterial communities during a bioremediation process. This technique is based on the direct extraction of genomic DNA from soil samples, the amplification of 16S rRNA genes (V3 region) by using the specific primers and the separation of the PCR products by DGGE. The soils sampled in different places and different depths can be analysis in the same DGGE profiles. After the genomic DNA of samples were extracted. 16S rDNA fragments (16S rRNA gene V3 region) were amplified by using two sets of specific primers F341GC, R907 with a GC clamp in 5'end of the forward primer and F341. Initial denaturation was at 94 °C for 45 s, amplification was carried out using 30 cycles including denaturation at 94°C for 45 s, annealing at 55°C for 45 s and DNA extension at 72°C for 60 s, and final extension at 72°C for 6 min. An aliquot of 5μL of the PCR product was run in 1.5% agarose gel at 120 V for 45 min. DGGE was performed using a D-Code 16/16-cm gel system with a 1-mm gel width (Bio-Rad, Hercules, Calif.) maintained at a constant temperature of 60°C in 7L of 1xTAE buffer. The acrylamide concentration in the gel was 6% and gradients were formed between 35% and 65% denaturant. Gels were run at a constant voltage of 110 V for 11 h, at 60°C. The gels were then stained with 0.5 mg L-1 ethidium bromide solution for 30 min, destained in 1xTAE buffer for 15 min, and photographed. Pieces of the DGGE bands of total community DNA to be sequenced were cut out with a sterile scalpel and placed in Eppendorf tubes containing 50μL of sterilized milli-Q water. The DNA of each band was allowed to diffuse into the water at 4°C overnight. After centrifugation at 10,000g for 6 min, 10μL of the solution was used as the DNA template in a PCR reaction using the same conditions. PCR products were analyzed using DGGE to confirm that the expected products were isolated.

The PCR products are separated by DGGE respectively. DGGE patterns from environmental samples were compared with the migration of reference clones of known sequence, and the major environmental bands were excised, reamplified, and sequenced to investigate their identities further. Similar bands were matched after normalising the bands using Gaussian modelling and background subtraction. The matched band profiles were converted into binary data using Quantity One Software. The similarity matrix was calculated based on the Dice coefficient (Dice, 1945). The clustering algorithm of unweighted pair-group method with arithmetic averages (UPGMA) was used to calculate the dendrogram.

4. Results and discussions

4.1. Relationship between microbial population and soil contamination levels

The reduction of PAH content was linked to the bacterial degrading activity of the soil, which was kept at an optimal level with respect to humidity and oxygenation during the treatment. Soils highly contaminated by hydrocarbons displayed different microbiological properties. In particular the higher/the lower the pollutant content, the smaller/the greater are the activities of some enzymes related to nutrient cycling and the viable bacterial cell numbers. The different microbiological properties of the soils probably reflect the different bacterial diversity as assessed. Studies showed that: PAH induce perturbations in the microbial communities in terms of density and metabolism; indigenous bacteria seem to have a high capacity of PAH degradation, depending on the physicochemical properties and the availability of substances present.

A number of bacterial species are known to degrade PAHs and most of them are isolated from contaminated soil or sediments. *Pseudomonas aeruginosa, Pseudomons fluoresens, Mycobacterium* spp., *Haemophilus* spp., *Rhodococcus* spp., *Paenibacillus* spp. are some of the commonly studied PAH-degrading bacteria. *Lignolytic* fungi too have the property of PAH degradation. *Phanerochaete chrysosporium, Bjerkandera adusta,* and *Pleurotus ostreatus* are the common PAH-degrading fungi. Among the PAH in petrochemical waste, 5 and more rings PAHs always have toxicity and harder to be degraded. Fungi have prevalent capability of degrading five-ring PAHs because of their recalcitrant and hydrophobic nature (Cerniglia, 1993). Both lignin-degrading white-rot and some non-white-rot fungi (*Aspergillus niger, Penicillium glabrum, zygomycete Cunninghamella elegans, basidiomycete Crinipellis stipitaria*) have been shown to degrade a variety of pollutants including PAH, DDT, PCP, and TNT (Haemmerli et al., 1986). Benzo(a)pyrene is considered as the most carcinogenic and toxic. And some kinds of bacteria are also found having the ability to degrade BaP. Ye et al. *Sphingomonas paucimobilis* strain EPA 505 can degrade BaP and also indicated that has the significant ability of hydroxylation and ring cleavage of the 7,8,9,10-benzo ring. Aitken et al. (1991) isolated 11 strains from a variety of contaminated sites (oil, motor oil, wood treatment, and refinery) with the ability to degrade BaP. The organismswere identified as at least three species of Pseudomonas, as well as *Agrobacterium*, *Bacillus, Burkholderia* and *Sphingomonas* species. and BaP has been reported to be degraded by other bacteria including *Rhodococcus* sp., *Mycobacterium*, and mixed culture of *Pseudomonas* and *Flavobacterium* species (Walter et al.1991). Small molecular weight PAHs such as 2-, 3- and 4-ring PAHs are considered to be degraded easier than 5 and more rings. Christine et al (2010) used by DGGE profiles of the 16S rDNA genes assessed the bacterial community in soil contaminated by mostly 2-, 3- and 4-ring PAHs produced by coal tar distillation, and found persistence of a bacterial consortium represented by Gram-negative bacterial strains belonging mainly to *Gamma-proteobacteria,* and in particular to the *Pseudomonas* and *Enterobacter* genera .These strains had a strong PAH-degrading capacity that remained throughout the biotreatment. Thus, the presence of *Pseudomonas* and *Enterobacter* strains in this type of PAH-contaminated soil seems to be a good bioindicator for the potential

biodegradation of 2-, 3- and 4-ring PAHs. Other species, such as *Beta-proteobacteria*, appeared over the course of time, when the PAH concentration was low enough to strongly decrease the ecotoxicity of the soil. Thus, the *Beta-proteobacteria* group could be a good indicator to estimate the end point of biotreatment of mostly 2-, 3- and 4-ring PAH-polluted soil to complement chemicalmethods.

In the study of Beijing Coking and Chemistry plant, the direct count of microbes from all 68 samples showed the microbes distribution varied in each borehole and increased with contaminates concentration. The minimum, 8.90×10^{6} CFU/g, appeared at the depth of 4.10m, the maximum, 1.422×10^{8} CFU/g, appeared at the depth of 6.80m. The reasons may be that the sites have been exposured to coal tar pollution for a long time, and indigenous microorganisms can survive after selected by the pressure of the coal tar-contaminated environment, that is "survival of the fittest". Microorganisms in this site survive well can be well adapted to the coal tar, suggesting that the number of indigenous microorganisms in the local underground environment may have a relationship with coal tar pollutants. There is obvious correspondence between the distribution rules of the number of microorganisms at vertical depth of the same drilling and the distribution rules of pollutant concentration. The numbers of microorganisms increase in the soil with higher concentrations of pollutants. In fact, the maintenance of an adequate microbial biomass in soil, with a high microbial activity, could be a mechanism for soil resistance to degradation factors and thus of paramount importance for ecosystem sustainability. We can find that there is a growing trend of the number of microorganisms as the concentration of PAHs increased (Fig.2) and it is consistent with the finding of the research of Duncan et al. (1997). The results of this study show that the characteristics of the distribution of microorganisms in the contaminated soil is an important biological factors affecting natural attenuation and biological degradation of organic pollutants of coal tar. The coal tar contamination on soil can enhance the adaptability of degrading microorganisms in soil and increase the number of indigenous degrading bacteria.

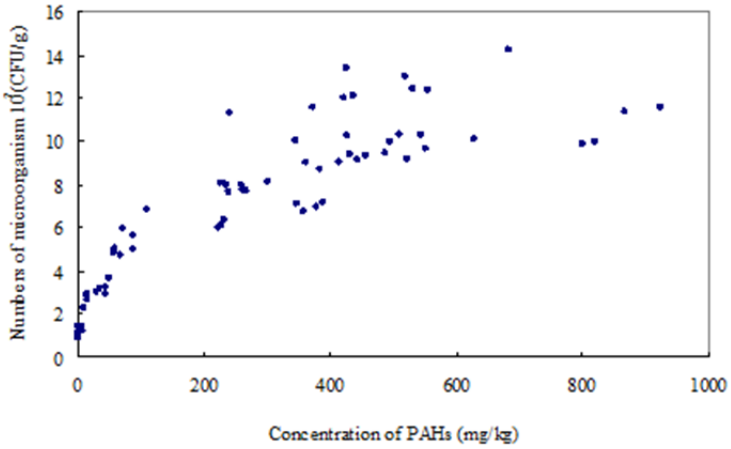

Figure 2. The relationship between the microorganisms quantities and concentrations of pollutants of sampling point

The determination and the analysis of the sequence of the advantages and the specific belt in DGGE fingerprints shows that 14 kinds of the indigenous prokaryotic microorganisms had been found in underground environment of pollution site, namely *Bacillus* sp. EPI- R1 (*Bacillus* genus EPI-R1 strains), *Bacillus* cereus.03BB102, *Bacillus thuringiensis (Bacillus thuringiensis*), *Bacillus* sp. SA, Ant14 *(Bacillus* SA Ant14 strains), the *Bacillus weihenstephanensis* KBAB4 (*Wechsler Bacillus* KBAB4), *Uncultured Bacillus* sp. (*uncultured Bacillus* certain bacteria) and *Ralstonia pickettii* (*Petri Ralston* bacteria), *Nocardioides* sp. (*Nocardia* certain bacteria), *Azoarcus* sp. BH72 (nitrogen-fixing Vibrio genus BH72 strain), *Erythrobacter litoralis* HTCC2594 (*Erythrobacter* genus HTCC2594 strain), *Magnetospirillum magneticum* (*Acidovorax Willems* genus *magnetotactic spiral* strains), *Acidovorax avenae* subsp. (food acid sub-species of the genus avenae strain), *clavibacter michiganensis* subsp. (a sub-species of *Michigan* stick rod-shaped bacteria) and an unknown new species has not been reported in GenBank.

In the research, the wildly existed bacteria underground environmental of contaminated sites can be classified as 4 groups (shows in Fig.3). (1) *Bacillus* cereus, the *Bacillus* genus, has an absolute advantage and are all found at different depth of the drilling. *Bacillus* sp. EPI-R1, the *Bacillus* genus; *Bacillus* cereus 03BB102, *Bacillus* sp.; *Bacillus thuringiensis*; *Bacillus* sp. SA, Ant14; *Bacillus weihenstephanensis* KBAB4, can also exist in various vertical depth in the venues. It means these five kinds of Bacillus genus bacteria share a common living space. However, in the *Bacillus* genus, there is a kind of bacteria named *Uncultured Bacillus* sp. different from the other five kinds. It survives in the depth range of 6.30 ~ 12.90m and is not grown in the laboratory and making the anaerobic living. (2) *Actinobacteria, Nocardioides* and *Actinobacteria Clavibacter* are the dominant *Actinobateria* to exist in this contaminated environment. *Nocardioides* sp. and *Clavibacter michiganensis* subsp are widely distributed at the vertical depth from the surface to the deepest. It indicates that these two actinomyces have the highly viability and can live in both aerobic and anaerobic environments. (3) *Alphaproteobacteria, Magnetospirillum* and *Alphaproteobacteria, Erythrobacter* are the dominant bacteria existing in the depth range of 12.20 ~ 12.70 m. The *Erythrobacter litoralis* HTCC2594 and *Magnetospirillum magneticum*, exist in the aqueous medium near the groundwater level, indicating that these two bacteria of the *Alphaproteobacteria* live an anaerobic life. (4)*Azoarcus* sp. BH72, *Ralstonia pickettii* and *Acidovorax avenae* subsp. are widely exsist in the whole of the vertical distribution of the contaminated sites, indicating that these three *Betaproteobacteria* (*Beta Proteobacteria*) bacteria can survive both in aerobic and anaerobic conditions and are facultativebacteria.

4.2. Relationship between catalase and dehydrogenase distribution and hydrocarbon contamination levels

The changes of soil catalase activities influenced by PAHs contamination are showed in Fig.4 from the study of Zhang et al. (2009). The soil catalase activities were stimulated at first and then inhibited slightly during PAHs injection within the range ~ 0.05mL (0.1M $KMnO_4$)/g (soil). Lin et al. (2005) also revealed that soil catalase activities were declined with the increase of oil concentration. At the beginning of the experiment the PAHs concentrations were low which could be as carbon sources to the indigenous

microorganisms, the catalase activities were higher than the original soil before day 30. With the accumulation of PAHs in the soil, its toxicity became significant and inhibited the growth of aerobic microorganisms, the catalase activities declined after 60 days, lower than those in the unpolluted soil. This is also indicated by the environmental conditions change from aerobic to anaerobic with the continuous input of PAHs contaminants.

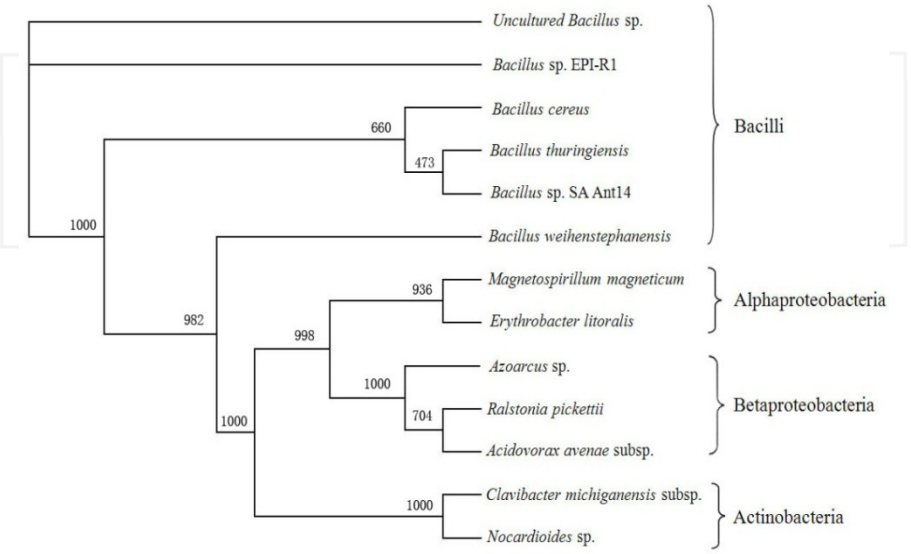

Figure 3. The 16S rDNA phylogenetic tree of dominant prokaryotic microorganisms in underground environmental of contaminated sites

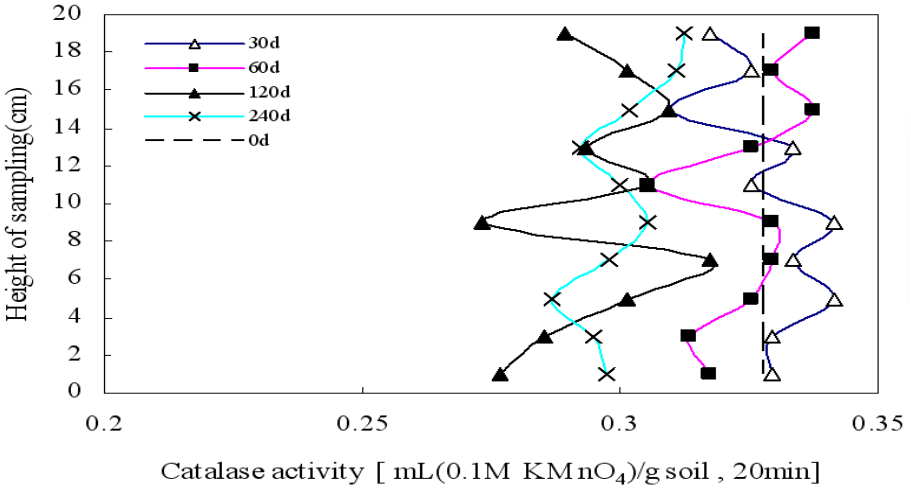

Figure 4. Change of soil catalase activities

In the present study, it was clearly shown that a positive correlation between dehydrogenase activity and the content of phenanthrene and also fluoranthene, chrysene and dibenzo[ah]anthraacene was observed. We can conclude that the effect of PAHs contamination on soil dehydrogenase activity. There was a progressive increase in the values obtained as the concentrations of the PAHs contamination increased and this was found to be significant between in soil contamination relative to the control value. Also dehydrogenase activity depends not only on the PAH type, but also primarily on the other compounds from this group and also their amount.

In the case of Beijing Coking and Chemistry plant, the spatial trends of the catalase activity at different depths of vertical drilling on the whole are the same. For each drilling, the catalase activity of the surface is higher and the catalase activity of the deep drillings is lower, the overall trend is that the catalase activity decreases with depth, indicating that the distribution characteristics of aerobic microorganisms. Catalase activity of the contaminated sites of all 68 sampling points range between 0.554 mL 0.02M $KMnO_4$ /g•h~6.230 mL 0.02M $KMnO_4$ /g•h; while the difference of catalase activity in the same formation of the entire site is not particularly large.

As to dehydrogenase in this case, the result of measurement show that the trend of dehydrogenase activity overall is the same at different depths drilling vertically, the range of the change is between 0.14 μg TF/g•h~2.54 μg TF /g•h. The differences of dehydrogenase activity are not particularly large, the reason maybe that the indigenous microorganisms of the different vertical depth of the contaminated sites can generate dehydrogenase and to some extent all have the ability to degrade organic pollutants of coal tar.

Distribution rules of dehydrogenase activity in vertical depth of the same drilling and the rules of the distribution of number of microorganisms have a certain correlation. Fig.5 shows the relationship between the dehydrogenase activities with numbers of microorganisms in this sampling site. Activities of dehydrogenase can be enhanced in the soil with more numbers of microorganisms. It means that the selection of coal tar contamination on soil microorganisms can enhance the adaptability of microorganisms and the ability of degradation.

In the vertical spatial scale, the distribution rules of the concentrations of pollutants in the site and the distribution of number of microorganisms and dehydrogenase activity has a certain correlation. The higher concentrations of pollutants in the soil, the more numbers of microorganisms and the greater the activities of dehydrogenase produced by indigenous microorganisms with of the degradation function.

4.3. Relationship of microbial diversity with soil contamination levels

PAHs present in soil may exhibit a toxic activity towards different plants, microorganisms and invertebrates. Microorganisms, being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. In general, they are very sensitive to low concentrations of contaminants and rapidly response to soil perturbation. An alteration

of their activity and diversity may result, and in turn it will reflect in a reduced soil quality (Schloter et al., 2003). Hydrocarbon contamination may affect soil microbial structure. However, in soil microbiology there is a lack of knowledge regarding the environmental determinants of microbial population variation in soil environments contaminated by complex hydrocarbon mixtures (Hamamura et al., 2006). In this sense, different authors have observed, by denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP), a decrease in the microbial diversity of fuel contaminated soils, leading to the predominance of well-adapted microorganisms (Ahn et al., 2006), and a change in the community structure (Labb´e et al., 2007).

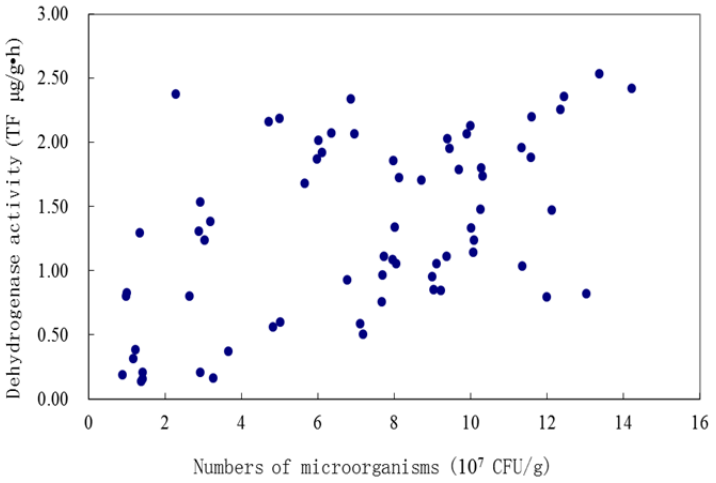

Figure 5. The relationship between the dehydrogenase activities with microorganisms quantities in the sampling sites

When bacterial diversity is analyzed by DGGE profiles of the 16S rDNA genes, the number of DGGE bands was taken as an indication of species in each sample. The relative surface intensity of each DGGE band and the sum of all the surfaces for all bands in a sample were used to estimate species abundance (Fromin et al., 2002; Sekiguchi et al., 2002). The different microbiological properties of the soils reflect the different bacterial diversity as assessed by DGGE profiles of the 16S rDNA genes.

The Shannon index, sometimes referred to as the Shannon-Wiener index or the Shannon-Weaver index, was used to evaluate the biodiversity of both soils and enrichment cultures. The Shannon-Weaver diversity index is a general diversity index which increases with the number of species and which is higher when the mass is distributed more evenly over the species. The evenness is independent of the number of species. Evenness is lower if a small number of bands are dominant and highest if the relative abundance of all bands is the same. The equitability correspondingly indicates whether there are dominant bands. The Shannon index of soils was calculated on the basis of the number and intensity of bands present on DGGE samples, run on the same gel, as follows: $H=-\sum P_i \log P_i$, where P_i is the

importance probability of the bands in a gel lane. P_i was calculated as follows: $P_i = n_i/N$, where n_i is the band intensity for each individual band and N is the sum of intensities of bands in a lane. *H* was used as a parameter that reflects the structural diversity of the dominant microbial community.

In particular the lower / the higher the parameter *H*, the higher/ the lower the pollutant content, the smaller/the greater are the activities of some enzymes related to nutrient cycling and the viable bacterial cell numbers. (Andreoni et al., 2004).

In the study of Beijing Coking and Chemistry plant, the relationship of microbial diversity with soil contamination levels was show in Fig.6. Generally there are more numbers of the microorganisms in contaminated soil. However the result shows the relationship between the microbial diversity of different depth of the drilling and the soil contamination levels is not clear. The trend of the microbial diversity is also not clear. It means that the microbial diversity not only decides by the contamination levels and it is the result of the coaction of various environmental factors. The relational graph of the relationship between the prokaryotic microbial community diversity and concentration of pollutants of 68 sampling points of the underground environment also shows that the correlation between the prokaryotic microbial diversity and concentration of pollutants is not obvious.

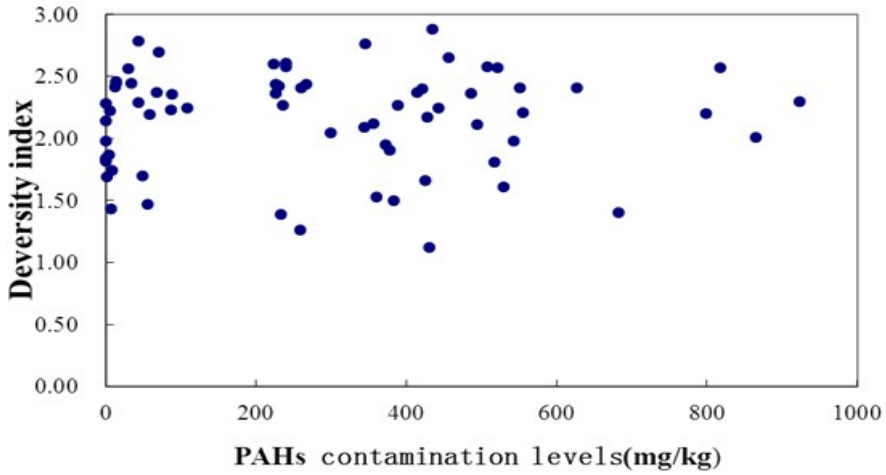

Figure 6. The relationship between the prokaryotic microbial diversity and concentration of pollutants

5. Conclusions

In the case of Beijing Coking and Chemistry plant, dehydrogenase and catalase activities were analysis to assess soil biological activity. Dehydrogenase activity (DHA), a very sensitive soil enzyme to pollutants, showed significant variation between soils. The research showed that the trend of dehydrogenase activity overall is the same at different depths drilling vertically, the range of the change is between 0.14 μg TF / g • h ~ 2.54 μg TF / g • h.

The higher concentrations of pollutants in the soil, the more numbers of microorganisms and the greater the activities of dehydrogenase activity produced by indigenous microorganisms with the degradation function. As to catalase, for each drilling, the catalase activity of the surface is higher and the catalase activity of the deep drillings is lower, the overall trend is that the catalase activities decreased with depth, indicating that the distribution characteristics of aerobic microorganisms.

The sequencing analysis of the specific belts in DGGE fingerprints showed that the prokaryotic microbial community structure had a certain degree of changes with vertical depth of the site and the DGGE finger-print of the six holes were so different. The analysis of the sequence of the advantages and the specific bands in DGGE fingerprints showed that 14 kinds of the indigenous prokaryotic microorganisms had been found in underground environment of pollution site. They could be classified into 4 classes by Phylogenetic analysis: *Bacilli, Alphaproteobacteria, Betaproteobacteria, Actinobacteria*.

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