Microbial Diversity and Activity of an Aged Soil Contaminated by Polycyclic Aromatic Hydrocarbons

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Introduction

PAHs are widespread environmental contaminants that result from various anthropogenic and natural activities and have proven to be carcinogenic, mutagenic and teratogenic to human and animal health. PAHs-organic matter bonding in soils or sediments reduces the mobility of PAHs and enables the contaminants more resistant to microbial degradation in the anoxic environment, especially for sites after decades of pollution. In this study, we analyzed the chemical profile of PAHs, the response of biological properties of soil with varying PAHs concentrations, and the microbial composition heterogeneity in aged PAH contaminated soils. The purpose is to assess the distribution patterns of endogenous microbiota as well as their diversity and activity in PAH contaminated soils, thus providing strategic direction and guidance for bioremediation practices of the contaminated soil in the future.

Materials and methods

Soil samples were collected from an abandoned coking-chemical plant in Beijing (China) contaminated with different levels of PAHs due to its nearly 50 years of operation. Representative soil samples were collected from three selected boreholes (A, B and C) to evaluate soil contamination level, microbial abundance, activity and diversity. In each borehole soil was sampled at depths from the surface down to 12 m deep below the ground surface and in each depth more than 2 kg of soil was collected integrally in original condition. The soil samples were freeze-dried and then subjected to ultrasonic solvent extraction. PAHs were analyzed by Ultimate 3000 HPLC equipped with a UV–visible detector. Soil enzymes, which are widely present in nature, can be an indicator of diverse microbial activities in soil correlated to the population of microorganisms and soil fertility. DHA in long-term contaminated soils was measured through the method as described by Szulc et al. The principle of the method is the transformation of 2, 3, 5-triphenyltetrazolium chloride (TTC) into triphenylformazan (TPF) and subsequent spectroscopic determination of TPF. A fingerprinting method, denaturing gradient gel electrophoresis (DGGE), was carried out to determine similarities and differences between the predominant bacterial communities in the soil samples. The total microbial cell numbers were estimated using the most probable number (MPN) method referred from Wrenn, Venosa. Biodiversity represents the variety and heterogeneity of organisms at all levels of the hierarchy of life, which commonly involves the measurement of richness and abundance of the species. The Shannon index (H') was used to evaluate the biodiversity in soil samples and enrichment cultures.

Results and discussion:

The distribution of total PAHs or three-ring PAHs showed a positive correlation with the number of microorganisms and DHA. On the other hand, the microbial diversity was closely correlated with the distribution of high-molecular-weight PAHs (4 or 5 rings). The loadings of microbial diversity was not as high as enzymatic activities and number of microorganisms, which may therefore imply its relatively independent behavior toward the extent of PAHs contamination. The clustering of samples from borehole A in left side when the depth were less than10 m is attributed to the lower PAHs concentration and microbial activities.

PAH concentrations, microbial cell numbers, dehydrogenase activity and microbial diversity in the vertical distribution of three boreholes were analyzed. Overall, an increased average PAH concentrations were observed from the sampling site A, B and C. From the vertical direction, the PAH content increased (from 0 to 430.6 mg/kg) with the increasing depth in site A while a decreasing trend in PAH levels (from 937.3 to 298.6 mg/kg) was found with the increase of depth in site C. PAH concentrations in site B were relatively stable (220~300

mg/kg) when it was less than 6 m below the ground surface. The PAH concentration then raised to 557.9 mg/kg at the depth of 8 m and dropped to 240.2 mg/kg at 12 m deep. The three sites with different PAH distribution patterns are representative contaminated sites resulted from multiple uncertainty factors. The possible causes may include the time, source and severity of original PAH contamination, and factors which have significant influence in pollutant migration and transformation, such as the profile of soil layers and the availability of oxygen for microbial PAH degradation.

The direct counting results of microbes from all the samples indicate that the microbial populations distributed unevenly in each borehole but are closely associated with contaminant concentrations. The minimum value of microbial number, 8.9×10^6 CFU/g, appeared at the depth of 4 m in site A while the maximum one, 1.2×10^8 CFU/g, appeared at the depth of 10 m in site B. The number of microbial cells is in good accordance with the measured contaminant levels in vertical direction. This may be attributed to the fact that the sites have been exposed to PAH contamination for a long period, and indigenous microorganisms survived from the effect of contaminants have thrived in the environment. Thus, the numbers of microorganisms increase in the soil with the presence of higher concentrations of pollutants. The correlation results between microbial numbers and contaminant levels are direct evidence that indigenous soil microorganisms are able to adapt to new substrates.

Soil microbial biomass acts as an important ecological indicator as well and an adequate microbial biomass is typically associated with high levels of microbial activity in soil. The maintenance and growth of the soil microbial biomass increase the soil fertility and favor the maintenance of the soil quality and environmental sustainability. The DHA results indicated that soil microbial activities accorded with the distribution of microbial numbers in the contaminated soil. DHA range was observed between 2.2 and 26.4 μg TF /g·h, in which relatively higher levels of DHA were found from site B (15.7 μg TF /g·h) and C (21.0 μg TF /g·h) than that of site A (10.2 μg TF /g·h). As dehydrogenases are intracellular enzymes involved in microbial respiration, they are closely linked to microbial populations and very sensitive to environmental perturbation. Similar research findings that DHA tended to increase with increasing concentration of PAHs were once observed. However, most research efforts are needed to investigate the correlation between DHA and the content of contaminants as well as its importance in the removal of these contaminants. Overall, from the vertical distribution analysis of the microbial characteristics, the concentration of pollutants was positively correlated with the number of microorganisms and soil enzyme activity in the contaminated site. Higher concentrations of pollutants induced higher numbers of microorganisms and activities of dehydrogenase produced by indigenous microorganisms. In contrast, the microbial diversity of soil samples was found with limited correlation with other factors discussed above. Soil bacterial communities are affected by specific environmental changes or disturbances which integrate the influences of biotic and abiotic factors. Soil characteristics including soil pH, Moisture, the Ca2+/Mg2+ ratio, Al3+ and phosphorus content, and etc, coupled with the degrees of human impact all have influences on the diversity and composition of soil bacterial communities. Thus, the content of PAHs alone were not necessarily in direct positive correlation with the microbial diversity. More investigations are needed to thoroughly interpret the patterns of microbial diversity.

PAHs are regarded as thermodynamically stable and recalcitrant compounds, which are resistant to microbial degradation due to their aromatic structure and hydrophobic nature. Despite these properties, PAHs can be decomposed to different extents via environmental biotic or abiotic processes after discharged into the soil. Subsequently, the residual PAHs are transferred to soil profiles and groundwater through runoff and leaching. Microbial degradation is the main process occurring in natural PAHs decontamination and a variety of bacterial species from various environmental media are found to degrade PAHs.

The bioaugmentation approach involves inoculation of endogenous or genetically engineered microorganisms with desired degradation capability into soil and has the potential to enhance biodegradability of toxic contaminants. Currently, the commercial microbial species (engineered microorganisms) used in bioaugmentation were always reported to be applied into much different environments and it's hard to justify their cost when considering the benefits of these products. The technique is more suitable for soils contaminated by compounds requiring long-term based acclimation or adaptation of microorganisms and it may be hard to deliver the exogenous microorganisms to the desired sites. Indigenous microbial communities are important for the success of bioremediation treatments, whereas most soil microorganisms cannot be cultivated by conventional laboratory culturing methods. Therefore, a thorough identification of the existing bacterial patterns on site were vital in determining further selection of remediation approaches. Even for the application of bioaugmentation, the use of native microflora is preferred because these microorganisms typically have

better ability to adapt to target specific pollutant than exogenous microorganisms. Particularly, the dominating indigenous microbial populations with PAH degradation capacity should be put in top priority.

Twelve kinds of indigenous prokaryotic microorganisms were identified by 16S rRNA analysis from all the sampling points of contaminated sites. According to the differences in genotypic and biological characteristics, the microorganisms can be divided into 4 categories: (1) Bacillus species. The Bacillus species are the most abundant and include Bacillus sp. EPI- R1, Bacillus cereus 03BB102, Bacillus thuringiensis, Bacillus sp. SA Ant14, Bacillus weihenstephanensis KBAB4, and uncultured Bacillus sp.. The Bacillus species are advantageous microbial populations and exist in various vertical depth in the venues. In particular, the uncultured Bacillus sp. are different from other Bacillus species stated previously. It mainly exists in the depth range of 8~10 m of borehole B and the anaerobic strain is not cultivable under conventional laboratory conditions. (2) Betaproteobacteria groups. Azoarcus sp. BH72 (nitrogen-fixing bacteria), Ralstonia pickettii, and Acidovorax avenae subsp. are spotted from scattered sources throughout the vertical dimensions of the contaminated sites, which indicates the three facultative Betaproteobacteria bacteria can survive both in aerobic and anaerobic conditions. (3) Actinobateria groups. Nocardioides sp. and Clavibacter michiganensis subsp. (a sub-species of Michigan stick rod-shaped bacteria) are representative Actinobacteria strains found in the contaminated sites. Nocardioides sp. is a mesophilic and aerobic bacterial genus with high viability which was mainly found various depths from borehole A and C. Clavibacter michiganensis is typically an aerobic plant pathogenic actinomycete within the genus Clavibacter, but it also can grow slowly in anaerobic condition. (4) Uncultured bacterium. An unknown new species not reported in GenBank was found and it has a similarity of 85% with Cytophaga hutchinsonii ATCC 33406.

The most abundant species are Bacillus cereus 03BB102, Bacillus thuringiensis, and Bacillus *sp.* SA Ant14. In contrast, the species of Ralstonia pickettii, Clavibacter michiganensis subsp. and Acidovorax avenae subsp. were only rarely observed. The microbial abundance in each sampling point varied over several ranges of magnitude while a diverse profiling of biomass composition was obtained as a result of the differences in soil properties and conditions. The relative abundance of individual bacterial taxa varied distinctly among different samples. Even though, Bacilli, which include a myriad of well-known hydrocarbon degraders, clearly dominated in nearly all the zones of long-term pollution. At class level, other bacteria are minority populations and sensitive to environmental disturbances. Specially, Actinobacteria (Nocardioides sp. and and Clavibacter michiganensis subsp.) which help to decompose the organic matter in soil are widely distributed both on the soil surface and at depths of of more than 10 m below ground.

Comprehensive assessment of indigenous microbial diversity and their activities is considered critical in determining the functional groups responsible for degradation of PAHs during in situ pools of contaminants. As degradation of PAHs is facilitated by multiple functional microorganisms, the specific microbial composition in natural environments determines the degradation potential and success of any bioremediation efforts.

B. cereus bacteria are facultative anaerobes and have been reported as PAH-degrading bacteria in several studies. A strain named *B. cereus* P21was found in active growth on phenanthrene and *B. cereus* P21 transformed pyrene to *cis*-4,5-dihydro-4,5-dihydroxypyrene, a degradation metabolite in the known pathway for aerobic bacterial mineralization of pyrene. Another strain *B. cereus* Py5 isolated from the sediment samples of Huian mangroves were observed consuming 65.8% of pyrene (50 mg/L) within three weeks although little pyrene biodegradation (14.8%) occurred in the first 12 days of incubation. Besides, a novel strain of *Bacillus cereus* (JQ178332) was isolated from an oil contaminated soil at Tehran refinery distillation unit and the degradation test revealed 65.5% decrease in asphaltenic, 22.1% in aliphatics and 30.3% in aromatics content of the vacuum distillation residue in MSM medium. *B. cereus* was also included in microbial consortium to degrade and mineralize different concentrations of anthracene, phenanthrene and pyrene in soil, during which over 90% of the different concentrations of PAHs were degraded in 70 days. All these studies imply that *Bacillus cereus* can utilize both aliphatic and complex aromatic structures as sole source of carbon and energy and has the potential of unique use in soil bioremediation or petroleum biological processing. As dominant bacterium, *Bacillus cereus* 03BB102 was found in appreciable amounts from different sampling points and the strain was believed to be the main resident microbe in bioremediation actions.

The *Bacillus cereus* group comprises several bacterial species including *Bacillus cereus, Bacillus thuringiensis, Bacillus weihenstephanensis,* and others. Bacillus thuringiensis species were reported to be isolated from petroleum-contaminated soil in Hilo, Hawaii and in surface soils collected from the uppermost soil horizon (0–20 cm) subjected to strong pressure of naphthalene and pyrene. In a recent study, when B.

thuringiensis was used to degrade phenanthrene in batch using Erlenmeyer flasks, an almost complete removal (97.3%) of the pollutant from 17.8 ppm was obtained after only 10 days. The author then concluded that B. thuringiensis has an enormous potential to mineralize a wide spectrum of emerging pollutants, such as PAHs and pesticides.

Bacillus weihenstephanensis KBAB4 were correlated with increasing concentrations of PAHs, especially PAHs with 4 or 5 rings. Another type of Bacillus species, Bacillus sp. SA Ant14 was also found in close relationship with the distribution of PAHs with 4 or 5 rings, which were largely present in borehole C. From the results of sludge consortium change in response to sequential adaptation to benzene, toluene, and o-xylene (BTX), Bacillus sp. SA Ant14 was observed from the adaptation process as a result of the BTX addition. This indicated that Bacillus sp. SA Ant14 was probably essential component of functional consortium in contaminated site and was actively involved in the degradation of PAHs as well.

Phenanthrene-degrading *Nocardioides sp.* strain KP7 was the most commonly reported PAHs degrader among *Nocardioides* species. This strain is characterized by capability of growing on phenanthrene but not utilizing naphthalene, which degrades phenanthrene via 1-hydroxy-2-naphthoate, o-phthalate, and protocatechuate. Based on our results, *Nocardioides sp.* was closely associated with the distribution of PAHs, which indicated its key role in phenanthrene degradation.

Strains of *R. pickettii* are capable of degrading many of aromatic hydrocarbons and using them as both a carbon and an energy source. Under carbon-limiting conditions, *R. pickettii* has been shown to utilize benzene in the presence of more easily utilizable substrate succinate. Bacterial strain identified as Ralstonia picketti BP 20 was isolated from petroleum hydrocarbon-contaminated soil following bioremediation treatment. The strain was found to have a surface hydrophobicity in the following order: aliphatic hydrocarbons, BTEX, and PAHs. As the ability to adhere to bulk hydrocarbon is mostly a characteristic of hydrocarbon-degrading bacteria, the bacterial isolate have good emulsification property to be used in the petroleum industry, e.g. in bioremediation processes. Co-cultures R. pickettii-Penicillium *sp.* exhibited synergism for phenanthrene removal of over 70% in 18 days is approximately the sum of individual phenantherene removal, 58% and 20% for Penicillium *sp.* And B. pickettii, respectively. Only low levels of *R. pickettii* were observed from the borehole samples while it was even not detected in some sampling points. This presence of *R. pickettii* in trace level could be attributed the fierce competition of available nutrients between bacterial species in the soil layers with varying geological characteristics and soil properties.

In summary, the functional groups in degradation of PAHs were found positively correlated with the distribution of PAHs. Although different combination patterns of microbial consortium were observed from the sampling points, *Bacillus cereus* 03BB102, Bacillus thuringiensis, *Bacillus weihenstephanensis* KBAB4, and Nocardioides *sp.* were recognized as main functional bacterial species of PAH degradation in most cases. They are very likely the functional PAH degraders when nutrients and oxygen (currently limiting factors in biodegradation) are added in subsequent biostimulation studies and recommended to be further utilized as microbial candidates in possible bioaugumentation applications. Other possible PAH degradation contributors could also be *Ralstonia pickettii* and Bacillus *sp.* SA Ant14. A positive correlation was found between the concentration of pollutants with the number of microorganisms and soil enzyme activity in the contaminated site. The microbial diversity was closely correlated with the distribution of high-molecular-weight PAHs (4 or 5 rings) as well. While the indigenous soil microorganisms were dominated by species from *Bacillus cereus,* Bacillus thuringiensis, *Bacillus weihenstephanensis, Bacillus sp., and* Cytophaga hutchinsonii, the possible functional PAH degradation species were discussed according to the literature. Main functional bacterial species in the microbial consortium of PAH degradation were defined as *Bacillus cereus* 03BB102, Bacillus thuringiensis, *Bacillus weihenstephanensis* KBAB4, and Nocardioides sp. from the contaminated site.

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